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DISSERTATION

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Sexual selection, social selection and individual quality: underlying mechanisms and ultimate consequences of ornamentation in a monomorphic species, the King penguin (*Aptenodytes patagonicus*)

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UNIVERSITÉ DE STRASBOURG

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Thèse

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Sélection sexuelle, sélection sociale et qualité individuelle : déterminisme et valeur sélective de l'ornementation chez une espèce monomorphique, le manchot royal (*Aptenodytes patagonicus*)

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Sexual selection, social selection and individual quality: underlying mechanisms and ultimate consequences of ornamentation in a monomorphic species, the King penguin (*Aptenodytes patagonicus*)

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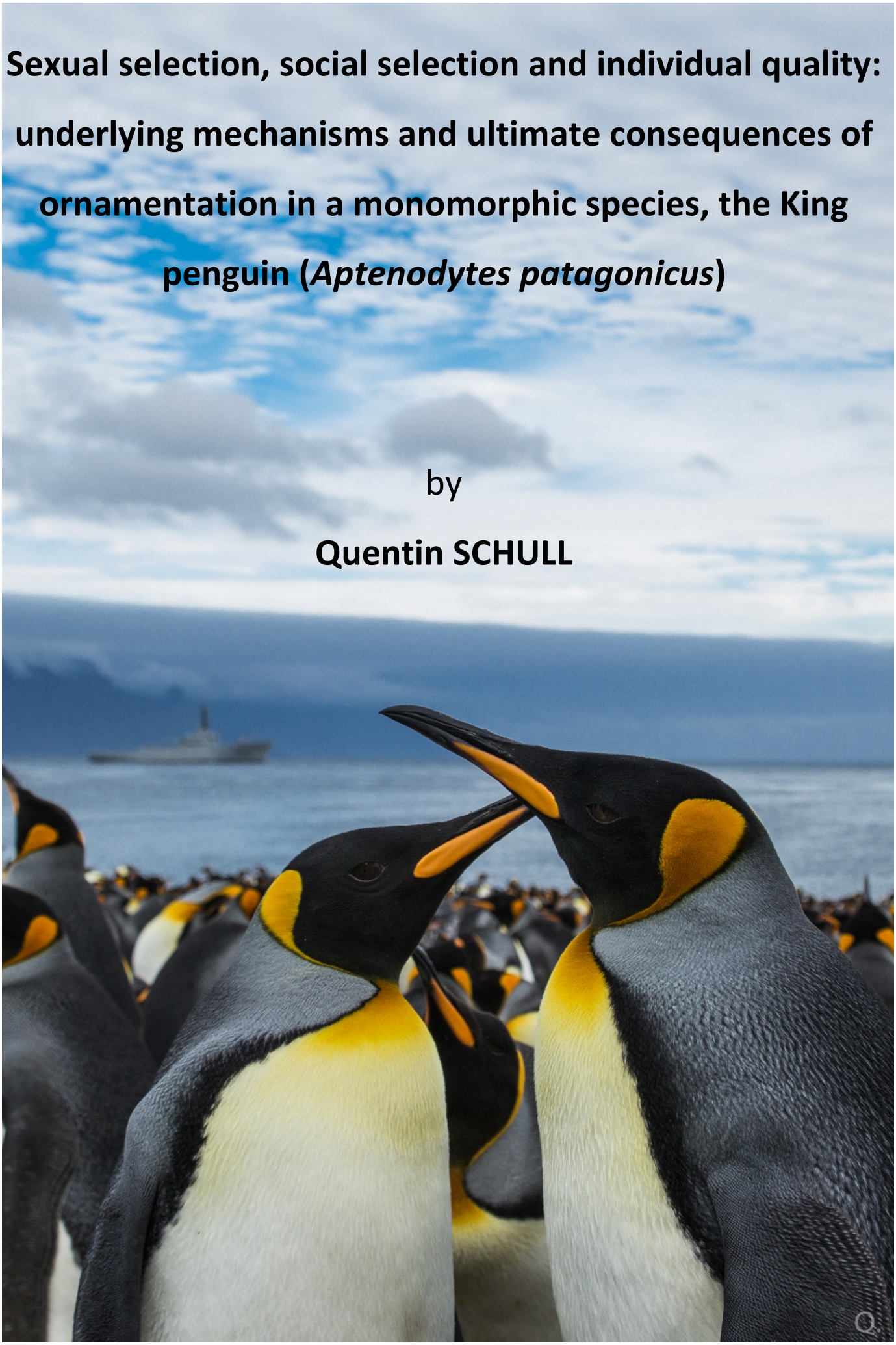

“Of the branches of biological science to which Charles Darwin’s life-work has given us the key, few, if any, are as attractive as the subject of sexual selection” Fisher 1915

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Toutefois, avant de commencer cette thèse il m'a tout de même fallu une année: 6 mois comme surveillant dans un lycée (merci à Stéphane et à mes deux acolytes, Sandrine et Jonathan, qui ont rendu cette année-là exceptionnelle), deux mois à étudier les mésanges (encore une fois, merci Sylvie !), une pelle brisée (encore désolé Sylvie). Cela a permis d'éviter que mon cerveau en manque de défis intellectuels ne s'ankylose et m'a décidé finalement à envisager un projet de thèse.

Et quel projet de thèse ! Encadré par papi Jean-Pas-Triste (désolé Emilio pour le plagiat) et papi Bize, la « dream team ». Un entretien incroyable, le lendemain de l' « Ecology and Behaviour » à Strasbourg (oui ce lendemain-là Emilio!) entre JP qui enchaîne un jeu de mots toutes les deux phrases et son voisin Suisse qui me demande comment je définirais le concept de qualité individuelle de manière exhaustive. Ce fut épuisant, mais combien stimulant. Merci JP, merci Pierre de m'avoir permis d'entreprendre ce projet avec vous.

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Vient le tour de notre ressortissant de la confédération helvétique qui s'en est allé au pays du haggis. Ce qui est sûr c'est que les Suisses ont la réputation d'être lents, par contre après avoir rencontré papi Bize on a la certitude qu'une fois lancé on ne les arrête plus (ici je ne fais aucune référence à un quelconque phénomène inertiel lié à la mise en mouvement d'un objet ou d'une personne d'une masse imposante). On m'avait vanté l'efficacité des franchises germaniques, autant dire que l'efficacité Suisse restera pour moi le nec plus ultra du chercheur. C'est comme partir avec son couteau (Suisse) faire de l'alpinisme, commencer une thèse avec Pierre Bize et tout tournera comme une horloge bien réglée. Un grand merci Pierre, que ce soit pour les enseignements (multiples, oui il y en aura eu beaucoup et je ne suis pas arrivé au bout) que j'ai appris à tes côtés, une leçon professionnelle mais également une leçon de vie. Un grand merci à toi et Sophie Cotting (!) pour les balades aux alentours de Rieddes, les fondues et autres dérivés de raclette, les bonnes bouteilles, les fou-rires, les batailles et tous les agréables moments passés autour de votre table. Merci également d'être si mauvais aux fléchettes, cela m'a souvent permis de retrouver un peu d'estime de soi après les batailles (très souvent contre moi-même) livrées sur le terrain de squash. Un grand merci encore, cette thèse de son début à sa concrétisation (encore merci pour le boulot énorme réalisé en un temps record) n'aurait pas été ce qu'elle est sans super papi Bize. Tu auras été (et le restera je n'en doute pas) extraordinaire. J'ai hâte de voir la tête de la marmaille du couple valaisan-vaudois!

Il est temps d'attaquer le prochain sujet. Je dirais même qu'il est temps de rendre à César ce qui appartient à César ! (j'ai eu un chat qui s'appelait Schull César… oui bon bah après 3 ans avec JP inexorablement on s'imprègne). VAV aka bob aka l'homme de toutes les situations, aka mon troisième directeur de thèse aka buddy. Il mériterait bien une page entière de remerciements tant sa contribution à ce travail est **ENORME** (et oui tu noteras que j'ai utilisé un superlatif de manière adéquat !). Vincent était là depuis le début, on s'est regardé de loin (très loin), on se repoussait, s'attirait, se jaugeait, comme les 6 premiers mois de toute relation, la passion, la tension, les moments forts. Puis, on s'est envolé pour le terrain, et là tout a basculé. De collègue on est devenus des partenaires de terrain, puis des potes et là cela s'emballe, on passe 23 jours dans la même cabine sur un bateau de 200 mètres de long, toi malade, patché, moi la forme frais comme un gardon… Que des bons moments passés allongés 14 heures par jours à bosser sur les bannettes du pont G entre les odeurs de gasoil (relativement masqué par l'odeur masculine d'un PAX, d'un VAV et d'un QS, selon les dire d'Agnès). Bref on débarque et 2 semaines après on emménage ensemble. On a eu la chance de partager des moments professionnels hors du commun, de refaire le monde autour d'un ou deux (ou trois) verres de whisky, et de se chamailler à maintes reprises autour de concepts auxquels nous ne trouverons probablement jamais de réponses définitives, et quand bien même on finirait par s'attaquer à savoir qui de la poule ou de l'œuf est arrivé en premier, pourvu que l'un puisse prendre le contre-pied de l'autre. Aujourd'hui encore, ni toi ni moi ne savons vraiment si c'est la meilleure ou la pire chose que l'on ait faite pour notre équilibre mental personnel, mais ce qui est certain c'est qu'aujourd'hui tu es pour moi un ami. Ce travail de thèse c'est également ton travail, autant sur les idées, la réalisation, le terrain, les analyses (et oui j'ai même réussi à te trainer une semaine entière

de 8h du matin à 8 h du soir tous les jours à la paillasse ! avec un peu de recul, une des victoires de ma thèse). En somme, on a travaillé main dans la main et on a mené ce bébé jusqu'à son terme ensemble (an amazing work you provided those last days, thanks you so much) et on est déjà en train d'en préparer un autre. MERCI COPAIN !!!!

Je tiens à remercier tous les chercheurs permanent du bâtiment 60 qui constituent le cœur de ce laboratoire et le font vivre, merci Yves, Thierry, Jean-Yves, Caroline, André, Céline, Yvon, Damien, Stéphane, et Audrey. Merci à Sandrine pour les dosages de télomères et à Mathilde A. pour l'aide et le temps passé à la paillasse. Merci à Martine pour les innombrables ordres de missions que je lui ai demandés, à Brigitte pour son efficacité à trouver les articles auquel Sci-hub lui-même ne permet pas d'accéder. Et un grand, grand merci à Claudine, pour sa joie de vivre et le nombre incalculable de fois où elle a trouvée des solutions qu'importe le problème rencontré, Super-Claudine !

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que j'ai hâte de retrouver sur le terrain cette année. Merci Carox !!!! Pour les sorties à Crozet, les manips à JJap, le bateau, les vacances à la réunion, les sorties sur Strasbourg et bien d'autres en perspective !!! Gracias Roger Colominas por los buenos momentos pasados en Montpellier. Robin, l'amour de ma vie, reviens moi vite. Tina, ma deuxième référence Suisse toujours aussi efficace ! Merci à Flo pour la visite de Kerguelen, les bons moments passés en Espagne. Merci à Sophie et Franky pour les moments à CapeTown, un élephant de mer à CAPETOWN !!! Merci à Malicia pour les moments passés à Gotland, à Montpellier, sur mer ou bien par skype, je t'adore ! Merci à toi Emilie G. pour ton soutien, ta passion, ton amitié et ton oreille attentive. Merci aux thésards et post-docs du CSM qui m'ont accompagné dans la rédaction de cette thèse ; Dyugu, Carinne, Leila et Vanessa. Et merci Céline de m'avoir accueilli pour cette période à Monaco. Victor gracias por los largo debate sobre la ciencia de la estadística.

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Papers presented in this thesis

Schull Q, Dobson FS, Stier A, Robin JP, Bize P, Viblanc VA. (2016). Beak color dynamically signals changes in fasting status and parasite loads in king penguins. Behav. Ecol. arw091

Schull Q, Viblanc VA, Stier A, Saadaoui H, Lefol E, Criscuolo F, Bize P, Robin JP. (in press). The oxidative debt of fasting: evidence for short to medium-term costs of advanced fasting in adult king penguins. J. Exp. Biol

Schull Q, Saadaoui H., Dobson FS, Robin JP, Viblanc VA, Bize P. (in prep). Experimental stress during moult suggests the evolution of condition-dependent and condition-independent ornaments in the king penguin (Aptendoytes patagonicus)

Schull Q, Durand L, Lefol E, Cilliard A, Robin JP, Bize P, Viblanc VA. (in prep). Offspring quality is foremost explained by ornamentation of biological parents early in life and of rearing parents late in the development in the king penguin).

Schull Q, Viblanc VA, Dobson FS, Robin JP, Zahn S, Bize P, Criscuolo F. (in review for Naturwissenschaften) King penguins telomere length: assortative mating and relationship with breeding success.

Schull Q, Boulinier T, Saadaoui H, Voisin E, Viblanc VA, Robin JP, Bize P. (in prep) Immunocompetence and bright colors: a preliminary test of the Hamilton-Zuk model in breeding king penguins.

Schull Q, Stier A, Viblanc VA, Romestaing C, Robin JP, Roussel D, Bize P. (in prep) Linking ornamentation to mitochondrial function in breeding king penguins.

Stier A, Romestaing C, **Schull Q,** Lefol E, Robin JP, Roussel D, Bize P. (submitted at Methods in Ecology and Evolution) How to measure mitochondrial function in birds using red blood cells.

Viblanc VA, Dobson FS, Stier A, **Schull Q**, Saraux C, Gineste B, Pardonnet S, Kauffmann M, Robin J-P, Bize P. (2016). Mutually honest? Physiological “qualities” signalled by colour ornaments in monomorphic king penguins. Biol. J. Linn. Soc. 118:200–214.

Other works

Schull Q, Cornioley T, Ménard JJ, Viblanc VA, Robin JP. (in prep). An integrative appraisal of the hormonal and metabolic changes in response to acute stress using energy-constrained king penguins as a model.

Bize P, Viblanc VA, **Schull Q**, Stier A, Pardonnet S, Gineste B, Kauffmann M, Massemin S, Criscuolo F, Robin JP. (in prep). Effects of tick infestation on the physiology, behavior and reproduction of king penguins.

Rojas ER, **Schull, Q,** Fohr, R, Massemin, S. (in prep). Negative genetic correlation between natal dispersal and morphometrics in the white-throated dippers (*Cinclus cinclus).*

Stier A, **Schull Q**, Viblanc VA, De Margerie E, Zahn S, Handrich Y, De Buffrénil V, Erbrech A, Guérin N, Martrette JM, Groscolas R, Criscuolo F, Bize P, Robin JP. (2015). How do adults and chicks of king penguins (Aptenodytes patagonicus) face nutritional constraints while breeding or growing? Acta Physiologica 214 (supplement) S700: 87.

Stier A, Bize P, Roussel D, **Schull Q**, Massemin S, Criscuolo F. (2014). Mitochondrial uncoupling as a regulator of life-history trajectories in birds: an experimental study in the zebra finch. J. Exp. Biol. 217:3579–3589.

Stier A, Bize P, **Schull Q**, Zoll J, Singh F, Geny B, Gros F, Royer C, Massemin S, Criscuolo F. (2013). Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. Front. Zool. 10:33.

Communications

Oral communications

Schull Q, Viblanc VA, Durand L, Lefol E, Bize P, Robin JP. (2016). Parent ornamental colors predicts offspring's early life growth and parental care in king penguin (*Aptenodytes patagonicus*). *9th International Penguin Congress*, Cape Town, South Africa.

Schull Q, Dobson FS, Robin JP, Bize P, Viblanc VA. (2015). Experimental and correlative evidence for condition-dependent sexual signals in breeding king penguin (*Aptenodytes patagonicus*). *2nd World Sea Bird Conference,* Cape Town, South Africa.

Schull Q, Viblanc VA, Dobson FS, Zahn S, Robin JP, Bize P, Criscuolo F. (2015). Sexual selection and individual quality in the king penguin (Aptenodytes patagonicus). *12es Journées Scientifiques du CNFRA*, Paris, France. Talk given by JP. Robin

Schull Q, Stier A, Criscuolo F, Viblanc VA, Bize P, Robin JP. (2015). Prolonged fasting: how king penguins cope with physiological oxidative stress. *Colloque d'Ecophysiologie Animale – CEPA 2e édition*. La Rochelle, France. Talk given by JP. Robin

Schull Q, Viblanc VA, Dobson FS, Stier A, Criscuolo F, Lefol E, Saadaoui H, Bize P, Robin JP. (2015). Sexual selection and individual quality in the king penguin (Aptenodytes patagonicus). *11es Journées Scientifiques du CNFRA*, Paris, France. Talk given by JP. Robin

Schull Q, Reichert S, Stier A, Zahn S, Bize P, Robin JP, Massemin S, Criscuolo F, Viblanc VA. (2015). What can telomeres tell us about life-history trade-offs in king penguins? *Telomere Dynamic Workshop 2015*. Drymen, Scotland, UK. Talk given by F. Criscuolo.

Viblanc VA, **Schull Q**, Dobson FS, Bize P, Robin JP. (2015). Advertising quality: condition-dependent signals in a monomorphic seabird, the king penguin (Aptenodytes patagonicus*). 10th Conference of the European Ornithological Union,* Badajoz, Spain. Talk given by VA. Viblanc

Stier A, **Schull Q,** Viblanc VA, Roussel D, Robin JP, Bize P, Criscuolo F. (2015). Thermogenesis, fasting and oxidative stress: new insights from model and non-model animals*. 9th International Congress of Comparative Physiology and Biochemistry*. Krakow, Poland. Invited by N. Metcalfe and J. Taylor. Talk given by A. Stier

Robin JP, Viblanc VA, Stier A, Gineste B, Reichert S, **Schull Q,** Kauffmann M, Pardonnet S, Durand L, Lefol E, Massemin S, Criscuolo F, Dobson FS, Handrich Y, Bize P. (2014). Phenotypic plasticity in the king penguin (Aptenodytes patagonicus) chicks and adults: effect of reproductive constraints. *10ème Journées Scientifiques du CNFRA*, Université de Rennes 1, France. Talk given by JP. Robin

Poster presentations

Schull Q, Bize P, Dobson FS, Robin JP, Zahn S, Criscuolo F, Viblanc VA. (2016). Mutual mate choice for partners with long telomeres and fitness consequences in king penguins. *9th International Penguin Congress.* Cape Town, South Africa.

Schull Q, Viblanc VA, Stier A, Durand L, Lefol E, Bize P, Robin JP. (2015). Sexual selection and individual quality: do parental color ornaments predict chick growth in monogamous king penguin, *Aptenodytes patagonicus*? *European Ornitologist Union.* Badajoz, Spain.

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RESUME EN FRANÇAIS

Contexte Scientifique

Afin d'expliquer l'existence de traits morphologiques extravagants pouvant apparaître désavantageux pour la survie de l'individu (tels que des couleurs vives chez de nombreux oiseaux ou un plumage exagéré comme la queue du paon), Darwin proposa en 1871 qu'ils puissent évoluer par sélection sexuelle. La théorie de la sélection sexuelle propose que de tels traits morphologiques puissent être conservés au cours de l'évolution s'ils procurent des avantages à leurs porteurs pour l'accès à la reproduction. Ces traits sont coûteux à produire/maintenir et doivent par conséquent «honnêtement» refléter la qualité du porteur, puisque seuls les individus capables d'investir à la fois dans leur survie et dans leur ornementation sont en mesure de revêtir de tels apparats. Bien que cette hypothèse ait été largement étudiée chez les mâles d'espèces présentant un dimorphisme sexuel marqué, moins d'études se sont focalisées sur l'évolution et le maintien de traits ornementaux chez les espèces dites monomorphiques, où l'investissement parental est souvent similaire entre les sexes, et où le choix d'un partenaire de bonne qualité est par conséquent primordial.

Le manchot royal (*Aptenodytes patagonicus*) est une espèce monomorphique pour laquelle l'investissement dans la reproduction est similaire entre les sexes. Au cours de son cycle reproducteur, cet oiseau marin longévif alterne entre périodes de garde de l'œuf ou du poussin à terre, durant lesquelles mâles et femelles font face à des périodes de jeûne prolongé, et périodes de recherche alimentaire en mer. Les deux parents sont alors fortement tributaires l'un de l'autre pour élever leur progéniture et mener le poussin avec succès jusqu'à l'émancipation. Une forte pression de sélection favorisant un choix mutuel (mâle et femelle) pour des partenaires de haute qualité est alors attendu.

Chez le manchot royal, mâles et femelles présentent de multiples ornements sexuels : de larges tâches auriculaires aux plumes jaune-orange vives, un dégradé pectoral avec des plumes allant du brun au jaune, ainsi que deux plaques mandibulaires jaune-orangées de part et d'autre du bec. Tandis que les plumes contiennent un pigment endogène dérivé de ptérine, les plaques mandibulaires semblent quant à elle contenir des pigments dérivés de caroténoïdes. Par ailleurs, le bec a également la particularité de refléter dans l'UV, une caractéristique due à l'agencement de structures de kératine à sa surface (i.e. couleur structurelle). Chez cette espèce, des études antérieures ont expérimentalement démontré un rôle important des tâches auriculaires et du bec dans le choix du partenaire. En effet, réduire la surface (tâches) ou la brillance (bec) de ces ornements induit un délai dans l'appariement, plaçant ainsi ces signaux directement sous influence de la sélection sexuelle. Toutefois, nous manquons de connaissances sur les mécanismes sous-jacents ces caractères sexuels secondaires et garantissant leur caractère honnête.

Ce travail de thèse considère les déterminants de l'ornementation sexuelle chez le manchot royal afin d'élucider i) dans quelle mesure les ornements colorés d'origine structurelle, pigmentaire endogène et pigmentaire exogène sont couteux à produire, ii) à quelle échelle temporelle cette information est transmise aux congénères, iii) quelles qualités intrinsèques à l'individus les ornements reflètent et iv) dans quelles mesures les ornements conditionnent le succès reproducteur de l'individu/du couple. Dans un contexte évolutif, il permet d'aborder de manière intégrative les différentes pressions de sélection qui ont conduit au maintien d'ornements colorés chez les espèces monomorphiques.

Développement de la thèse

I. L'ornementation chez le manchot royal est-elle condition-dépendante ou condition-indépendante ?

La diversité des histoires de vie est principalement expliquée par le concept clé que les organismes disposent d'une quantité limitée d'énergie et de ressources qu'ils vont devoir allouer entre les principales fonctions vitales telles que la croissance, la reproduction et la défense (immunité, maintenance). Cette idée est la clé de voute de la théorie des traits d'histoire de vie, et fais ressortir la notion compromis évolutifs.

Chez le manchot royal, le renouvellement des ornements s'opère en quelques semaines lors d'une mue critique où les oiseaux remplacent l'intégralité de leur plumage et leurs plaques mandibulaires, période durant laquelle l'apport énergétique à cette fonction est considérable. Afin de déterminer l'honnêteté de l'ornementation chez cette espèce, j'ai expérimentalement modulé ces compromis d'allocation des ressources au travers d'une première expérience de manipulation du statut physiologique des sujets. Pour cela, j'ai manipulé deux composantes clés des compromis évolutifs affectant les traits d'histoire de vie chez cette espèce coloniale : (1) l'efficacité du système immunitaire, élément crucial afin de résister aux pathogènes/parasites en milieu colonial; et (2) la capacité à gérer son métabolisme et son stress, élément crucial pour ces animaux faisant face à des jeûnes prolongés à répétition. Pour cela j'ai réalisé deux groupes d'oiseaux expérimentaux et groupe contrôles au cours de 2 saisons de reproduction différentes. Durant la totalité de la mue, les groupes expérimentaux avaient soit un niveau plasmatique basal d'hormone glucocorticoïdes (corticostérone) augmenté (Expérience 1), soit (2) un système immunitaire stimulé via une injection sous cutanée de lipopolysaccharides (Expérience 2).

Les deux traitements ont induit une diminution de l'investissement de pigment dans les plumes des taches auriculaires, suggérant ainsi un coût limitant de ces pigments endogènes alors soumis à un compromis d'allocation entre maintien et ornementation. De plus, la stimulation du système immunitaire s'est suivie d'une diminution de la réflectance du bec dans l'UV suggérant que les individus faisant face à ce challenge immunitaire réduisaient également leur efficacité dans la production de cet ornement structurel. Par ailleurs, la taille de la tâche auriculaire tout comme la teinte UV du bec (UV hue) est resté non seulement insensibles au traitement, mais également fortement corrélé avant et après la mue, respectivement. Ces résultats suggèrent que ces ornements n'étaient pas condition-dépendants, reflétant principalement des caractéristiques génétique et/ou sociales.

II. L'ornementation chez le manchot royal est-il un signal dynamique ?

Les signaux ornementaux dynamiques – qui varient au fil des minutes, heures ou des semaines – permettent d'accéder à une information continue sur la condition de l'individu (par exemple modification des réserves énergétiques ou du statut immunitaire), et peuvent donc être soumis à une forte pression de sélection sociale et/ou sexuelle. Chez les vertébrés, la coloration des téguments est souvent considérée comme un ornement dynamique. Chez les oiseaux les mêmes phénomènes peuvent s'opérer dans le bec. J'ai donc étudié la dynamicité de la couleur du bec chez le manchot royal. Pour cela, je me suis à nouveau focalisé sur deux aspects cruciaux de la réussite d'un épisode de reproduction chez cette

espèce : la capacité à jeûner et à faire face aux pathogènes. Au travers d'une approche corrélative, j'ai suivi l'évolution de la couleur du bec chez 3 groupes de manchots, correspondant à trois étapes différentes du processus de reproduction : (1) Lors de la parade qui correspond à la première période de jeûne prolongé, (2) lors de la première période d'incubation du mâle où l'on observe la plus longue période de jeûne à laquelle cette espèce fait face et enfin (3) lorsque les couples sont en incubation durant leur 3ème et 4ème tour de garde, période qui est associée à une phase plus avancée de la reproduction où l'investissement entre les mâles et les femelles est similaire. Ceci m'a permis d'explorer d'éventuelles différences entre les sexes. Dans un deuxième temps, je me suis focalisé sur l'évolution de la couleur du bec suite à un traitement antiparasitaire réduisant la prévalence de parasites endogènes et exogènes.

Sur une échelle de temps de quelques jours à quelques semaines, la couleur du bec est modifiée en réponse au jeûne et en réponse à la modification de la charge parasitaire. Pour les oiseaux en parade, la couleur jaune-orange représentative de la présence de pigments diminue après un jeûne de 24 jours. Chez les individus en incubation la couleur structurale du bec (UV) diminue après 10 jours de jeûne seulement. Les oiseaux qui ont été traités avec une solution antiparasitaire montre une augmentation de la coloration structurelle (UV) après élimination des parasites. Ces résultats sont les premiers à démontrer la nature dynamique de cet ornement qui reflète directement l'évolution de l'état physiologique de l'individu.

III. L'ornementation, reflet de la qualité individuelle ?

Le choix du partenaire s'opérant en début de reproduction, je me suis alors intéressé au lien existant entre ornementations et caractéristiques physiologiques/comportementales des individus en début de reproduction. Par ailleurs, les femelles et les mâles diffèrent en termes de contraintes physiologiques ce qui laissait suggérer que les compromis sous-jacents pourraient être différents pour les deux sexes, ceci ayant pour conséquence d'engendrer des signaux reflétant des informations différentes. J'ai donc participé à une étude explorant un vaste ensemble de variables physiologiques chez 31 couples en début d'incubation. Pour les deux sexes, l'immunité innée, le taux métabolique au repos (proxy de la dépense énergétique), et la capacité à répondre au stress aiguë (réponse à une capture) sont signalés par divers aspects de la coloration du bec ou de la taille de la tache auriculaire. Cependant, il apparait également des relations significatives et contrastées entre les sexes. La condition corporelle et la balance oxydative sont reflétées par la coloration du bec, bien que dans des directions opposées chez les mâles et les femelles. Sur un ensemble exhaustif de variables physiologiques, nos résultats suggèrent que l'émission de l'information des caractéristiques de l'individu est honnête pour cette espèce monomorphique, mais diffère en fonction des sexes.

Dans une seconde série d'analyse j'ai pu relier l'expression de la coloration des ornements à la réponse immunitaire humorale des individus suite à l'injection d'un vaccin. Des résultats préliminaires chez les mâles, montrent que la brillance de l'UV du bec (UV brightness) prédit la quantité d'anticorps produits suite à une primo infection.

Enfin l'idée communément admise des compromis d'allocation entre différentes fonctions se réfère en premier lieu à l'investissement énergétique. Récemment, il a été proposé que l'activité énergétique de l'organisme entier, y compris les compromis d'investissement énergétique dans l'ornementation ou autre fonction de maintenance, dépende avant tout de l'activité mitochondriale à sa plus petite échelle : l'échelle cellulaire. Dans l'optique de tester expérimentalement cette théorie qui n'a pour le moment aucun

support expérimental, nous avons mesuré l'activité mitochondriale de manchots lors de la en début de reproduction, et avons relié cette activité à la production de l'ornementation. Il en ressort que la brillance de l'UV du bec est positivement corrélée à l'efficacité mitochondriale. Ces résultats sont les premiers à démontrer un lien entre respiration cellulaire et production de traits ornementaux et ouvrent la porte à un secteur de recherche primordial dans la compréhension de l'évolution et du maintien de signaux ornementaux coûteux dans le règne animal.

IV. L'ornementation chez le manchot royal prédit-elle le succès reproducteur ?

Le choix d'un partenaire de bonne qualité est crucial pour la réussite de la reproduction. Ce choix s'appuie vraisemblablement sur la qualité de l'ornementation chez cette espèce. Nous avons vu que celle-ci reflète un grand nombre de caractéristiques intrinsèques de l'individu. Dans cette dernière partie j'ai souhaité étudier en quoi la qualité de l'ornementation des parents prédit la qualité des soins parentaux en mesurant la condition du poussin tout au long de sa croissance. Afin de séparer les effets maternels (génétiques mais également l'ensemble des facteurs transmis dans l'œuf) des effets de l'environnement d'élevage, j'ai réalisé une expérience de « cross-fostering ». Celle-ci a consisté en un suivi de 120 couples s'étalant sur une période de deux ans pour lesquels l'ensemble des œufs a été changé entre couples avant éclosion. Les paramètres de croissance des poussins ont été relevés en tout début de vie (10 jours après éclosion), puis après 35 jours, ainsi qu'en fin de croissance (105 jours après éclosion).

Cette expérience m'a permis de démontrer que la qualité des ornements des parents prédit le développement du poussin. Ceci est consistant avec l'idée d'un signal honnête, reflétant les capacités du porteur, ainsi que d'un choix du partenaire s'appuyant sur ce signal. Bien que les caractéristiques physiques du poussin soient liées aux ornements des parents génétiques en tout début de vie, celles-ci sont davantage corrélées aux ornements de leurs parents adoptifs en fin de croissance. Ceci souligne un effet mixte entre effets maternels et qualité de l'environnement précoce. Tous deux reflétés par l'ornementation des parents, joue un rôle prépondérant dans le développement du poussin.

Conclusion générale

Cette thèse a permis de déterminer le caractère honnête de l'ornementation chez une espèce monormorphique, le manchot royal. Ces signaux sexuels secondaires reflètent directement l'aptitude de l'individu à faire face aux contraintes environnementales et prédisent un ensemble de qualités intrinsèques en tant que reproducteur. L'ornementation apparait donc comme un support clé pour le choix du partenaire et le succès de la reproduction. Par ailleurs, le coût de production de certains traits apparaît négligeable, ce qui suggère que l'évolution et le maintien de ces traits relève de pressions sélectives sociales. Ce travail a également permis de mettre en évidence le caractère dynamique de l'ornementation à l'échelle d'une saison, permettant de signaliser des changements rapides et transitoires du statut de l'organisme. Ce dernier résultat soulève une question intéressante qui reste en suspens : dans quelles mesures le partenaire est-il capable d'intégrer des variations du signal à fine échelle temporelle et dans quelle mesure est-il capable d'utiliser cette information pour ajuster son comportement afin d'optimiser son succès reproducteur ?

Content

Introduction

Over 150 years of on-going studies on sexual selection: What have we learned?

1 What is natural selection?

Adenine, Cytosine, Guanine, Thymine. A-C-G-T, four letters that combined together constitute the alphabet of life. In the same way that the 26 letters of our alphabet will form words, then sentences, pages and eventually an entire paper, book, or PhD thesis, those fundamental DNA units provide the template from which is built the incredible diversity of phenotypic traits we may observe in nature. Their combination and the way in which those combinations are translated is almost unlimited (Mortimer 2000; Glinka et al. 2003; Feuk et al. 2006; Clark et al. 2007; Durbin et al. 2010). External factors and replication mistakes during cell replication might induce random genetic mutations (Loeb et al. 1974; Cairns et al. 1988; Lenski et al. 2003) the main source of genetic variability undoubtedly results from the sexual reproduction process. Indeed, for species presenting sexual reproduction, the intermingling of male and female genomes and genetic recombination processes that occur (which primary function is to repair genetic damage and eliminate deleterious mutations) will favour genetic diversity and sustaining natural phenotypic variation within a population or species (Meselson and Radding 1975; Modrich and Lahue 1996; Otto and Lenormand 2002; Bengtsson 2003). Phenotypic variants may either be advantageous in fitness terms, detrimental or neutral, and the real consequences of genetic heterogeneity may only occur in light of natural environmental conditions. Indeed, from the first stage of its development to the end of its life, the individual's genome will interact with its environment. The ability of a genotype to produce distinct phenotypes when exposed to different environments throughout its ontogeny (named as "phenotypic plasticity") will affect the way that the individual later deals with perturbations that occur (Via et al. 1995; DeWitt et al. 1998; Figure 1). From an evolutionary perspective, what ultimately matters is the reproductive output over the total lifetime reproduction of the organism. This enlarges the concept of fitness, which may be viewed as the adaptive value of a gene or a genotype. Simply, this can be described as the average contribution to the gene pool of the next generation through offspring and kin (Fisher

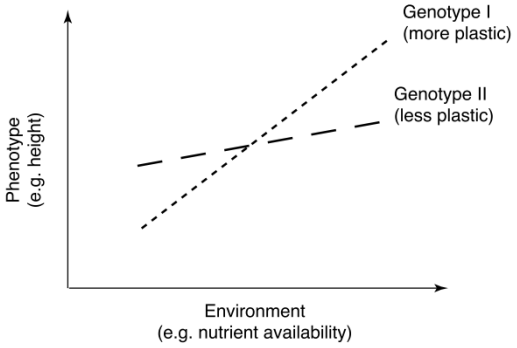


Figure 1. The Gene x Environment interaction. Illustration on how the across-genotypes phenotypic mean of a trait changes with the environment. Extracted from Pigliucci 2005)

1930; Hamilton and Zuk 1982; Stearns 1989; Stearns 1992). Nevertheless, Natural selection acts on phenotypes and not directly genes and will ultimately favour those being best adapted to their environment at a given time (Darwin 1859). From the expression of a particular gene to complex social behaviour, through the basic but not less complex regulation of physiological and metabolic processes, every step will be determined by the intrinsic properties of the individual (its genome) in interaction with its environment (Via and Lande 1985; Pigliucci 2005). This complex interaction between genes and the environment (GxE interaction), will lead to specific phenotypes and may even lead to speciation, as a consequence of divergent natural selection on traits between environments (Dieckmann and Doebeli 1999; Schluter 2001; Coyne 2004; Silvertown 2008). Genes encoding for traits maximizing individual fitness under variable environments, i.e. "good genes", are expected to spread in the population (Moore 1994; Kokko 1998; Iwasa and Pomiankowski 1999; Møller, Alatalo, et al. 1999; Neff and Pitcher 2005). Such genes might encode for traits or functions involved in organism development, metabolism, self-maintenance or behaviour (Hoelzer 1989; Iwasa and Pomiankowski 1999).

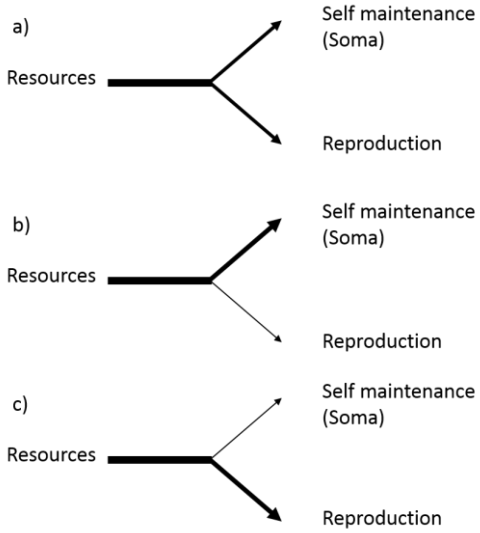


Figure 2. Y models of the cost of reproduction. The apportionment to reproduction, at the expense of limited resources available for the soma, forms the basis of the cost of reproduction (Harshman and Zera 2007). b) Resources available for the organism is equally invested in both function. b and c) Investment in one or the other function occur at the expense of the other.

However, because resources in the environment are generally limited (in time, space or global quantities), not all functions or traits can be simultaneously maximised or expressed (Stearns 1989; Stearns 1992; Zera and Harshman 2001; Ardia 2005). Individuals are often constrained by investment trade-offs into various functions, such as that between self-maintenance and reproduction (Calow 1979; Linden and Møller 1989; Nilsson and Svensson 1996; Harshman and Zera 2007, Figure 2), or various traits (e.g. pigment allocation to immunity or ornamentation). Inter-individual variation in investment trade-offs provides scope for inter-individual heterogeneity in fitness and highlights the concept we must now discuss, that of individual quality (Ardia 2005; Wilson and Nussey 2010; Bergeron et al. 2011; Lailvaux and Kasumovic 2011).

2 The concept of Individual Quality

The concept of Individual quality has increasingly captured the interest of the evolutionary biology community over the past two decades (Figure 3). Individual quality is a term that is used for explaining differences in survival and reproductive traits between individuals, or more generally,

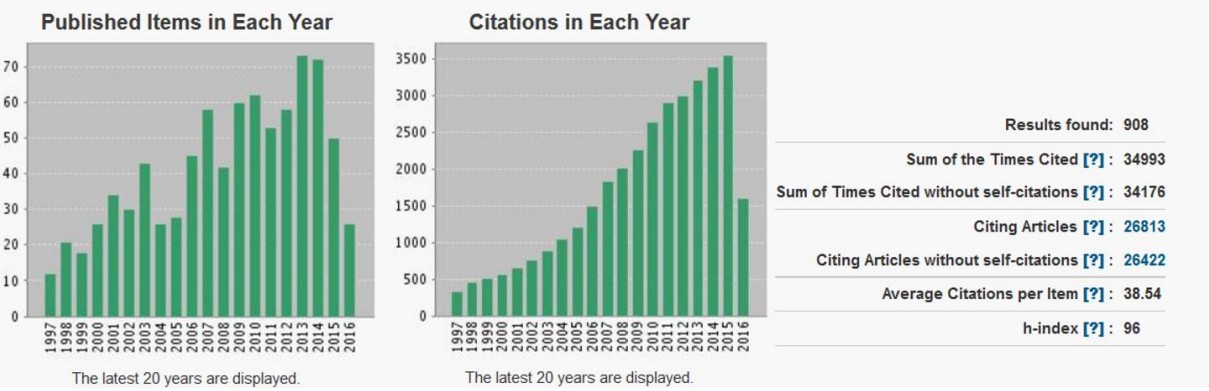


Figure 3. Trends in the number of published studies and citation for the field of "individual quality" in evolutionary biology.
Those trends are based on a literature search performed in ISI web of Science in July 2016. Specified key word was "Individual quality".

describing heterogeneity between individuals (Wilson and Nussey 2009). However, the term "individual quality" lacks a common and consistent definition (Cam et al. 2004, Wilson et al. 2009). Wilson and Nussey (2009) argue that the concept of differences between individuals is central, since natural selection acts on individual differences in phenotypes. Yet, the relation between phenotypic traits, fitness and individual quality is unclear. Studies have used isolated traits as indicators of individual quality (Ardia 2005; Bauch et al. 2012), sets of traits (Hamel, Côté, et al. 2009; Hamel, Gaillard, et al. 2009) or have treated individual quality as a trait itself, amongst other actually measurable traits.

Depending on the scale, the term individual quality can have different meanings. At the population level, the quality of an individual (the phenotype of the individual as one entity all over its life) is linked to its fitness in that population (Fisher 1930; Hamilton and Zuk 1982; Stearns 1992). However, measurements are mostly taken when the individual is still alive and the long-term fitness of the phenotype in terms of offspring survival and reproduction is unknown. Thus, estimating individual quality at a given time of its life is challenging. In their review, Wilson and Nussey (2009) suggest to use multivariate statistics, more specifically principal component analyses in which individual quality is treated as the "the axis of phenotypic variation that best explains variance in individual fitness". Given that an indicator for individual fitness exists, their approach allows individual quality to continuously vary over time or different conditions for an individual. Lailvaux and Kasumovic (2010) take the discussion to the next level, and discuss not only the need for studies

to include the environment of individuals when studying individual quality, but also the relevance of the time scale over which the study is conducted to account for changes of this very environment.

Thus, because individual performance is best explained as multidimensional concept including traits related to growth (Monaghan 2008), metabolism (V. A. Viblanc et al. 2012; V. A. Viblanc et al. 2014; V. A. Viblanc et al. 2014), immunity (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003; Bize et al. 2008; Palacios et al. 2009; Schulenburg et al. 2009), stress (Holberton and Wingfield 2003; Wingfield and Sapolsky 2003; Lendvai et al. 2007; Bókony et al. 2009; Bonier, Martin, et al. 2009; Bonier, Moore, et al. 2009; Schmid et al. 2013), oxydative stress (Costantini 2008; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010) and behaviour (Rhoades 1976; Ketterson 1992; Lind and Cresswell 2005; Wiegmann et al. 2010) a better understanding of the « individual quality concept » requires the integrative study of those different traits throughout different life history stages (e.g. during moult, during reproduction, after reproduction). This is the approach I explore in the present thesis, by questionning whether individual quality may be insure the honesty of ornamental signals in a monomorphic seabird, the king penguin (*Aptenodytes patagonicus*). The evolution of condition-dependent (quality-dependent) ornamental features is contingent hinges on specific cases of natural selection, i.e. sexual and social selection, fundamental notions we must now discuss.

3 Social and sexual selection

It is easy to see how phenotypic traits favouring individual survival might evolve. For instance, traits related to the efficiency of energy acquisition and processing (Lack 1947; van Noordwijk and de Jong 1986; Berner et al. 2008) transportation (e.g. a strong musculature allowing fast locomotion; Domenici and Blake 1997; Veasey et al. 2000; Hedenstrom and Rosen 2001) or organism defence against predators (e.g. cryptic plumage; Stevens and Merilaita 2009; John Endler 2012) are generally expected to be favoured by natural selection. Surprisingly however, some phenotypic features that seemingly appear to decrease individual survival (known as 'handicaps'; Zahavi 1975; Grafen 1990; Johnstone 1995) are also preserved over the course of evolution. Well-known examples for instance include the cumbersome antlers of some ungulates that are renewed on a yearly basis (Figure 4), or extravagant plumage features such

Figure 4. Two male red deer (*Cervus elaphus*) fighting during the breeding season.

as the plumage train of male peafowls (*Pavo cristatus*) (Figure 5), or the exaggeratedly long tail of widowbirds (*Euplectes progne,* Figure 6*).* Those few examples provide a good illustration of conspicuous and potentially costly traits (in terms of production, maintenance, survival) that appear to be maintained over the course of evolution. It is by trying to reconcile such observations with his theory of evolution by means of natural selection that over a century ago, Charles Darwin laid the grounds for a field of investigation that has persisted ever since (Darwin 1859). The idea behind it all was that of sexual selection (Darwin 1871). Costly and potentially handicapping features might

Figure 5. Picture of a male asian peafolws (*Pavo cristatus*) while parading. ©Dylan O'Donnell

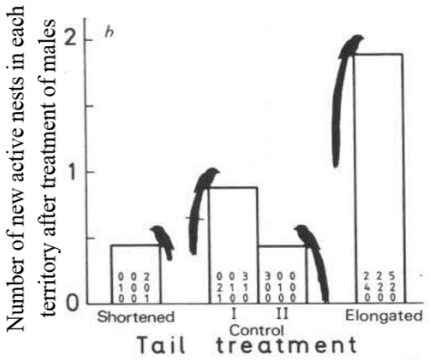


Figure 6. Mating success in male Jong-tailed widows subjected to different tail treatments. (From Anderson 1982)

evolve if they provide benefits in terms of access to mating partners (Zahavi 1975; Grafen 1990; Johnstone 1995) via two different forms of selection: *intra*-sexual and *inter*-sexual selection relying on the use of secondary sexual signals.

In many species, intra-sexual competition is stronger in males than females, likely due to an imbalanced investment in reproduction. Investment in reproduction is described as any investment by the parent in the current reproduction that increases the offspring's chance of surviving (and hence reproductive success) at the cost of the parent's ability to invest into other (somatic) functions and future reproductions (Trivers 1972). At the most basic level, it considers the metabolic investment into gametes which is two to four orders of magnitude higher in females (Hayward and Gillooly 2011) any parental care investment (incubating, lactating, feeding, territorial defence and offspring protection) that benefits the young. The sex providing less parental care has a higher potential rate of reproduction, and hence more to gain from mating with multiple mates. Sexual selection is therefore expected to be stronger in the sex providing less parental care. This is especially well known in birds where males exhibit elaborate colourful ornamental traits, and females are drab by comparison (G. E. Hill and McGraw 2006). In the second half of the twentieth century, whereas a plethora of studies had examined the evolution of costly handicapping signals by means of **sexual selection**, researchers realized that ornaments could also be used in competition for non-sexual resources, such as access to food and territories during and outside

reproduction (West-Eberhard 1979; Rohwer 1985; G. E. Hill 2014). Such selective forces may also favour the evolution of ornaments out of a mating context, and has generally been termed **social selection**. The production of social ornaments should increase their bearer's fitness by improving access to limiting resources via social interactions (Tanaka 1996; Wolf et al. 1999; McGlothlin et al. 2010; Moore et al. 2015). For instance, in many migratory bird species that flock in winter, individuals of both sexes resolve conflicts over food with plumage signals that enable them to determine fighting ability and establish social dominance without direct physical contests (Rohwer 1977; Marra 2000; Senar 2006; Santos et al. 2011). Thus, individuals may display social signals allowing opponents to assess the profitability of engaging in social contests. The appropriate turning point of continuing or breaking-off from social contests is expected to be set by social selection (Maynard Smith 1974; Taylor and Elwood 2003). Individuals may then use social signals to determine whether the costs of social contests outweigh long-term fitness benefits (West-Eberhard 1979), and avoid or pursue in conflict escalation. Of course, it is likely that ornaments indicating social attributes are also used in **mate choice** since higher social competitiveness may also present an advantage in reproductive contexts. Thus *sexual* and *non-sexual social* selection should not be considered as independent modalities, and studies should aim at understanding their respective contributions in the evolution of animal ornaments/armaments.

4 Mate choice and the honesty of ornaments

Honest signals reliably reflect information on the quality of the bearer, assisting the individual expressing them in acquiring a mate (Johnstone et al. 1996; Kokko and Johnstone 2002; Hooper and Miller 2008) might reflect (a) direct information about genetic features (b), or indirect honest information of bearing them.

4.1 Genetic-based hypotheses and the evolution of ornaments

Hamilton and Zuk (1982) were the first to suggest that an individual's capacity to resist parasites may be genetically inherited and a central evolutionary pressure in sexual selection. Based on their findings published in 1982 on the relation between blood parasites and ornamentation in North American passerine birds, they proposed that host resistance to parasites is heritable and that the genes involved in host resistance should evolve over time with parasite resistance, leading to a variation in host fitness. Indicators of resistance as suggested by Hamilton and Zuk would be ornamental features. Female preference for mates would be based on those ornamental traits differing between individuals (Figure 7). Since then, a wide range of studies in different taxa has

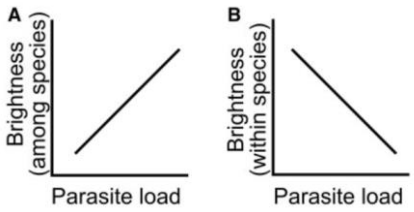


Figure 7. Predictions of the Hamilton–Zuk hypothesis of parasite-mediated sexual selection. A) At the interspecific level, it predicts that sexual selection pressure (leading to ornaments as signal of parasite infection) is positively linked to the parasite load of the species itself. B) At the intraspecific level, the brightest, most ornamented males will have the lowest parasite loads. Extracted from Balenger and Zuk 2014.

provided evidence that ornamental features may reflect individual immune responses to pathogens. For instance, Dufva and Kallander (1995) infected great tit males with blood parasites and found that those individuals exhibiting a more intense belly colouration had a higher number of heterophils, which play a role in inflammation and disease resistance. Similarly, Doucet and Montgomerie (2003) focused on UV plumage colouration in satin bowerbirds and its relation with an infection with blood parasites in juvenile and adults. Juvenile males with higher UV saturation in their plumage had a lower amount of blood parasites, whereas in adult males, plumage UV brightness negatively predicted blood parasites. Ornaments might thus reflect 'good genes' for parasite resistance and more generally broad genetic quality and, by choosing such mates, females therefore insure genetic benefits to her offspring that inherit favourable alleles from their father (Kokko et al. 2003; Mead and Arnold 2004; Neff and Pitcher 2005; Anderson 2006; but see Bonduriansky and Rowe 2005). Moreover, if ornaments reflect genetic features, there might also be non-additive genetic benefits for an individual to choose a mate with alleles that are complementary to its own (Zeh and Zeh 1996; Group 2000; Colegrave et al. 2002; Mays and Hill 2004; Johnson and Hill 2013; G. E. Hill 2014). This is for instance the case for major histocompatibility complex genes for which polymorphism in MHC alleles may provide offspring with higher immune resistance to parasites (Whittingham et al. 2015).

4.2 Condition-dependent signals and the evolution of ornaments

Honest signals implicate either direct (e.g. handicap in daily routine, predation evasion, etc.) or indirect (e.g. energy and time to produce and maintain such signals not available to invest in other processes) survival costs. The former are rather obvious, e.g. bearing a conspicuous plumage or an oversized tail increases predation risk by decreasing the aptitude to hide or escape. The latter are subtler. Energy investment is linked to energy availability. Indeed, even if resources are present ad-libitum, the time and mechanisms necessary to collect, process, and transform them into usable energy for the whole organism are limited and might rely on several interrelated processes. Ornaments and their production vary highly between species and should be linked to system functionality: the entity of vital and non-vital processes of an organism characterized by specific mechanical and physiological structures, biochemical pathways and molecular compounds. The use of the musculoskeletal (e.g. horns) or the integumentary (e.g. feathers) system for instance often involves the use other physiological systems including the immune system, the nervous system or the

respiratory system. It can for example involve. Hill (2011) reviewed the four possible hypotheses explaining how those systems are not mutually exclusive.

4.2.1 *The mediator hypothesis*

A substance that has a positive effect on ornamentation production may have a negative effect on other functional systems (Figure 8). This hypothesis flows from "The immunocompetence handicap hypothesis" as first proposed by Folstad and Karter (1992) who suggested that the link between immunity and ornament production is mediated by testosterone secretion. Whereas testosterone secretion promotes the production of ornaments, it suppresses immune system functions. Hence, only individuals able to withstand a down-regulation of their immune system may fully invest into ornamentation. This hypothesis has been continously refined, emcompasing hormones in a broader context (Westneat and Birkhead 1998). Indeed, just as the hypothalamic–pituitary–adrenal axis may have a positive effect on the immune system on the short term but negative effects if stress is maintained (Buchanan 2000) hormones too may have pleiotropic effects. As a consequence, ornamentation appears as an honest signal by which only individuals with a well-functioning immune system can afford the production of testosterone-linked high quality ornaments, making ornaments an honest signal for immune system functionality. However, the handicap theory fails as a general explanation of the honesty of ornaments since many ornaments are not regulated by testosterone or other steroid hormones, and testosterone is not always immunosuppressive (for a review see Hasselquist et al. 1999; Peters 2000; Roberts et al. 2004, for a review see Kimball and Ligon 1999).

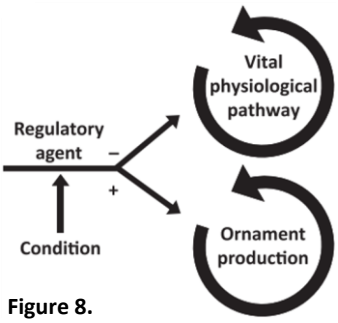


Figure 8. The mediator Hypothesis. A regulatory agent that promotes ornament production but depresses a vital pathway such as testosterone on the immune system. (Hill 2011)

4.2.2 *The Shared Pathway Hypothesis*

The shared pathway hypothesis (Figure 9) suggests that not just a substance but an entire physiological pathway is shared between ornamentation production and system functionality. Examples include elongated feathers and skeletal features, such as the tail of male Long-tailed Widowbirds (*Euplectes progne*) and the horns of Bighorn sheep (*Ovis canadensis*) as extensions of structural materials that make up the organism's body. For instance, Badyaev & Landeen (2007) linked the concentration of carotenoid-based pigmentation of the plumage with the efficiency of the feather growth process in House finches (*Haemorhous mexicanus*). The production of the

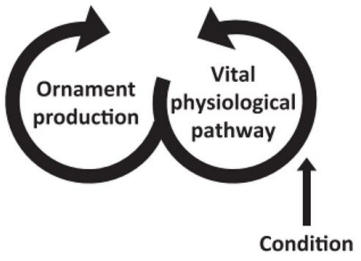


Figure 9. Shared Pathway Hypothesis. Ornament production shares an entire physiological pathway with another vital process (Hill 2011).

ornament in those examples is only possible if the systems for feather-keratin synthesis or bone growth are well functioning. If ornament production is inexorably linked to fundamental biochemical pathways (Figure 9), then ornament expression will be linked to condition – and remain honest.

4.2.3 *The Resource Trade-off Hypothesis*

More recently, Morehouse (2014) proponed the resource trade-off hypothesis (Figure 10) as an explanation for how ornaments may reliably reflect immune system functioning. A substance of limited quantity (e.g. carotenoid pigments) is used both for ornament production and in another functional system. Thus, only individuals with sufficient resources can afford to allocate ressources to ornamentation as opposed to self-maintenance systems like immune function (Lozano 1994; von Schantz et al. 1999; McGraw and Hill 2000; Alonso-Alvarez et al. 2008). Energy has to be allocated between processes such as growth, maintenance and reproduction (Slobodkin 1962; Brown et al. 2004). Hence, any investment of resources into physiology or behaviour that increases reproductive success, for example territory defence, growth or parental care is done at the expense of somatic maintenance and future reproduction and/or survival (Stearns 1992). This is classically referred to as the cost of reproduction (Williams 1966; Höglund et al. 1998). For instance exogenous carotenoid pigments acquired from the diet are known to function in immunocompetence (Bendich 1989; Chew 1993), oxidative balance (Edge et al. 1997; McGraw 2005; but see Costantini and Møller 2008) and ornamentation colouration (reviewed in Simons et al. 2012). However, as a consequence for ornamentation Getty (2006) pointed out that with such a one-dimensional signal, individuals can, over a short term, produce high ornamentation by over-investing in ornament production, thus diluting the honesty of the signal.

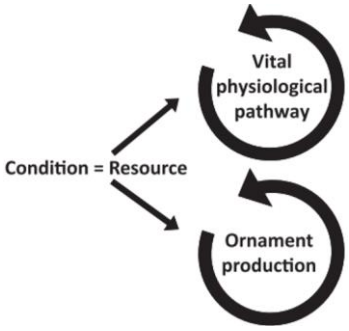


Figure 10. Resource Trade-off Hypothesis. Limiting resources are traded off between vital pathways and ornament production. (Hill 2011)

4.2.4 *The Pathway Functionality Hypothesis*

The pathway functionality hypothesis (Figure 11) suggests that ornament production is limited by the outcome substance of a physiological pathway. Unlike the resource trade-off hypothesis, the pathway functionality hypothesis relies on the common use of endogenous substances that can be allocated to ornaments or other functions (Bendich 1989; Chew 1993; Edge et al. 1997; Costantini and Møller 2008; Simons et al. 2012). When ornaments are made up of

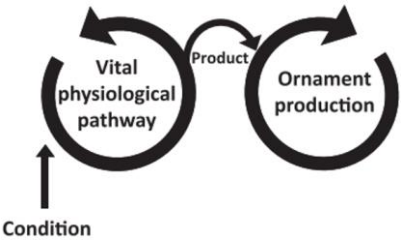


Figure 11. Pathway Functionality Hypothesis. Ornament production is proportional to a product of a vital pathway (Hill 2011).

endogenously synthetized pigment or structural cell types (see below section 6.3.2 on structural colouration), pigment availability and/or pathways of allocation are harder to define. The limiting factor is likely to be considered at its more basic level, i.e. the usable energy form available to the whole organism Adenosine-tri-phosphate (ATP) (Lane 2005; Wallace and Fan 2010). This transformation mainly happens in the mitochondria through the oxidative phosphorylation process (OXPHOS) (Hatefi 1985; Shutt and McBride 2013). Hence, the efficiency of individuals to transform metabolic resources (carbohydrates, lipids and proteins) into usable energy (ATP) has been suggested as the ultimate underlying mechanisms explaining condition-dependent ornaments (Hill 2011; G. E. Hill 2014).

The four above hypotheses highlight different mechanisms ensuring signal honesty. However, it is important to note that they are not mutually exclusive and might occur simultaneously within the same species or depending on the ornament considered. Moreover, sexual displays are often highly complex, involving many different signal components. Thus, it has been suggested that mate choice likely relies on several multimodal signals (reviewed in Candolin 2003). According to the multiple messages hypothesis, different signals provide information on different mate qualities (Møller and Pomiankowski 1993; Johnstone 1997). Taking those signals together into account might allow to access the general quality of the mate, or alternatively, different receivers may pay attention to different signals (Wedekind 1992; Johnstone 1996). Multiple signalling may thus reduce mate choice errors or the cost at which information is gained by reducing the time or energy spent, or mortality risk, when looking for a mate (Candolin 2003; Hebets and Papaj 2005).

4.3 Biased selection

Fisher pointed out that selection under mate choice differs from that involving real or ritualized male combats in that there is the potential for a "runaway" process that could rapidly lead exaggerated unconstrained signals not reflecting the true quality of their bearers (other than in the ability to signal per se Fisher 1915; Fisher 1930). Thus, alternative hypothesis that do not rely on signal honesty have been proposed to explain female preference for conspicuous ornaments which do not rely on ornament as honest signal.

A first hypothesis based on the Fisher's hypothesis, considered that a genetic coupling between ornamental productions might exist, so that any indicator used by females in mate choice soon becomes an advantage in itself, due to the increased attractiveness of its bearers. Males showing the most developed expression of such traits are more successful at obtaining mates, and females mating with them gain an advantage through the greater attractiveness and mating success of their sons. This genetic coupling might lead to self-reinforcing coevolution between trait expression and

preference for traits under the Fisherian sexy sons' hypothesis (Lande 1981; Kirkpatrick 1982; Pomiankowski and Iwasa 1998; Kirkpatrick and Hall 2004; Mead and Arnold 2004). However, this process has been difficult to demonstrate empirically, due in part to the difficulty of detecting the genetic mechanism and the process by which it is initiated (Andersson 1994; Andersson and Simmons 2006), although molecular genetics offer new interesting possibilities (Wayne and McIntyre 2002; Erickson et al. 2004; Thomas and Klaper 2004). On the other hand, for ornaments used in intra-sexual competition, selection against dishonest signallers should eventually limit the evolution of signals not indicating a true underlying ability or willingness to fight (see Zahavi, 1977; West-Eberhard, 1979).

Female choice might also evolve under some bias as a result of resistance to direct costs imposed by males (Holland and Rice 1998; Chapman et al. 2003; Pizzari and Snook 2003; Arnqvist 2004). For instance female preference favouring a male ornament can initially evolve under natural selection for other reasons, e.g. in the context of foraging or predator avoidance, thus males evolving traits that exploit this sensory bias then become favoured by mate choice. (see Endler and Basolo 1998; Boughman 2002; Ryan 2008).

5 Monomorphic species and shared ornamentation

Mate choice has been largely studied in dimorphic species where it is often males that display conspicuous ornament (Hill 2006; Kraaijeveld et al. 2007; Jones and Ratterman 2009). However, in some species, both males and females exhibit ornamental traits (Kraaijeveld et al. 2007). Those cases of mutual ornamentation provide a contrast to ornamentation that occurs dimorphically, and begs the question of what the evolutionary advantages might be. Sexually shared ornamental traits might have evolved because of a variety of selective advantages (reviewed by Kraavijeveld et al. 2007).

5.1 Genetic correlation

The genetic correlation hypothesis suggests that the ornament is sexually selected in one sex and occurs in the other simply due to genetic correlation between the sexes (Lande 1980; Lande 1987; Mank 2009; Figure 12)—what we might today call 'shared genetic architecture'(Tobias et al. 2012) and implies that one of the sexes gains no selective benefits or even might pay a cost from the expression of elaborate characters. For instance, several dimorphic bird species have evolved sex-specific ornamental traits depending either on the absence or presence of sexual hormones (i.e. oestrogen, testosterone or luteinizing hormone Owens and Short 1995; Kimball and Ligon 1999). This

shows that the genes coding for the ornament are present in females, but are suppressed under normal circumstances through the action of hormones. In contrast, Price (1996) showed opposite relationships for male (+) and female (-) between ornamentation intensity and reproductive success, suggesting that evolutionary pressure would favour males with redder bill, but females with lesser red bills. Using an original cross-fostering experiment he demonstrated that heritability of bill colouration was significant (from 0.23 to 0.58) and genetic correlation between sexes was high (r = 0.81). This suggests that genetic correlation between the sexes might create a genetic load preventing both sexes from independently evolving towards their separate selective optima, thereby maintaining mutual ornamentation.

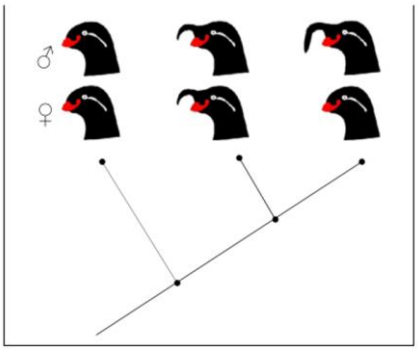


Figure 12. Hypothetical scenario for the evolution of sexual dimorphism from a dull monomorphic ancestor. If the genetic differentiation of the sexes may not have occurred both sexes appear mutually ornamented. (Kraaijeveld et al. 2007)

5.2 Social selection

Monomorphic ornament may have evolved under social competition over non-mate resources in both sexes as badges of social status (West-Eberhard 1983; Lyon and Montgomerie 2012). As an example, dusky moorhen (*Gallinula tenebrosa*) displaying larger shields are more likely to win inter-individual contests, regardless of their age and sex (Crowley and Magrath 2004). Game-theory models, exaggerated badges of status are likely costly and therefore opposed by natural selection might still be evolutionary stable (Maynard Smith 1974; Johnstone and Norris 1993). When the sexes experience the same selection pressure, the resulting signal traits are likely to be monomorphic (Tanaka 1996).

5.3 Natural selection

Other forms of natural selection than sexual selection might also produce ornaments in both sexes. For instance, ornaments may be used to signal predators prey unsuitability. This appears to be the case in the poison-dart frog family (*Dendrobatidae*), where both males and females are thought to signal distastefulness by adorning bright colours (Daly and Myers 1967). Nonetheless, evidence also suggests these ornamental features are used in mate choice (Summers et al. 1999).

5.4 Sexual mimicry

In socially dense group-living species, frequent interactions with courting males might be detrimental for females. Thus selection for females able to conceal their sex and avoid sexual harassment might occur (Burley 1981; Butcher and Rowher 1988). The theory predicts that the cost of producing an otherwise non-functional ornament in females could conceivably be outweighed if

the pressure imposed by male is high enough, but this idea has little empirical support (Kraaijeveld et al. 2007).

5.5 Mutual mate choice

Several experiments have shown that mutual mate choice occurs in a variety of taxa, including birds (Monaghan et al. 1996; Faivre et al. 2001; Saether et al. 2001), amphibians (Verrell 1995), fish (Rowland 1982; Wong et al. 2004), amphipods (Hua Wen 1993), termites (Shellman-Reeve 1999), fruit flies (Chenoweth and Blows 2003) and rotifers (Gomez and Serra 1996). In species where extended cooperation between both parents is necessary to successfully raise offspring, mutual mate choice for high quality partners is expected to occur (Burley 1986; Johnstone et al. 1996) and may lead to the emergence and maintenance of honest signals in both sexes. Alternatively, sexual selection on both sexes at once through mutual mate choice could produce monomorphic ornaments (Huxley 1914; Kokko and Johnstone 2002). Male and female competition for mates is expected to be stronger when the potential reproductive rate is similar for both sexes (Clutton-Brock and Parker 1992), neither sex representing a scarce resource (operational sex ratio close to 1; Emlen and Oring 1977). Mutual choosiness is further promoted in systems with high mate encounter rates where rejected mates can be rapidly replaced (Johnstone et al. 1996; Kokko and Johnstone 2002). Thus relying on honest signal mutual mate choice leads to the emergence and maintenance of honest signals in both sexes. Species subjected to those evolutionary pressures naturally appear as monomorphic, both sexes showing the same secondary sexual traits. However, those ornaments may signal either the same or a different aspect of individual quality in males and females. Indeed, males and females often have differing physiological constraints making unclear which proximate physiological pathways guarantee the honesty of male and female signals in similarly ornamented species (López et al. 2008). For instance, in goldfinches (*Spinus tristis*), monomorphic bill coloration is correlated with acquired immunity in females but not males, and such a difference has been suggested to be linked to the different functional roles of beak coloration in male and female social communication (Kelly et al. 2012).

6 Colour as a signal

Signals are used to transmit information from an emitter to a receiver. In a biological context, the emitted signal has to be perceived and integrated by the receiver.

6.1 The emitted signal: Physical properties of light and colour

Light is a radiation consisting of electromagnetic waves: synchronized oscillations of electric and magnetic fields that propagate, and are characterized by their wavelength. In nature, light is mostly emitted by the sun and the light spectrum reaching the Earth's atmosphere spans a range of 100 nm to about 1 mm (1,000,000 nm) in wavelength. The particles that make up our atmosphere (mostly ozone, dioxygen, water and carbon dioxide) absorb part of the energy and filter the light before it reaches the ground (Figure 13 a).

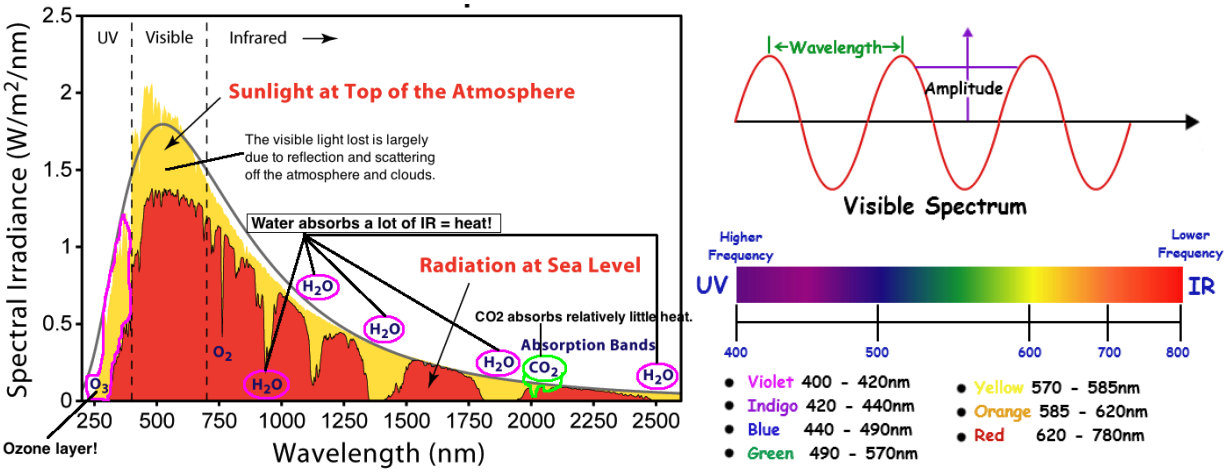


Figure 13. a) Solar Radiation Spectrum: The spectral irradiance (in W/m2/nm) of the light emitted by the sun that enters the atmosphere is figured in yellow, and the irradiance of light that reaches the surface of the earth is figured in red. Note that specific atoms/molecules preferentially absorb specific wavelengths of electromagnetic energy (Reproduced from Ballachey 2014).

The spectrum (the full range of all frequencies of electromagnetic radiation) is usually divided in 3 regions. The “visible” spectrum spans 400-700 nm and corresponds to the wavelength that humans can perceive and identify as colours ranging from violet to red (Figure 13 b). The infra-red wavelength, extending from the nominal red edge of the visible spectrum at 700-1000 nm, is invisible to humans. It is known as heat radiation and mostly absorbed by water molecules in the atmosphere. Ultra-violet (UV) wavelengths, from 10-400 nm, are mostly absorbed by ozone molecules present in the atmosphere with only a small fraction reaching the surface of the planet. Unlike humans and most other mammals (Hunt et al. 2001), many fish (Losey et al. 1999), insects (Brunton and Majerus 1995) and birds can see and respond to ultraviolet light (Burkhardt 1989; Rajchard 2009).

6.2 Colour in nature

Colour is abundant in nature. Some organisms are able to produce light using chemical reactions leading to the generation of bioluminescence, i.e. a form of chemiluminescence (Hastings 1996; Wilson and Hastings 1998). This phenomenon occurs widely in marine vertebrates and invertebrates, as well as in some fungi, microorganisms including some bioluminescent bacteria, and terrestrial invertebrates such as fireflies (Nealson and Hastings 1979; Lloyd 1983). The principal chemical reaction in bioluminescence involves the light-emitting pigment luciferin and the enzyme luciferase, assisted by other proteins such as aequorin in some species (Hastings 1996). The enzyme catalyzes the oxidation of luciferin. In some species, the type of luciferin requires cofactors such as calcium or magnesium ions, and sometimes also the energy-carrying molecule adenosine triphosphate (ATP) (Wilson and Hastings 1998).

However, most organisms display colour by reflecting different wavelengths of light from the ambient environment. Two main processes are involved: the presence of pigments and the fine structure of teguments (Figure 14).

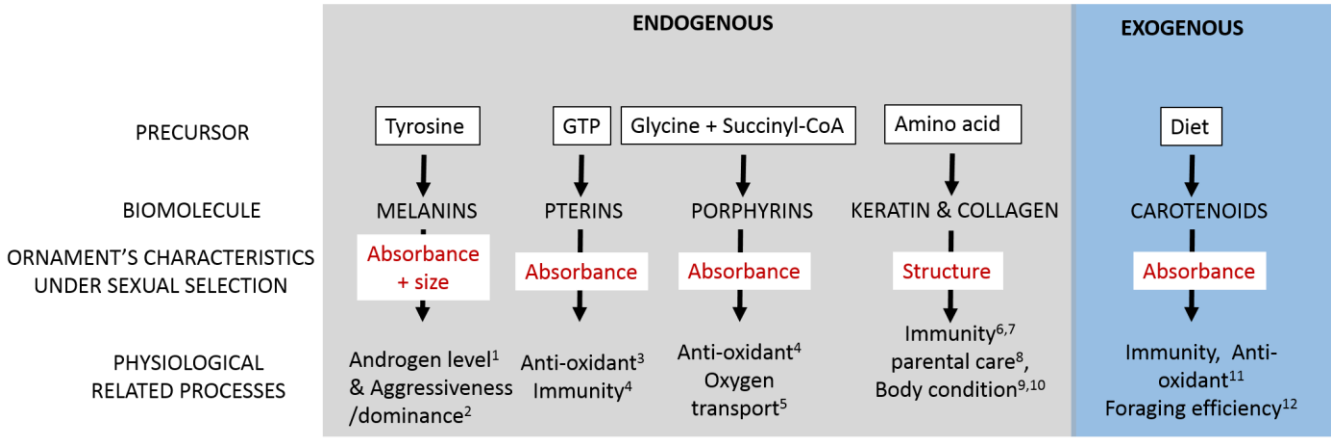


Figure 14. Review of the principal biomolecules involved in bird coloration. The figure illustrates the metabolic precursors, the physical processes involved in the transmission of the signal and the relation of the ornament with physiological processes. **1**. Gonzalez et al. 2001. **2**. Gonzalez et al. 2002. **3** Karl et al. 2004. **4**. McGraw 2005. **5**. Ponka 1999. **6**. Bize et al. 2006. **7**. Shawkey et al. 2004. **8.** Siefferman 2003 **9.** Bize 2006 10**.** Dobson 2008 **11**. Simons et al. 2012. **12**. Møller et al. 2000

6.3 Absorbance and reflectance

When reaching matter, part of the light is be absorbed. The process in which the energy of a photon is absorbed (typically the electrons of an atom) leads to the transformation of electromagnetic energy into internal energy of the absorber (e.g. thermal energy, (Parker 2002).

Each matter will absorb particular wavelengths, reflecting the wavelengths that are not absorbed. A pure white surface will reflect 100% of the wavelengths it receives, whereas a black structure will absorb all visible colours. The reflectance of the substrate is its effectiveness in

reflecting radiant energy. The reflectance spectrum or spectral reflectance curve is the plot of the reflectance R (in percent) as a function of wavelength (nm) following the equation:

$$R_\lambda = \Phi_e{}^r{}_\lambda / \Phi_e{}^i{}_\lambda$$

where Φer is the radiant flux reflected by that surface; Φei is the radiant flux received by that surface. Those reflected wavelengths, constitute the perceived colour of an object. For example, an object that absorbs wavelengths between 400 and 600 nm will absorb the violet to yellow wavelengths and reflect the red wavelengths. Thus this object will be perceived as red.

6.3.1 *Pigments*

Pigments are defined as materials that change the colour of a structure by wavelength-selective absorption. The most abundant pigments in living organisms are chlorophylls, melanin and carotenoids, which absorb light at different wavelengths (Figure 15a). Higher quantities of pigment in a structure result in higher light absorption and therefore less light is reflected (Figure 15b). The colour of a structure results from its molecular structure and concentration of its pigments.

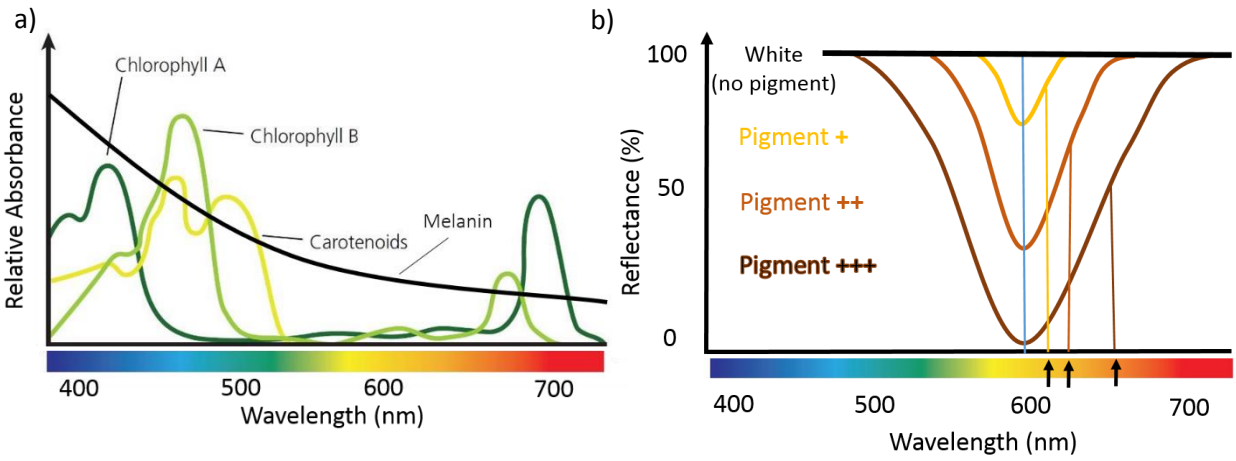


Figure 15. **a) Relative absorbance of Chlorophyll a and b, Carotenoids and Melanin** over the visible spectrum **b) Relation between the amount of an example pigment absorbing wavelengths around 600 nm and the amount reflected light:** a higher quantity of pigment results in higher light absorption, i.e. less light is reflected. Therefore, the perception of color (indicated by arrows) changes with the amount of pigment concentration.

6.3.1.1 Pigments in birds

Mate choice in songbirds has been largely studied, investigating how colourful ornaments reflect the quality of their bearer (Kodric-Brown and Brown 1984; Dufva and Allander 1995; Owens and Short 1995; Roulin et al. 1998; Westneat and Birkhead 1998; Buchanan 2000; McGraw and Hill 2000; McGraw 2005; Badyaev and Landeen 2007; Biernaskie et al. 2014; G. E. Hill 2014; Morehouse 2014; Roulin 2016). By their property to absorb light, pigments are the basic unit of colour ornaments. Pigments can be exogenous which means that they cannot be synthetized *de novo* and have to be provided by the diet (exogenous), or they can be endogenous and synthetized by the organism itself (Hill and Brawner 1998; Delgado-Vargas et al. 2000; Weiss et al. 2011; G. E. Hill 2014).

The honesty of **exogenous pigment-based ornaments** first relies on the ability of the individual to acquire such pigment (e.g. through foraging) (van Noordwijk and de Jong 1986), whereas for **endogenous pigment-based ornaments** it is the producing pathways that are limiting (Pathway Functionnality and Shared Pathway Hypothesis; Hill 2011). However, the cost associated with producing endogenous pigments is relatively unknown and studies on melanin-based ornaments (an endogenous pigment) suggest that the production cost may be low in specific circumstances (see below; Møller 1987a; Gonzalez et al. 2002)

Regardless of which pigments an ornament contains, underlying trade-offs in allocating those pigments to colour production or other vital functions insure signal honesty (see above; Ressource Tradeoff Hypothesis; Hill 2011). Indeed, pigments play crucial roles in different physiological functions, such as immunocompetence (Bendich 1989; Chew 1993), oxidative balance (Edge et al. 1997; Oettl and Reibnegger 2002; Karl et al. 2004; McGraw 2005; Weiss et al. 2011; but see Costantini and Møller 2008) and ornamentation colouration (reviewed in Simons et al. 2012). Their investment in colouration occurs at the expense of other functions (Ponka 1999; von Schantz et al. 1999; Oettl et al. 2004; Morehouse et al. 2007; Weiss et al. 2011). The amount and wavelengths of light reflected by the ornament, as well as the size of the ornament, are directly linked to the type and amount of pigments it contains (Saks et al. 2003; McGraw and Gregory 2004). Thus, ornaments transmit direct information on the aptitude of the bearer to deal with the underlying mechanisms that pigments are linked with. Behavioural ecologists have devoted the most attention to carotenoid- (exogenous) and melanin- (endogenous) based ornaments, however pterins and porphyrins are two other endogenous types of pigments involved in colour ornamentation (G.E. Hill and McGraw 2006).

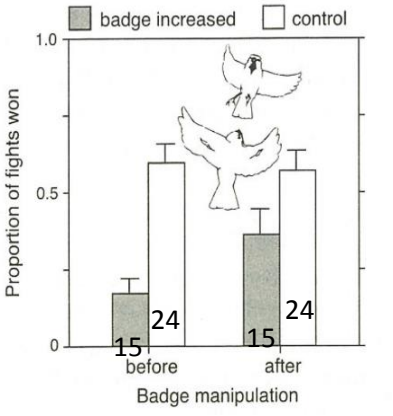


Figure 16. Proportion of total fights won before and after artificially increasing the breast patch surface in male house sparrows Sample sizes are given in the barplots. (reproduced from Gonzalez et al. 2002)

M**elanin-**based ornaments, mostly defined as badges of social status, the larger the ornament, the larger the amount of pigment that is invested. Size has been linked to androgen secretion (Gonzalez et al. 2001) and directly to individual aggression and dominance status (Gonzalez et al. 2002). Experimentally increasing black throat patches of male house sparrows leads to a significant increase in the proportion of fights won and enhances their dominance rank (Gonzalez et al. 2002). Thus, by comparing the size of male ornamental patches, females might gain information about which individual has the best ability to defend a territory (Figure 16; Møller 1987; Johnstone and Norris 1993; Qvarnstroöm 1997; Gonzalez et al. 2002). The expression of many (but not all) melanin-

based colour traits is weakly sensitive to the environment but strongly heritable suggesting that these colour traits are relatively cheap to produce (Roulin 2016) and that the real cost that constrain the evolution and the honesty of such ornament is delayed and relies on pleiotropic effects (Ducrest et al. 2008; Roulin 2016).

Porphyrins are found in egg shells and the brown feathers of busards (*Circus sp*.), nightjars (*Caprimulgus sp.*) and owls (reviewed in With 1975; With 1978) as well as the blood-engorged red parts of many birds (Laruelle et al. 1951). Porphyrins, such as hemoglobin, contribute to oxygen transport in blood (Ponka 1999) and porphyrins derivates found in bird ornaments and eggshells also present antioxidant properties (e.g. Williams et al. 1995; Sahoo et al. 2002). Little is known about porphyrin-based ornaments and their relation with individual quality. However, Moreno & Osorno (2003) first discussed the antioxidant properties of porphyrins as they related to eggshell coloration to maternal quality in birds. Holveck and collegues (2012), using the Blue tit (*Cyanistes caeruleus*) as a model species, showed that eggshell coloration did not relate to yolk carotenoids, but that both the quantity and distribution of brown pigments reflected the transfer of maternal immune compounds to egg yolks.

Carotenoid and **Pterins** are known to play important roles in immune function and as anti-oxidants (Christoph et al. 1984; Bendich 1989; Edge et al. 1997; Oettl and Reibnegger 2002; Karl et al. 2004; McGraw 2005; Weiss et al. 2011; but see Costantini and Møller 2008). Indeed, carotenoids directly bolster the immune system of vertebrates by stimulating effector T-cell functions, enhancing macrophage and cytotoxic T-cell capacities, as well as stimulating T- and B-lymphocyte proliferation (Bendich 1989; Jyonouchi et al. 1994; Chew 1996). Carotenoids and pterins act as free radical scavengers contributing to the organisms' ability to face the free radicals produced during daily metabolism and immunological responses (Burton 1989; Stahl and Sies 2003; Oettl et al. 2004). Thus, only individuals able to support immune and antioxidant functions should also be able to allocate pigments to their ornaments. For instance, individuals more efficient in foraging (Møller et al. 2000) or in better condition should require fewer carotenoids for maintenance (i.e. immune function) and should therefore be able to allocate a larger portion of their limited stores to ornamentation. Empirical studies directly highlight those relationships. Studies in blackbirds (*Turdus merula*) and American goldfinches (*Spinus tristis*) have shown that the expression of colour ornaments may be reduced in individuals with high parasite loads, or in immunologically-challenged individuals (Faivre et al. 2003a; Rosenthal et al. 2012, respectively). More colourful ornaments have been associated with higher immune system function (Blount et al. 2003; Mougeot 2008; Kelly et al. 2012; Whittingham et al. 2015), higher resistance to pathogens (Lindström and Lundström 2000; Hill and Farmer 2005) and maternal capacity to invest antioxidant and pro-immune pigments (e.g. carotenoids) to egg yolk under challenging conditions (Midamegbe et al. 2013). In a meta-analyses

on 357 study on 88 different species, Simons et al. (2012) found that immunocompetence was positively related to carotenoid levels (r = 0.20) and trait colour ornament intensity (r = 0.17).

6.3.2 *Structural colouration*

Structural colour is produced by the physical and optical interactions of light waves with the structure of an organism. Fine and dense structures allow light to pass through them, however a small amount of light (few percent) is always reflected. When those structures are accumulated the reflected energy increases. Thus, the nanostructural organization of tissues can modulate the amount of light reflected.

Coherant Light Scattering

Most structural colours are produced by light scattering, which results from the physical interactions of light waves that are scattered at the interfaces of biological materials of different refractive indices (Figure 17). The scattered wave length at a given angle ϑ and a distance d between the two scattering planes is given by Bragg's law (Bragg 1915):

$$\lambda\,(nm) \;=\; 2d\,sin\theta$$

In other words, the light waves remain in phase if the difference between the path length of the two waves is equal to an integer multiple of the wavelength (Bragg 1915). This phenomenon is known as coherent scattering caused by constructive interference and leads to the emission of colour depending on the distance d separating the layers and the angle ϑ of the entering light (Figure 17 a). If in contrast, the interference is destructive (opposite phase) no light is emitted (Figure 17 b) (Bragg 1915).

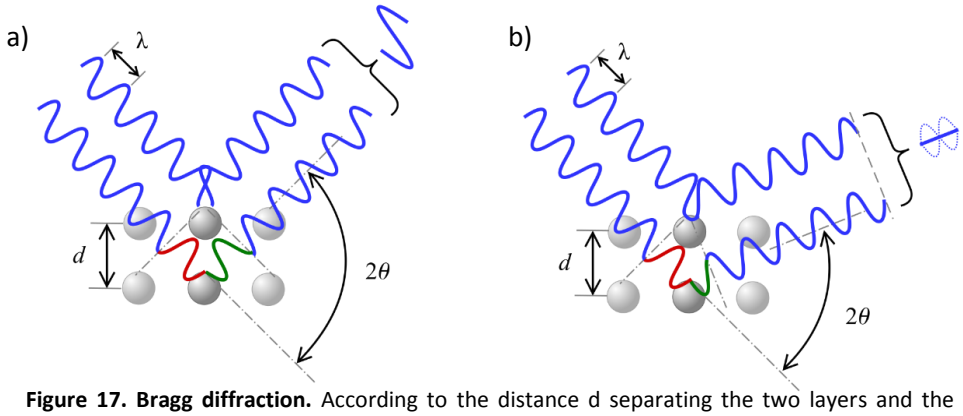


Figure 17. Bragg diffraction. According to the distance d separating the two layers and the 2θ deviation, the phase shift causes constructive (a) or destructive (b) interferences (Bragg 1915).

Iridescence (also known as goniochromism) is a particular phenomenon where the colour produced by the surfaces appears to change as the angle of view or the angle of illumination changes (Shawkey et al. 2015). Iridescence is a relatively common feature in some groups of invertebrates, particularly in arthropods and molluscs (Doucet et al. 2009) and insects (Seago et al. 2009). Among vertebrates, the evolution of iridescence has apparently been confined to birds, fish, reptiles and amphibian (Doucet et al. 2009). In birds, the nanostructural organization of keratin, melanin and air in feather barbules can produce iridescent coloration (e.g. Greenewalt et al. 1960; Durrer and Villiger 1966; Durrer and Villiger 1970; Land 1972; Zi et al. 2003; Doucet et al. 2006; Shawkey et al. 2006). Iridescence is broadly distributed throughout *Aves*, and appears to have evolved independently in a number of different groups (Prum 2006).

6.3.2.1 Structural colour in birds

The colouration of feathers, skin or iris (eye) integuments can also be caused by the nano-scale organization of keratin and collagen tissues (i.e. **structural colouration**) that usually reflect ultraviolet wavelength (UV) and are mostly associated with the presence of melanin pigments (Prum 2003; Prum 2006). The homogeneity and resolution of those structures at the moment they are produced determines the property of the emitted colour (Doucet et al. 2006; Dresp and Langley 2006). The skin of Alpine swifts (*Tachymarptis melba*) and European starlings (*Sturnus vulgaris*) for instance presents a structural based UV reflectance (Jourdie et al. 2004; Bize et al. 2006), and individuals in better body condition (body mass and size) also reflect more UV light (Bize et al. 2006). More interestingly, artificially increasing the UV reflectance of offspring skin was shown to affect parent allocation of food preferences, and the effect was opposite depending on whether they started to breed early or late in the season (Bize et al. 2006). This highlights that the UV signal is integrated by the parents and reliably linked to chick condition. In wild turkeys (*Meleagris gallopavo*) the quality of UV feather ornaments has been related to individual's immune status during the moult (Hill et al. 2005). When challenged with intestinal parasites, birds produced ornaments that were dull, suggesting a condition-dependence of those traits at the moment they were renewed (Hill et al. 2005). Moreover, Harper et al. (1999) presented correlative results linking feather-mite prevalence and feather colour reflectance in male house finches (*Carpodacus mexicanus*). Also, for some soft structures like feathers, the aptitude of an individual to preserve the integrity of the structure from external attacks such as parasite or environmental conditions (e.g. by preening, applying uropygial oil) will preserve the signal across time (Shawkey and Hill 2004).

7 Motivation and scope of the thesis

In this thesis, I investigate the underlying mechanisms linking ornamental displays to individual condition, health and fitness. My aim is to contribute to our understanding on the evolution and function of ornaments, a topic that has kept evolutionary biologists marvelling since Darwin (Darwin 1859). Specifically, the originality of this thesis, is not only to experimentally test but move beyond the classical theories of condition-dependence (immuno-competence, stress-linked ornamentation) by bringing new light on the importance of mitochondrial functioning in the establishment of ornamentation. This work further considers the importance of sexual vs. social selection in the evolution of mutual ornamentation by jointly investigating structural and pigment-based ornaments in a monomorphic species where mutual mate choice is known to occur: the king penguin *Aptenodytes patagonicus*. This thesis is divided in 4 main chapters that respectively question: (1) the production cost of ornamentation insuring signal honesty, (2) the maintenance cost of ornamentation and the dynamicity of both structural and pigment-based ornaments, (3) the existence of allocation trade-offs between ornamentation and immunity, and the links between ornament production and energy availability at its most fundamental level: the mitochondrion, (4) the contribution of environmental vs. genetic factors insuring signal honesty in the evolution of mutual ornamentation.

Methods

1 The king penguin: a monomorphic species with mutual mate choice

1.1 Description of the species

The king penguin (*Aptenodytes patagonicus*) is one of the 18 known penguin species (Miller 1778) and is the second largest after the Emperor penguin (*A. fosterii*) that lives in Antarctica (Williams 1995). Those semi-altricial seabirds (entirely dependent on their parents for food and warmth during the first period of growth) reproduce in vast colonies of several thousands of pairs on most of the islands and shores between latitudes of 45° and 58° South (Stonehouse 1960; Figure 18).

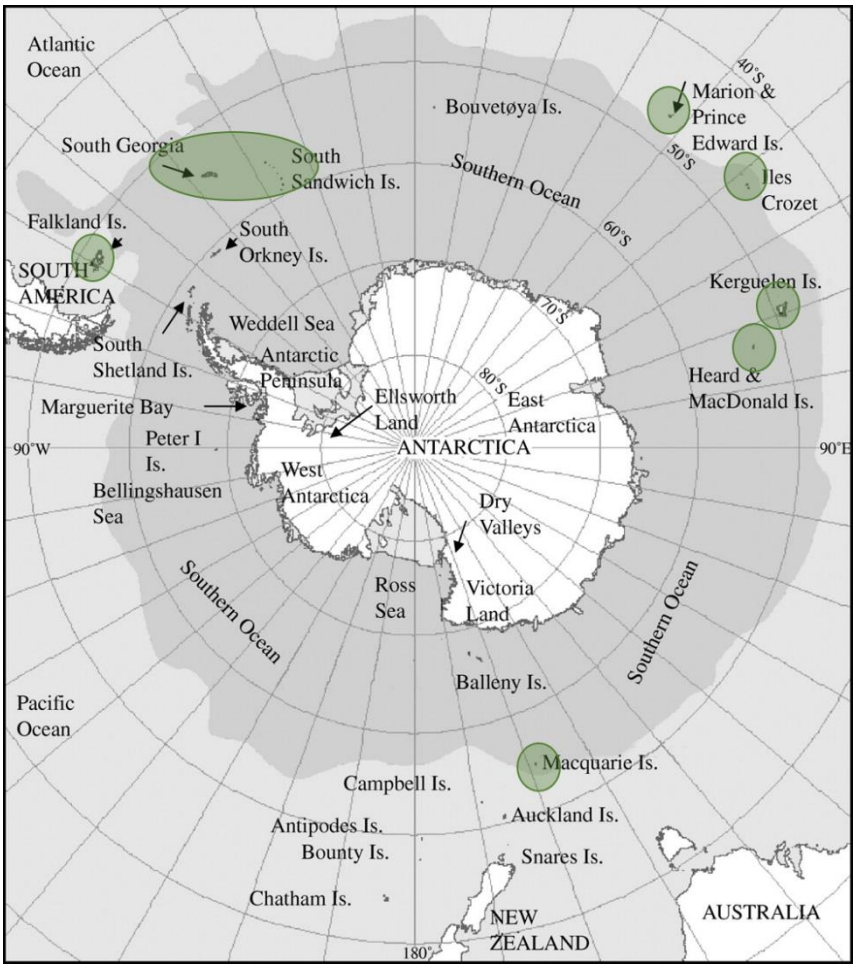


Figure 18. Map of the global distribution of king penguin colonies. The species is divided in two subspecies, depending on their geographic localisation. *A.p. patagonicus* occurs in South Georgia, the Sandwich Islands, and the Falkland Islands. *A.p. halli* occurs in the Prince Edward and Marion, Crozet, Kerguelen, Heard and Macquarie Islands (Rogers 2012).

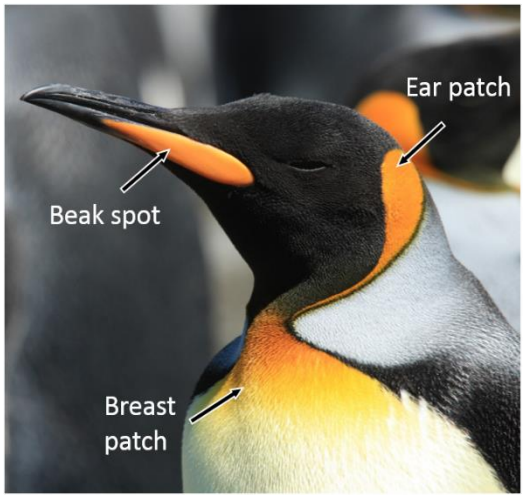


Figure 19. Photograph of an adult king penguin (*Aptenodytes patagonicus*). At adulthood they display conspicuous ornaments on both sides of their head, both sides of their beak and on their breast.

The king penguin is a monomorphic species, i.e. both males and females share conspicuous colour ornaments thought to have evolved under sexual selection (Jouventin et al. 2008; Pincemy et al. 2009; Nolan et al. 2010; Dobson et al. 2011). Both sexes have yellow to orange feather patches on both sides of the head (called "auricular patches"), a breast feather patch that grades from brown on the throat to light yellow on the upper quarter of the breast (the breast is white beneath the coloured patch), and beak spots on both sides of the mandibles that are yellow-orange and ultra-violet (UV) in colour (Stonehouse 1960; Dresp et al. 2005; Jouventin et al. 2005; Thomas et al. 2013) (Figure 19). King penguin adults can reach up to 85 to 95 cm in height and weigh between 12 and 14 kg. They are long-lived seabirds that exhibit high fidelity to their breeding colony (Olsson 1997a; Gauthier-Clerc et al. 2003). Their inter-annual survival rate is high (i.e. 90 %; Weimerskirch et al. 1992) and their life expectancy is around 20 years. They feed on pelagic invertebrates (squid) and myctophid (lantern) fish (Cherel and Ridoux 1992; Olsson and North 1997) in waters along the polar front (a zone of high primary productivity; Laubscher et al. 1993; Moore and Abbott 2000) that is situated some 300 km from their colonies during the breeding season. During winter, they forage as far as the marginal ice zone, ca. 1000 km away from the colony and seldom return to their breeding grounds during that period (Charrassin and Bost 2001; Pütz 2002). King penguins are exceptional divers, reaching depths of ca. 300 m and lasting up to 7.5 minutes (Handrich et al. 1997). They reach sexual maturity at around 3-4 year old after their first breeding attempt after a 2 years post-fledging foraging trip at sea (Barrat 1976; Saraux, Viblanc, et al. 2011). The first successful breeding attempt mostly happens when the birds are around 6 years old (Weimerskirch et al. 1992).

1.1.1 *Breeding cycle*

The reproductive cycle of the king penguin extends for over a year (14 months). As most seabirds, king penguins forage at sea but breed on land. On land, their entire metabolism relies on the reserves they accumulate during foraging trips at sea (Y. Cherel et al. 1994). Thus, periods on land are associated with long-term fasting, a situation that individuals face repeatedly throughout the season making this life cycle highly constraining from an energy perspective (Cherel and Le Maho 1985; Cherel et al. 1987; Cherel, Robin, et al. 1988; Cherel, Leloup, et al. 1988; Le Ninan et al. 1988; Y Cherel et al. 1994; Yves Cherel et al. 1994). The breeding cycle of king penguins (Figure 20) begins by

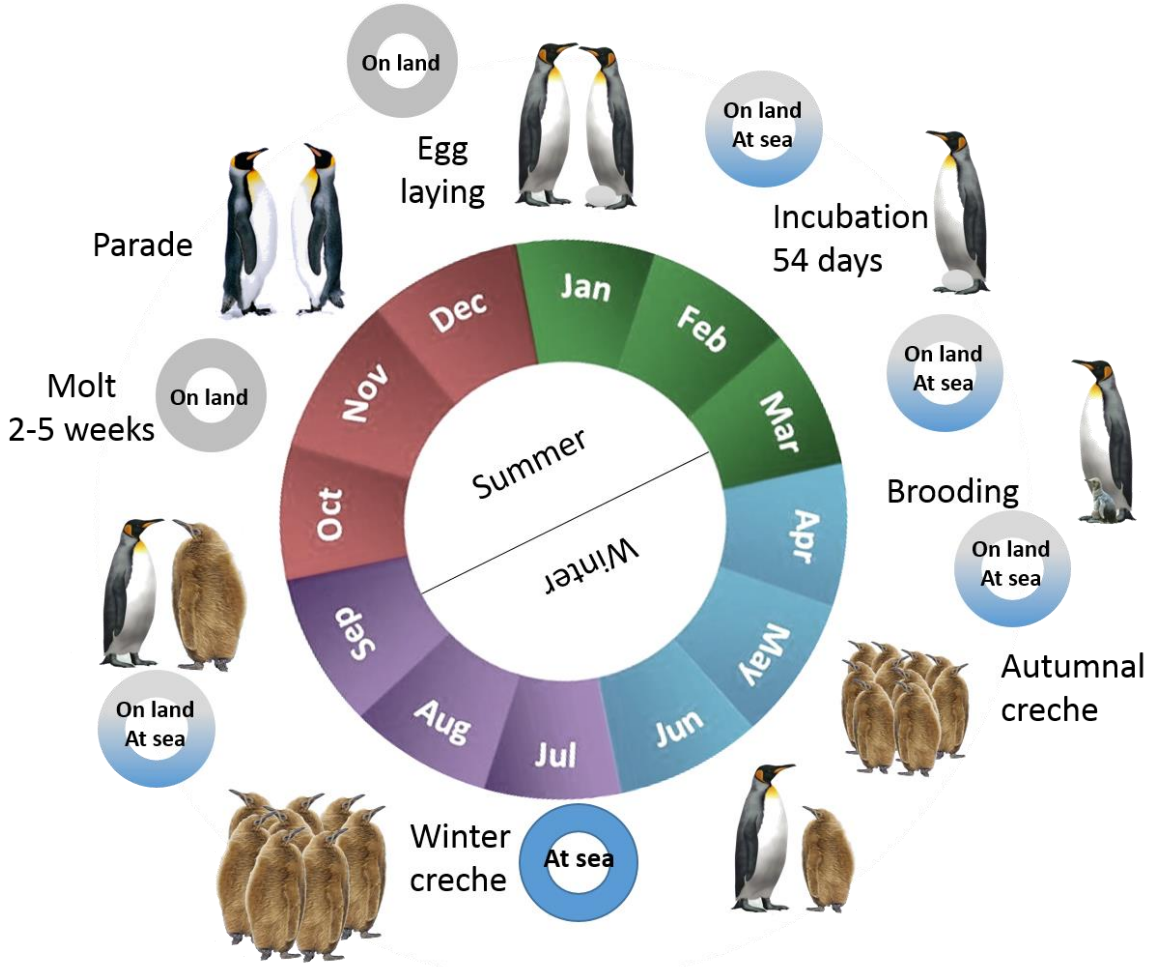


Figure 20. Schematic representation of the king penguin's breeding cycle.
The cycle presented here is for a king penguin pair that did not raise a chick the previous year. Proportional time spent by the adults foraging at sea (blue) or on land (grey) is represented as circle. The egg typically hatches around mid-January. During the autumnal creche parents still regularly alternate sojourns at sea with chick-feeding on land (Stonehouse 1960). During the winter parental-feeding is scarce and king-penguin chicks undergo long periods of starvation (Cherel and Le Maho 1985). Feeding is resumed in the spring, at the end of which the chick is finally fledged.

a critical pre-nuptial moult that lasts for 2 to 5 weeks, during which the complete plumage and the beak spots – and thus ornamental features – are renewed (Stonehouse 1967; Cherel et al. 1988a; Bourgeon et al. 2007; Figure 21). After a foraging trip at sea, king penguins start their moult ashore with an initial body mass of 17-18 kg, while renewing their feathers and eventually losing up to 58 % of their initial body mass (Cherel, Leloup, et al. 1988). This challenging period is followed by a foraging trip to replenish energy reserves. The first adults then arrive at the colony to breed in early November. During the subsequent courtship phase, mating partners carefully choose each other during ritualized courtship displays. As any monomorphic species, king penguin's male and female outer appearance is similar.

Figure 21. The molt of king penguins (*Aptenodytes patagonicus*). During the molt, king penguins renew their entire plumage (left panel) and their beak spot on each side of the beak (middle panel). The old keratin based beak spot are shed (right panel)

However, calls differ in their structure and frequencies between sexes (Jouventin 1982; Robisson 1992) allowing individuals to identify the opposite sex. Both potential mates exhibit colourful ornaments to one another in a ritualized way (Stonehouse 1960, Jouventin et al. 2008, Nolan et al. 2010, Dobson et al. 2011a; see below section 1.1.1 on mate choice in king penguins). Once the pair has bonded, the couple settles down on its breeding territory and the egg is laid a few days later. The partners exchange their individually distinct calls before the female leaves the colony to forage at sea and the male starts incubating the egg (first incubation shift) (Stonehouse 1960). Facing a critical depletion of their energy stores, parents may abandon the egg or young chick in order to go and re-feed at sea before their partner can return, leading to the failure of the current reproduction (Olsson 1997b; Groscolas et al. 2000; Gauthier-Clerc et al. 2001). The incubation usually lasts about 54 days (Stonehouse 1960), and the first eggs in the colony hatch at the beginning of January. At that time, the male is usually in charge of taking care of the chick and feeds the chick with undigested food, which is preserved by an beta-defensin antimicrobial peptide (named "spheniscin") in its stomach for weeks (Thouzeau et al. 2003; Landon et al. 2004). During incubation and until the chick is fully thermally emancipated (around 6 weeks later), the parents take turn caring for the single egg or young chick on their feet, thus undergoing prolonged periods of fasting ashore while the partner is foraging at sea (Stonehouse 1960; Descamps et al. 2002). Chicks will then group into crèches and both parents will go back and forth between the colony and sea to provision their chick (Saraux et al. 2012). The growth of the chick is split into two phases. The first growth phase lasts until the beginning of the austral winter (early May), during which it will grow and accumulate a large amount of fat reserves. During the austral winter (from May to September) adults are compelled to move to feeding grounds at the marginal ice zone (1000 km; Weimerskirch et al. 1992; Charrassin and Bost 2001; Descamps et al. 2002), and only seldom returning to feed the chick that will face its first long term fast (Cherel and Le Maho 1985; Saraux et al. 2012). This period is critical for the chick and many do not survive that long starvation period, during which they also face high predation by Giant petrels (*Macronectes giganteus*) (Stonehouse 1960; Descamps et al. 2005). In September, when climate conditions are favourable once again, adults begin the second intensive feeding period until mid-November/December. At that time, after moulting into its waterproof adult plumage, the successfully raised chick fledges and leaves to sea to forage by itself. It will only return to the colony 2 to 3 years later (Saraux, Viblanc, et al. 2011). At this point, the adults start a new breeding season. However, for those individuals that succeeded in their past reproductive event, the initiation of the following breeding cycle is delayed. This explains the occurrence of two reproduction peaks (early in November and late in January), with the latter rarely successful (1 %; Weimerskirch et al. 1992; Olsson 1996). King penguins have to defend and secure their breeding territory (1m²) against other conspecifics, representing 14% of daily spent time-budget (Viera et al. 2011) and a mean of 100

interactions per bird per hour is observed (Côté 2000) during incubation and brooding periods representing a important source of social stress for the birds (V. A. Viblanc et al. 2012). Predation occurs during the entire breeding cycle. Brown skuas (*Stercorarius antarcticus*) predate on eggs and small chicks and giant petrels on heavier chicks (Descamps et al. 2005). Thus to successfully raise a chick, both parents face prolonged periods of food depletion, harsh weather conditions, high predation risk and must coordinate their efforts for over a year. Such strong environmental pressures are expected to underlie mutual mate choice for high quality partners, leading to the evolution of mutual ornamentation (Johnstone et al. 1996; Kokko and Johnstone 2002; Kraaijeveld et al. 2007; Hooper and Miller 2008).

1.1.2 *Finding a good partner: Mutual mate choice in king penguin*

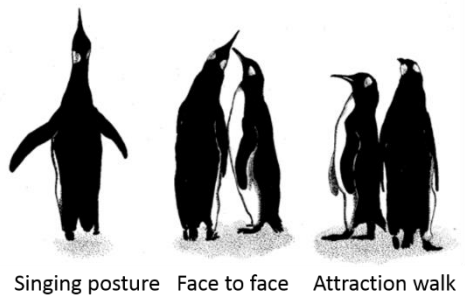


Figure 22. King penguin's parading behavior (modified from Jouventin 1982)

King penguins remain in pairs during the entire breeding season, however only about 20% of pairs breed again with the same partner in the following season (Olof Olsson 1998). Thus, most individuals have to find a new mate before initiating reproduction. Finding a high quality partner is crucial given the high and shared parental investment required to fledge a chick. The courtship period lasts for 12-29 days in males and 8-18 in females (Stonehouse 1960). First, calls are directed to the colony as a whole, and serve to announce the presence of a bird in breeding condition, also providing information about its sex (Stonehouse 1960). Once a tentative pair has come into contact, both birds face each other (somewhat laterally) and stretch out as tall as possible, slowly lifting their heads and pointing their beak to the sky with half close eyes ("face to face"; Figure 22). After spending a few minutes in this position, birds gradually relax back to a normal position. One of the potential mates (usually the male) then leads off with a characteristic strut well suited to pushing and also edging rivals away from a coveted partner; the "attraction walk". The back is arched, the chest and abdomen are held out and the neck feathers are ruffed. During the walk the leading individual exaggeratedly swings is head from side to side. Occasionally, small groups (mostly trios) of multiple individuals competing for the same mates are formed. Keddar and colleagues found that trios almost always consisted of a female trailed by two fighting males (19/20 cases; Keddar et al. 2013). Potential pairs will form and split until they definitely choose their mate and settle in the breeding colony. The sex ratio in the "Baie du marin" king penguins colony appears to be slightly biased in favour of males at their first return at the colony (at the age of 2-3 years) (Saraux et al. 2011) and potentially showing a similar pattern for

adults (Bried et al. 1999). Olsson and Van der Jeugd 2002 showed that in adult parading king penguin of South Georgia the sex ratio was 56% males and 44% females.

Several studies have investigated the particular role of sexual ornaments in king penguin mate choice. A pioneering study conducted by Stonehouse (1960) showed that birds were unable to acquire mates when their auricular patch was covered by hair dye in black until the paint wore-off (Stonehouse 1960). Less drastic reductions of auricular patch size highlighted that this ornamental trait plays an important role for female choice, since artificially reducing males' auricular patch size significantly delayed their time of pairing (Jouventin et al. 2008; Pincemy et al. 2009; Nolan et al. 2010; Dobson et al. 2011; Figure 23). Thus, sexual selection favoured larger auricular patches in males. In addition, UV reflectance by the beak spot appears to be an important component in mate choice. Indeed, experimental reductions of UV reflectance of the beak spot by 30 % showed similar results for both sexes by delaying their pairing by more than a week, suggesting mutual mate choice based on this ornament (Nolan et al. 2010).

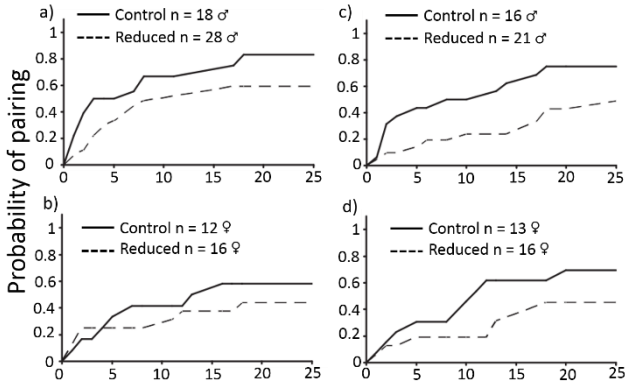


Figure 23. Probability of pairing in (a) male and (b) female king penguins after experimental reduction in auricular patch size and (d) male and (e) female king penguins after experimental reduction of ultraviolet (UV) reflectance from beak spot.

1.1.3 *Honesty of the signal in king penguins: what is known so far*

Altering auricular patch size and UV reflectance of the beak spots delays pairing in king penguins, suggesting that those ornaments are used in mate choice (Jouventin et al. 2008; Pincemy et al. 2009; Nolan et al. 2010). Several studies have considered the mechanisms involved in the production of colour ornaments in this species. For instance, in 2005 and 2006 Dresp and collaborators highlighted the structural basis of the UV component of the beak spot (Dresp et al. 2005; Dresp and Langley 2006). In 2013, Thomas and collaborators (Thomas et al. 2013) characterized the chemical classes responsible of feather colouration, i.e. pterin-like pigments. Whereas those studies contributed to our understanding of the mechanisms underlying colour production, very little is actually known on what information those ornaments convey to conspecifics, whether it is in sexual or other social contexts. In 2008, Dobson and colleagues found indirect links between body condition and colour parameters: early male breeders displayed higher UV reflectance than early females and late breeders of both sexes. Those males were also heavier and in better condition. As male king penguins are the ones that endure the longest reproductive fast

of the cycle (Stonehouse, 1960), including courtship and the first incubation shift, it is possible that the honest signalling of body condition by UV reflectance could be more important in males than in females. Further, Nolan and collaborators (2006) found that the hue of the breast patch was negatively related to the inflammatory response male penguins mounted following the experimental injection of a novel mitogen (phytohaemagglutinin, PHA). Those results suggested that individuals that invest more in their ornaments also show a lower cellular immune response (Nolan et al. 2006). Finally, the size of the auricular patch in king penguins has been suggested to act as a badge of status. Birds with larger auricular patches (regardless of sex) are more aggressive and appear to be able to secure central breeding spots in the colony (Viera et al. 2008; Keddar, Jouventin, et al. 2015), which are thought to be better in terms of reproductive success (Weimerskirch et al. 1992; Côté 2000 but see Viblanc et al. 2014a in terms of individual stress).

1.1.4 *How is colour produced in king penguins?*

King penguins display ornaments that rely both on structural and pigmentary coloration. On each side of the head and on the chest they exhibit yellow to orange feathers that contain a pterin-like pigment (Thomas et al. 2013). This pigment reflects above 450nm and absorbs all wavelengths below. The beak spot reflects both UV and yellow-orange wavelengths (Jouventin et al. 2005). The UV colouration of the beak results from structural coherent scattering: king penguins possess stacks of elongated lamellae in the horn layer of the beak (Figure 24a). Those structures form a photonic microstructure which reflects light in the UV to violet wavelengths (Dresp et al. 2005; Dresp and Langley 2006). Based on Bragg's law, the distance (in nm) separating those double-folds predicts the wavelength reflected in the UV (Figure 24b).

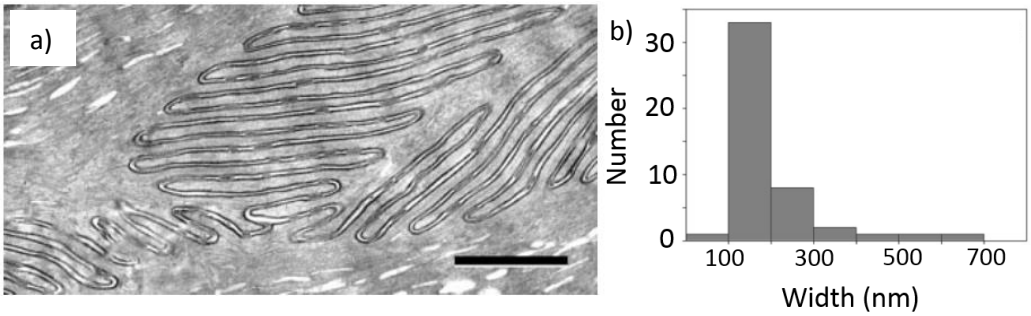


Figure 24. a) Electron micrograph of the microstructure present in the upper-layer of king penguins beak spots. This keratin-based tissue is composed of interconnected microstructures, constituted of multiple layers of folded membrane doublets. The Scale bar = 1000 nm (from (Dresp and Langley 2006) **b) Distribution of the widths of the microstructures (in µm)**

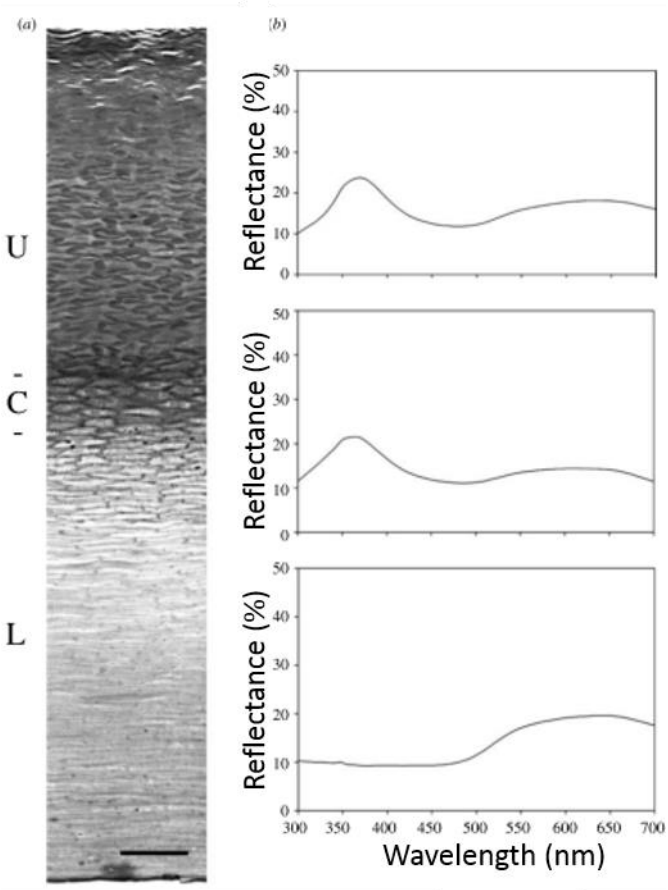


Figure 25. Histological transverse section of a king penguin's beak spot stained with Toluidine blue. U: upper layer, C: Central layer, L: lower layer. The joined spectrum have been measured after the removal of first the upper layer followed by the central layer. Scale bar = 25µm

After rehydrating dry beak spots for 24 hours by immersion in water, the wavelength showing the maximum reflected intensity increased from ca. 370 to 420 nm, and the percentage of maximum reflectance approximately doubled. Two mechanisms could be involved. First, hydrating the tissue could lead to a dilatation and a resulting increase in the distance between the two layers of the lamellae. Secondly, water shows a higher reflective index than air. Thus both processes could lead to a higher diffraction and a reflectance of higher wavelengths. Finally, removing 17% of the beak spot upper-layer results in a decrease of 10% in maximum reflectance. When the horn thickness is reduced by 38% the UV reflectance disappears but a yellow-orange reflectance remains (Figure 25). Indeed, the beak also contains an orange-yellow pigment that reflects the same wavelength as the auricular patch and is likely caused by carotenoid pigments assimilated through diet that are only present in the deeper parts of the beak (McGraw et al. 2007).

Sexual selection may promotes the development and maintenance of conspicuous traits on which mate choice occur in king penguin (Jouventin 2001; Pincemy et al. 2009; Nolan et al. 2010; Dobson et al. 2011). However little is known on how the ornaments reflect individual quality. There is thus an urging need for experiments testing for potential links between ornaments and condition-dependent traits (including immune and stress responses, the management of the energy reserves and oxidative balance) to identify how which underlying mechanism insure the honesty of ornamental signals in this species.

2 The study site

Most of the data was collected over 4 field seasons, from November to March, during the austral summers 2011-2012, 2012-2013, 2013-2014 and 2015-2016. I was directly involved in coordinating the field studies and collecting the data for 3-5 months each year in 2013, 2014 and 2015. Subsequent lab analyses for the various immune and oxidative stress markers presented here took me approximately 8 months in total to perform.

2.1 Geography

The field site where the different studies presented in the thesis occurred is located in the sub-antarctic Crozet Archipelago (46°25'S, 51°45'E), in the Southern Indian Ocean (Figure 27). The Archipelago, consisting of five islands, is one of the five districts of the French Southern and Antarctic territory. The five islands, all of volcanic origin and basalt, are Pig Island (67 km²), Auk Island (3 km²), Apostle Islets (2 km²), East Island (130 km²) and Possession Island (150 km²). Only the latter is inhabited at the research station Alfred Faure (Figure 26). The only access to Possession Island for visitors, staff and researchers is via the research vessel "Marion Dufresne", named after the French explorer who discovered the islands in 1772, and a short helicopter flight.

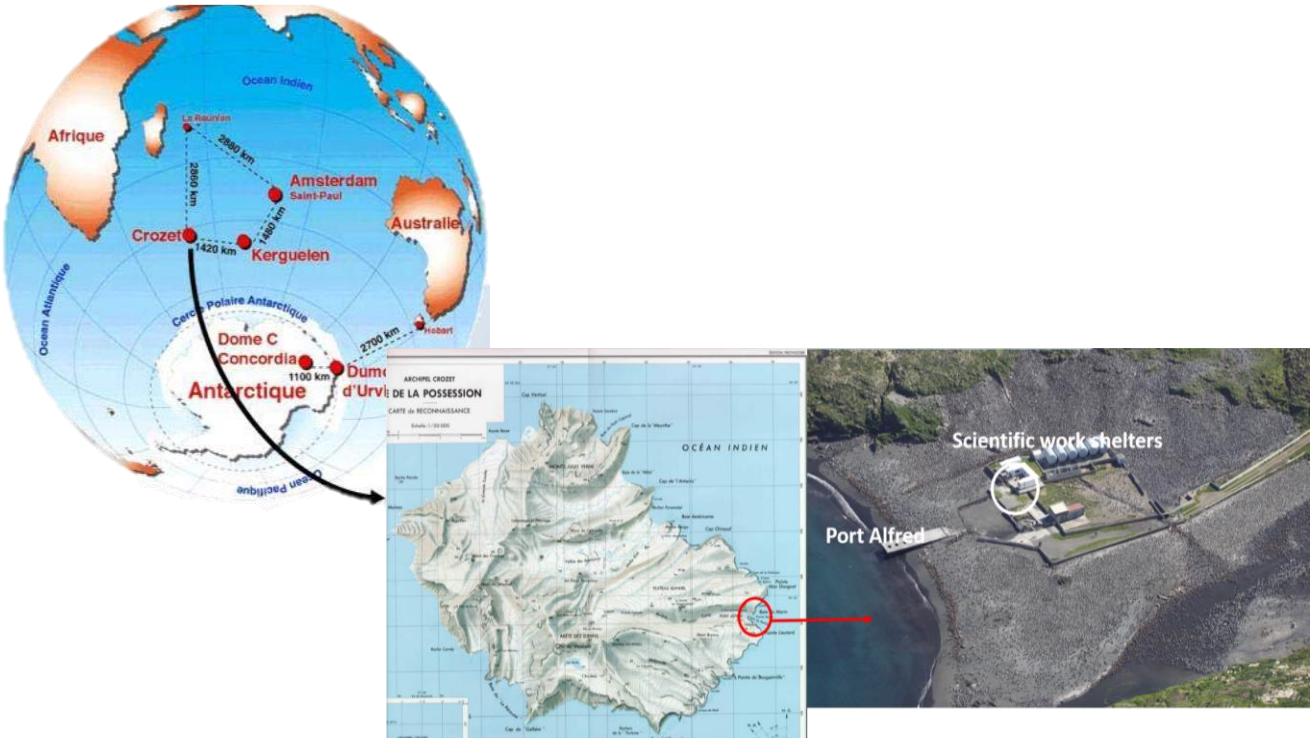


Figure 26. Location of the French Subantarctic Islands (Crozet, Kerguelen, Amsterdam & St Paul), detailed map of Possession Island (Crozet archipelago) and aerial photography of the "Baie du Marin" king penguin colony. Note that the scientific facilities are installed in the vicinity of the colony.

2.2 Climate

The island's climate can be classified as tundra-like with average temperatures of 3 °C in winter and 8°C in summer and rarely exceeding 20°C. Precipitation is high (over 2000 mm per year) and the island is constantly subjected to strong winds often exceeding 100 km/h (Bougere and Bougere 1998; Van de Vijver et al. 1999; Duriez et al. 2005).

2.3 Flora and Fauna

Plants that can be found are mainly different species of grass, moss and lichen. Wildlife is rich, including insects (snails, spiders), fish (Patagonian toothfish, marbled rockcod), mammals (fur seals, southern elephant seals, killer whales) and 22 bird species including an endemic duck species (*Anas eatoni*), southern giant petrels, six different species of albatrosses (Weimerskirch et al. 2008) and four different species of penguins: macaroni penguin, Eastern rockhopper penguin, Gentoo penguin and king penguin (Duriez et al. 2005). With 700,000 breeding pairs, half of the world's population of king penguins live in colonies in Crozet.

2.4 Conservation

In 2007, the Crozet islands became a French Natural Reserve. Scientific research and visitation of the island is limited and authorized by specific ministerial decree.

2.5 Research on king penguins – research station and facilities

Research on king penguins at Possession Island is mostly conducted in one colony, the Baie du Marin (BDM from herein), which consists of 25.000 breeding king penguin pairs and is thus the second largest breeding colony after Jardin Japonais (some 34,200 pairs, Delord et al. 2004). Several research programs are working within this colony and each of them has a work shelter available (Figure 26), while sharing instruments and equipment, such as a -20°C freezer and a centrifuge. The shelters are located within the king penguin colony (historical reasons of construction). At the Alfred Faure Station, which is located about 1.5 km away from BDM, further storage, equipment and material is readily available (-80°C freezer, biochemistry lab, Oroboros, microscopy, etc.)

3 General methods on ornament measurements

In this section, I rapidly present the general methods used to measure ornaments in king penguins. This general methodology has been used throughout the different studies performed in the thesis, and will be re-detailed where appropriate.

3.1 Auricular patch size

To estimate auricular feather patch size, known to be important in mate choice (Jouventin et al. 2005; Pincemy et al. 2009) and likely also in non-sexual social contexts (Viera et al. 2008; Keddar, Jouventin, et al. 2015), we measured the length (L) and width (w) of the ear patch to the nearest 1mm using a flexible tape-ruler as detailed on Figure 27. We then estimated the surface of auricular feather by calculating the surface of the ellipse defined as:

$$Surface = \pi \, x \, L/2 \, x \, w/2$$

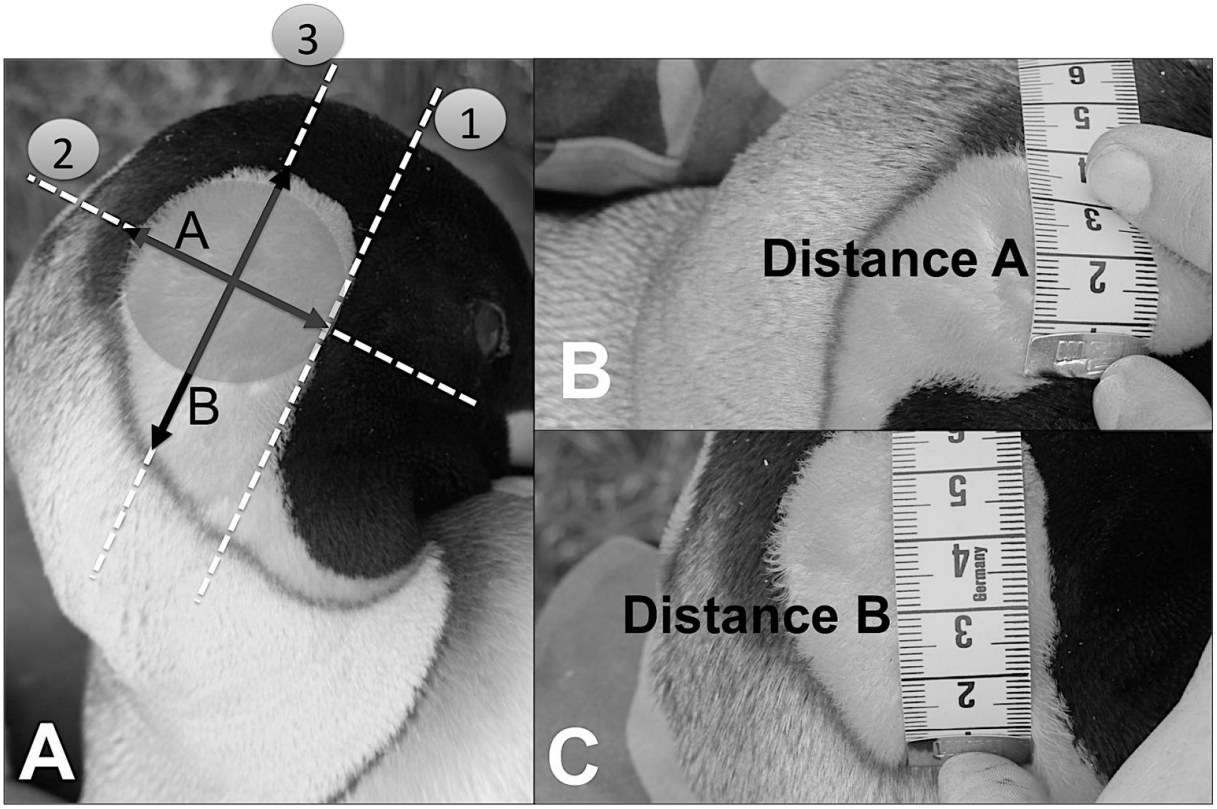


Figure 27: Standardized measures of the auricular patches of breeding king penguin (*Aptenodytes patagonicus*). The head of the bird was held such that its beak rested on the shoulder opposite to the side of the body where the auricular patch was measured (Fig. 27A). A virtual line was pictured along the side of the auricular patch closest to the eye (line 1; Fig 27A). Then, a second perpendicular line reaching the most distant point of the circle (diameter) was pictured (line 2; Fig 27A), and the width of the auricular patch was measured (distance A; Fig 27B). From the center of distance A (line 3; Fig 27A), the height of the auricular patch was measured at a 90° angle (distance B; Fig 27C). Reproduced from Viblanc et al. 2016)

3.2 Colour measurement and spectrometry

To measure the colour ornaments of king penguins (coloration of the beak spots and auricular patches), we relied on spectrometry. Optical spectrometers emit light of constant intensity over 100 to 900 nm. An analyser then captures the reflected light and a spectrum is returned as reflected light intensity as a function of wavelength. In our studies, we used a portable JAZ spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) with a spectral resolution of 0.3 nm across the spectral range of 320–700 nm. Compared to classical bench top spectrometers, this set-up was ideal, as it allowed measuring colour on the birds directly in the breeding colony. The JAZ contains a pulsed-xenon light and was calibrated against a white standard (Ocean Optics Spectralon). Measures were repeated 3 times across each ornament on each side using a 200-µm fiber-optic probe with a 90° angle window. A typical reflectance/absorbance spectrum for king penguins is presented Figure 28.

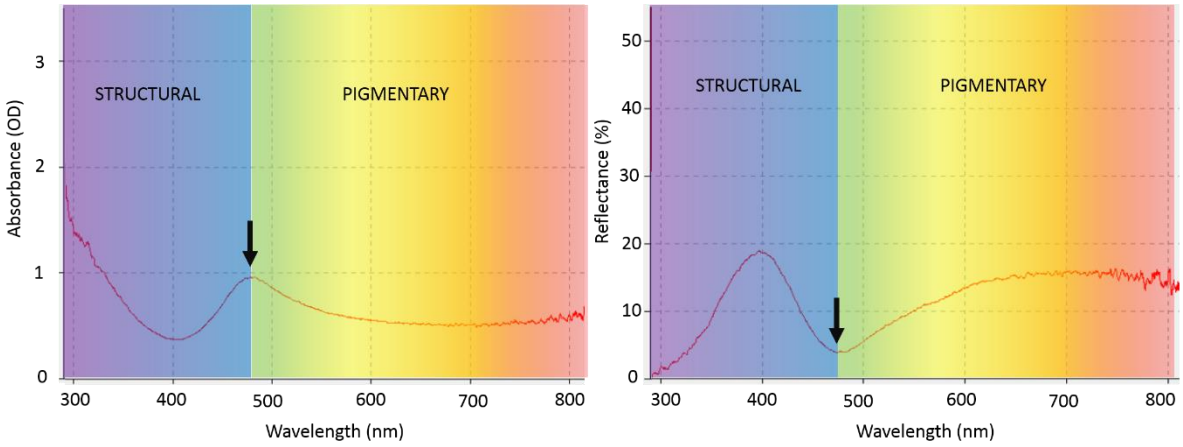


Figure 28. Absorbance and reflectance spectrum of a king penguin beak spot. The absorbance, as the Optical Density (OD) and the Reflectance, as the percentage of light reflected are presented for each wavelength (nm). Note that the spectrum are complementary to each other, the wavelengths absorbed (e.g peak at 490 nm, left panel) are the less reflected (right panel indicated by arrows). The reflected wavelength in the UV to Violet part (300-490) are due to structural properties and the Yellow to Orange (491-700nm) rely on a pigment based absorbance.

3.3 Analyses

Once obtained, the reflectance spectra were smoothed and averaged using an R script adapted from Montgomerie 2008. From those spectra, we calculated variables in the HSB tristimulus colour space, i.e. hue, saturation and chroma, over the spectral range of 320–700 nm, which corresponds to the full range of spectral sensitivity in birds (Cuthill 2006). The reflectance spectrum of king penguin beak spots is composed of a peak in the UV-violet region (320-490 nm) and a plateau in the yellow-orange region (491-700 nm) of the spectrum whereas the auricular patch feathers present only the yellow-orange region of the reflected spectrum (350-700) (Figure 28).

3.3.1 *Colour mean brightness*

The spectral intensity of the ornament, described as mean UV-violet brightness ($UV_{brightness}$) or mean yellow-orange brightness ($YO_{brightness}$) were calculated by averaging reflectance over the 3 different regions defined above (Montgomerie 2006).

$$Mean\ brightness = (\int_{\lambda a}^{\lambda b} Ri\ )/\ nw$$

where a and b are the boundaries of the spectrum, Ri Percentage reflectance at the ith wavelength and n the wavelength intervals between a and b.

3.3.2 *Colour hue*

For the yellow-orange plateau portion of the spectrum, hue (YO_{hue}) was calculated as the wavelength at which reflectance is halfway between its maximum and minimum over 491-700 nm for the beak spot and 350-700 for the auricular patch (Keddar et al. 2013). Thus, we used the formula

$$Hue = \lambda Rmid$$

where Rmid is the wavelength at the reflection of the midpoint between Rmax and Rmin.

For the UV-violet peak of the beak spot, hue (UV_{hue}) was calculated as the wavelength of maximum reflectance between 320 and 490 nm (Rmax).

$$UV\ hue = \lambda Rmax$$

3.3.3 *Colour chroma*

Finally, chroma was calculated within the region of interest (UV_{chroma} and YO_{chroma}) as the difference between maximum (Rmax) and minimum (Rmin) reflectance over the mean reflectance for that particular region (formula S8 in Hill and McGraw 2006, p. 108).

$$Chroma = \frac{(Rmax - Rmin)}{Mean\ brightness}$$

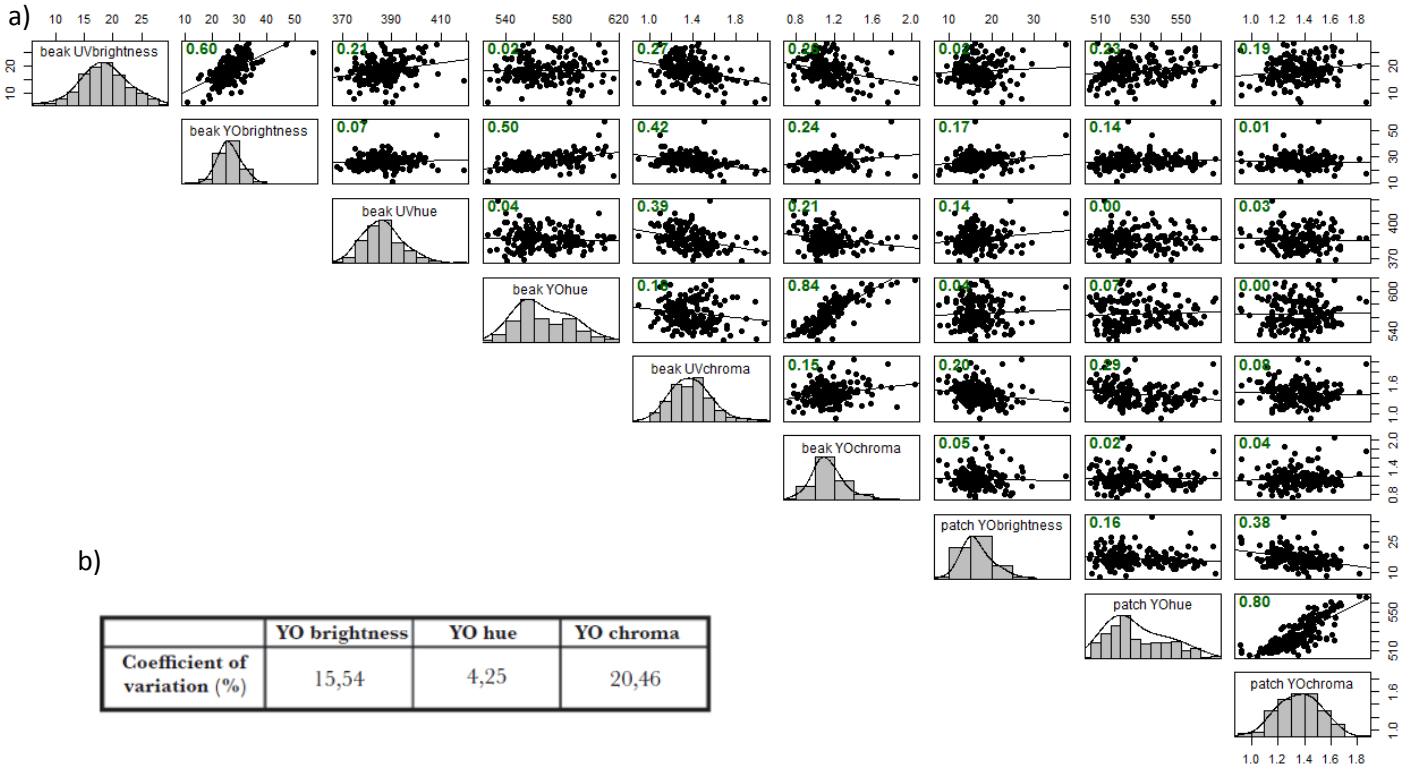
Over all studies, the repeatability of the colour measurements were high (between 0.70 and 0.91, Table 1). Correlations between those spectral parameters are presented in Figure 29a. Briefly, the correlations between UV and YO colour parameters of the beak spot are very low, consistent with the fact that those colours in the king penguin are produced by 2 independent mechanisms (structural for the UV and pigmentary for YO colours; see above section 1.1.4; Dresp et al. 2005; Dresp and Langley 2006). For UV colour, brightness, hue and chroma parameters were independent and therefore we considered them separately in further analyses. In contrast, yellow-orange colour parameters were highly correlated both in the beak and auricular feather patches. Thus, we chose to

Colour parameter	R	95% CI	P-value
$UV_{brightness}$	0.70	0.68 – 0.73	< 0.001
$YO_{brightness}$	0.74	0.71 – 0.76	< 0.001
UV_{hue}	0.91	0.90 – 0.92	< 0.001
YO_{hue}	0.81	0.80 – 0.83	< 0.001
UV_{chroma}	0.75	0.73 – 0.77	< 0.001
YO_{chroma}	0.83	0.82 – 0.85	< 0.001

Table 1: Repeatability of colour measurements. We used the rpt.aov function from the R-package 'rptR' (Nakagawa and Schielzeth 2010) to test (i) if inter individual variability is higher than intra-individual variability (i.e. between the 6 repeated measurements: 3 on each side of the beak of 1 individual; n = 6 for N=1), and (ii) the r coefficient of repeatability. Data are from individuals used in different from birds at different life history stage (moult, parading and breeding) for a total of n= 4595 measurements; N= 810 individuals. Measures collected on the same individuals but at different points of time were considered as independent when computing repeatability.

focus on YO_{chroma} for the beak, as this measure was the one presenting the highest among-individual variation, thus containing the most information (Dale 2006, Figure 29b), and is known to direct reflecting ornament pigment concentrations in several bird species (Saks et al. 2003; McGraw and Gregory 2004). For the auricular patch, YO_{hue} and YO_{chroma} were highly correlated. We thus chose to focus only on YO_{chroma} and $YO_{brightness}$ for the auricular patch analyses.

a)



b)

	YO brightness	YO hue	YO chroma
Coefficient of variation (%)	15,54	4,25	20,46

Figure 29. a) Pairwise correlation plots between the different color parameters in king penguin beak spots. Spearman correlation coefficients are provided and the distribution of the each color parameter is presented. Note that UV and YO color parameters are weakly correlated. b) Coefficient of variation of the different YO color parameters. Measurements of the 181 individuals are represented (36 were in courtship and 145 at the beginning of their breeding shift).

Chapter 1

Experimental stress during moult suggests the evolution of condition-dependent and condition-independent ornaments in the king penguin (Aptenodytes patagonicus)

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1 Abstract

The importance of ornaments in sexual and social selection has been well studied. However, how environmental and physiological factors during development influence the showy nature of sexual and social ornaments, and whether these are similarly affected, remains overlooked. We experimentally manipulated physiological stress and immunity status during the moult in adult king penguins (*Aptenodytes patagonicus*). We studied the consequences of our treatments on the size and colour of auricular feather patches and on the colour of beak spots, which are ornaments under social and sexual selection in this species. We found that some ornamental features show strong condition-dependence (yellow auricular feather chroma, yellow and UV chroma of the beak). This indicates that they are costly to produce and may be honest signals used in mate choice. Other features were condition-independent and remained highly correlated before and after the moult (auricular patch size and beak UV hue), which suggests that their honesty may require social mediation. Notably, because auricular patch yellow chroma and beak UV chroma have been reported as used in mate choice and auricular patch size as reflecting social dominance in king penguins, our study provides a rare examination of the links between ornament determinism and selection processes.

2 Introduction

How conspicuous ornaments, perhaps costly to produce, evolve and are maintained has long been a central question for behavioural ecologists and evolutionary biologists (Andersson 1994; Jones and Ratterman 2009; Kuijper et al. 2012). Darwin (Darwin 1871) laid the groundwork for this topic by observing that conspicuous ornaments could enhance access to sexual partners and thus reproduction, and that their evolution might thus be explained by mate choice and sexual selection. In the second half of the twentieth century however, researchers realized that ornaments could also be used in competition for non-sexual resources, such as access to food and territories during and outside reproduction (West-Eberhard 1983; Rohwer 1985; Tobias et al. 2011; G. E. Hill 2014). Explanations for the evolution of ornaments in a non-sexual context have elegantly been put forward by West-Eberhard who pointed out in her theory of social selection that ornaments can evolve whenever they enhance gene replication due 'to differential success in social competition, whatever the resource at stake' (West-Eberhard 1983). Consequently, sexual selection is a 'subset of social selection in which the resource at stake is mates' (West-Eberhard 1983; Lyon and Montgomerie 2012). However, drawing the line between ornaments that are under sexual versus 'non-sexual social' selection (hereafter 'social selection') can be complicated, because many ornaments have multiple functions. For example, ornaments used for courtship behaviour may also be used for year-round territory defence (Tobias et al. 2011; Tobias et al. 2012).

Regardless of function or selective process, ornaments evolution may depend on how honestly they reflect the health, vigour or dominance of the bearer (Zahavi 1975; Kodric-Brown and Brown 1984; Tanaka 1996). Two alternative mechanisms have been proposed to guarantee signal honesty: condition-dependence and social mediation (Berglund et al. 1996; G. E. Hill 2014). Whereas the condition-dependent signalling hypothesis states that only individuals in prime condition can afford to produce conspicuous ornaments (Kodric-Brown and Brown 1984; Hill 2011; Biernaskie et al. 2014), the social mediation hypothesis suggests that since signals of social status are constantly assessed, only competitive individuals able to defend themselves in aggressive contests can afford to bear them (Rohwer 1977). Social mediation is frequently invoked to explain the occurrence of condition-independent trait production (Hill and Brawner 1998), with the costs being defrayed only after trait production. Hence, an overlooked and intriguing question is whether sexual and social ornaments show condition-dependence or condition-independence in their production.

We addressed this question in the king penguin (*Aptenodytes patagonicus*) which is a brightly coloured bird breeding in large colonies (>10,000 breeding pairs) throughout the sub-Antarctic islands. King penguins display showy ornaments including an orange beak spot containing

carotenoids (McGraw et al. 2007) and specialized stacks of elongated lamellae that also reflect structural UV colours (Dresp and Langley 2006), and orange auricular feather ornaments containing pterin pigments (Thomas et al. 2013). Experimental reductions of beak UV reflectance and auricular patch size decrease the likelihood of pairing and initiating a reproductive event (Jouventin et al. 2008; Pincemy et al. 2009; Nolan et al. 2010), demonstrating their use in mate choice. Correlative studies have also reported that individuals with larger auricular patches are more aggressive (Viera et al. 2008) enabling them to occupy more central breeding territories in the colony expected to be of greater reproductive value (Viera et al. 2008; Keddar, Jouventin, et al. 2015). Those results suggest that auricular patch size may function as a social signal of dominance. There is also support for links between ornament colours and size, and various condition indices, including body condition, stress status, metabolic rate, and innate immunity (Dobson et al. 2008; Keddar et al., 2015b; Schull et al., 2016; Viblanc et al., 2016). Finally, the beak spot ornament is a dynamic signal, reflecting short to medium-term physiological changes in parasite loads and fasting status (Schull et al. 2016). However, experimental studies are now required to test whether trait production (i.e. beak spot and auricular patch coloration and size) is condition-dependent or not. Indeed, a positive association between indices of condition and social ornaments may be explained by social dominance and an increased access to resources rather than by a cost of ornament production itself (e.g. Gonzalez et al., 1999).

Remarkably, in the king penguin, both feather and beak ornaments are renewed each year during a catastrophic moult of the entire plumage (Groscolas and Cherel 1992; Schull et al. 2016). This particular context of a complete renewal (i.e. production) of all ornaments over a short period of time provides an ideal opportunity to investigate the costs of production of ornaments used in sexual and social contexts, and thus to examine whether sexual and social ornaments may differ in their costs of production. To do so, we experimentally subjected moulting birds to increased chronic stress (elevated glucocorticoid levels) or to an immune challenge (lipopolysaccharide LPS injection). Both chronic and immune stress are energy costly processes that can divert resources investment from the production of showy ornaments (Folstad and Karter 1992; Faivre, Grégoire, et al. 2003; Bortolotti et al. 2009), so we predicted that increasing stress through glucocorticoid manipulation or immune stimulation should hinder the ability of treated birds to invest in showy ornaments during the moult when compared to control individuals.

3 Material and Methods

3.1 Study species and experimental procedures

Experiments were performed in the breeding colony of "La Baie du Marin" on Possession Island, Crozet Archipelago (46° 26' S, 51° 52' E, South Indian Ocean) over two consecutive field sessions: from November 2014-February 2015 and from November 2015-February 2016.

In 2014-2015, we subjected 30 moulting birds (15 treated, 15 controls) to an experimental increase in baseline glucocorticoid levels. Treated birds were implanted with a subcutaneous corticosterone (CORT, G-111) pellet in the middle of their back, just above the hip-line. Control birds were implanted with a placebo (SHAM implants, C-111, ©Innovative Research of America) (see Spée et al., 2011; Thierry et al., 2013 for a description in Adélie penguins). Both implants were designed to diffuse over a 21-day period. For CORT implants, this represented a 100 mg CORT release over the 21-day period. Similar implants in the Adélie penguin have been shown to result in a 2.4 times increase in baseline CORT levels thus staying within the natural range observed in penguins, but mimicking a late fasting stage in this species (Spée et al. 2011; Thierry et al. 2013). Breeding Adélie penguins present similar circulating CORT levels as king penguins during the moult (Bourgeon et al. 2007).

In 2015-2016, we subjected 30 birds (15 treated, 15 controls) to an experimental immune challenge. Treated birds were injected 4 times (once every third day) with 2mg of lipopolysaccharide (LPS) diluted in 1mL of physiological serum (LPS from *Escherichia coli* 0111:B4 © Sigma Aldrich). The LPS dose injected (ca. 0.13 mg/kg) was 38 times lower than that typically used in poultry studies (5mg/kg; Cheng et al. 2004), yet local inflammation (swelling) at the site of injection was systematically observed. Control birds were injected with 1mL physiological solution only (SHAM).

3.2 Morphometric and ornamental measures

In both years, we obtained measurements of body mass and colour ornaments at the beginning and end of the experiment. Both experimental and control birds were initially measured at the start of the moult prior to providing the implants, and were measured a second time when caught after returning from their post-moult foraging trip in order to court and breed.

When initially caught, birds were transported to a nearby dry shelter (within 10 m of the colony) and their body mass was measured to the nearest 2g using an electronic scale. Flipper length (indices of structural size) was also measured to the nearest 1mm using a solid metal ruler. We then regressed body mass on flipper size ($F = 5.24$, $P = 0.036$, $R^2 = 0.25$ in 2015; $F = 1.32$, $P = 0.262$, $R^2 = 0.05$ in 2016) and used the residuals as an index of body condition.

Colours reflected by the beak spot and the auricular patches were measured using a portable

JAZ spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) containing a pulsed-xenon light with a spectral resolution of 0.3 nm across the spectral range of 320-700 nm, and was calibrated against a white standard (Ocean Optics Spectralon). Measures were repeated 3 times on each ornament (on both sides of the bird) using a 200 µm fibre-optic probe with a 90° angle window. Reflectance spectra of given ornaments were smoothed and averaged using an R script adapted from Montgomerie (Montgomerie 2008). The obtained spectra were used to calculate mean brightness, hue, and chroma (see below) over the spectral range 320-700 nm, which corresponds to the full range of spectral sensitivity in birds (Cuthill 2006). King penguin beak spots show a reflectance peak in UV-violet (320-490nm) and a plateau in the yellow-orange portion (491-700 nm) of the spectrum (Schull et al. 2016), and we calculated colour variables separately over those 2 regions. In contrast, feathered auricular-patches contain a pterin based pigment (Thomas et al. 2013), only reflective above 450 nm. The spectral intensity, mean brightness ($UV_{brightness}$ and $YO_{brightness}$) was calculated by averaging reflectance over wavelengths 320-490 nm and 491-700 nm for the beak; and 450-700 nm for the auricular patch (Montgomerie 2006). Hue is a measure of colour appearance (e.g. 'blue', 'yellow', etc.). For the yellow-orange plateau portion of the spectrum, YO_{hue} was calculated as the wavelength at which the reflectance was halfway between its maximum and minimum (Keddar et al. 2013). For the UV-violet colour of the beakspot, UV_{hue} was calculated as the wavelength of maximum reflectance between 320 and 490 nm. Finally, chroma is a measure of colour purity and was calculated within the region of interest (UV_{chroma} and YO_{chroma}) as the difference between maximum and minimum reflectance over the mean reflectance for that particular region (formula S8; Hill and McGraw 2006, p. 108). In the king penguin, correlations between beak UV colour parameters based on a large sample in previous experiments show that brightness, hue and chroma signal different information to breeding birds (for a discussion, see Schull et al. 2016 and ESM 1 therein). In contrast, yellow-orange colour parameters are highly correlated both in the beak and ear feather patches (see Schull et al. 2016 and ESM 1 therein). We thus chose to focus on YO_{chroma} for both beak and auricular patch analyses, as this measure was the one presenting the highest among-individual variation (thus containing the most information; Dale 2006), and directly reflects ornament pigment concentrations in several bird species (Saks et al. 2003; McGraw and Gregory 2004).

3.3 Statistical analyses

All analyses were run in the statistical computing software R (v. 3.1.1; R development Core Team 2013). Differences between treated and control groups at the beginning of the moult and when returning from the post-moult foraging trip for breeding were investigated using linear models (LMs). We succeeded in recapturing 10 CORT and 8 control birds in 2015, and 13 LPS and 13 control birds in 2016, explaining the slight variation in sample sizes in our various analyses. Whereas only males were used in the LPS experiment (2016), 2 out the 10 CORT individuals and 2 of the 8 control birds in 2015

were females. Preliminary analyses show no significant effects of the factors sex, alone (LMs; 0.012 < F < 2.416, 0.142 < P < 0.914) or in interaction (LMs; 0.078 < F < 2.873, 0.114< P < 0.785) with the CORT treatment; thus the factor sex was not retained in the final analyses. Birds were sexed using morphological and behavioural traits. We found no evidence of a bias in size or coloration before the moult between individuals that were subsequently recaptured or not when returning from the post-moult foraging trip (LM models testing for effect of recapture [yes/no] alone or in interaction with treatment: 0.003 < F < 2.304; 0.139 < P < 0.955). Birds' initial body condition index at the entrance of the moult was considered as a covariate in the analyses to account for individual initial condition effects on colour parameters and on the size of the auricular patch. For analyses on the size of the auricular patch, individual flipper size (as a size proxy) was also controlled for as a covariate in the analyses. Relations between body condition and colour parameters were investigated using linear models with body condition index as the response variable and all ornamental features as fixed factors. We then used Akaike´s information criterion corrected for small sample size (AICc) for model selection following a backward stepwise procedure. Correlations between colour variables (hue, chroma and brightness) before and after moult were investigated using Pearson correlation tests. F-statistics for fixed effects (tests of differences from zero) and P-values are given. Effects were considered significant for P < 0.05. Residuals were visually inspected for normality using qqplots (opposing theoretical quantiles to sample quantiles).

3.4 Ethical statement

All experiments were approved by independent ethics committees (Comités d'éthique Midi-Pyrénées et Alsace pour l'expérimentation animale) and comply with the current laws in France. Authorizations to enter the breeding colony and handle the birds were provided by the "Terres Australes et Antarctiques Françaises" (permit n°2014-127 issued on the 15th of October 2014 and APAFIS#375 issued on the 17th of July 2015).

4 RESULTS

4.1 Experiment 1: Corticosterone manipulation

At moult onset, CORT-treated and control birds did not differ significantly in terms of ornamental features (LMs; treatment: 0.02 < F < 0.93, 0.341 < P < 0.964; Fig. 1A) or body condition (LM; F = 0.18, P = 0.67). When returning from their post-moult foraging trip to court, birds treated with a CORT implant had significantly lower YO_{chroma} of auricular patches (LM; F = 7.16; P = 0.043) (Fig. 1B&C). The CORT treatment had no effect on body condition (LM; F = 0.48, P = 0.500), beak spot colouration (LM; 0.066 < F < 1.812, 0.198 < P <0.801) and auricular patch size (LM; F = 0.12; P = 0.736; Fig. 1B&C). The correlation between beak UV_{hue} before and after the moult was high (r = 0.59, t = 2.85, P =

0.012), as was that of auricular patches size (r = 0.67, t = 3.53, P = 0.003); other relations were non-significant (0.099 < P < 0.997) (Fig. 2).

Birds' initial body condition was only related to the YO_{chroma} (estimate = 7.33 ± 1.88; F = 28.93, P < 0.001) and size (estimate = 0.391 ± 0.113; F = 14.37, P = 0.002) of the auricular patch. No significant links were found between body condition at their return from the post-moult foraging trip and ornamental features (LM; 0.01 < F < 1.92, 0.199 < P < 0.979).

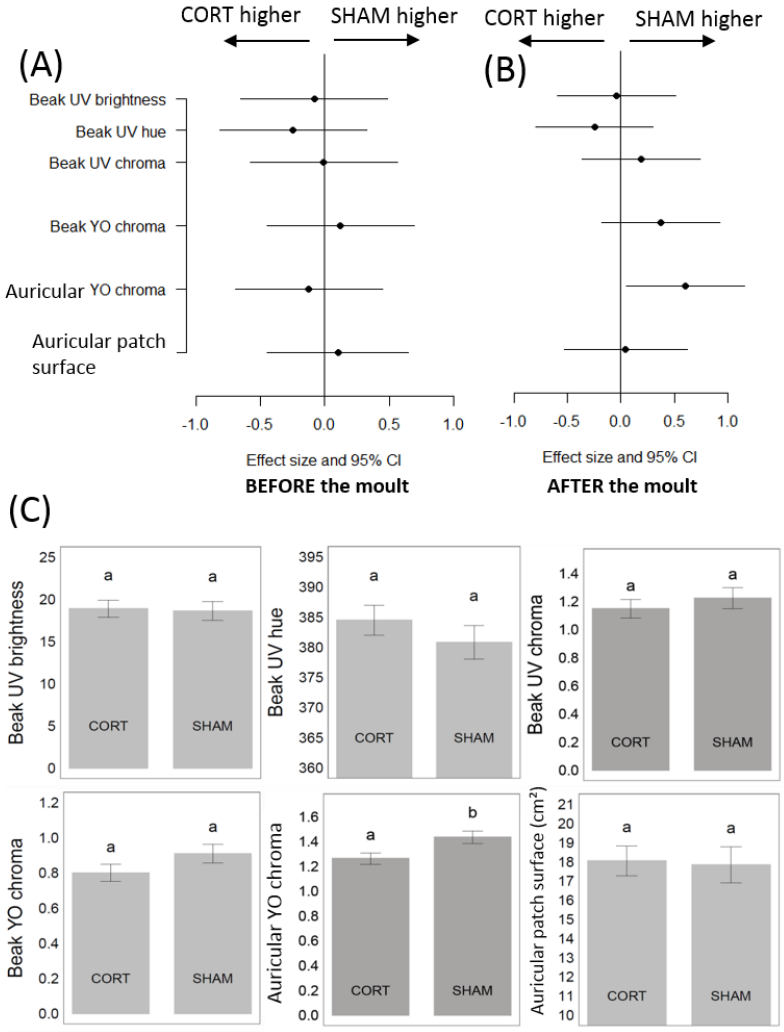

Figure 1. Comparison of beak and auricular patch colour variables (controlled for body condition at the beginning of the moult), and auricular patch surface (controlled for structural size & body condition) for king penguins (*Aptenodytes patagonicus*) treated at moult initiation with a corticosterone (CORT) implant or a sham implant. Effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007 for colour variables measured at (A) moult initiation and (B) after the moult. Effects are considered significant if their 95% CI do not overlap zero. Panel C represents marginal means (± SE) of colour variables measures after the moult in CORT and sham treated penguins.

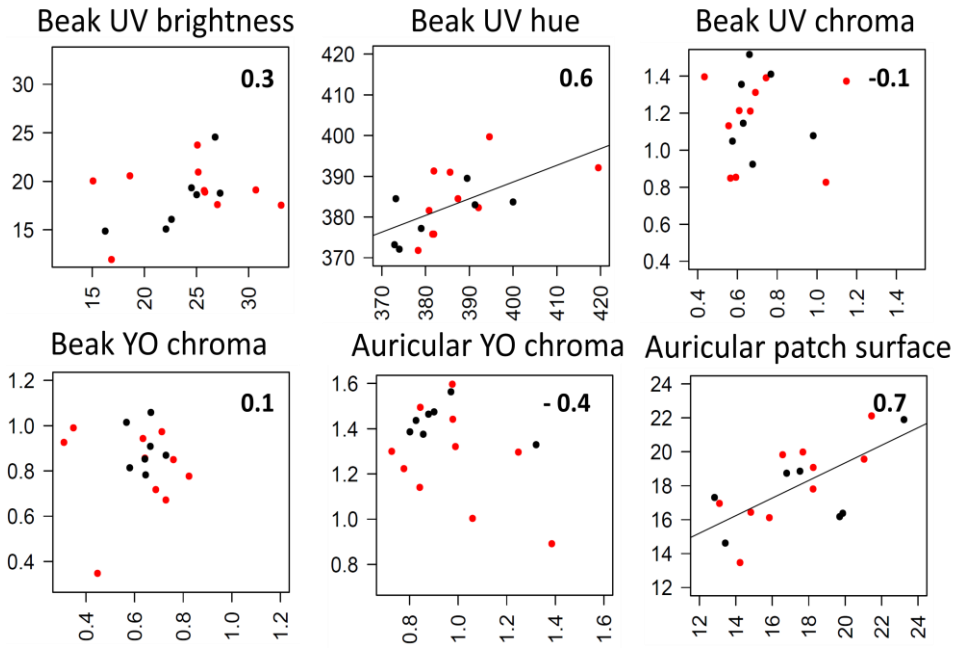

Figure 2. Correlation of beak, auricular patch colour variables, and auricular patch surface before (x-axis) and after the moult (y-axis), for the same king penguin (*Aptenodytes patagonicus*). Pearson correlation coefficients are given in the top right corner. CORT and sham treated birds are highlighted with red and black dots, respectively. A regression line is presented when the association is significant.

4.2 Experiment 2: LPS immune challenge

At moult onset, both treated (LPS) and control birds were similar in terms of ornamental features (LMs; treatment: $0.89 < F < 2.67$, $0.12 < P < 0.36$, Fig. 3A) and body condition (LM; $F = 0.45$, $P = 0.509$). When returning from their post-moult foraging trip to court, both beak UV_{chroma} and the YO_{chroma} of the auricular patches were lower in LPS treated birds compared to control birds (LMs; $F = 5.98$; $P = 0.023$ and $F = 9.60$; $P = 0.005$, respectively, Fig. 3 B&C). The LPS treatment has no significant effect on body condition (LM; $F = 0.642$, $P = 0.432$), on the other colour parameters, and on the size of the auricular patches (LMs; treatment: $0.11 < F < 1.02$, $0.324 < P < 0.743$; Fig 3. B&C). Here again, the correlations for beak UV hue and auricular patch size measured before moult and after the moult were high ($r = 0.65$, $t = 4.25$, $P < 0.001$ and $r = 0.44$, $t = 2.41$, $P = 0.024$ respectively); other relations were non-significant ($0.123 < P < 0.698$) (Fig. 4).

Initial body condition was related to auricular patch YO_{chroma} (LM; F = 4.67, P = 0.041). However, other parameters including the previous significant relation with auricular patch size (see Experiment 1) were not significantly related to initial body condition (LMs; 0.26 < F < 4.67; 0.157 < P < 0.609). No significant links were found between body condition at their return from the post-moult foraging trip and ornament features (LM; 0.12 < F < 1.37, 0.257 < P < 0.738)

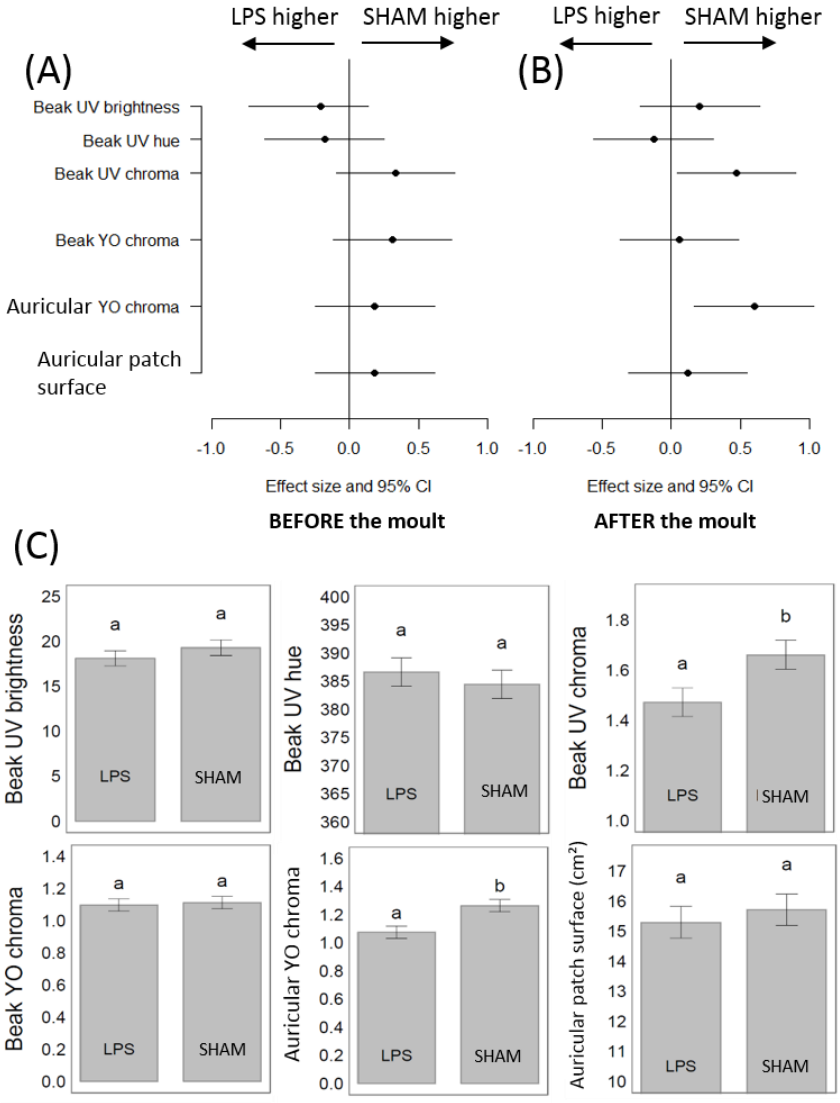

Figure 3. Comparison of beak and auricular patch colour variables (controlled for body condition at the beginning of the moult), and auricular patch surface (controlled for structural size& body condition) for king penguins (*Aptenodytes patagonicus*) treated at moult initiation with Lipopolysaccharide (LPS) or physiological serum (sham). Effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007 for colour variables measured at (A) moult initiation and (B) after the moult. Effects are considered significant if their 95% CI do not overlap zero. Panel C represents marginal means (±SE) of colour variables measures after the moult in LPS and sham treated penguins

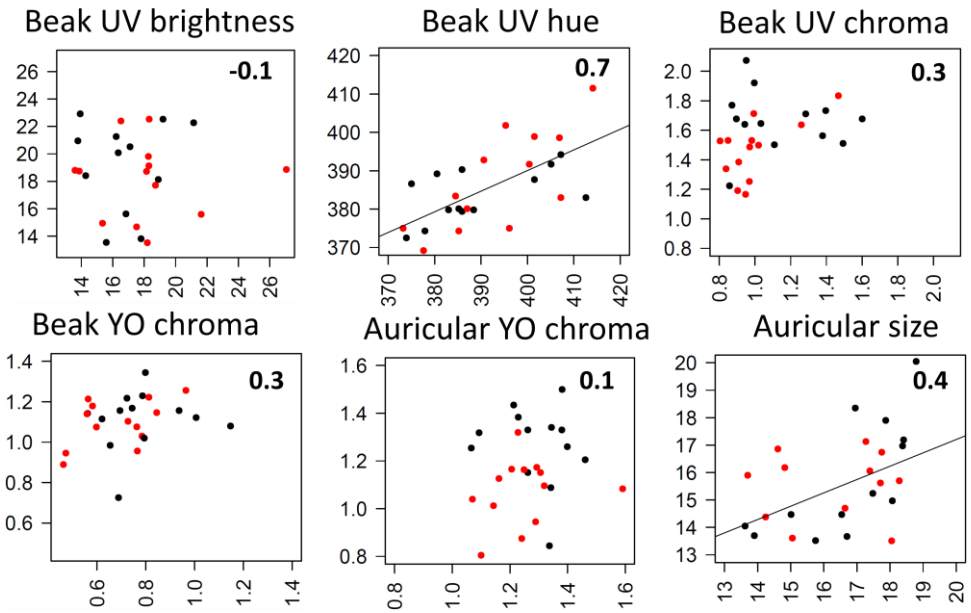

Figure 4. . Correlation of beak, auricular patch colour variables, and auricular patch size before (x-axis) and after the moult (y-axis), for the same king penguin (*Aptenodytes patagonicus*). Pearson correlation coefficients are given in the top right corner. LPS and sham treated birds are highlighted with red and black dots, respectively. A regression line is presented when the association is significant.

5 Discussion

Ornaments can be used in sexual and/or social interactions (West-Eberhard 1983; Rohwer 1985; Tobias et al. 2011; G. E. Hill 2014) and regardless of the selective process are expected to honestly reflect the individual health, vigour or social status in competitive contexts (Zahavi 1975; Kodric-Brown and Brown 1984; Tanaka 1996). Ornament honesty can be enforced by production costs (condition-dependent signalling hypothesis; Biernaskie et al., 2014; Hill, 2011; Kodric-Brown and Brown, 1984) or by the costs of bearing them in a social group (social mediation hypothesis; Rohwer, 1977). In the first case, ornaments are expected to be condition-dependent whereas costs are expected to be defrayed after ornament production in the second case. Those mechanisms highlight the existence of condition-dependent and condition-independent ornaments (in terms of production costs), where the costs may be best explained by the use of ornaments in sexual or social interactions, respectively. Here, we investigated production costs of ornaments in adult king penguins by manipulating physiological stress and immunity status during moult. This bird species undergoes a moult where all the ornaments are regrown over a short time period (Cherel, Leloup, et al. 1988; Bourgeon et al. 2007; Schull et al. 2016). After the moult, penguins return at sea to forage and rebuild their body reserves before engaging into courtship and a new breeding cycle (Stonehouse 1960; Weimerskirch et al. 1992). Our experiments challenging moulting penguins with

CORT or LPS treatments show that some ornamental features were strongly affected by our treatments (auricular patch YO_{chroma} and beak spot UV_{chroma}), while no evidence for an effect of our treatments were found on other ornamental features (auricular patch size and beak spot UV_{hue}). Those results support the common view that ornaments can be divided into condition-dependent and condition-independent features (Berglund et al. 1996; G. E. Hill 2014). More notably, because auricular patch and beak spot UV features have been reported to be used in mate choice (Nolan et al., 2010; Pincemy et al., 2009) and auricular patch size to reflect social dominance in king penguins (Keddar et al., 2015a; Viera et al., 2008; but see also Jouventin et al. 2008; Pincemy et al. 2009; Nolan et al 2010 for links between patch size and rate of pairing), our results suggest that the dichotomy in ornamental costs could be linked to the main use of ornaments in sexual or social interactions.

5.1 Condition-dependent ornaments

The condition-dependent signalling hypothesis points out that the honesty of ornaments can come from unavoidable physiological or developmental costs of production (i.e. preventing dishonest signals to be (Kodric-Brown and Brown 1984; Hill 2011; Biernaskie et al. 2014). It predicts the existence of a trade-off in energy or resource allocation between ornament and self-maintenance. Thus, challenging individuals during the production of ornaments (e.g. the moult) by increasing the energy demand into self-investment is expected to highlight trade-off investments between those processes. Accordingly, ornaments that changed in response to CORT and LPS treatments showed a decrease in the purity of ornamental colours. Birds treated with CORT moulted new auricular patches for which feather YO_{chroma} was lower than controls. Similarly, individuals repeatedly injected with LPS moulted beak spots and ear feather patches for which UV_{chroma} and YO_{chroma}, respectively, were both lower than for controls.

The so called 'stress' hormone CORT is under the regulation of the hypothalamic-pituitary-adrenal (i.e. HPA) axis, and its release modulates energy allocation to allow individuals to overcome challenging environmental conditions (Angelier et al. 2010). At short time scales, CORT release induces a rapid mobilisation of energy resources preparing the organism to cope with challenging situations (Exton et al. 1972; Exton 1979; Xu et al. 2009; Peckett et al. 2011). Chronically elevated CORT leads to a re-allocation of the reserves into survival instead of other functions (Axelrod and Reisine 1984; Johnson et al. 1992; Angelier and Chastel 2009; Spée et al. 2010), for example by inhibiting vocalizations (Spencer et al. 2005; Macdougall-Shackleton et al. 2009), coloration (Roulin et al. 2008), courtship (Moore and Miller 1984) and breeding behaviour (Groscolas et al. 2008; Angelier et al. 2009; Spée et al. 2010). In this study, penguins experimentally treated with a CORT implant may

have invested less in the endogenous synthesis of pterin resulting in a decreased allocation of this pigment in their ornament reflected by a lower YO_{chroma} of the auricular patch feathers. Although changes in YO_{chroma} have been directly linked to carotenoid pigment concentrations in feathers in other bird species (L Saks et al. 2003; McGraw and Gregory 2004), yellow-orange coloration in the auricular patch of the king penguin (Thomas et al. 2013) is due to pterins (and not carotenoids). However, the costs associated with pterin endogenous synthesis and allocation remain unclear. Because pterins have been proposed to have antioxidant or immune functions (Oettl and Reibnegger 2002; Oettl et al. 2004; McGraw 2005; Weiss et al. 2011), those pigments could have been devoted to protection against CORT-induced oxidative stress (Costantini et al. 2011) at the expense of auricular patch coloration.

The immunocompetence handicap hypothesis (which is a sub-hypothesis of the condition-dependent signalling hypothesis) proposes that the production of ornaments comes at the expense of resistance to disease and parasites (Hamilton and Zuk 1982; Folstad and Karter 1992). Several studies have shown positive links between immune efficiency and ornaments, notably ornamental colouration relying on pigment availability (Blount et al. 2003; Faivre, Préault, et al. 2003; Aguilera and Amat 2007), and reported a decrease in pigment based colouration in immune-challenged individuals (Faivre, Grégoire, et al. 2003; Rosenthal et al. 2012). Thus, a trade-off in the allocation of pigments towards colourful ornaments or immune functions may explain the honesty of some ornamental features. In this study, stimulating the immune function of moulting king penguins lead to decreased purity in the yellow-orange colour of auricular patch feathers (lower YO_{chroma}), which presumably came from the allocation of pterins to immune functions at the expense of auricular patch coloration. The LPS treatment also induced a decreased in purity of the UV colouration of the beak spot (UV_{chroma}). Conversely to YO_{chroma} coloration of the auricular patch feathers that is due to pigments, beak spot UV reflectance in the king penguin has a structural basis resulting from crystal-like photonic microstructures in the horny layer of the beak (Dresp et al. 2005; Dresp and Langley 2006). Our results suggest that the production of that photonic structure may be costly, especially in response to an immune challenge. A previous study where king penguins were treated with an antiparasitic solution outside the moulting period (i.e. during breeding) showed a strong increase in beak spot UV hue and brightness and a weak decrease in UV chroma after parasite removal (Schull et al. 2016). Although those results support a link between beak spot UV coloration, immunity and parasitism, we would have expected an increase in UV chroma in response to parasite removal in the Schull et al. (2016) and/or a decrease in UV hue and brightness in response to LPS treatments. This calls for a deeper understanding of the physiological and structural mechanisms leading to changes in UV hue, brightness and chroma, notably the importance of production and maturation of photonic

structures during the moult versus maintenance and modulation of those structures outside the moult (i.e. during breeding).

5.2 Condition-independent ornaments

Previous studies have shown that environmental factors encountered during ornament production (parasites, food shortage or other stressors) do not necessarily strongly impact their expression (Hill and Brawner 1998; Roulin et al. 1998). In other words, the production/expression of those ornaments may be viewed as condition-independent, raising questions about what factors enforce their honesty (G. E. Hill 2014; Roulin 2016). Here, we found no evidence that auricular patch size and beak spot UV hue (UV_{hue}) were affected by CORT and LPS treatments, and both traits show similar expression before and after the moult. A similar finding for beak spot UV_{hue} has already been observed in Schull et al. 2016. Those findings indicate that the expression of auricular patch size and beak spot UV_{hue} is maintained over time, which in turn suggests that their expression is largely determined by genetic or by developmental factors encountered early in life that have lifelong consequences (i.e. silver spoon effects; Metcalfe and Monaghan, 2001; Minias et al., 2015; Tilgar et al., 2010). One hypothesis is that the honesty of condition-independent ornaments is enforced by social mediation (Rohwer 1977; West-Eberhard 1983). This hypothesis predicts that such ornaments should mirror social status and be constantly assessed during competitive interactions (Rohwer 1977; Rohwer 1985). Accordingly, auricular patch size in king penguin has been previously suggested to act as a social status badge, signalling individual competitiveness (Viera et al. 2008; Keddar, Jouventin, et al. 2015). Individual penguins bearing larger auricular patches appear to be more aggressive and defend central breeding areas (Viera et al. 2008; Keddar, Jouventin, et al. 2015) thought to be of higher quality (Bried and Jouventin, 2008; but see Descamps et al., 2009; Viblanc et al., 2014). The role of beak UV hue in social or sexual interactions is unknown. Experimental manipulation of auricular patch size (or beak UV hue) is now required to demonstrate whether wearing large auricular patches (or high UV wavelengths on the beak) entails socially mediated costs.

An alternative hypothesis for the evolution of condition-independent ornamental expression in response to environmental challenges is that ornaments reflect the genetic quality of their bearer (i.e. the good gene hypothesis Hamilton and Zuk, 1982; Hill, 2014a; Roulin et al., 1998). However, the mechanisms linking individual genetic and epigenetic variation to trait production remain elusive. It has been recently proposed that ornamental production could be constrained by the efficiency of vital cellular processes, with genes encoding for cellular metabolic pathways playing a key role in enforcing signal honesty (Hill 2011; G. E. Hill 2014). Because the mitochondrion is the powerhouse of the cell, variation in mitochondrial and nuclear genes, epigenetic status, and mito-

nuclear interactions could account for inter-individual genetic variation in ornament expression (Hill and Johnson 2013; G. E. Hill 2014). We have investigated elsewhere whether ornament expression mirror mitochondrial function in red blood cells of breeding king penguins (see below Chapter 3b). Results based on 36 breeding males revealed links between mitochondrial endogenous respiration and beak UV brightness but not UV hue (see below Chapter 3b). Work using larger sample sizes, individuals of both sexes and investigating mitochondrial function during and outside ornamental production may provide insightful information on the honesty of UV signals in the king penguin, and more generally on the importance of mitochondrial efficiency as a mediator of signal honesty in the animal kingdom (Hill 2011; Johnson and Hill 2013; G. E. Hill 2014).

6 Conclusion

Taken together, our study provides evidence for the evolution of condition-dependent and condition-independent ornament expression in the King penguin, and suggests that variation in the cost of ornament expression could be rooted in their main use for mate attraction or for signalling social status. The expression of condition-dependent ornaments was revealed by the fading in yellow-orange auricular feathers, most likely explained by a low deposition of pterin pigments in those feathers. Despite pterins being endogenously synthetized pigments, this finding suggests the evolution of a trade-off in the allocation of pterin pigments to stress responses, such as antioxidant and immune functions, at the expense of colourful ornaments.

Chapter 2 – The beak spot, a dynamic ornament

1 Preface

An intriguing observation one can make when observing king penguins walking out of the cold (5-6°C) Southern Ocean is that they display a particularly colorful red-pinky beak spot (Figure 1) that rapidly changes to yellow-orange once the birds are on dry land. The study described in the following chapter is based on that observation.

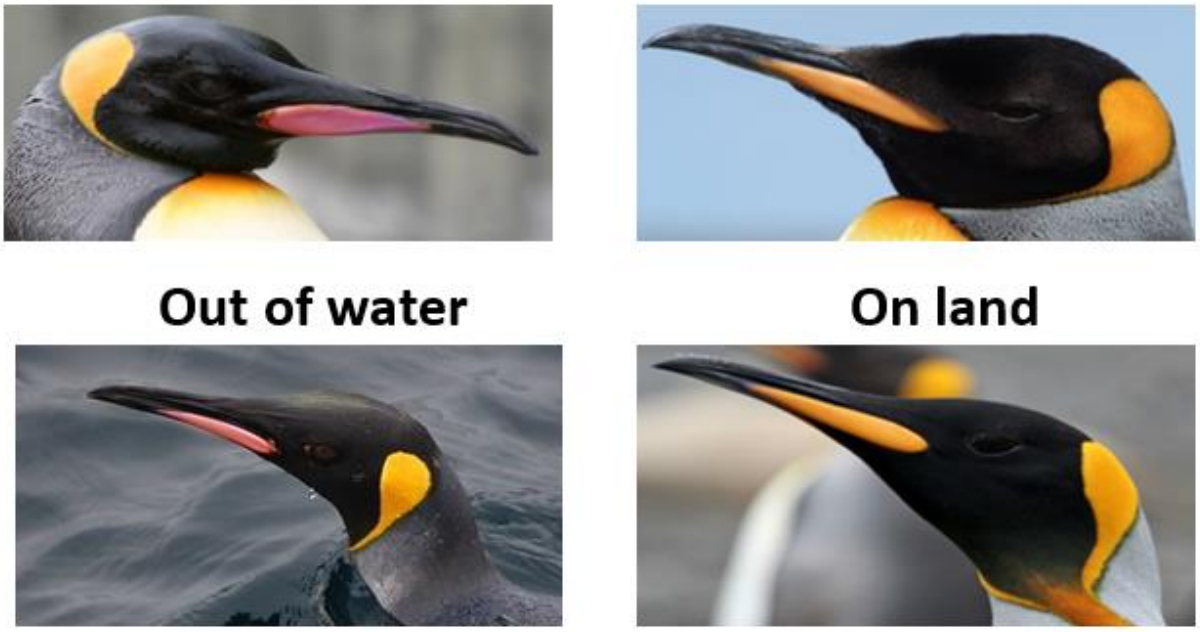

Figure 1: Pictures of adult king penguins when they arrive ashore (left panel) and in the breeding colony (right panel). Penguins alternate between a cold (5-6 °C) and wet marine environment for foraging and a dry and temperate terrestrial environment. King penguins that just arrive ashore have deep red-pink to violet beak spots, and birds in the colony always show bright yellow-orange beak spots.

1.1 Short-term changes in beak spot colouration after arrival on-shore

During reproduction, king penguins face stressful situations induced by the negative social interaction they constantly perform to defend their territory (V. A. Viblanc et al. 2012; Vincent A Viblanc et al. 2014). Being exposed to repetitive stress as well as immune infections can raise the body temperature level (Long et al. 1990; Gray et al. 2013). Previous study on crested caracara (*Polyborus plancus*) and the hooded vulture (*Necrosyrtes monachus*) showed that rapid changes in integument colouration may signal information to conspecifics related to blood flow and

vasoconstriction (Negro et al. 2006). The beak of king penguins is formed of conjunctive vascularized tissue covered by a keratin based coloured structure (Dresp et al. 2005; Jouventin et al. 2005). Body temperature of king penguin is often challenge by alternating between cold water and temperate air onshore. Hence, changes in beak colouration after individuals emerged from Antarctic waters might have two explanation: 1) changes in beak dryness or 2) a thermoregulation basis, explained by change in blood flow to the beak as birds warm-up onshore. We studied variation in beak spot colouration in response to proximal abiotic factors in an in vivo situation to investigate if those mechanisms are relevant. For that, we considered both the effects of moisture and temperature on beak colouration, possibly linked to changes in body temperature regulation.

Short-term changes in beak colouration were investigated in 12 adults that were measured within 5.25 ± 0.65 min after their arrival on shore (i.e. at T_0) in a shelter located in the vicinity of the colony. The birds were then kept captive in a pen for an hour and 2 more measures of beak colouration were taken at 30 and 60 minutes (subsequently; T_{30} and T_{60}). Following T_{60}, we investigated whether changes in colouration were explained by changes in beak blood flow as individuals warmed-up. We experimentally cooled-off the beak by applying a bag of ice for 5 minutes to the beak-spot and took a final spectral measure (T_{cold}). If exposure to cold water (and in turn vasoconstriction) explains changes in beak colouration, we expected that our cold treatment should reset T_{cold} beak colouration to values similar to the T_0 beak colouration. To investigate the effect of moisture on beak colouration, we performed a second experiment on two groups of twelve birds that were captured immediately as they came out of the water and assigned either to a drying group or a control group. In the drying group, we collected an initial measure of beak colouration shortly after the birds came out of the water (T_0), we then enhanced beak drying by gently blowing ambient air (using an air dryer at ambient temperature) on the beak spot for 5 minutes before taking a second measure (T_5). We took a final measure after leaving the birds to rest undisturbed for 30 minutes (T_{30}). Birds in the control group were handled and measured as birds in the drying group with the exception that we did not experimentally enhance the drying of their beak. There was no difference between birds in the drying and control groups in the time of their initial measure after coming out of the water (2.92 ± 0.23 min versus 3.25 ± 0.35 min; *t*-test: $t = -0.79$, $df = 22$, $P = 0.43$).

Changes in beak colouration after arrival on shore were investigated by specifying beak colour variables (hue, chroma and brightness) as dependent variables in separate LMMs. The time period for colour measurements was entered as a discrete ordinal independent variable (e.g. T_0, T_5, T_{30}, T_{60}, T_{cold}). Bird ID was entered as a random effect to account for repeated measurements on individual birds.

1.2 Beak colouration changes under local environment

Following bird arrival on shore, there were substantial changes in some of the colouration features of the beak spot (Fig. 2). Whereas $YO_{brightness}$, and $UV_{brightness}$ remained relatively constant (LMMs; 1.32 < F < 1.82, df = 2, 0.18 < P < 0.29, n = 36, N = 12), UV_{hue} and YO_{hue} decreased significantly in the 30 first

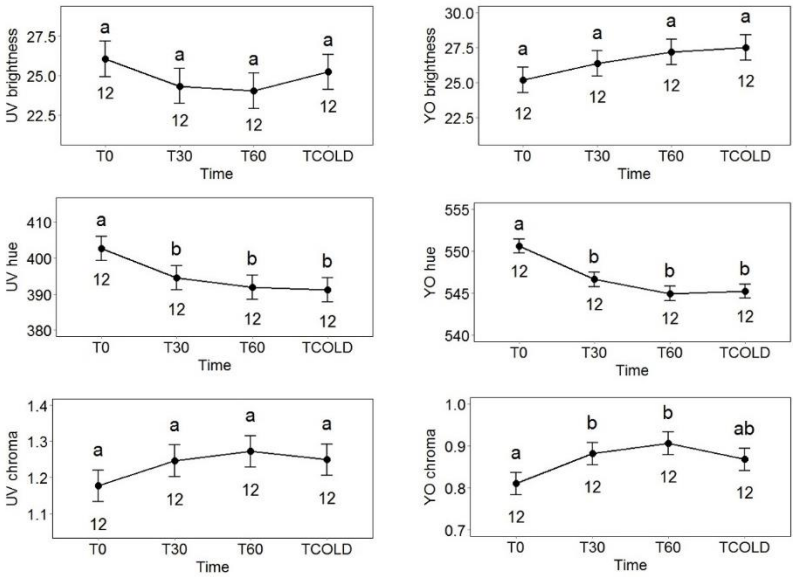

Figure 2. Changes over time in the beak coloration of 12 adult king penguins that were caught as they came out of sea and measured at times 0, 30, 60 and subsequently cooled-off for 5 minutes. The changes in raw spectral data for one individual are presented for illustrative purposes. Note the overall general shift of the spectra to lower wavelengths as time goes by. Changes in brightness, hue and chroma over time over all birds were assessed using LMMs, with bird ID specified as a random variable, and time passed between capture and first spectral measurement as a covariate. Least-Square means ± SE are presented. Values not sharing a common letter are significantly different for P < 0.05.

minutes of bird arrival on shore (F = 37.61 and 19.25, respectively, df = 2, P < 0.001, n = 36, N = 12). Running these analyses with time specified as a continuous variable yielded identic results. UV_{chroma} and YO_{chroma} showed an increase in the first 30 minutes following the arrival of birds on-shore (F = 7.67 and 11.06, respectively, df = 2, P < 0.001, n = 36, N = 12). Cooling-off the beak spot for 5 minutes following the T_{60} measurement did not affect beak colouration (LMMs between T_{60} and T_{COLD}: 0.01 < F < 1.48, df = 1, 0.31 < P < 0.78, n = 24, N = 12; Figure 2).

Notably, beak spot colouration of the 12 air-dried birds showed a more rapid shift in UV_{hue} and YO_{hue} (other colour parameters did not substantially change compared to controls; LMMs; all P > 0.07). Between 0 and 5 minutes of drying, air-dried birds showed shifts in UV_{hue} and YO_{hue} that were twice as big as those that dried naturally (Figure 3; t-tests; t = -3.8, df = 22, P < 0.001, and t = -2.8, df = 22, P = 0.01). Changes in colouration between T_{+0} and T_{+30} were similar for birds air-dried or that dried naturally (t-tests; -1.04 < t < 0.24, 0.31 < P < 0.81).

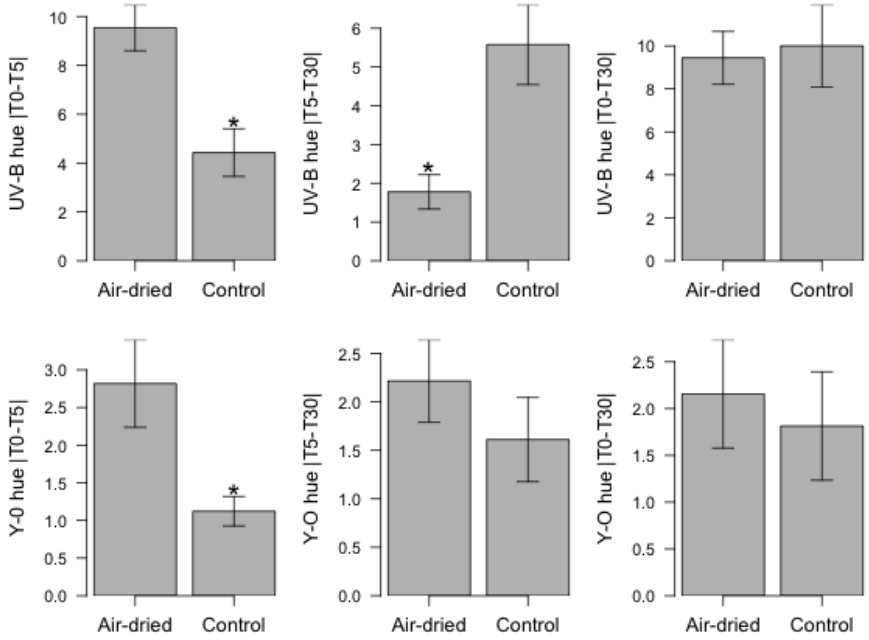

Figure 3. Absolute change in UV and yellow-orange hue in the beak colouration of 24 adult king penguins (*Aptenodytes patagonicus*) that were caught as they came out of sea and measured at times 0, 5, and 30 minutes afterwards. The figure compares the changes in hue for birds that dried naturally and birds for which beak drying was enhanced by blowing air on it for 5 minutes. Changes in brightness, hue and chroma were assessed using LMMs, with bird ID specified as a random variable. Least-Square means ± SE are presented. Significant differences are indicated by an asterisk* for $P < 0.05$. The sample size is given below the means.

For birds (Negro et al. 2006) and mammals (Stephen et al. 2009; Walton and Bennett 1993), rapid changes in integument (skin) colouration may signal information to conspecifics related to blood flow and vasoconstriction. Because of the conjunctive vascularized nature of beak spot tissue, and a drastic change from the cold oceanic environment to the temperate terrestrial environment, we expected an influence of local temperature (via vasodilatation) on modulations of beak colouration. However, contrary to our expectation, experimentally cooling the beak with bagged ice after an hour of natural drying did not appear to push the reflectance spectrum of the beak back to its original value of when the birds emerged from the sea. Instead, experimentally air-drying the beak rapidly accelerated changes in beak UV hue, i.e. by twofold within 5 minutes of drying (Figure 2). Thus, any effect of warming of the beak on-shore likely resulted from exposure to air and augmentation of drying. Interestingly, changes in UV colour, specifically hue, were much greater than changes in the yellow-orange part of the beak. Dresp and Langley (2006), previously showed that rehydrating dry beak spots for 24 hours by immersion in water leads to an increase of the UV hue from ca. 370 to 420 nm and the percentage of maximum reflectance approximately doubled. Beak UV reflectance results from structural coherent scattering based on the reflection of stacks of elongated lamellae (multiple layers of doubly folded membranes) in the horny layer of the beak (Dresp and Langley 2006). The lattice dimension of the photonic crystals (i.e. distance in nm separating those double-folds) is responsible for UV hue and the homogeneity of those distances is mostly reflecting by the UV chroma (Dresp and Langley 2006, see section 1.1.4 of the general methods). This photonic property can be explained by Bragg's law, with $\lambda = n2d \sin\theta$, where λ is the wavelength of reflected light, n is the refractive index of the tissue, d is the separation of the layers (lattice dimension), and θ is the angle of incidence of the light (Bragg 1915). Thus two mechanisms could explain the rapid change of the UV hue in those monitored penguins. First, hydrating the tissue could lead to a dilatation and a

resulting increase in the distance between the two layers of the lamellae (distance d). Secondly, water has a higher reflective index than air ($n_{water}>n_{air}$) and full filling the interspace of the beak structure with water instead of air would modify the reflecting property, and thus both processes would lead to a higher diffraction and a reflectance of higher wavelengths. Those results suggest that the production of UV colour by light scattering from dermal collagen or keratin structures in animals (Prum 2006), including king penguins (Dresp et al. 2005), may be conditioned by the water content of those structures. This also raises questions on whether YO colours may convey more reliable information than UV colours, and whether similar phenomena occur in other tissues (Prum 2003) and in other species. Indeed, YO colours are often produced by exogenous carotenoid pigments which are acquired from the diet and may reflect yearly environmental forage conditions (Linville and Breitwisch 1997; McGraw et al. 2009; Slagsvold and Lifjeld 2009). In king penguins, similar mechanisms might explain variation in YO colour production (i.e. higher YO colour production in years of high resource availability; Keddar et al. 2015b). In contrast, it would appear that UV structural colours are more sensitive to short-term fluctuations in local environmental changes (i.e. humidity and moisture). Thus, those findings stimulate our desire to know more about the potential information that could be reflected by beak spot colour changes at different time scale.

2 Beak colour dynamically signals changes in fasting status and parasite loads in king penguins

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2.1 Abstract

Dynamic ornamental signals that vary over minutes, hours or weeks can yield continuous information on individual condition (e.g. energy reserves or immune status), and may therefore be under strong social and/or sexual selection. In vertebrates, the colouration of integument is often viewed as a dynamic ornament, which in birds can be apparent in the beak. King penguins (*Aptenodytes patagonicus*) are monomorphic seabirds that possess conspicuous yellow-orange and ultra-violet beak spots that are used by both males and females in mate choice. We studied the dynamicity of beak spot sexual traits, and to what extent they reflected changes in individual condition in fasting king penguins and in penguins treated with an anti-parasitic drug. We also describe for the first time the maturation of this colourful ornament during the yearly catastrophic moult. On a time-scale of days to weeks, beak spot colouration changed in response to fasting and experimental changes in parasite load. Beak spot $UV_{brightness}$ decreased over a 10 days fast in breeding birds. For birds caught during courtship and held in captivity YO_{chroma} decreased after a 24 day fast. Birds that were treated with an anti-parasitic solution showed an increase in UV colouration after parasite removal. Altogether, our results show that beak spot colouration is a dynamic ornament that reflects multiple dimensions of changes in individual condition in breeding-fasting penguins.

2.2 Introduction

Darwin's theory of sexual selection has been central to evolutionary biology, providing scientists with a framework for understanding mechanisms that might lead to the evolution of individuals selecting mates that produce fitter offspring (Darwin 1871). When assessing mate or competitor condition, animals often rely on ornamental signals that are costly to produce and/or maintain, and are therefore expected to honestly reflect individual quality (Zahavi 1975; Grafen 1990; Cotton et al. 2004; Walther and Clayton 2004). Mates may use such ornaments to assess the direct and/or indirect fitness benefits (e.g. paternal care, genetic benefits; Møller and Thornhill 1998; Mays and Hill 2004; Fromhage et al. 2009) that arise from mating with partners able to bear their cost.

In species where interactions with mates and/or social competitors occur repeatedly, there should be strong selection for dynamic signals that allow to continuously track changes in individual condition over extended periods of time (Velando et al. 2006; Ardia et al. 2010; Rosenthal et al. 2012). Dynamic changes in integument colouration have been reported in fish and amphibians (Sköld et al. 2013), reptiles (Weiss 2002), mammals (Stephen et al. 2009), and birds (Velando et al. 2006; Ardia et al. 2010). In birds, studies have suggested that beak colouration may serve as a dynamic signal of individual condition (Blount et al. 2003; Faivre, Grégoire, et al. 2003; Navarro et al. 2010; Rosenthal et al. 2012). In contrast to feathers that are replaced only during moult and constitute an inert (non-vascularized) tissue, the beak is a vascularized part of the integument (Lucas and Stettenheim 1972). Thus, rapid changes in beak colouration may reflect more dynamic changes than feathers in the deposition or mobilization of pigments (e.g. carotenoids; Alonso-Alvarez et al. 2004), or rearrangement of local microstructures (e.g. keratin; Dresp and Langley 2006) linked to modifications in individual condition over time.

One important trade-off shaping the evolution of honest signals is that between sexual ornaments and immune function, and by extension resistance to parasites (Hamilton and Zuk 1982). Differential allocation of pigments to ornaments or immune function is thought to act as a constraint, so that only high quality individuals are able to invest pigments at the same time both into coloured ornaments and efficient immune defences (Blount et al. 2003; Faivre, Préault, et al. 2003; Aguilera and Amat 2007). In addition, pigment-based colouration appears to change rapidly under stressful conditions or immune stimulation (Faivre, Grégoire, et al. 2003; Rosenthal et al. 2012) raising questions on the potential role of pigmented ornaments in reflecting rapid changes in body condition and energy depletion. In addition to pigmented ornaments, structural colours such as ultra-violet (UV) may also provide information on individual quality. For instance, inter-individual variation in

integument UV colouration has been linked to inter-individual variation in body condition in several species (Bize et al. 2006; Jacot and Kempenaers 2007; Dobson et al. 2008; Viblanc et al. 2016). However, because of the structural nature of UV colours, it is unclear whether this trait is labile and reflects intra-individual changes in condition over short to long time periods.

Using king penguins (*Aptenodytes patagonicus*) as a study species, we studied the dynamicity of beak colouration and tested whether it responded to ecological factors (parasites, long-term fasting) that might produce changes in beak colouration. Both male and female king penguins display colourful yellow-orange beak spots that also reflect ultraviolet (UV) (Dresp et al. 2005; Jouventin et al. 2005). In particular, beak UV appears to be an important signal of individual quality used in mutual mate choice. For instance, experimental studies have shown that reducing beak $UV_{brightness}$ decreases the pairing likelihood in both males and females (Nolan et al. 2010). Further, beak UV is associated with indices of condition. For instance, beak $UV_{brightness}$ was positively correlated to body condition in breeding males, but negatively correlated in breeding females (Dobson et al. 2008; Viblanc et al. 2016). Beak UV_{hue} is negatively related to total oxidative damage in breeding females but not in males, and is negatively related to individuals' responsiveness to acute stress in both sexes (Viblanc et al. 2016).

Picture 1: Photograph of the beak spots of an adult king penguin at the end of moult. Note that the superficial keratin layer is renewed entirely and a new beak spot is produced. The black horn material of the maxilla and mandible are not replaced after moult, but the seemingly articulated yellow-orange and UV-violet horn of the beak spots are shed and replaced from fresh tissue beneath the old horn. The old colour horn surface of the beak spots is lost after feather moult is complete, and these likely drop off the birds on the beach after feather moult, as evidenced by the prevalence of shed beak spot horn on the shore. None of our 25 moulting birds lost their beak spots before feather moult was complete. If one considers the shedding of the old beak horn a part of the moult process, then it appears to be the final stage of the moult. (Photograph copyright ©Anaïs Rameau)

UV colouration of penguin beaks is structural, resulting from the reflection of light off stacks of elongated lamellae in the horny layer of the beak, forming a photonic microstructure that reflects light in the UV to violet wavelengths (Dresp and Langley 2006). Removing 17% of the beak spot upper-layer results in a decrease of 10% in maximum reflectance. When the horn thickness is reduced by 38% the UV reflectance disappears but the YO orange reflectance remains (Dresp et al. 2005). The remaining reflectance (starting after 450 nm) is that of the yellow-orange beak colour, and is likely caused by carotenoid pigments assimilated through diet that are only present in the

deeper parts of the beak (McGraw et al. 2007). Indeed, yellow-orange beak colours appear to be constrained by the availability of environmental resources, birds displaying higher yellow-orange beak hue in good years (Keddar, Couchoux, et al. 2015). Because previous studies of beak spot colouration in this species have relied on measures of beak colouration taken on different individuals at a single point in time, it remains unclear whether beak colouration may signal changes in bird condition during breeding, and what underlying factors might trigger short-term changes in colouration. Here, we used repeated measures on breeding adult king penguins to investigate changes in beak colouration focusing on two original aspects of variation in beak colour. First, we examined the development of beak colouration following moult. King penguins (and probably the closely related emperor penguin, which has similar UV and yellow-orange beak spots; Jouventin et al. 2005b) entirely renew the coloured keratin superficial layer on both sides of the beak at the end of process of feather moult (see Picture 1), a feature that appears unique in birds. Renewed beak spots are present by the time birds return to land after the post-moult foraging trip at sea, and are displaying for mates on the beach adjacent to the breeding colony.

Second, we studied variation in beak spot colouration in response to physiological constraints (fasting and parasite load) of key importance to king penguins during reproduction. King penguin fast on-land, facing repetitive long-term fasting periods (up to 3-5 weeks for the male during the first shift of incubation) (Groscolas and Robin 2001) and as mainly colonial animal cope with parasites which made of these two essential aspects of reproduction in king penguins. Breeding birds will abandon reproduction if their energy reserves are critically depleted (Groscolas and Robin, 2001), and As bi-parental investment is an obligate condition for reproductive success, ornamental signals that dynamically reflect individual energetic reserves or parasite loads should be of importance for assessing partner condition throughout the season, and to allow birds to adjust their reproductive effort accordingly.

2.3 Methods

2.3.1 Study species

We studied king penguins in the ‘Baie du Marin’ colony on Possession Island, Crozet Archipelago (46°25’ S, 51°45’ E) during the breeding season (November to March) in 2011-12, 2012-13 and 2014-15. King penguins are long-lived seabirds with a unique breeding cycle. After having moulted and replenished their energy stores at sea, males and females will court and establish a breeding territory during a period of ca. 11 days before the female lays a single egg (Weimerskirch et al. 1992). Pairing takes place after ritualized interactions with several potential partners that include

calling and exposing coloured ornaments (including the beak spot) to tentative partners in stereotyped postures (sky-pointing of the head in unison with a potential partner; Jouventin 1982). Once the egg is laid, males take duty for the first incubation shift; and must continue what is already a prolonged fasting period. The female relieves them some 16 days later, and males then return at sea to forage (Weimerskirch et al. 1992). Alternated incubation continues until the egg hatches on average 54 days later (Stonehouse 1960). Birds followed in these studies were at least 3-4 years old, the age at first reproduction in king penguins (Stonehouse 1960), but their exact age in unknown.

2.3.2 *Measures of beak spot colouration*

Colours measurements have been taken as described previously section 3.2 of the general methods

2.3.3 Changes in beak spot colouration following the moult

For adult king penguins, beak spots are renewed each year at the end of the period of moult of the entire plumage (P). Moulting of feathers and beak spots occurs before the start of the breeding season in November-January and the whole process takes ca. 32 days (Groscolas and Cherel 1992). To study the maturation of the new beak spot following the moult process, 25 moulting males were caught shortly before the end of moult and kept captive in a pen. Birds were checked daily for moult completion, and the colouration of their beak spot was measured before the moult started and after the moult was completed. Seventeen birds were kept captive for an extra 2 days, (5 were released because close to reaching a critical body mass, i.e. phase 3 of fasting; Groscolas and Robin 2001) and the colouration of the beak spot was measured a second time. All birds were released and departed to forage at sea. Before being released, birds were identified by marking them on the breast with a unique letter/number combination using a non-toxic human hair dye (Franck Provost, blue-black 2.1). The beach was walked every day to search for birds that returned to the colony to start breeding after having being at sea to feed and replenish their body reserves. Eighteen of the 25 followed birds were caught on the beach within a few hours after returning from their foraging trip and beak measurements were taken a final time. At the end of the moult, 3 individuals presented no reflectance signal whatsoever in the UV part of the beak (completely flat spectrum) but a classical YO reflectance spectrum, clearly due to a lack of maturation of the UV component. These 3 individuals were not taken into account in the analyses of UV colour parameters, explaining the variation of the sample size between UV and YO colour analyses (i.e. N = 22 vs. 25).

We studied changes in beak colouration following the moult by specifying beak colour variables (hue, chroma and brightness) as dependent variables in separate Linear Mixed Models (LMMs). The time period of colour measurement was entered as a discrete ordinal variable with 3 levels: the day the old beak spots were shed (day 0), two days later (day 2), and the day birds

returned from their post-moult foraging trip at sea (courtship). Bird ID was entered as a random effect to account for repeated measurements on individual birds. Correlation between colour parameters before moult and after their post-moult foraging trip (courtship) were investigate using Spearman's rank test on 16 birds for which we had both measurements.

2.3.4 Changes in beak colouration during fasting

We investigated the effect of fasting on beak spot colouration using either breeding penguins that were naturally fasting while incubating their egg or captive penguins that were forced to fast. We ran three different studies that covered different fasting and duration periods for this species.

In a first study performed in 2011-12, we studied 22 males and 22 females during the third and fourth incubation shift, respectively. We measured beak spots directly in the colony while birds were incubating their eggs. Birds were measured on their second day of incubation and again 6 days later (incubation day 8). We waited 2 days before the first measure to insure that individuals had settled on their eggs, thus avoiding any risk of breeding abandonment. Those birds had all just returned from their foraging trip before taking their incubation shift, and thus this study covers changes in beak colouration during the first days of fasting.

In a second study performed in 2014-15, we investigated changes in beak colouration of 36 breeding males during their first incubation shift. Beak colouration was measured on the third day after the start of incubation and again 10 days later, i.e. on day 13 of incubation. Males do not return at sea to feed between the period of courtship display and their first incubation shift, and thus they had already endured at least 10-days of fasting before our first measurement of colouration. Hence, this study covers changes in beak colouration during the second half of a long (> 20 days) fasting period.

Finally, in a third study performed in 2013-14 and 2014-15, we caught 20 males during courtship on land (10 birds each year) and kept them captive in a pen. These individuals experienced a forced fasting period (0-24 days) covering the natural fasting periods of our first (2-8 days) and second (ca. 13-23 days) studies presented above. Such prolonged fasting periods are well within the natural range of fasting observed in this species. In these birds, we measured body mass and beak spot colouration every 6 days to investigate changes in colour as fasting progressed. Upon release, all birds were observed departing to sea to feed and subsequently seen returning at the colony to breed.

Within each experiment, monitored birds were all caught at same day of the same breeding shift and thus had comparable breeding status. Changes in beak colouration during fasting were investigated by specifying beak colour variables (hue, chroma and brightness) as dependent variables

in separate LMMs. The time period of colour measurement was entered as a discrete ordinal variable (e.g. day 2 and day 8 in the first study). Bird sex was entered as a fixed factor in the models, and for the first experiment (the only one where both sexes were monitored), we considered the interaction of sex and time. However, as the interaction was not significant, we removed it from the final model. In addition, we accounted for body girth, a proxy to body condition (V. A. Viblanc et al. 2012), as a covariate in the models. Again however, as body girth never significantly affected colour parameters, we removed it from the final models. Bird ID was entered as a random effect to account for repeated measurements on individual birds. In the third study, captive birds were measured in two different years (2013-2014 and 2014-2015), and thus we entered the year as random factor to control for potential year effects on colouration (Keddar, Couchoux, et al. 2015).

2.3.5 Response of beak colouration to experimental parasite removal

In 2011-2012, we investigated the effects of seabird parasite loads on beak colouration by experimentally removing parasites in an experimental group of 20 breeding pairs using the anti-parasitic solution Eprinex Pour-On®. This solution is commonly used in cattle and poultry and known to remove a large spectrum of ecto- and endo-parasites including worms, lice, ticks, mange mites and grubs) (Shoop et al. 1996). A control group of 20 pairs was treated with a solution of propylene glycol, which is the solvent used in Eprinex Pour-On®. We obtained information on changes in beak spot colouration in 27 treated birds (14 males, 13 females) and 33 control birds (16 males, 17 females). To control for possible confounding effects of breeding timing and localization in the colony on inter-individual variation in beak spot colouration, we applied our treatments so that treated and control pairs did not differ in their breeding onset (all were early breeders) or where they were breeding in the colony. The anti-parasitic and control solutions were deposited on the skin of the birds just below the feathers at the base of the neck at the beginning of the first incubation shift and of the third incubation shift in males and at the beginning of the second and fourth incubation shift in females. The efficiency of the anti-parasitic treatment was controlled by counting tick loads (*Ixodes uriae*) on a small part of the body (the head) of monitored birds. At the start of the treatments (i.e. beginning of shift one of males and shift two of females), there was no difference in the number of ticks on the head of birds treated with the anti-parasitic versus sham solutions (mean ± s.e. = 2.26 ticks ± 0.62 versus 1.39 ticks ± 0.59; Wilcoxon's test: $chi^2 = 0.21$, $df = 1$, $P = 0.64$). The Eprinex Pour-On® solution was efficient at removing parasites as reflected by lower tick loads after treatment on the head of birds treated with the anti-parasitic versus sham solutions (0.05 ticks ± 0.64 versus 3.15 ticks ± 0.62; *Wilcoxon's test*: $chi^2 = 27.23$, $df = 1$, $P < 0.001$). Effects of our treatments on changes in beak spot colouration were measured during shift 3 and 4 of males and females, respectively, by

taking a first measure of beak colouration on the second day after the start of incubation and again 6 days later, i.e. on day 8 of incubation.

Changes in beak colouration in response to our treatments were investigated by specifying beak colour variables (hue, chroma and brightness) as dependent variables in separate LMMs. The effect of the experimental anti-parasitic treatment on beak colouration was tested from the significance of the interaction between treatment (anti-parasitic vs. sham) and time period (day 3 vs. day 8) in the model. Sex was added as a fixed factor in the model to test for potential sex differences in beak colouration variation and its interaction with treatment was also considered. Again, body girth was added as a covariate in the model and its interaction with treatment and period considered, but as it never showed any significant effect on colour parameters, it was removed from the final models. Bird ID was entered as a random effect to account for repeated measurements on individual birds.

2.3.6 Statistics

Statistical analyses were run with R v.3.1.1 (R development core team). F-statistics for fixed effects (tests of differences from zero), the total number of observations (n) and corresponding number of individuals (N) are given. Effects were considered significant for $P < 0.05$. When appropriate, significant differences between groups were assessed using Tukey's Honest Significant Difference (HSD) tests for least square means. We insured residuals followed a normal distribution using qqplots (opposing theoretical Quantiles to Sample Quantiles).

2.3.7 Ethical statement

All experiments were approved by an independent ethics committee (Comité d'éthique Midi-Pyrénées pour l'expérimentation animale) commissioned by the French Polar Institute and comply with the current laws of France. Authorizations to enter the breeding colony and handle the birds were provided by the "Terres Australes et Antarctiques Françaises" (permit n°2010-65 issued on the 3rd of September 2010, n°2011-96 issued on the 14th of October 2011, n°2012-116 issued on the 29th of October 2012, n°2013-72 issued on the 29th of October 2013 and n°2014-127 issued on the 15th of October 2014).

2.4 Results

2.4.1 Changes in beak spot colouration following the moult

Birds showed pronounced changes in the colouration of their beak spot following the moult. The new beak spot appeared particularly pinkish and changed from day to day (Figure 1). $UV_{brightness}$ of the new beak decreased by 14% within the first 2 days of shedding the old beak spot, and continued to decrease by another 23% during the post-moult foraging trip at sea (LMM; F =79.21, df = 2, P < 0.001, n = 55, N = 22). Freshly moulted birds exhibited a rapid decrease in the UV_{hue} of their new beak spot (by 20.7 nm on average) within the first 2 days of shedding the old beak spot (Figure 1). UV_{hue} continued to decrease during the post-moult foraging trip (another 10.7 nm on average), albeit less substantially (F = 30.20, df = 2, P < 0.001, n = 55, N = 22). We observed increases both in UV_{chroma} (+19% within 2 days post-moult and another 23% by the time the birds returned for courtship; F = 51.04, df = 2, P < 0.001, n = 55, N = 22) and YO_{chroma} (+24% within 2 days post-moult, and another 13% by courtship; F = 23.88, df = 2, P < 0.001, n = 60, N = 25).

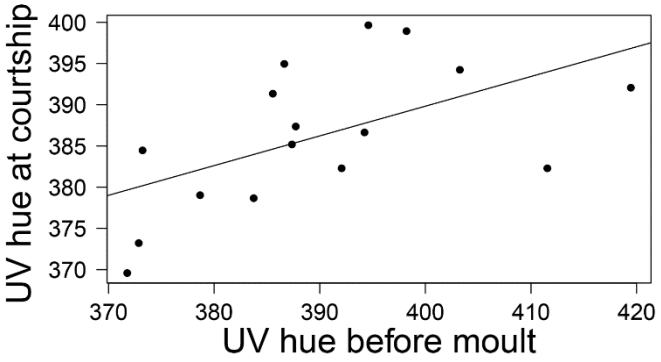

Figure 2: Correlation between beak UV_{hue} before the moult and after the post-moult foraging trip in king penguins (*Aptenodytes patagonicus*) at the time the birds come for courtship. Correlation were assessed using Spearman's rank correlation test (*rho* = 0.64; *S* = 246.7; *P* = 0.008).

When comparing colour parameters before the moult and after birds came back from their post-moult foraging trip, UV_{hue} was significantly correlated (Spearman's rank correlation; *rho* = 0.64, *S* = 246.7, *P* = 0.008, *n* = 30, *N* = 15, Figure 2). Other parameters, however, remained uncorrelated (-0.36 < *rho* < 0.38, 422 < *S* < 992, 0.148 < *P* < 0.51, *n* = 30, N = *15*).

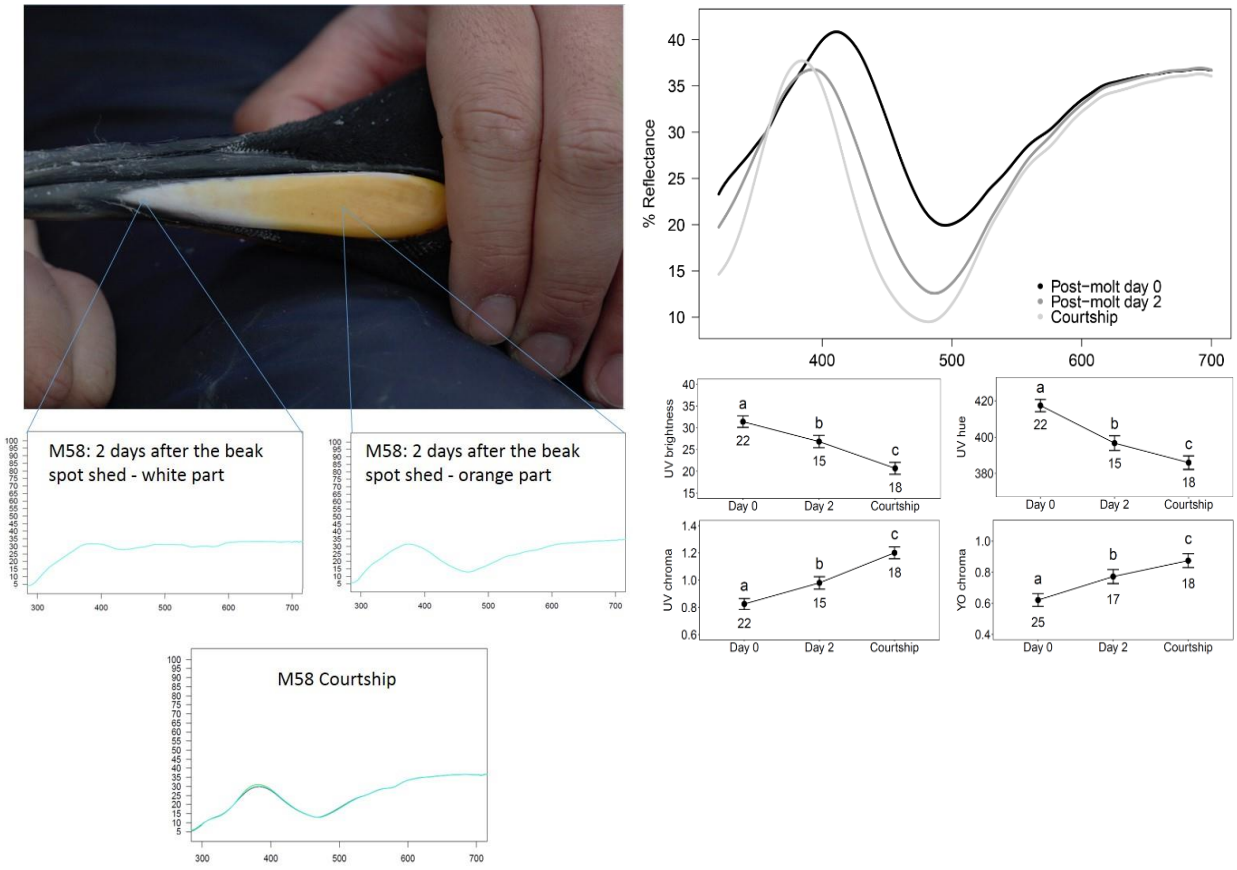

Figure 1. a) Picture of an unmatured beak and associate colour spectrum compared to the spectrum taken for the same individual after its post-moult foraging trip. During the maturation of the beak spots, the brightness of both UV and YO significantly decrease. Moreover, as you can see on the picture below from a particularly unmatured beak spot, it appears white, both visually and spectrally (reflectance measurement). After they come back from the sea to parade all birds express a colourful but less bright beak spot than just after moult.

b) Changes in the beak colouration of adult king penguins (*Aptenodytes patagonicus*) following the moult. Average changes in raw spectral data over all birds are presented for illustrative purposes. Beak colour was measured on the day the old beak spot was shed (day 0), 2 days later (day 2), and after birds returned from their post-moult foraging trip for breeding (courtship). Changes in brightness, hue and chroma were assessed using LMMs, with bird ID specified as a random variable. Least-Square means ± SE are presented. Values not sharing a common letter are significantly different for *P* < 0.05. The sample sizes are given below the means.

2.4.2 *Changes in beak spot colouration during fasting*

In breeding penguins naturally fasting in the colony, we found no significant changes in beak spot colouration during the first days of fasting (between day 2 and 8 of males in shift three and females in shift four) (LMMs; 0.01 < F < 0.51, 0.48 < P < 0.98, n = 88, N = 44).

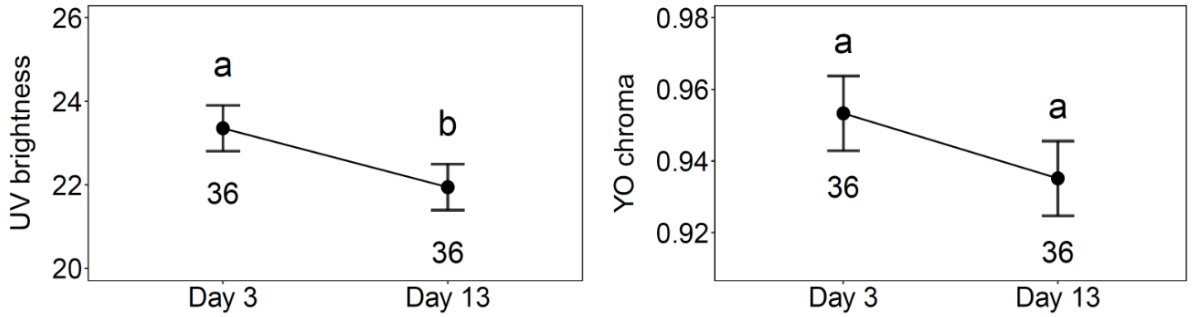

Figure 3: Changes in beak $UV_{brightness}$ and YO_{chroma} for 36 male king penguins (*Aptenodytes patagonicus*) that were measured on day 3 of their first incubation shift and on day 13 after having experienced a 10-day fast while breeding in the colony. Changes in brightness were assessed using LMMs, with bird ID specified as a random variable. Least-Square (LS) means ± SE are presented. Values not sharing a common letter are significantly different for P < 0.05. The sample sizes are given below the means.

In contrast, we found a significant 6% decrease in $UV_{brightness}$ in breeding male penguins at the end of a long natural fasting period (between day 13 and 23 of the first incubation shift) (LMM; F = 7.90, P < 0.01, n = 72, N = 36, Figure 3) and no significant changes for the other colour parameters (LMMs; 0.16 < F < 2.54, 0.12 < P < 0.69, n = 72, N = 36).

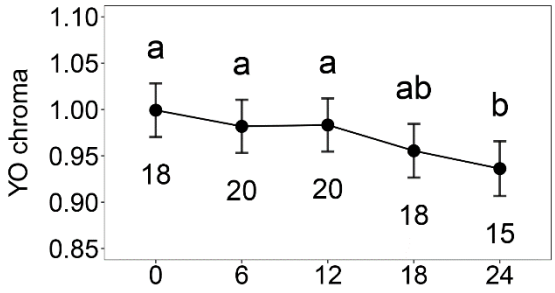

Figure 4: Changes in yellow-orange beak chroma (YO_{chroma}) in 20 males that endured a prolonged fast in captivity. Changes in chroma were assessed using LMMs, with bird ID and year specified as random variables.

In captive birds that fasted up to a similar body mass of that naturally occurring at partner relief (24 days), fasting duration did not affect UV beak colouration (brightness, hue or chroma) (LMMs: 0.92 < F < 2.04, df = 4, 0.10 < P < 0.46, n = 91, N = 20). In contrast, YO_{chroma} decreased (F = 4.23, df = 4, P < 0.05) with increased fasting (Figure 4), and showed a strong significant decrease at the end of the fasting period (Tukey's HSD: -3.63 < Z < -2.77, 0.003 < P < 0.044, Figure 5).

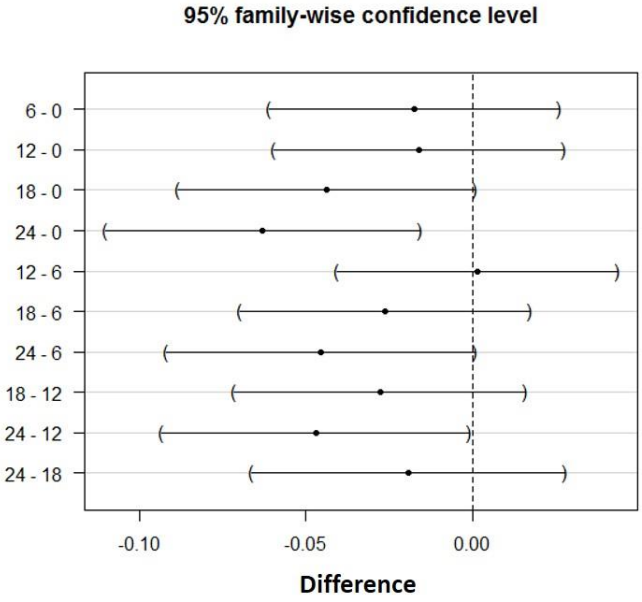

Figure 5: Changes in yellow-orange beak chroma (YO_{chroma}) in 20 males that endured a prolonged fast in captivity. 95% interval confidence of a multipair comparison test for differences between duration days of fasting. Differences in chroma between time periods were assessed using LMMs, with bird ID and year specified as a random variable and Tukey's Honnest Significant Difference posthoc test.

2.4.3 *Response of beak colouration to experimental parasite removal*

Six days after the experimental treatments (Eprinex® or sham), birds treated with the anti-parasitic solution showed significant increases in beak $UV_{brightness}$ and UV_{hue} , and a decrease in UV_{chroma} compared to before the treatments (Table 1; Figure 6). Control birds did not show significant changes in beak colouration after having received the control treatment (Tukey's HSD; $0.80 < P < 0.99$, n =120, N = 60). Bird sex, whether considered independently or in interaction with treatments and/or time period, never significantly affected beak colouration ($0.12 < P < 0.96$ n = 120, N = 60). The effect of sex was removed from the final models presented in Table 1.

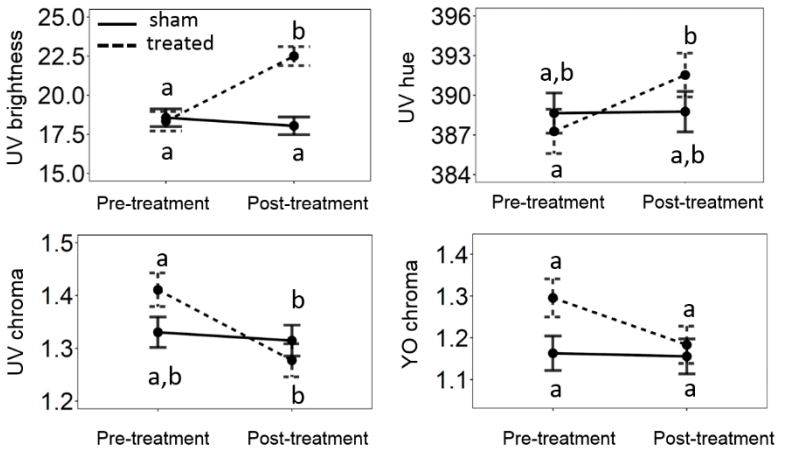

Fig. 6. Interactive effects of an experimental anti-parasitic treatment on the beak colouration of adult kings penguins (*Aptenodytes patagonicus*) freely incubating in the colony. Changes in brightness, hue and chroma were assessed using LMMs, with bird ID specified as a random variable, the treatment (treated vs. control), the time period (pre-treatment vs. post-treatment) and the interaction between those factors specified as independent variables. Least-Square (LS) means are presented. Values not sharing a common letter are significantly different for $P < 0.05$.

Table 1. Mixed model estimates (± SE) for the effects of an anti-parasitic treatment on beak colouration in breeding king penguins (*Aptenodytes patagonicus*). The control level for the treatment factor is tested against the treatment level [T]. The time period [post-treatment] is tested against the level. Bird ID were entered as a random factor in the model to account for repeated measures on the individual. Significant values for $P < 0.05$ are indicated by an asterisk. Note the significant interaction terms revealing different treatment effects on pre- and post-treatment measures of treated vs. control birds. n = 120 observations, N = 60 birds.

	Term	Estimate ± SE	t Ratio	Prob > |t|
$UV_{brightness}$	Intercept	18.56 ± 0.57	32.99	<0.001*
	Period[Post-treatment]	-0.52 ± 0.66	-0.78	0.437
	Treatment [T]	-0.23 ± 0.84	-0.27	0.787
	Period*Treatment	4. 68 ± 0.98	4.79	<0.001*
UV_{hue}	Intercept	388.65 ± 1.51	256.57	<0.001*
	Period[Post-treatment]	0.12 ± 1.10	0.11	0.915
	Treatment [T]	-1.34 ± 2.26	-0.61	0.54
	Period*Treatment	4.15 ± 1.62	2.56	<0.001*
UV_{chroma}	Intercept	1.33 ± 0.03	46.21	<0.001*
	Period[Post-treatment]	-0.02 ± 0.04	-0.43	0.666
	Treatment [T]	0.08 ± 0.04	1.87	0.064
	Period*Treatment	-0.12 ± 0.05	-2.20	0.032*
YO_{chroma}	Intercept	1.16 ± 0.04	28.27	<0.001*
	Period[Post-treatment]	-0.01 ±0.06	-0.13	0.898
	Treatment [T]	0.13 ±0.06	2.16	0.032*
	Period*Treatment	-0.10 ±0.08	-1.249	0.217

2.5 Discussion

Dynamic ornamental signals can provide continuous information on individual condition, and are expected to be under strong sexual and social selection (e.g. Velando et al. 2006). In birds, beak colouration has been proposed to function as a dynamic ornament (Faivre, Grégoire, et al. 2003; Navarro et al. 2010; Rosenthal et al. 2012) as indeed appears to be the case in our species. King penguins showed a rapid maturation of beak colouration following the moult and we found dynamic changes in colouration in response to changes in individual condition (fasting status and parasite load) in breeding birds over the scale of a single breeding season.

2.5.1 Changes in beak spot colouration following the moult

Although studies on avian moult are numerous, there is virtually no information on moulted structures other than feathers (King and Murphy 1990). Horn, claw and beak material are thought to grow continuously in response to wear, but king penguins appear to be an exception in that the entire horny material of their beak spot is shed every year at the end of the moult, while the rest of the black beak horn is not. Thus, beak spots are replaced each time these long-lived seabirds breed, most of the time attracting a new breeding partner (Bried et al. 1999; Olsson 1998). After the moult, beak spots colouration becomes less bright changing to a deeper (decrease in hue) and purer UV colour (Figure 1). As previously mentioned, the UV colouration of penguin beaks is structural, resulting from the reflection of stacks of elongated lamellae (multiple layers of doubly folded membranes) in the horny layer of the beak (Dresp and Langley 2006). The distance (in nm) separating those double-folds is responsible for UV_{hue}, i.e. the lattice dimension of the photonic crystals (Dresp and Langley 2006). This photonic property may be explained by Bragg's law, with $\lambda\ max = n2d\ sin\theta$, where $\lambda\ max$ is the peak wavelength of reflected light, n is the average refractive index of the tissue, d is the separation of the layers (lattice dimension), and ϑ is the angle of incidence of the light (Bragg 1915). Thus, our results suggest that as the beak matures, there is a decrease in the distance between the doubly folded membrane structures that compose the upper-layer of the beak. Simultaneously, although the magnitude of change is weaker, the yellow-orange colour of the beak also becomes purer as chroma increases. These latter colour changes occur as the bird prepares to mate, over a period of 13-20 days and are likely explained by the deposition of carotenoid pigments in the deeper layers of beak spots (higher chroma), as is the case in many bird species (L Saks et al. 2003; McGraw and Gregory 2004). Interestingly, comparing colours before and at courtship for the same birds, UV_{hue} appeared strongly correlated (Figure 2). This suggest that UV_{hue} (the distance between doubly-folded membranes) is determined largely genetically, but some scope for variation does exist (i.e. time since the last moult, time spent at sea during the past year, time spend preening, etc.).

2.5.2 *Beak spot colouration and fasting*

Nutritional status and body condition are important information that may be used by conspecifics both in reproductive and social contexts. In the Alpine swift and the European starling for instance, parents adapt their feeding effort to the UV colouration of chick skin, which is positively correlated with body mass and structural size, and is used by parents to adapt their feeding behaviour to the intensity of the reflected colour of their chicks (Bize et al. 2006). In the same way, in blue-footed boobies, rapid experimental changes in male foot integument colouration (reflecting nutritional status) elicited rapid adjustments in female reproductive strategies, i.e. facilitation of brood reduction by laying smaller eggs compared to controls (Velando et al. 2006). In our study, male beak colouration appeared to reflect changes in nutritional status. Over 24 fasting days, captive males showed a progressive decline in YO_{chroma} but no change in UV colouration. However, when fasting was prolonged for even longer periods in colonial conditions (over >24 days including 11-15 days of courtship followed by 13 days of incubation; Figure 4 & 5), $UV_{brightness}$ appeared to decrease. Those results suggests that UV and YO colouration may signal fasting status on different time-scales. A progressive decrease in YO_{chroma} in fasting birds may reflect a re-allocation of beak pigments to antioxidant defences, in line with the hypothesis of costly signalization by (limited) carotenoid-dependent structures (e.g. in birds: Alonso-Alvarez et al. 2004; in fish: Pike et al. 2010; but see Cote et al. 2010 in reptiles). This suggestion is consistent with our recent data suggesting that plasmatic anti-oxidant defences indeed increase over the course of fasting (Schull et al. 2016b; Appendix 1). Yellow-orange colours are often produced by exogenous carotenoid pigments, which are acquired from the diet and may reflect yearly environmental forage conditions (Linville and Breitwisch 1997; McGraw et al. 2009; Slagsvold and Lifjeld 2009). In king penguins, similar mechanisms might explain variation in YO colour production between years (i.e. higher YO colour in years of high resource availability; Keddar, Couchoux, et al. 2015). Decreases in $UV_{brightness}$ only at advanced fasting stages may suggest a cost for UV maintenance and the inability to maintain high UV reflectance when energy is critically limiting. For instance, preening and associated comfort behaviour (keeping the beak clean) is generally costly in birds (Walther and Clayton 2004), including king penguins (Viblanc et al. 2011), and reducing those behaviour may allow substantial savings at advanced stages of fasting. Whether such behavioural changes linked to fasting status might indirectly affect the maintenance of beak colouration remains to be tested. Furthermore, as captive birds and free-living breeders also differed in their social environmental and breeding status, we cannot exclude that such differences also affected changes in beak colouration differently between the two groups. Further investigations are needed to clarify how beak spot dynamics are conditioned by the rate of nutrient reserves mobilization and availability of dietary antioxidants such as carotenoids. This could be

achieved by concomitantly following plasma carotenoid availability and changes in beak coloration over the course of fasting.

2.5.3 Beak spot colouration and parasites

Parasites drain resources (including pigments and nutrients) from their hosts, which might otherwise be partly allocated to producing ornaments. Furthermore, in fighting parasites, hosts also mount immune responses that divert resources from other functions such as ornamentation (Rosenthal et al. 2012; Velando et al. 2014). Regardless of the underlying mechanism, parasitism should affect ornamental signals (e.g., parasite-mediated sexual selection; Hamilton and Zuk 1982). Consistent with this hypothesis, we found that removing parasites from males and females during incubation produced significant changes in the UV component of beak colouration. While not influencing YO colours, removing parasites resulted in beak spots of higher $UV_{brightness}$, higher UV_{hue} and lower $UV_{saturation}$ (Figure 6). Birds relieved of parasites should have a less stimulated immune system, and may therefore invest more into beak colouration. Accordingly, several studies in birds have highlighted strong links between UV ornamentation and parasite load (Hõrak et al. 2001; Mougeot et al. 2005) or in a broader context immune-competence (Griggio et al. 2010).

Here again, the independent changes we observe in UV or YO colouration support previous findings that in king penguins, UV and YO colours of the beak are produced by distinct mechanisms (Dresp and Langley 2006), and appear to change over the course of the breeding season. Indeed, YO carotenoid-based colouration is produced by pigments embedded in the deeper beak layers and was relatively unaffected in our anti-parasitic treatment. In contrast, changes in beak UV suggest structural modifications. Since UV reflectance depends on the thickness of the upper beak layer composed of the doubly folded membrane structures, which result from the differentiation of basal cells into dead keratin (as is the case for skin renewal) (Dresp and Langley 2006), an increase in UV reflectance following parasite removal suggests either an increase in cell division or a reorganisation of those structures. However, whether the thickness of the upper beak layer can be increased after the moult (i.e. complete renewal of the structure), or whether it can be remodelled is currently unknown and requires further investigations. Moreover, ectoparasites are known as disease vectors, as for instance Lyme disease carried by ticks (Gauthier-Clerc et al. 1999), which would not be cured with our anti-parasitic treatment and could limit the effect of our experimental treatment. Whereas other proximal mechanisms are likely, our results point to parasitism as an important influence on structural based UV colour signalisation in the king penguin.

2.5.4 Concluding remarks

The beak colouration of king penguins can be highly flexible, with both components of colouration (UV and YO) modified independently. Both beak UV hue and chroma, and YO chroma appear to have a maturation process, with an associated decline in beak spot brightness that continues through pre-breeding moult, subsequent feeding at sea, return to the breeding grounds, and mating. Importantly, due to its dynamicity, beak colouration may serve as an important signal of short-term changes in individual condition over the course of a single breeding season. This supports the idea that the information conveyed by sexual ornaments is not restricted to the single time period when mate choice occurs (e.g. Velando et al. 2006; Ardia et al. 2010).

Previous studies have found that higher beak $UV_{brightness}$ is associated with higher mating prospects (Nolan et al. 2010) and different body condition in males and females (Dobson et al. 2008; Viblanc et al. 2016). Higher beak UV_{hue} is negatively related with hormonal stress responses and oxidative damages in female (Viblanc et al. 2016). In line with these studies, our current results highlighted positive associations between $UV_{brightness}$ and YO_{chroma} and fasting, that is, a decrease in beak coloration with a decrease in individual body condition. On the contrary, when parasites were removed during breeding, the condition of the birds increase as well as $UV_{brightness}$. The next step is to focus on how such variations might be perceived by the mate at the time it takes over egg or chick-guarding duties, how beak colouration may help parents coordinate their efforts throughout the breeding season, and how dynamic changes in beak colouration may be perceived by social conspecifics.

2.6 Acknowledgements

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Chapter 3

How ornamentation reflects humoral immune system and mitochondrial functions in the King penguin

1 Preface

The aim of this preface is to provide the reader with the necessary background information and hypotheses about immunity system function and mitochondrial function that pertain to the studies presented in **Chapter 3**.

1.1 The immune system function and its links with ornamentation

The immune system is classified into two subsystems according to the specificity and time-scale of the response: the innate and the adaptive immune system (Ahmed and Gray 1996). ***The innate immune response*** is a short-term response triggered on a short time-scale (minutes) when the organism is faced with a forging agent such as a pathogen (Janeway and Medzhitov 2002; Beutler 2004; Akira et al. 2006; Kawai and Akira 2006; Takeuchi and Akira 2010). This type of response includes the activation of stem cells (granulocytes, monocytes, lymphocytes) triggered by general chemical properties of the antigen (Janeway and Medzhitov 2002; Beutler 2004; Akira et al. 2006; Kawai and Akira 2006; Takeuchi and Akira 2010). **T*he adaptive immune system*** is antigen-specific (thus also known as the specific immune system) and provides the organism with long-term protection against a pathogen that may be encountered again, owing to an "immunological memory" (Ahmed and Gray 1996). The cells involved in the adaptive immune response are also lymphocytes (white blood cells) that can be divided into T-cells and B-cells (Ahmed and Gray 1996). B-cells are the major component in the process of the creation of antibodies and are involved in one type of adaptive immune response, the *humoral immune response* (Ahmed and Gray 1996; McHeyzer-Williams 2005; Ramos et al. 2014). T-cells mature from thymocytes and play a role in the second type of adaptive immune response, the *cell-mediated response*, which does not involve antibodies (Ahmed and Gray 1996; Barry and Bleackley 2002).

McGraw and Ardia (2003) considered the two different immune response types by challenging male zebra finches (*Taeniopygia guttata*) with (1) a phytohemagglutinin skin test (PHA) triggering the cell-mediated immune response, and (2) a sheep red blood cell hemagglutination assay (SRBC) triggering the humoral immune response. Additionally, they supplied the birds with increased levels of carotenoid pigments in their diet. Their results showed that males with higher levels of carotenoid pigments had elevated levels of carotenoid in their blood and showed an increase in both cell-mediated and humoral immune responses. Furthermore, the cell-mediated response was higher in males with more colourful beaks. Similarly, Dunn et al. (2010) found that in common yellowthroats (genus *Geothlypis*) males plumage yellow coloration and mask size signalled humoral immunity in two geographically isolated populations. Whereas most of the studies linking ornaments and immune function have focused on males (Møller, Christe, et al. 1999; Faivre, Grégoire, et al. 2003; Faivre,

Préault, et al. 2003; Lauri Saks et al. 2003; Hill and Farmer 2005; Mougeot 2008; Sild et al. 2011; Rosenthal et al. 2012). Kelly et al. (2012) tested whether shared bill and plumage ornamentation in American Goldfinches (*Spinus tristis*) communicated the same aspects of immunity in males and females. They found bill coloration to be positively correlated with the functioning of the adaptive immune system, and plumage colour to be positively correlated to the functioning of the innate immune system in females but not males, highlighting that multiple ornaments in the same sex may signal different components of immunity (Kelly et al. 2012).

Similarly to American Goldfinches, king penguins are mutually ornamented birds (Stonehouse 1960). They face high parasite loads when breeding in their colony, which are known to impact the health of both adults and chicks (Gauthier-Clerc et al. 1998; Mangin et al. 2003; Bize et al., unpublish data). Nolan et al. (2006) was the first to test for links between ornament production and immunity in king penguin, though their study was only conducted in males. They found that males with lighter coloured feather breast patches had stronger cellular immune responses to an experimentally injected mitogen (phytohaemagglutinin). Apart from this study however, no consideration has been given to the other components of the immune response or whether they might be associated with the production of other ornamental features in this species. Thus, in **Chapter 3a**, we test whether sexual ornaments signal the individual's ability to mount a humoral response after exposure to a novel antigen (Newcastle disease Vaccine, NDV) and whether the tentative links between ornament production and humoral immunity differs between sexes. This work expanded from our correlative study (Viblanc et al. 2016) considering the links between constitutive innate humoral immunity and ornamentation in king penguin (see **Box 1**).

1.1.1 *Box 1. Constitutive innate humoral immunity and ornamentation in king penguins*

In the following box, I present results from our 2016 study to which I contributed by laboratory analyses and writing parts of the manuscript. The full version of the article can be found in the appendix 2.

In Viblanc et al. (2016), we investigated the physiological information ornaments may signal to conspecifics. We considered key physiological mediators of individual condition hypothesized to underlie the honesty of ornamental signals. Those were: birds' body condition, immune status, metabolic rate, HPA and adrenergic stress axes, and oxidative status (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003; Bize et al. 2008; Costantini 2008; Monaghan et al. 2009; Palacios et al. 2009; Schulenburg et al. 2009; Groscolas et al. 2010; Metcalfe and Alonso-Alvarez 2010; V. A. Viblanc et al. 2012; Vincent A Viblanc et al. 2014; Vincent A. Viblanc et al. 2014). Regarding measures of innate immunity, those were related to bird ornamentation both in males and females.

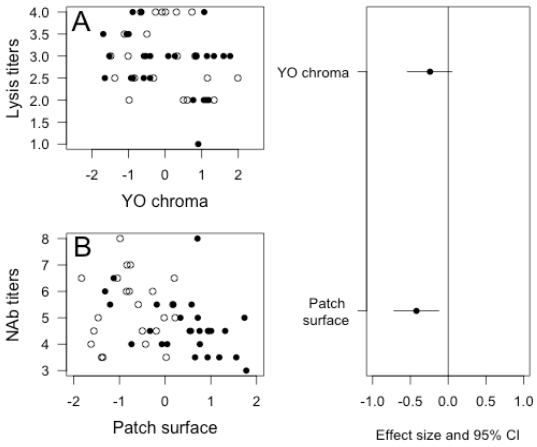

Figure 5. Relationship between beak coloration, auricular patch surface and innate immunity in breeding king penguins. Relationships are given for (A) plasma lysis titres and yellow-orange chroma, and (B) plasma NAb titres and auricular patch surface. On the left panel, males are depicted by filled circles, females by open circles. The right panel provides effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap

We found weak to moderate negative associations between lysis titers and beak YO chroma, and between NAb titers and auricular patch surface, respectively, suggesting that investments into ornamental traits (larger auricular patches and more YO beaks) may incur a cost in terms of immunity (both in terms of hemagglutination and complement activity). This idea is consistent with the suggestion that, given limited resources (energy, nutrients, protein), increased investments into sexual ornaments might compromise investments into humoral immunocompetence (Saino et al. 1997, Verhulst et al. 1999, Norris and Evans 2000). NAb titres were negatively related to auricular patch surface suggesting that displaying larger auricular patches came at a cost in terms of immunity. Unlike the PHA test that measures a wide range of immune responses involving both innate and acquired immunity (Tella et al., 2008), NAb titres reflect a well-defined component of the innate immune response not induced by an experimental infection (Matson et al., 2005). These findings support the notion that different ornaments may signal different components of immunity in breeding birds (Kelly et al., 2012).

1.2 Mitochondrial function and its links with ornamentation

Our previous study (Viblanc et al. 2016) noted a positive correlation between individual resting metabolic rate and beak UV brightness, suggesting a metabolic cost to UV production/maintenance (see **Box 2**). However, the energy spend at the level of the entire organism mostly results from the function of the mitochondria at the cellular level. This convinced us to study the direct link between ornament production and cellular respiration efficiency at the cellular level, focusing on red blood cells for which our recent work in birds has shown that they possess functional mitochondria (Stier et al. 2013; **Chapter 3b**). In the following paragraph, I aim to provide basic knowledge on mitochondrial functioning and involvement in life history trait theory as an introduction of **Chapter 3b**.

1.2.1 Box 2. Resting metabolic rate and ornamentation in king penguins

Beak UV brightness was positively (medium effect size) associated with resting HR levels (a proxy for resting metabolic rate; Viblanc et al., 2014) in both sexes.

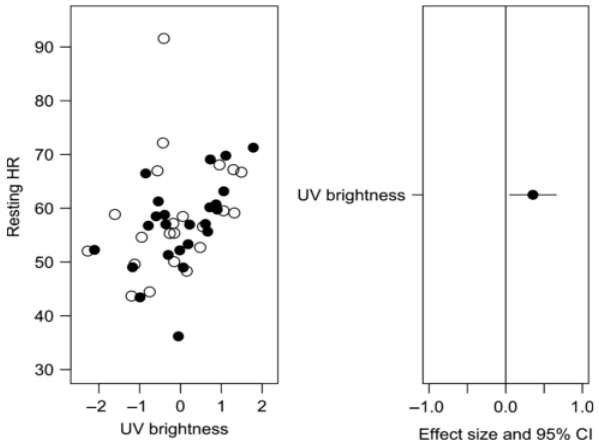

Figure 6. Relationship between beak UV brightness and daily resting HR levels (bpm) in breeding king penguins. On the left panel, males are depicted by filled circles, females by open circles. The right panel provides effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero.

High resting metabolic rates may reflect increased capacities to engage in a suite of challenging activities such as foraging, caring for the young or competing for resources, and might be honestly reflected by colour ornaments (Biro & Stamps, 2010; Kelly et al., 2012). The links between UV coloration and metabolic rate may lie within the energy costs of producing/maintaining structural colours (Siefferman & Hill, 2005; Doutrelant et al., 2012). For example, Siefferman & Hill (2005) showed that experimentally reducing the energy cost of reproduction by reducing brood size in bluebirds (*Sialia sialis*) allowed males to increase their investment into plumage UV in the subsequent year. Rather than a long-term energy trade-off between competing functions (conserving energy for ornament production vs. expanding it for current reproduction), our results suggest possible indirect metabolic costs, such as keeping the beak clean, for UV maintenance.

***The mitochondria: powerhouse of the cell*:** Depending on cell type, mitochondria can have different shapes and size, but are essentially constructed in the same way in five parts: (1) an outer membrane, (2) an inner membrane, (3) the inter-membrane space in-between, (4) the cristae space formed by the convolutions of the inner membrane, and (5) the matrix: the interior of the mitochondria (Lodish et al. 2008) The first step of converting nutrients into usable energy occurs in the cytoplasm of the cell. Glycolysis provides the first elementary component to the mitochondria (Lodish et al. 2008; Figure 1). The Tricarboxylic acid (TCA) or Krebs cycle is the key metabolic pathway that occurs inside the mitochondria and during which nutrient molecules (carbohydrates, fats, and protein) are oxidized along the OXidative PHOSphorylation pathway (abbreviated "OXPHOS"). The OXPHOS is the last step of energy conversion into Adenosine Triphosphate (ATP), the basic energy unit of the life (Ebenhöh and Heinrich 2001). The TCA cycle takes place in the inner membrane of the mitochondria and consists of 8 chemical reactions (Zhang and Bryant 2011). "Nutrient molecules" are broken into acetyl groups. A two-carbon acetyl group is transferred by the enzyme acetyl coenzyme A to form citrate. Citrate, a six-carbon molecule, undergoes several reactions to a four-carbon molecule leading eventually to the reduction of three nicotinamide adenine dinucleotide (NAD+) and one flavin adenine dinucleotide (FAD+) to three molecules NADH and one FADH2. The NADH and FADH molecules are the link between the TCA cycle and the OXPHOS pathway (Meléndez-Hevia et al. 1996).

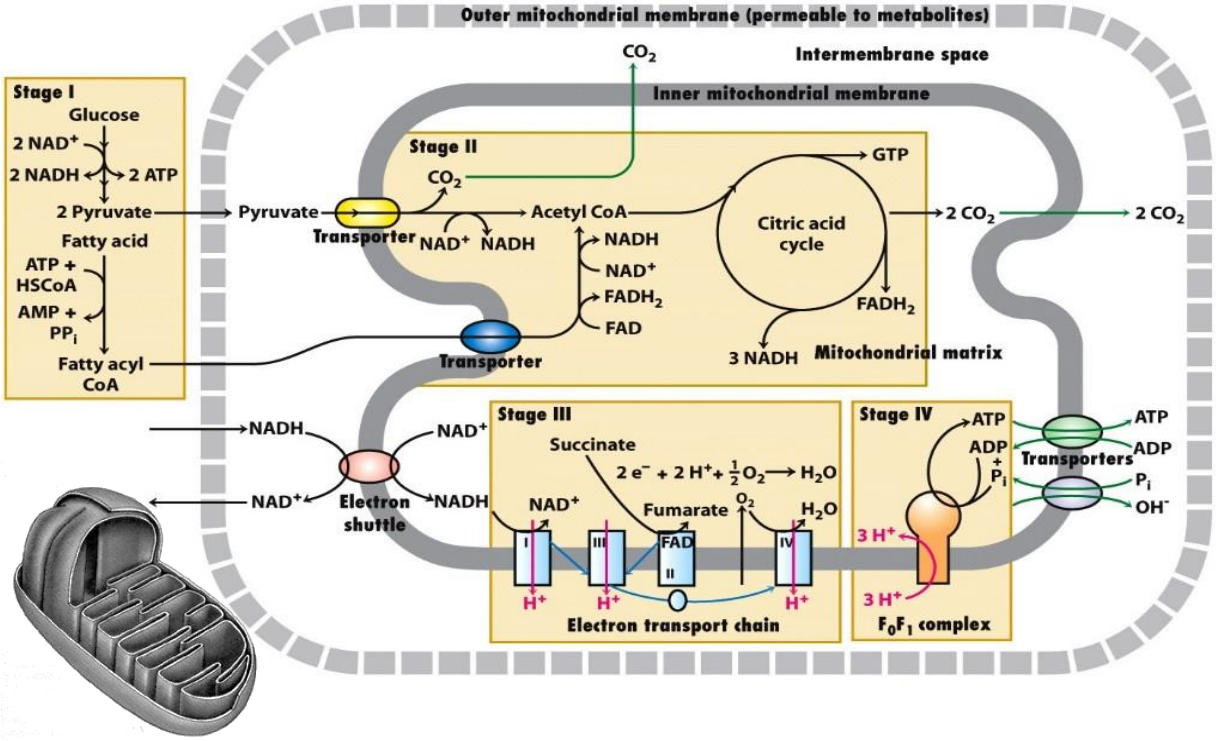

Figure 1. The mitochondrion, from the Glycolysis to the ATP production. Adapted from (Lodish et al. 2008)

At the beginning of the electron transport chain (Figure 1), which involves membrane-imbedded proteins and organic molecules that can be grouped into the four complexes I to IV, NADH is oxidised to NAD+ + H+, moving electrons generated from this process through the complex I (Schultz and Chan 2001). FADH2 molecules undergo the same reaction but at complex II (Schultz and Chan 2001). The electrons from both reactions are transferred to an organic compound that serves as an electron carrier and is called ubiquinone (Q) (Crane 2001). Its reduction to QH2 lets the molecule travel through the membrane donating the electrons to complex III (Crane 2001). As electrons move through complex III, protons are pumped from the mitochondrial matrix to the intermembrane space. Electrons are passed on to complex IV by another electron carrier (cytochrome C) (Scott Mathews 1985). At complex IV, the reduction of oxygen in the mitochondrial matrix leads to the production of water molecules (Mitchell and Moyle 1967; Scott Mathews 1985; Schultz and Chan 2001, Figure 1 & 2).

Overall, the electron transport chain builds a pH gradient, with higher concentration of H+ molecules in the intermembrane space and a lower concentration in the matrix. This gradient drives the synthesis of ATP as protons travel along the gradient, i.e. back into the matrix through the enzyme ATP-synthase. Driven by this proton flow, ADP is turned into ATP in a phosphorylation reaction (Porter and Brand 1995). However, some protons "leak" across the membrane without contributing to ATP production but leading to heat production (Schultz and Chan 2001, Figure 2). The

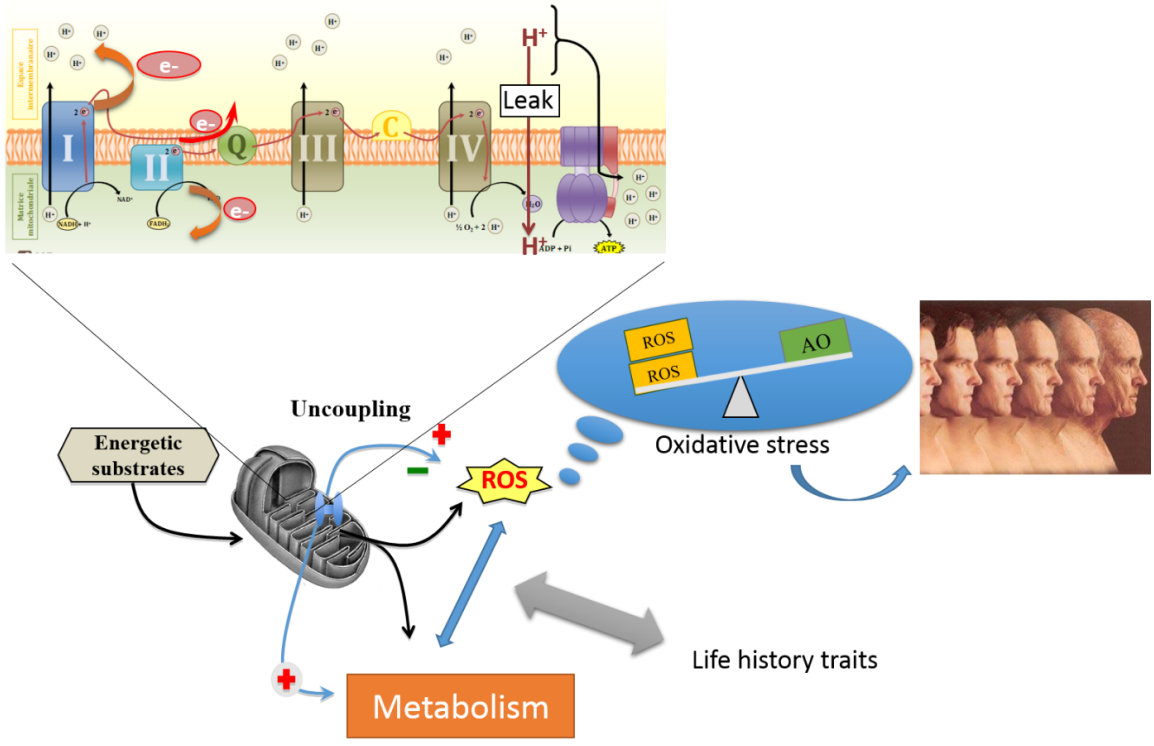

Figure 2. The mitochondria, at the heart of the life history traits theories. (Stearns 1989; Finkel and Holbrook 2000; Balaban et al. 2005; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010; Selman et al. 2012; Costantini 2014)

mechanisms involved in the proton leakiness might involve specific transmembrane transporters (i.e. Uncoupling proteins; Nedergaard et al. 2001; Criscuolo et al. 2005) or passive transport influenced by the membrane structure (Hulbert et al. 2007). The efficiency of OXPHOS is thus evaluated by the number of ATP molecules produced per O2 molecule consumed during this process (the P/O ratio)(Porter and Brand 1995).

Although the mitochondrial OXPHOS is obviously a vital biochemical pathway, it also produces reactive oxygen species (ROS) (Hansford et al. 1997; Figure 2): the reduction of oxygen by electrons that exit the OXPHOS chain generates potentially harmful intermediates such as superoxide and hydrogen peroxide if instead of four electrons that result in the production of water, one or two electrons are transferred (Murphy 2009). ROS can cause significant damages to the cell, including DNA and RNA damage known to contribute to aging and carcinogenesis (Aruoma 1998; Finkel and Holbrook 2000; Halliwell 2007). The negative impacts of ROS to the cell are referred to as oxidative damages. Oxidative stress occurs when the production of ROS overweighs the antioxidant defences (Aruoma 1998; Metcalfe and Alonso-Alvarez 2010; Costantini 2014). The mitochondrial theory of aging proposes “that accumulation of damage to mitochondria and mitochondrial DNA (mtDNA) leads to aging of humans and animals” (Finkel and Holbrook 2000; Balaban et al. 2005) and oxidative stress is therefore an important factor in the life history of an individual (Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010; Costantini 2014; Speakman et al. 2015, Figure 2).

2 Chapter 3a: Immunocompetence and bright colors: a preliminary test of the Hamilton-Zuk model in breeding king penguins

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[#]These authors share seniorship.

2.1 Introduction

One fundamental hypothesis for the evolution and maintenance of ornamental traits is that ornaments may convey honest information to the choosing sex on the quality of prospective mates. Hamilton and Zuk (1982) were the first to suggest that an individual's capacity to resist parasites may be genetically inherited and a central evolutionary pressure in sexual selection. During the following thirty years, a number of evolutionary biologists have tested the Hamilton-Zuk hypothesis, investigating relationships between ornamental traits and various aspects of an individual's immune system. For instance, studies in blackbirds (*Turdus merula*) and American goldfinches (*Spinus tristis*) have shown that the expression of colour ornaments may be reduced in individuals with high parasite loads, or in immunologically-challenged individuals (Faivre et al. 2003a; Rosenthal et al. 2012, respectively). More colorful ornaments have also been associated with higher immune system function (Blount et al. 2003; Kelly et al. 2012; Whittingham et al. 2015), higher resistance to pathogens (Lindström and Lundström 2000; Hill and Farmer 2005) and higher maternal investment of antioxidant and pro-immune pigments (e.g. carotenoids) to egg yolk under challenging conditions (Midamegbe et al. 2013).

Ornament coloration can result from exogenous pigments collected through the diet, such as carotenoids causing yellow to red colorations, or endogenous pigments that are synthetized *de novo*, such as melanins (brown and black coloration) and other less common pigments including pterins and porphyrins (Masello and Quillfeldt 2004; McGraw and Nogare 2005, see also introduction section 6.3.1). The *Ressource Tradeoff Hypothesis* relies on the idea that pigment allocation to either ornaments or immune function acts as a constraint, so that only high quality individuals are able to affoard investing pigments both into coloured ornaments and efficient immune defences (Blount et al. 2003; Faivre, Préault, et al. 2003; Aguilera and Amat 2007, reviewed in Møller, Christe & Lux, 1999; Hill, 2006). Ornamental colours can also be caused by other structures than pigments. Typically, reflective tissues at the nano-scale formed by keratin and collagen usually producing ultraviolet (UV) and iridescent coloration (Gill 1995). In birds, structure remains the least studied aspect of ornament coloration (Griggio et al. 2010a). Nevertheless, some studies have highlighted

negative links between structural colours and parasite loads (Doucet and Montgomerie 2003a; Hill et al. 2005; Mougeot et al. 2005; Soler et al. 2007), individuals with brighter structural colours either showing lower levels of parasite infestation, or, more generally, having higher immune-competence (Anne Peters et al. 2004; Griggio et al. 2010a).

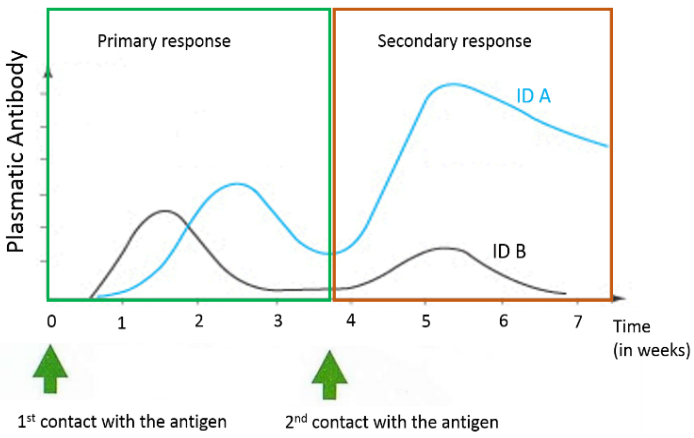

Figure 1. Theoretical diagram of the humoral immune response in term of antibody production along time (weeks) after a first and a second contact with a novel antigen for two different individual (A & B) presenting different immunocompetence.

The immune system is divided into two compartments, the innate and the adaptive function, the latter being itself separated in humoral and cellular responses. The innate immune function is the constitutive and immediate response to pathogen exposure and mediated by a variety of generalist immune cells such as dendritic cells, macrophages and granulocytes (Akira et al. 2006; Kawai and Akira 2006; Takeuchi and Akira 2010), natural antibodies (Nabs) and proteins of the complement system (Janeway and Medzhitov 2002; Beutler 2004; Matson et al. 2005). On top of this generalist immune response, when a pathogen enters the organism for the first time, an adaptive immune response is rapidly triggered within days (McHeyzer-Williams 2005). This *primary immune response* is largely mediated by B and T lymphocytes, respectively named the humoral adaptive response (production of specific antibodies; McHeyzer-Williams 2005) and the cellular adaptive response (Barry and Bleackley 2002). Both processes then lead to the generation of memory cells that remain quiescent, and will allow mounting a higher and even more rapid response, i.e. the *secondary immune response*, the second time the same pathogen is encountered by the organism (Ahmed and Gray 1996; Figure 1).

In their review, Staszewski & Boulinier (2004) underline that vaccines may be used as powerful experimental tools allowing to investigate the organism's ability to respond to infection from parasites. Indeed, measuring the organism's primary humoral response after injection of a novel antigen is largely used by immune-ecologists to evaluate individual immune-competence (reviewed in Schmid-Hempel 2003; Staszewski and Boulinier 2004). Studies have for instance shown that a first immunization with keyhole limpet hemocyanin (KLH) in red-winged black- birds (*Agelaius phoeniceus*) elicits a primary immune response peaking around 12 days after injection (Hasselquist et al., 1999). Broggi et al. (2013) have shown that nonbreeding adult house sparrows (*Passer domesticus*) develop a significant humoral response to New-castle Disease Vaccine (NDV) within 1 week of injection. In this sense, the use of inactivated vaccines to simulate immune responses to

novel antigens may provide a useful means for experimentally testing the trade-off of resource allocation between immunity and sexual ornamentation.

In the present study we sought to test Hamilton and Zuk's model of parasite-mediated sexual selection in king penguins. Specifically, we investigated whether sexual ornaments predicted the individual's ability to mount a humoral immune response after first exposure (primary response) to a novel antigen (NDV).

King penguins face strong ectoparasite loads (including ticks *Ixodes uriae* and lice *Austrogoniodes and Nesiotinus sp.*) during their breeding cycle, known to diminish the health of both adults and their offspring (Gauthier-Clerc et al. 1998; Mangin et al. 2003; Bize et al., unpublish data). Ticks are known to be pathogen vectors (e.g. Gauthier-Clerc et al. 1999) responsible of disease transmission (Gauthier-Clerc et al. 1999). Hence, the ability of birds to mount rapid and strong immune response to a novel antigen might favour individual survival and reproductive success, and may be sexually selected if it provides heritable disease resistance to the offspring. Although it is unknown whether disease resistance is heritable in penguins, mutual mate choice occurs relies on the use of colourful ornaments displayed to tentative mates (Dobson et al. 2011, Nolan et al. 2010, Stonehouse 1960; Bried and Jouventin 2008). Specifically, birds display two yellow to orange feathers patches on each side of their head and a brown to yellow feather patch on their breast (Stonehouse 1960; Bried and Jouventin 2008). They also possess two yellow-orange keratin based spots on each side of their beak that also reflect UV colours (Dresp et al. 2005; Jouventin et al. 2005; Dresp and Langley 2006). Of particular interest is the finding that breast plumage coloration is a reliable indicator of the cellular immune response to a novel mitogen (phytohaemagglutinin, PHA) in the form of foot-web swelling measured 24 h later. Also, Viblanc et al. (2016) recently found a negative but weak association between the purity of yellow-orange beak colours (yellow-orange beak chroma) and the innate ability of the complement system. Further, the size of auricular patches, also known to be important to mate choice (Jouventin et al. 2008; Nolan et al. 2010; Dobson et al. 2011), has been moderately and negatively related to the amount of natural antibodies (NABs) present in the plasma (Viblanc et al. 2016). Those studies suggest that beak and feather ornaments in king penguins may indeed provide information on the two compartments of the immune system, namely the innate and the cellular adaptive responses.

The results presented in this box are based on preliminary data acquired on 16 males only since not all samples have yet returned from the field site for lab analyses in France. Of those 16 males, only 15 were caught during the following incubation shift (see below) since 1 couple failed during reproduction.

2.2 Methods

2.2.1 General methods and immune stimulation

This study was conducted in the king penguin colony of “La Baie du Marin “, Possession Island, Crozet Archipelago (46°250S, 51°450E) during the 2014–2015 breeding season. From December to March, we monitored 20 pairs of breeding adults. All birds were immunologically challenged during their first incubation shift (overall incubation shift 1 for males, incubation shift 2 for females) with the subcutaneous injection of a novel antigen: an inactivated New Castel Disease (NDV) vaccine (Nobivac Paramyxo P201, Intervet, France). For each bird, we injected 25µL of vaccine at the base of its neck and subsequently collected blood samples from the marginal flipper vein during the current incubation shift before the bird left the colony to feed at sea (3 and 10 days following the injection). To insure that peak antibody levels had been reached when the birds returned from their foraging trip, we took three additional blood samples during the subsequent incubation shift (shift 3 for males and 4 for females), each separated by 2 days and compared the antibody levels. Blood samples were taken using 2 mL syringes and stored at 4°C. Within a few hours, samples were centrifuged and plasma was stored at -80°C until serological analyses. Measures of specific anti-NDV antibody levels were performed twice for each sample using an indirect enzyme-linked immunosorbent assay (ELISA) test (ID Screen®Newcastle Disease Indirect, IDvet, France) and are expressed as percentage of inhibition (PI). We corrected anti-NDV antibody levels after vaccination for initial PI values measured at day of vaccination (by subtracting initial PI values) to account for cross-reactions.

2.2.2 Colour measurements

Colour measurements of the beak spot and auricular feather patch of each bird were taken at the start of the experiment before the vaccine was administered. The colour reflectance of those structures was measured using a portable JAZ spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) and color parameters (hue, chroma and brightness) for UV and yellow-orange colors were calculated as described in the general methods section 3.2. Colour of the breast patch has been related to immune-competence in a previous study (Nolan et al. 2006). Thus, we took and additional colour measurement in the brown region of the breast feather patch and included the YO chroma (highly correlated to both brightness and hue) of this ornament in our statistical analysis.

2.2.3 Statistical analysis

All analyses were run in the free statistical software R (v. 3.1.1; R development Core Team 2013). First, we determined the repeatability of the anti-NDV antibody levels (%) when birds came back from their foraging trip over the 3 following measurements (days 2, day 4 and day 6 post foraging trip, Figure 2). Inter-individual variation being higher than intra-individual variation (LM; $F = 45.78$, $P < 0.001$) and repeatability being high (0.94), we calculated the mean between those 3 measurements for each individual and defined it as the response at "ca. day 30". We used a linear mixed model (LMM) to investigate changes in anti-NDV antibody levels (%) over the whole period by specifying the different blood samplings days as categorical variables (a: day of vaccination, b: 3 days post vaccination, c: 10 days post vaccination, d: ca. 30 days post vaccination) and accounting for repeated measures on the same individuals (ID as random factor). The immune response showing a significant increase only after ca. 30 days, we investigated the relationship between ornamental traits (size of the auricular patches, colour parameters of the beak spots, auricular and breast feather patches) and plasmatic antibody level (PI) at ca. 30 days. Since the number of independent variables we tested was important and our current sample size was small, we ran two independent linear models for beak spots and feathers patch ornaments. We added body condition and day of return at the colony as covariates in the model to account for tentative condition effects on bird immunity, however since those two parameters did not explain a substantial amount of variation in antibody levels, we removed them from the final models.

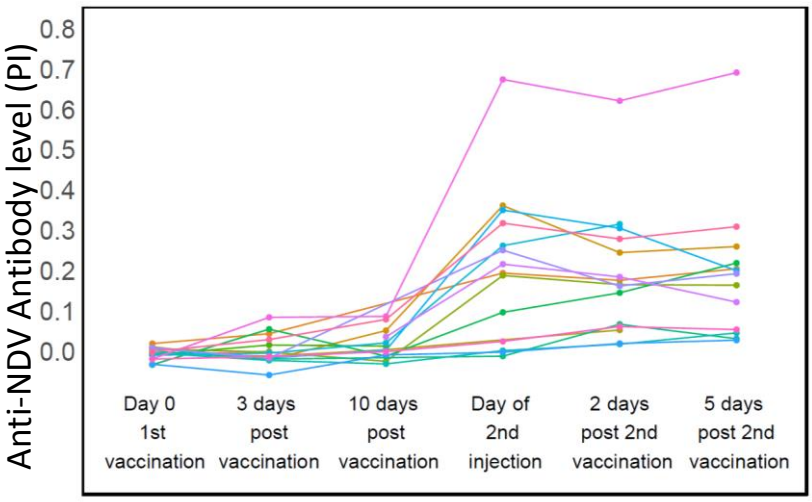

Figure 2. Specific anti-NDV antibodies level (PI) per individual following the first and the second vaccination.

2.3 Results

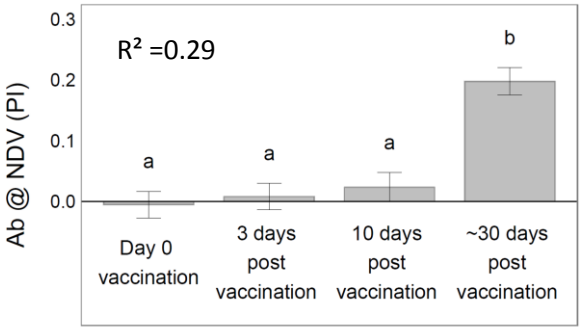

Figure 3. Specific anti-NDV antibody levels (PI) following a New castle disease vaccine injection. Estimate means ± SE extracted from the mixed linear model are represented. Letters reflect statistical differences (HSD Tukey's test, threshold= 0.05).

In the first 10 days following the NDV vaccination, antibody levels increased by 2.2 % on average, though the increase was not significant (day 10-day 0: HSD'Tukey; P = 0.776 N = 16; Figure 3). After ca. 30 days, antibody levels had significantly increased by 20 ± 2.2 % on average. We thus investigated whether bird ornamental features at the start of the experiment predicted the antibody response at ca. 30 days (see Table 1). Beak UV brightness was significantly positively related to antibody levels at ca. 30 days (Figure 4), all other relation being non-significant (Table 1).

	ca. 30 post-vaccination			
Variable	**Estimate**	**std error**	**F**	**Pvalue**
Beak UV Brightness	0.035	0.017	5.654	**0.039***
Beak UV hue	0.004	0.004	2.4540	0.148
Beak UV chroma	-0.434	0.364	0.5938	0.459
Beak YO chroma	1.158	1.222	0.8973	0.366
Auricular YO brightness	-0.007	0.024	0.0007	0.980
Auricular YO chroma	0.714	0.845	0.129	0.726
Surface of the auricular patch	-0.257	0.241	0.923	0.359
Breast YO chroma	-0.001	0.001	0.233	0.640

Table 1. Outputs of the two linear models investigating relationships between ornamental traits and specific anti-NDV antibody levels (PI) at ca. 30 days post-vaccination. Significant relationships are indicated by an asterisk. n = 15 male breeding king penguins.

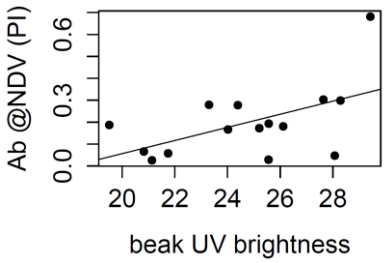

Figure 3. Relationship between UV brightness of the beak and the amount of specific anti-NDV antibody at 10 days (LM; F=5.654, P = 0.039; n = 15).

2.4 Discussion

The vaccination of breeding male king penguins resulted in a slow increase of anti-NDV antibody levels during the first ten days that peaked within 26 days. Contrary to what has been observed in small passerine birds – for which the humoral immune response peaks around 10 days (Hasselquist et al. 2001; Broggi et al. 2013) – the immune response in king penguins took longer to reach is maximum (Figure 1). Nevertheless, we observed that after 30 days individuals differed highly in the amount of antibodies they produced, suggesting this trait to be highly variable.

The humoral response at ca. 30 days reflected the synthesis of antibodies in response to a novel antigen, reflecting the intensity (maximal antibodies secreted) of the humoral immune response. Our preliminary results show that beak UV was associated with the intensity of the immune response (amount of specific anti-NDV antibodies produced) suggesting that beak UV

brightness might reflect the individual's ability to mount a humoral immune response when facing a novel antigen.

In past studies on king penguins, Nolan et al. (2010) have experimentally shown that higher beak UV brightness is associated with higher mating prospects (greater likelihood to successfully pair), and body condition has also been positively linked to UV brightness in males (Dobson et al. 2008; Viblanc et al. 2016). We recently showed that experimentally removing parasites from males and females during incubation resulted in significant changes in the UV component of beak colouration (increase in UV brightness and UV hue, decrease in UV chroma), while not influencing YO colours (see Chapter 2; Schull et al. 2016). In the present investigation UV brightness of the beak also appeared positively related to immune-competence. It is interesting to note that the UV beak coloration was positively influenced by an anti-parasitic solution (Schull et al. 2016) and positively related to immune-competence. This suggests that this ornament may be costly to produce, and that parasite-mediated selection on this sexual feature in penguins might occur.

Due to the small sample size, our results need to be interpreted with caution. However, those preliminary results suggest a link between UV light reflection in the king penguin's beak and immunity. Despite indications of a connection between immune function and ornamental traits, the physiological and biochemical mechanisms that can make the expression of these ornaments honest signals of immune system function remain an open question (Sild et al. 2011; G. E. Hill 2014). The immunocompetence handicap hypothesis as first proposed by Folstad and Karter (1992) suggested the link between immunity and ornament to be under testosterone secretion, with the production of larger/more colorful ornaments resulting in immunosuppression. Hence, only individuals able to withstand a down-regulation of their immune system may fully invest into ornamentation. This hypothesis has been refined to include hormones in a broader context (Westneat and Birkhead 1998). Indeed, just as the hypothalamic–pituitary–adrenal axis may have a short-term positive effect on the immune system but long-term negative consequences if stress is chronic (Buchanan 2000), hormones too may have pleiotropic effects. However the handicap theory fails as a general explanation for the honesty of ornaments since many are not regulated by testosterone or other steroid hormones, and testosterone is not always immunosuppressive (for a review see Hasselquist et al. 1999; Peters 2000; Roberts et al. 2004, for a review see Kimball and Ligon 1999). More recently, Morehouse (2014) proponed the resource trade-off hypothesis as an explanation for how ornaments may be reliable indicators of immune system functionning. Only individuals with sufficient resources can afford to allocate ressources to ornamentation as opposed to self-maintenance processes like immune functions (Lozano 1994; von Schantz et al. 1999; McGraw and Hill 2000; Alonso-Alvarez et al. 2008). However, the later-mentioned studies have relied mostly

on the investment of limiting carotenoids – known to have pleiotropic functions in immunocompetence (Bendich 1989; Chew 1993), oxidative balance (Edge et al. 1997; but see Costantini and Møller 2008) and ornament colouration (reviewed in Simons et al. 2012) – which are exogenous pigments that need to be acquired from the diet. When ornementation relies on endogenously pigments, synthetize *de novo*, or on structural colours (as appears to be the case in the present study), pigment availability and/or pathways of allocation are harder to define. The limiting factor is likely to be considered at its more basic level, i.e. the usable energy form available to the whole organism Adenosine-tri-phosphate (ATP) (Lane 2005; Wallace and Fan 2010). Hence, the efficiency of individuals to transform metabolic resources (carbohydrates, lipids and proteins) into usable ATP has been suggested to explain condition-dependent ornaments under the Shared Pathway Hypothesis (Hill 2011; G. E. Hill 2014). This transformation mainly happens in the mitochondria through oxidative phosphorylation (OXPHOS) (Hatefi 1985; Shutt and McBride 2013). Thus, the much-needed next step is to study the links between ornamental features and energy availability at the site of energy production: the mitochondrion and its efficiency.

3 Chapter 3b : Linking ornamentation to mitochondrial function in breeding king penguins

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[#]These authors share seniorship.

3.1 Introduction

A central aspect of sexual and social selection theories is that ornaments provide reliable (i.e. honest) information on the current condition of their bearer (Zahavi 1975; West-Eberhard 1979; Grafen 1990; Cotton et al. 2004; Walther and Clayton 2004). However, the mechanisms that may enforce the honesty of ornaments remain debated. The 'condition-dependent signalling hypothesis' proposes that honesty is enforced by physical, developmental or physiological constraints that cannot be cheated (Smith and Harper 1995; Maynard Smith and Harper 2003), and a universal mechanism was recently proposed by Hill who highlighted that mitochondrial function may underlie the associations between ornamentation, condition and performance for a broad range of traits across taxa (Hill 2011; G. E. Hill 2014). Mitochondria are cellular organelles responsible for the transduction of energy from food into the cell and whole organism during oxidative phosphorylation (OXPHOS) in aerobic organisms (Mitchell 1961). In other words, OXPHOS is the final metabolic pathway that uses enzymes to oxidize nutrients, thereby releasing energy that is used to synthesize adenosine-tri-phosphate (ATP) or is dissipated as heat (Hatefi 1985; Shutt and McBride 2013). Because mitochondria are the main site of ATP-production, they are often defined as "the powerhouse of the cell" (Nicholls 2002). Notably, mitochondria are also the primary producer of reactive oxygen species (ROS) that have important roles in cell signalling and immunity but that can also, when produced in excess, damage cellular components (Balaban et al. 2005; Hekimi et al. 2011; Kirkwood and Kowald 2012). Mitochondrial ROS production is suspected to contribute to the ageing process (Balaban et al. 2005; Hekimi et al. 2011; Kirkwood and Kowald 2012). Thus, mitochondrial function is characterized by the amount of nutrients and O_2 used over time, as well as the amount of ATP and ROS produced. In addition, decreasing mitochondrial efficiency to produce ATP (i.e. mitochondrial "uncoupling" between O_2 and ATP production) could be useful for endotherms to produce heat as observed in the brown fat of mammals (Nedergaard et al. 2001). Uncoupling oxygen consumption from ATP production may also help slowing down ageing by reducing ROS production

and exposure to oxidative stress (Brand 2000; Mookerjee et al. 2010; Stier et al. 2014). In contrast, increasing mitochondrial efficiency might be beneficial when energetic resources are limited (i.e. during fasting periods) or to optimize physical performances (Monternier et al. 2014; Conley 2016). Consequently, optimizing mitochondrial coupling state according to the biological needs of the organism at a given time is likely to condition individual performance, ultimately affecting fitness. Therefore, if ornament production relies on mitochondrial function and ATP production, we expect that ornament quality should mirror mitochondrial function.

Using breeding king penguins, we report here on the first investigation of the links between mitochondrial function and ornamentation. Adult king penguins display conspicuous yellow to orange feathers on both side of the head (auricular patches) and on the breast, and yellow-orange beak spots that also reflect UV (Jouventin et al. 2005). Experimental manipulations of the UV beak colour and auricular patch size have confirmed that these ornaments are used in mate choice (Jouventin et al. 2008; Pincemy et al. 2009; Nolan et al. 2010). Experimental stress during the production of these ornaments (i.e. during the moult) or during the breeding period indicate costs of production or maintenance for the colouration of auricular patches and the beak (see Chapters 1 & 2; Schull et al. 2016). Hence, we measured mitochondrial function and coloration in breeding king penguins and tested for links between these two traits.

3.2 Methods

3.2.1 General procedure

We studied free-living breeding king penguins in the 'Baie du Marin' colony on Possession Island, Crozet Archipelago (46°25' S, 51°45' E) during the breeding season of November 2013 to March 2014. To ensure that birds where at the same breeding and fasting stage, we sampled 36 males on the third day of their second incubation shift. At capture, we collected in the following order a blood sample from the brachial vein, measured the beak and auricular patch colouration using a portable JAZ spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA), and measured the flipper length and body girth at the nearest 1 mm using a metal ruler and a flexible ruler, respectively. To obtain an index of body condition, we regressed body girth on flipper size ($F_{1,40}$ = 11.69, P = 0.001, r^2 = 0.23) and used the residuals of this regression as an index of body condition which yields condition indices very similar to classical regression of mass on size (correlation, r = 0.92; Viblanc et al., 2012a).

3.2.2 Colour measurements

Colour parameters (hue, chroma and brightness) for UV and yellow-orange colours were calculated as described previously in the general methods Chapter 3.2 and 3.3

3.2.3 Mitochondrial respiration measurements

Birds possess functional mitochondria in their red blood cells (RBCs) that are by far the most abundant cell type in the blood (Stier et al. 2013; Stier et al. 2015). Hence, we used this opportunity to study mitochondrial function in a minimally invasive way by blood sampling breeding penguins (see appendix 3). A blood sample (see above) was collected in the flipper-vein within 5 minutes after capture, kept on crushed ice in the field before being centrifuged within ½ hour after blood collection at 3000*g* for 10 min to separate RBCs from the plasma fraction. 100µL of RBCs were transferred into a new tube containing 1mL of ice-cold phosphate buffer saline (PBS). After gentle homogenisation, RBCs were washed a first time by centrifuging samples at 600*g* for 5 min to pellet the cells, and discarding the supernatant. RBCs were then washed two times in 1mL of ice-cold PBS and finally re-suspended in 1mL of respiratory buffer MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L), pH 7.1).

We investigated the response of intact RBCs to mitochondrial agonists and antagonists, reflecting several aspects of mitochondrial function (Brand and Nicholls 2011). Measurements were obtained by adding 1mL of RBC suspension to 1mL of MiR05 buffer equilibrated at 38°C in the respirometry chamber of one Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). We recorded baseline O_2 consumption after 5-10min of stabilization, we then inhibited ATP-dependent O_2 consumption by adding oligomycin (1 $\mu g.mL^{-1}$), an inhibitor of ATP synthase. Maximal uncoupled O_2 consumption was measured after adding the mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone) at a final concentration of 1µM. Subsequently, we inhibited mitochondrial O_2 consumption by adding antimycin A (5 µM), an inhibitor of mitochondrial complex III. Finally, we added 2.5 mM of ascorbate and 0.5mM TMPD (N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride) to evaluate the maximal activity of complex IV, namely the cytochrome c oxidase (COX). Due to a quick and important release of O_2 during the last step (probably resulting from changes in haemoglobin-O_2 affinity within RBCs), we opened transiently the chamber during 5 min before measuring O_2 consumption. One typical mitochondrial measurement run is illustrated in Figure 1.

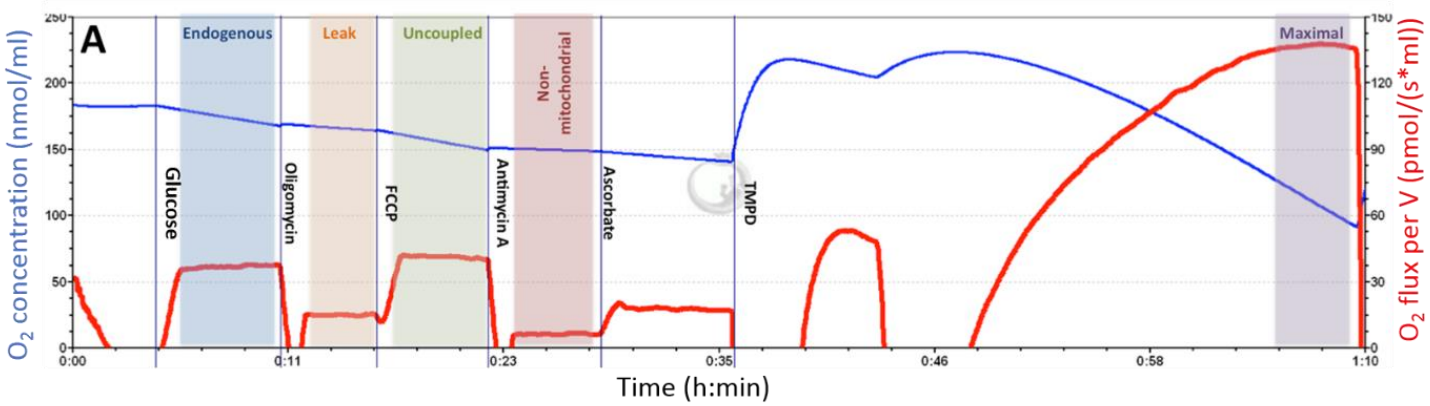

Figure 1. : Bioenergetics assessment of intact red blood cells: (A) Typical mitochondrial measurement run (O2 concentration: blue line and O2 consumption: red line) Following baseline respiration, 1 µg.mL-1 of oligomycin was added to inhibit ATP synthesis. 1 µM of the mitochondrial uncoupler FCCP was then added to stimulate respiration, followed by 5 µM of the complex III inhibitor antimycin A to inhibit mitochondrial respiration. 2.5 mM of ascorbate was then added followed by 0.5 mM of TMPD to evaluate the maximal mitochondrial oxidative activity by providing electrons directly to complex IV.

3.2.4 Mitochondrial respiration parameters

Mitochondrial O_2 consumption rates were obtained by subtracting residual non-mitochondrial O_2 consumption (measured after antimycin-A inhibition; Brand and Nicholls 2011). The respiration parameters obtained and their mechanistic meaning are given in table 1. Endogenous mitochondrial respiration can be divided in two different fractions: the ATP-dependent respiration corresponding to the fraction of O_2 used for ATP synthesis and by extension the overall energy available without any stimulation/inhibition, and leak respiration corresponding to O_2 consumption linked to mitochondrial proton leak. Maximal uncoupled respiration reflects the maximum O_2 consumption of the electron transport chain limited by the available resources within the cells. In contrast, maximal COX respiration is not limited by the available resources within the cells and reflects the ultimate potential respiration rate. We also computed two different respiratory control ratios (RCR) to evaluate mitochondrial coupling between O_2 consumption and ATP synthesis (Brand & Nicholls 2011). We computed the ratio between endogenous and leak respirations ($RCR_{endogenous/leak}$) that provides information about the actual coupling state (i.e. the efficiency in converting ATP for a fixed amount of O_2), and the ratio between maximal uncoupled and leak respirations ($RCR_{uncoupled/leak}$) that provides information about the coupling state of the mitochondria under stimulated conditions (i.e. not limited by ATP turnover). Hereafter, we retained in our statistical analyses the endogenous respiration, ATP-dependant respiration, maximal COX respiration, maximal uncoupled respirations, and the two ratios ($RCR_{endogenous/leak}$ & $RCR_{uncoupled/leak}$) as biologically relevant parameters (see Table 1).

Table 1. Measurement of mitochondrial functioning and the related mechanisms

Respiration type	Measurement	Information
Endogenous	Baseline O_2 consumption under cellular physiological conditions	Mitochondrial O_2 consumption without any stimulation/inhibition
ATP-dependent	Decrease due to ATP synthase inhibition (corresponding to the fraction of O_2 used for ATP synthesis)	Ability of mitochondria to produce ATP via OXPHOS
Leak	Insensitive to ATP synthase inhibition (O_2 consumption linked to mitochondrial proton leak)	Proton leakiness of the mitochondria
Maximal uncoupled	FCCP	Maximal respiration under the current cellular state (i.e. availability of substrates)
Maximal COX	Ascorbate+TMPD (subtracted from residual O_2 consumption and obtained in the presence of ascorbate only)	Maximal potential of the Electron Transport Chain to use O_2 as a final acceptor of e-
$RCR_{endogenous/leak}$	Ratio between Endogenous and Leak respiration measurements	Coupling efficiency of the mitochondria between O_2 consumption and ATP production under **Endogenous conditions**
$RCR_{uncoupled/leak}$	Ratio between Maximal uncoupled and Leak respiration measurements	Coupling efficiency of the mitochondria between O_2 consumption and ATP production under **stimulated conditions**

3.2.5 Statistical analyses

All analyses were run in the statistical computing software R (v.3.1.1; R development Core Team 2013). We used linear models (LMs) to investigate whether ornaments reflected mitochondrial function by running separate models for each mitochondrial trait (endogenous respiration, maximal uncoupled respiration, maximal COX respiration, RCR1 and RCR2) and specifying beak colour traits (hue, chroma and brightness) and auricular patch size as predictor variables in our models (see also Viblanc et al. 2016). Because larger birds are likely to have larger auricular patch sizes, we included flipper size as a covariate to control for structural size. Independent variables (i.e. colour traits) were standardized prior to analyses, so that model estimates were comparable (Schielzeth 2010). We checked for variance inflation factors (VIFs) in the full model and none of the included variables showed VIF > 3.39 (suggested cut-off = 5; Zuur et al. 2007). We ran statistical analyses by multi-model inference with Akaike's Information Criterion corrected for small sample size (AICc) to identify the best models with the lowest AICc, and averaged equally probable models with a ΔAIC < 2 ('MuMIn' package in R; Barton 2016). Diagnostic plots and the Shapiro–Wilk normality test were used to inspect model residuals for normality and potential outliers. Considering that all models included in a confidence set are assumed to be equally well-supported models, and following the principle of parsimony, we retained none of the predictors if the intercept-only model was included in the set of models (Keddar, Altmeyer, et al. 2015). We calculated effect sizes (ES, z-transformed r) and their associated 95% confidence intervals based on z-statistics of the averaged model using equations 11 and 19 in Nakagawa and Cuthill (2007). We used the benchmarks Zr = 0.1, 0.3, 0.5 to discuss weak, moderate and strong effects (Nakagawa and Cuthill 2007).

3.3 Results

3.3.1 *Endogenous respiration*

Beak UV brightness was moderately negatively related to endogenous respiration (*Zr* = -0.42; CI95 = [-0.79, -0.06] Figure 2a&b). Although all the other colour parameters were retained in our model selection procedure, those relationships were weak and their effect sizes largely overlapped zero (Figure 2a).

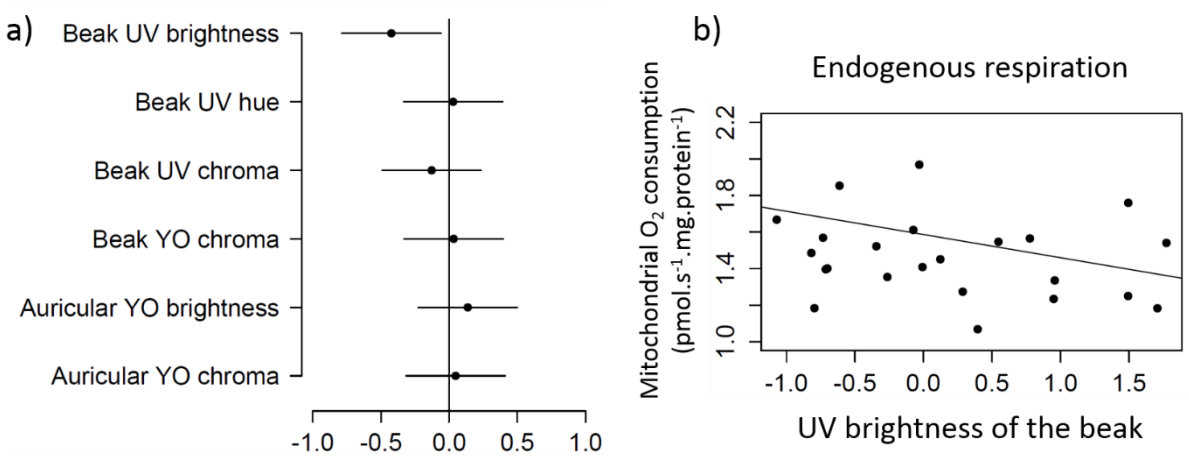

Figure 2: Links between ornamental features and endogenous respiration a) Effect sizes and 95% confidence intervals for ornaments calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero. B) Regression plot between UV brightness of the beak spot and the endogenous O2 consumption (pmol.s^{-1}.mg.protein^{-1}).

3.3.2 *Other respiration parameters*

Our model selection procedure and averaging based on the AICc indicates that the intercept-only model was included in the best models explaining variation in ATP-dependent respiration, maximum uncoupled respiration, maximum COX, $RCR_{endogenous/leak}$ and $RCR_{uncoupled/leak}$. Adding body condition in the starting models did not alter our results on the links between ornament and mitochondrial function. However, this revealed a significantly moderate positive association between body condition and $RCR_{uncoupled/leak}$ (*Zr* = 0.41; CI95 = [0.05, 0.77]).

3.4 Discussion

In agreement with the recently proposed hypothesis that ornamentation may reflect mitochondrial functioning (Hill 2011; G. E. Hill 2014; Koch, Josefson, et al. 2016), we show in breeding king penguins that the UV brightness of the beak spot is negatively linked to mitochondrial endogenous respiration measured in red blood cells. A puzzling question that arises from this result, however, is why should beak UV brightness and mitochondrial endogenous respiration be interrelated and why should it be adaptive for a mate to pay attention to this signal?

In the present study, individuals were sampled 3 days after the beginning of their incubation shift, preceded by the travel back from their foraging grounds hundreds of kilometres away from the colony (Charrassin and Bost 2001). During that period, carbohydrates are rapidly exhausted and lipids become the principal energy resource (Robin et al. 1987; Cherel, Robin, et al. 1988). King penguins are then in fasting phase II (Goodman et al. 1981; Le Maho et al. 1981; Cherel, Robin, et al. 1988; Robin et al. 1988; Belkhou et al. 1991), a metabolic state where energy expenditure is minimized and energy stores preserved to endure the prolonged fast separating them from the return of their partner feeding at sea. Hence, metabolic adaptations to efficiently manage energy stores and energy expenditure during incubation are under strong selective pressure. Indeed, adults generally abandon reproduction if stores are critically depleted (Gauthier-Clerc et al. 2001; Robin et al. 2001; Groscolas et al. 2008). Thus, finding a partner that is able to manage the entire breeding cycle, and notably long fasting periods, is crucial. By lowering their activity and metabolism, individuals might extent their fasting capacity. Accordingly, in a pilot study on fasting king penguins we show that RBC mitochondrial endogenous respiration decreases during fasting (Box 3), which suggests that low endogenous respiration may enable individuals to fast longer.

UV brightness appears to be negatively linked to endogenous respiration in birds starting their incubation. Moreover (1) we previously showed that beak UV brightness decreases at the end of a prolonged fast, likely highlighting a cost to UV maintenance when energy stores are critically depleted (Chapter 2; Schull et al. 2016), and (2) high UV brightness has been associated with increased chances to mate for both sexes (Nolan et al. 2010). Those two results suggest that UV brightness is an important condition-dependent signal used in mutual mate choice. When prospecting for a partner, individuals may thus take this ornamental feature into account to gauge the efficiency of energy management crucial to endure prolonged fasting periods.

1.1.1 Box 3. Endogenous mitochondrial respiration during a prolonged fasting period.

King penguins repeatedly face prolonged periods of fasting during their breeding cycle (Stonehouse 1960). While on land, adults depend entirely on energy reserves during fasting periods of up to 3-5 weeks (Groscolas and Robin 2001). During fasting bouts on-land, individual metabolism first relies mostly on carbohydrates as the main energy substrate, and body mass loss per day rapidly drops during the so-called fasting phase I. Glycogen stores are depleted and lipids become the principal energy resource. During this period, energy expenditure decreases to a minimum, and individuals enter a long energy-sparing period, the fasting phase II (Goodman et al. 1981; Le Maho et al. 1981; Cherel, Robin, et al. 1988; Robin et al. 1988; Belkhou et al. 1991). We followed adult king penguins at the time they initiated their fast associated with the courtship period. We measured mitochondrial red blood cell endogenous respiration at day zero (courtship), after 12 days and after 24 days of fast of captive individuals. Birds showed a significant decrease between the two first measurements. The endogenous respiration remains stable after 24 days. Reducing metabolism and physical activity helps extending the period during which energy stores may sustain metabolism. We suggest that the decrease in cellular respiration reflects the decrease of the metabolic rate at the individual level related to the energy-sparing fasting phase II period.

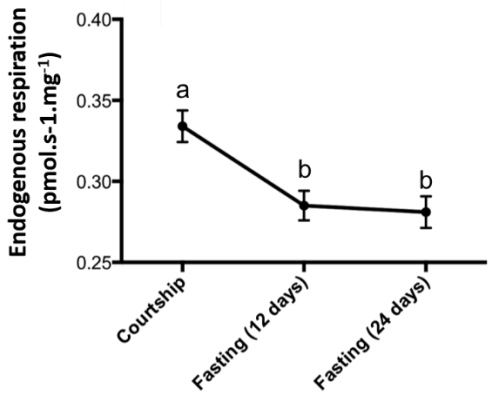

Figure box 3. RBC endogenous respiration (in pmol.s-1.mg-1 of 10 parading king penguins kept in captivity during 24 days of a fasting period. Letters represent significant differences between groups (Generalized Estimating Equation; Wald Chi Square = 13.38, 0=0.01, $\alpha < 0.05$)

Cell respiratory control ratios (RCRs) are believed to be the most reliable test of mitochondrial functioning using intact cells (Brand and Nicholls 2011). The $RCR_{endogenous/leak}$ ratio reflects the coupling between O_2 consumption and ATP production in the maximal potential stage of the electron transport chain, i.e. the quantity of ATP produced per O_2 unit when the electron transport chain is not limited by ATP turnover. If ornaments function as signals of cellular respiration efficiency (Hill 2011; Hill 2014; Koch et al. 2016) we would expect individuals presenting higher RCR to also adorn more colourful ornaments. However, in our study we found no evidence of links between ornaments and RCRs. This might be explained by at least 3 different reasons.

First, beak UV reflectance results from structural coherent scattering based on the reflection of stacks of elongated lamellae (multiple layers of doubly folded membranes) in the horny layer of the beak (Dresp and Langley 2006). The yellow-orange colouration is likely to be explained by the deposition of pigments in the deeper layers of beak spots and the feathers as it is the case in many

other bird species (L Saks et al. 2003; McGraw and Gregory 2004). In the king penguin, pigments present in the feathers belong to the pterin family (Thomas et al. 2013) which are endogenously synthetized from purines, as well as from other intermediate products of adenine or guanine metabolism by the organism (Needham 1974; Mcgraw 2006). Renewing the beak spots (high cellular division and keratinization) as well as synthetizing pterin pigments de novo are expected to be energy consuming processes and thus to be constrained by cellular respiration efficiency. However those ornament are produced within the short time scale of the moult (3-4 weeks; Cherel et al. 1988a; Bourgeon et al. 2007; Schull et al. 2016) and the relationships between O2 consumption and ATP production are not constant, can vary within and among individuals at the mitochondrial level (Brand 2005; box 1), and in response to a number of environmental parameters, including diet and temperature (Salin et al. 2015). A better understanding of the relationships between mitochondrial efficiency and ornamentation will thus require longitudinal measurements along the life cycle of the individual, with specific focus on important life history transitions such as during the establishment of ornamentation in young individuals, the renewal of ornaments during the annual moult, and during the different incubation/brooding shifts which vary in terms of fasting duration. In addition, it accounting for environmental sources of variation affecting energy expenditure (e.g. parasitism, climate, colonial density; Wiersma and Piersma 1994; Martin et al. 2003; Viblanc et al. 2012) should allow refining the links between cellular respiration efficiency and ornamentation.

Second, mitochondrial function express tissue-specific efficiency (Veksler et al. 1995; Holmström et al. 2012; Karamercan et al. 2014) and we are more interested to conclude at the scale of the organism rather than at the scale of the organ/cell. However, a preliminary study suggests that mitochondrial parameters are in some extent correlated between organs, and consequently that mitochondrial parameters measured in RBCs are likely to have relevance at the whole organism scale (box 1; appendix 3). Moreover, as mitochondrial function is tightly linked to ROS production (Balaban et al. 2005; Hekimi et al. 2011; Kirkwood and Kowald 2012), cellular respiration efficiency might directly be involved as a mediator of life history trade-offs (Costantini 2008; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010) and the ageing process (Balaban et al. 2005; Hekimi et al. 2011; Kirkwood and Kowald 2012). Given that the expression of sexual/social ornaments appear to be rather sensitive to ROS, it has been suggested that oxidative stress may provide a mechanism linking ornament expression to variation in genetic fitness (von Schantz et al. 1999). Interestingly, in king penguins the UV component of the beak spot (UV hue) appears to be positively and negatively related to oxidative damage in males and females, respectively (Viblanc et al. 2016). Thus, future studies should try to tease apart the respective contributions of ROS production and mitochondrial efficiency in determining ornament expression, in an attempt of defining at the finest scale the underlying trade-offs that occur.

Finally, it is important to keep in mind this study is based on a small sample size of 36 incubating males and recently developed measures of mitochondrial function in red blood cells (Stier et al. in prep; appendix 3). As discussed above, more work is now needed, with refined methods and greater sample sizes, to gain on knowledge on the importance of mitochondrial function as a key mechanism enforcing the honesty of ornamentation.

Chapter 4

Offspring quality is foremost explained by ornamentation of biological parents early in life and of rearing parents late in the development in the king penguin

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1 Abstract

Secondary sexual and/or social ornaments are expected to help their bearer to attract mates and/or deter rivals to engage in aggressive interactions for access to vital ecological resources, respectively. Hence, ornamentation can influence fitness through two major routes: (i) transmission of high quality genes and early maternal effects; and (ii) access to high parental care and better ecological resources. By swapping eggs between breeding pairs in the monomorphic king penguin (Aptenodytes patagonicus), we investigated how ornamentation of biological and foster parents relate to measures of offspring quality (size, body condition and oxidative balance) both early in life (at 10 days post hatching) and late in the development (after 105 days post hatching). In this species, considerable investment by both sexes is required for successful reproduction, and recent studies suggest a role for reciprocal sexual and social selection in the evolution of the coloured beak spots and auricular patches of male and female king penguins. We showed that chick early growth characteristics (at 10 days) where mostly explained by ornamental features of their biological parents, while later in life (at 105 days) foster parent ornaments became better predictors of the chick phenotype. Hence, our findings highlight that the links between ornaments and fitness can arise through cumulative effects of genetics and early maternal effects and high parental care throughout development

Key words: Sexual ornaments, honest signal, oxidative stress, King penguin.

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2 Introduction

Secondary sexual characteristics, often viewed as the prerogative of males, are thought to provide females with honest information on the quality of tentative mates (Andersson 1994; Walther and Clayton 2004; G. E. Hill and McGraw 2006; G. E. Hill 2014). Indeed, in many species males provide little to no parental care, transmitting only genes to their offspring. Females may then choose breeding partners of high genetic quality on the basis of costly secondary sexual characteristics, thereby transferring good or compatible genes to offspring that may directly affect offspring phenotype and fitness, i.e. a *direct* contribution to fitness (Mays and Hill 2004; Neff and Pitcher 2005). For instance, the colourful plumage of many bird species (Fisher 1930; Cronin 1991) or the horns and antlers of large mammalian herbivores (Clutton-Brock et al. 1979; Clutton-Brock et al. 1980; Clutton-Brock 1982), are often thought to evolve by means of sexual selection providing honest information to females on the genetic quality of their bearer. In birds, elaborate ornamental features have indeed been associated with individual body condition (Velando et al. 2001; Doucet 2002; Dobson et al. 2008; McGraw et al. 2009; Viblanc et al. 2016), stress (Eraud et al. 2007; Almasi et al. 2010; Mougeot, Martínez-Padilla, et al. 2010; Viblanc et al. 2016), or immune-competence (Møller, Christe, et al. 1999; Faivre, Grégoire, et al. 2003; Mougeot, Martinez-Padilla, et al. 2010; Sild et al. 2011; Rosenthal et al. 2012).

In contrast to species where males only contribute genes to the next generation, many species have evolved sophisticated parental care in both sexes. Parental care that occurs after fertilization is best described as an *indirect* contribution to fitness. In such cases, selection may favour the evolution of ornaments, providing information not only on direct genetic effects but also on indirect social contributions that occur long after mating, for instance via parental care that in most cases is associated with territorial defence (Rohwer 1985; Senar 2006; Santos et al. 2011) or the capacity to efficiently provision offspring (Burley 1988; de Lope and Møller 1993; Raouf et al. 1997; Siefferman and Hill 2003; Préault et al. 2005). Such forms of social selection on ornamentation are especially likely to occur in social or colonial species with extended parental care (Trivers 1972; Møller and Thornhill 1998; Iwasa and Pomiankowski 1999; Kokko and Johnstone 2002), where partner's direct and indirect contributions to fitness are strong for both sexes, and may promote the evolution of mutual ornamentation (Johnstone et al. 1996; Kokko and Johnstone 2002; Hooper and Miller 2008; Jones and Ratterman 2009; Schaedelin et al. 2015). Indeed, mate choice in monomorphic species is expected to be influenced by both sexual and social selection since the pay-off for choosing an ornamented partner is likely to include access to foraging territories, nest sites and the quality of parental care in addition to access to mating (West-Eberhard 1979; Lyon and Montgomerie 2012; Tobias et al. 2012).

Whereas several studies in monomorphic species have considered the honesty of ornamental traits linked to genetic quality during mate choice (Dunn et al. 2013), little consideration has been given as to whether ornamentation also reflects individual quality in social contexts after the mating period. This is perhaps not surprising since teasing apart the use of ornaments in sexual vs. social contexts is rather complex (West-Eberhard 1979; Lyon and Montgomerie 2012). In this study, we propose to use an alternative and easier approach by questioning the direct and indirect links between ornamentation and fitness, and by testing the respective contributions of sexual (via high quality genes and early maternal effects) and social (via access to high parental care and better ecological resources) selection in shaping ornamentation in a monomorphic seabird, the king penguin (*Aptenodytes patagonicus*). We do so by performing a cross-fostering experiment swapping eggs between biological and foster pairs at egg-laying to dissociate genetics and early maternal effects from environmental effects and explore the links between ornamental features and chick development.

King penguins are long lived colonial seabirds that breed on land but feed at sea several hundreds of kilometres away from their colony (Charrassin and Bost 2001). Their chicks rely entirely on the nutrients in the egg during embryonic development, on parental care for thermoregulation and nourishment early in life (until ca. 35 days), and on extended parental care for nourishment during the first year of life (Stonehouse 1960; Weimerskirch et al. 1992). Successful breeding requires the full cooperation and coordination of both parents for over a year (Stonehouse 1960; Cherel and Le Maho 1985; Weimerskirch et al. 1992). Mutual mate choice for high quality partners is therefore expected to be strong in this species (Johnstone et al. 1996; Kokko et al. 2000; Kokko and Johnstone 2002; Kraaijeveld et al. 2007; Hooper and Miller 2008), and experimental manipulations of the beak spot and ear patch ornaments (colourful yellow-orange and UV features) have confirmed that these ornaments are used in mate choice (Jouventin et al. 2008; Pincemy et al. 2009; Nolan et al. 2010). Further, experimental stress manipulations during the production of these ornaments (i.e. during the moult), or later in time (during the breeding period) indicate costs of production and maintenance for ear patch and beak coloration (Chapter 1 & 2; Schull et al. 2016). King penguins are therefore an interesting model species to investigate whether mutual mate choice may constrain offspring development through parental genetic quality/maternal investment into the eggs, or through parental care of the chick related to individual quality in social or foraging contexts. On top of chick growth rates, we consider how ornamental features predict chicks' oxidative stress status during development as oxidative stress is suggested to be a central mechanism underlying the trade-off between growth and self-maintenance (Nowicki et al. 2002; Alonso-Alvarez et al. 2007). Indeed, one of the costs of rapid growth is the production of damaging reactive oxygen species (ROS) – including in king penguins (Geiger et al. 2012) – which damaging effects may be mitigated by organisms' anti-

oxidant (Finkel and Holbrook 2000) and conditioned by female investment of anti-oxidant into the egg (Midamegbe et al. 2013) or parental ability to supply the chick with exogenous antioxidants, especially since chicks rely exclusively on parental provisioning until fledging at 14-mo. of age (Stonehouse 1960; Weimerskirch et al. 1992).

If chick development is mainly determined by genetic parent quality, we predict stronger relationships between biological parent ornamental features and chick developmental variables. In contrast, if chick development is mainly determined by parental care and/or environmental factors, we predict stronger relationships between foster parent ornamental features and chick developmental variables. Finally, because effects of parental care and environmental factors can overcome genetics and early maternal effects as chicks grow older (Bize et al. 2002), we predict that chick developmental variables will be primarily related to biological parent ornaments early in the development (at 10 days) and with foster parent ornaments later in the development (at days 105).

3 Materials and methods

3.1 Study species and general methods

This study was performed in the breeding colony of "La Baie du Marin" (approx. 20,000 breeding pairs), Possession Island, Crozet Archipelago (46° 26' S, 51° 52' E) over two breeding seasons: Nov. 2012 to May 2013 and Nov. 2013 to May 2014. Each year, we followed 54 breeding pairs from the moment they settled in the colony on their final breeding territory to the moment their chick reached 105 days of age, corresponding to the start of the Austral winter. Both males and females were first marked on the belly from a 1-m distance using spray animal dye (Porcimark®, Kruuse, Lageskov, Denmark). We then monitored those pairs daily at a distance using binoculars. The first time one member of the pair was observed alone incubating an egg, it was identified as a male at day 1 of incubation (Stonehouse 1960; Weimerskirch et al. 1992). The bird was then flipper-banded (semi-rigid P.V.C. Darvic bands; 25.8 mm wide, 1.9 mm thick, 7.4 g) during our first intervention (i.e. cross-fostering; see below) allowing its identification and follow-up during the study. Females were later caught and flipper banded when returning from their foraging trip at sea to relieve their partner on incubation duty. All flipper-bands were removed from the birds at the end of the study.

3.2 Cross-fostering experiment

Three days after the egg was laid, i.e. during the first incubation shift of the male, we performed a cross-fostering experiment and swapped eggs between penguin pairs that had laid their egg on the same day. The procedure required 3 persons. First, two males were caught and rapidly

hooded to minimize stress. Their respective egg was carefully removed from the brood pouch (king penguins incubate their single egg on their feet against a featherless skin region known as the brood pouch; Handrich 1989) and replaced by a warm dummy plaster egg during the exchange. Eggs were weighed to the nearest 1 g. One person then proceeded to exchange the eggs while the 2 other persons remained by the birds at all times to ensure the procedure went smoothly. Once the eggs were swapped and individuals released, we monitored bird behaviour to ensure it settled down once again on its breeding territory. We never witnessed breeding abandonment by the birds at this stage.

3.3 Morphometric and ornamental measures

During the incubation period, both partners were caught for morphometric (flipper and beak length) and ornamental (beak and ear feather patch) measurements. Females were caught during the second incubation shift when they returned from their foraging trip at sea to relieve their partner. Males were caught during the 3rd incubation shift. Both males and females were caught 2 days after having started their incubation shift, to ensure they were motivated to stay on the egg. Thus, those animals were of comparable fasting status. For males, we decided against performing measurements at the time of egg swapping since they had already undergone a 15-day fasting period during courtship (Stonehouse 1960; Weimerskirch et al. 1992) and thus differed in their nutritional status from females. In addition, this alleviated the stress caused to the bird at the time of egg swapping (king penguins become very agitated if their brood pouch is manipulated; QS, VAV, PB, JPR; *personal observations*). Chicks were caught, weighed, measured (flipper, tarsus and beak length) and blood sampled at 10 and 105 days post-hatching.

3.3.1 Structural size and body condition measurements

Adults: Flipper length was measured (± 1mm) from the sternum to the tip of the flipper using a solid metal ruler (for details; see Fahlman et al. 2006). Pectoral body girth was measured beneath the flipper (± 1mm) as a surrogate measure of body mass (for a validation; see Viblanc et al. 2012a). We used flipper size as an index of structural size (SSI). We then regressed body girth on flipper size ($F_{1,250}$ = 12.31, P < 0.001 , R^2 = 0.05) and used the residuals as an index of body condition. This method yields condition indices very similar to classical mass/size regressions (correlation, r = 0.92; Viblanc et al., 2012a), but is more practical than weighing birds in the breeding colony.

Chicks: For chicks, beak size and flipper length were measured as described above. In addition, we measured tarsus length (± 1mm) from the base of the heel to the end of the longest toe. Body mass was measured (± 5g) using a Pesola® spring-slide scale. Structural size was then defined as

the first axe (PC1 explaining 91 and 71 % at day 10 and 105, respectively) of a principal component analysis between beak length, flipper length and tarsus length ($SSI_{day\ 10}$ = 0.56 x beak length + 0.58 x flipper length + 0.59 tarsus length; $SSI_{day\ 105}$ = 0.57 x beak length + 0.61 x flipper length + 0.54 tarsus length). We then regressed body mass on PC1 ($F_{1,108}$ = 42.42, P < 0.001 , R^2 = 0.29 and $F_{1,80}$ = 85.06, P < 0.001 , R^2 = 0.52 at respectively day 10 and day 105) and used the residuals as an index of body condition.

3.3.2 Ornamental measurements in parents

For all birds, we calculated auricular patch surface as the surface of the ellipse (S = π x L/2 x w/2) defined by the length (L) and width (w) of the auricular patch measured to the nearest 1-mm using a flexible tape-ruler (see Figure S1).

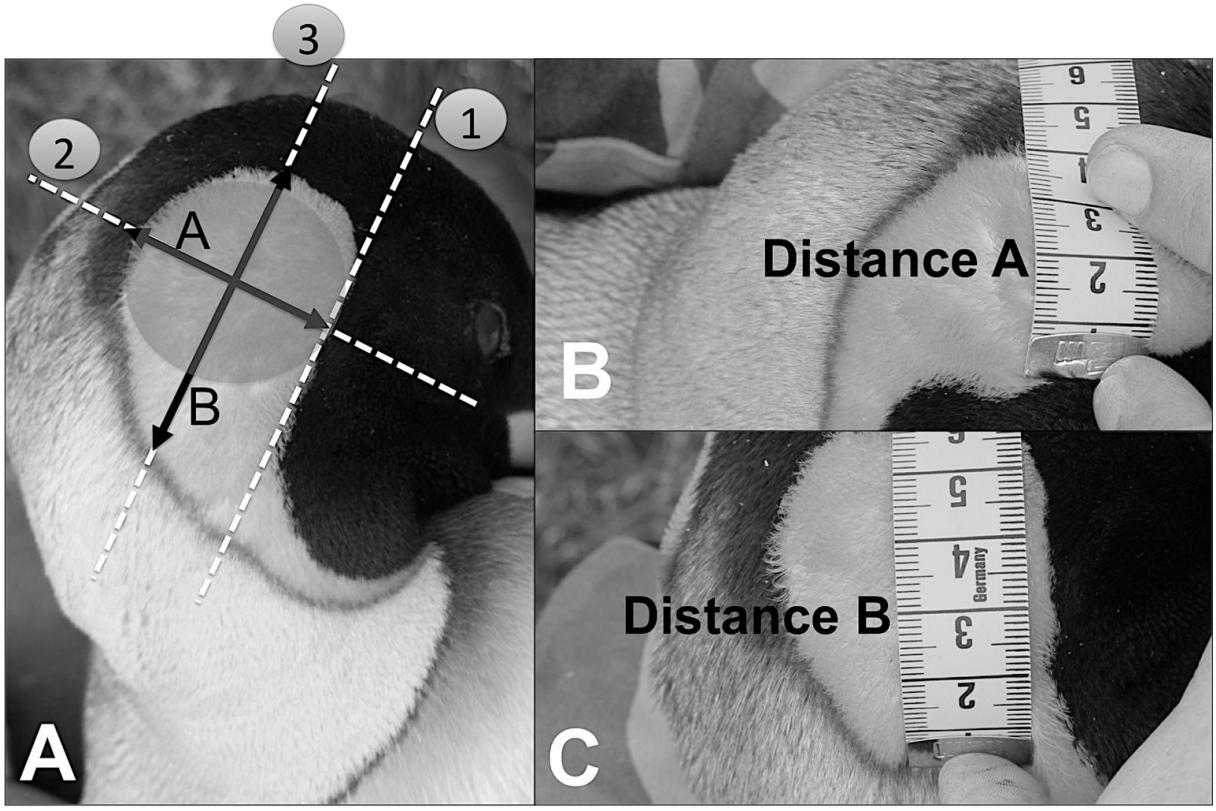


Figure S1: Standardized measures of the auricular patches of breeding king penguin (*Aptenodytes patagonicus*). The head of the bird was held such that its beak rested on the shoulder opposite to the side of the body where the auricular patch was measured (Fig. S1A). A virtual line was pictured along the side of the auricular patch closest to the eye (line 1; Fig S1A). Then, a second perpendicular line reaching the most distant point of the circle (diameter) was pictured (line 2; Fig S1A), and the width of the auricular patch was measured (distance A; Fig S1B). From the center of distance A (line 3; Fig S1A), the height of the auricular patch was measured at a 90° angle (distance B; Fig S1C). Extracted from Viblanc et al. 2016)

We also measured the colours reflected by the beak spot and the auricular patch using a portable JAZ spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA). This spectrophotometer contained a pulsed-xenon light with a spectral resolution of 0.3 nm across the spectral range of 320-700 nm, and was calibrated against a white standard (Ocean Optics Spectralon). Measures were repeated 3 times across each ornament using a 200 µm fibre-optic probe at a 90° angle window. Using an R script adapted from Montgomerie (Montgomerie 2008), the obtained spectra were smoothed and averaged before calculating mean brightness, hue, and chroma (see below) over the spectral range 320-700 nm, corresponding to the full range of spectral sensitivity in birds (Cuthill 2006).

In the king penguin, beak spots reflect light both in UV-violet (UV) (320-490 nm) and yellow-orange (YO) domains (491-700 nm). In contrast, ear feather patches only reflect light in the yellow-orange domain (over 350 nm). We thus calculated colour variables (brightness, hue and chroma) separately for UV and YO domains. Brightness is a measure of spectral intensity and was calculated by averaging reflectance over UV ($UV_{brightness}$) and YO ($YO_{brightness}$) regions separately (Montgomerie 2006, see Schull et al. 2016). Hue is a measure of colour appearance and was calculated as the wavelength of maximum reflectance between 320 and 490 nm for the UV region (UV_{hue}). For the YO region, YO_{hue} was calculated as the wavelength at which the reflectance was halfway between its maximum and minimum over 491-700 nm for the beak spot and 350-700 for the auricular patch (Keddar et al. 2013, Schull et al. 2016). Finally, chroma, a measure of colour purity, was calculated within the region of interest (UV_{chroma} and YO_{chroma}) as the difference between maximum and minimum reflectance over the mean reflectance for that particular region (formula S8; Hill and McGraw 2006, p. 108). In a previous study based on a large sample (n = 181), the analysis of UV colour parameters showed that brightness, hue and chroma are independent parameters that may signal different information to breeding birds (for a discussion, see Chapter 2; Schull et al. 2016 and supplementary material therein). We therefore considered them separately in subsequent analyses. In contrast, YO colour parameters were highly correlated in the beak. Thus, we chose to focus on YO_{chroma} for beak, as this measure was the one presenting the highest among-individual variation (thus containing the most information; Dale 2006), and directly reflects ornament pigment concentrations in several bird species (Saks et al. 2003; McGraw and Gregory 2004). Similarly, YO_{chroma} and YO_{hue} of the ear patches were highly correlated, thus we focused on YO_{chroma} and $YO_{brightness}$ for analyses of the auricular patch.

3.3.3 Chick blood sampling and oxidative balance

3.3.3.1 Blood sampling

When blood sampling the chicks, we covered the bird's head with a hood to minimize stress and agitation. Blood samples (ca. 0,1mL at 10 days and 1 mL at 105 days post hatching) were taken from the brachial vein. We used a heparinised capillary for the chick at day 10 and a G22-1 ½ needle fitted to a 1 mL heparinized syringe for the chick at day 105. All blood samples were obtained within 3 min of handling. After centrifugation (3000 g for 10 min), plasma was kept frozen at -20 °C and moved to a -80°C ultra-cold freezer at the end of the day until assayed.

3.3.3.2 Total plasma antioxidant capacity

We measured total plasma antioxidant capacity using the OXY Adsorbent test (5 µL of 1:100 diluted plasma) (Diacron International©, Grosseto, Italy) in accordance with methods reported in previous studies (e.g. Costantini and Dell'Omo 2006; Stier et al. 2013). The OXY adsorbent test quantifies the ability of the plasma to buffer massive oxidation through hydroperoxyde acid. All sample measurements were duplicates. Intra-individual variation was 5.18 % and inter-plate variation based on a standard sample repeated over plates was 6.29 %.

3.3.3.3 Reactive oxygen metabolites (ROMs)

Plasmatic ROMs levels were measured using the d-ROMs test (8 µL of plasma) (Diacron International©, Grosseto, Italy), in accordance with methods reported for previous studies (e.g. Costantini and Dell'Omo, 2006; Stier et al., 2013). The d-ROMs test measures mostly hydroperoxydes, as a marker of oxidative damage and is expressed as mg of H2O2 equivalent/dL. Intra-individual variation based on duplicates was 7.86 % and inter-plate variation based on a standard sample repeated over plates was 7.91%.

3.3.3.4 Thiobarbituric Acid Reactive Substance (TBARS)

Lipid peroxidation plasma levels were measured using a commercial Thiobarbituric Acid Reactive Substance assay (20µL plasma) (Oxitek ZeptoMetrix© New York, USA). This method reveals the degradation product of lipid peroxidation, i.e. malondialdehyde (MDA), which reacts with TBA under conditions of high temperature to generate a coloured adduct measured by spectrometry. TBARS are measured in nmol/mL. Intra-individual variation based on duplicates was 2.44 % and inter-plate variation based on a standard sample repeated over plates was 6.85%.

3.4 Statistical analyses

All analyses were run in the statistical computing software R (v.3.1.1; R development Core Team 2013).

We used LMs to investigate the links between (1) egg mass and biological parent ornaments; and (2) chick developmental traits (at day 10 and day 105) and biological or foster parent ornaments. Statistical analyses were performed independently for each parent (biological female, biological male, foster female and foster male). Egg mass and developmental traits (body mass, size index, body condition, ROMs, TBARS, OXY levels) were specified as dependent variables in separate LMs. Parent ornamental traits (beak and auricular patch colours, auricular patch size) were specified as independent variables. When analysing chicks at 105 days, we controlled for initial chick condition by adding size, body mass, body condition, and oxidative balance parameters at day 10, as covariates in the respective models. Year was included as a fixed factor in all models. To control for potential variable social environment, we tested for colony density (high or low) as covariates. Indeed, a relationship between density and aggressivity has previously been highlighted (Viblanc et al.; Steeve D. Côté 2000), however this factor never showed significant effect on chick feature and have been removed during model selection procedures. Independent variables were standardized (centered and scaled) prior to analyses (Schielzeth 2010). We checked for variance inflation factors (VIFs) in the full model (suggested cut-off = 5; Zuur et al. 2007) and used a multi-model inference procedure to identify the best subset of candidate models where parent ornamental traits explained observed variation in egg mass and chick developmental traits. We ran all possible models ('MuMIn' package in R; Barton, 2016), and selected models with the lowest Akaike's Information Criterion (AIC). Models with a $\Delta AIC < 2$ when compared to the model with the lowest AIC were assumed to be equally well-supported models (Burnham and Anderson 2002). Parsimoniously, if the intercept-only model was included in the set of models presenting a $\Delta AIC < 2$, we considered that none of the ornamental variables explained substantial variation in in egg mass or chick developmental traits. Models with a $\Delta AIC < 2$ were averaged (full-model averaging; 'MuMIn' package in R; Barton, 2016), and the effect sizes (ES; z-transformed r) and 95% confidence intervals for retained independent variables were calculated based on the z-statistics of the averaged models (see equations 11 and 19 from Nakagawa & Cuthill, 2007). Diagnostic plots and the Shapiro–Wilk normality test were used to inspect model residuals for normality and potential outliers. We use the benchmarks r = 0.1, 0.3, 0.5 to discuss small, medium and large effect sizes (Nakagawa & Cuthill, 2007). To improve the reading of the results we only present only independent variables presenting a relatively high level of contribution in the averaged model (Relative Variable Importance > 0.7).

4 Results

4.1 Biological parent ornamentation and egg mass

Egg mass was only related to the coloured ornaments of biological males. Model selection retained auricular $YO_{brightness}$ and YO_{chroma}, beak $UV_{brightness}$ and UV_{chroma}, and male structural size. However, only auricular $YO_{brightness}$ had a relative importance higher than 0.7 (RVI=1, Table 1 & 2) in the averaged model. Indeed, the mass of the egg was positively related to male's auricular patch $YO_{brightness}$ (*Zr* = 0.32; *CI95* = [0.11, 0.54], Figure 2, Table 1).

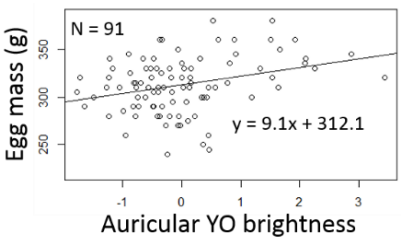


Figure 2. Relationship between Genetic male Auricular $YO_{brightness}$ and egg mass. The equation is provided by the full-averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016), Sample size is given in the top left corner.

4.2 Parent ornamentation and chick developmental parameters

4.2.1 Chick body mass

At 10 days of age, chick mass appeared to be related to the ornamental features of biological parents (male and female) and to the ornamental features of the foster male. Model selection on biological males retained auricular patch YO_{chroma}, beak UV_{chroma}, male structural size and body condition. For biological females, model selection retained auricular patch $YO_{brightness}$, beak YO_{chroma} and UV_{chroma}, and female body condition. However, only the YO_{chroma} and $YO_{brightness}$ of the auricular patch had an RIV > 0.7 (RVI=1) for biological male and biological female models respectively (Table 1 & 2). Regarding foster males, model selection retained, beak $UV_{brightness}$, beak UV_{hue}, beak UV_{chroma}, auricular $YO_{brightness}$, auricular YO_{chroma}, patch surface and male structural size as well as body condition (Appendix). Auricular YO_{chroma} of biological males and auricular $YO_{brightness}$ of biological females and foster males, were negatively (weakly to moderately) related to chick body mass at 10 days (Table 1; Figure 3). Female foster ornaments and morphometric parameters showed no pattern of association with chick mass at 10 days (intercept-only model included in the subset of models with a ΔAIC < 2).

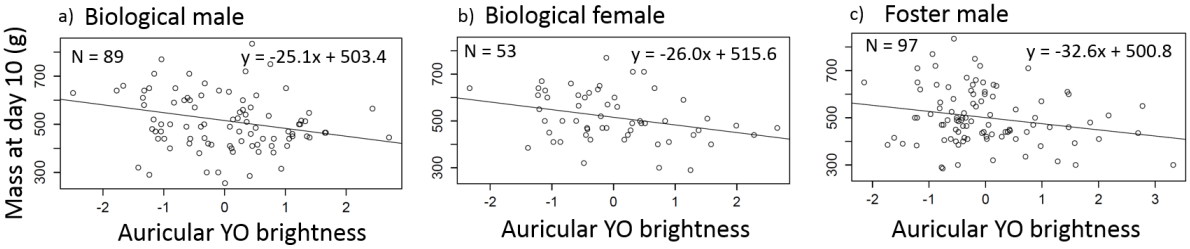


Figure 3. Relationships between a) genetic male auricular YO_{chroma} b) genetic female and c) foster male auricular $YO_{brightness}$ and chick body mass at 10 days. The equation is provided by the full-averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016). Sample sizes are given on the top left corner of each panel.

At 105 days, biological male ornaments or morphometric parameters were not related to chick body mass, nor were foster female variables (intercept-only model included in the subset of models with a ΔAIC < 2). Model selection on biological females retained beak UV_{hue}, UV_{chroma} and YO_{chroma}, as well as female structural size and body condition. Beak UV_{chroma} was the only parameter with a high RVI (0.74, Appendix). However the 95% confidence intervals (CI_{95}) overlapped zero, thus the effect remained non-significant (Table1). Model selection on foster males retained auricular beak UV_{hue}, and auricular $YO_{brightness}$, YO_{chroma}, and patch surface as well as male structural size. Despite the three later parameters presenting RVI = 1; 0.88; and 0.83, respectively, only auricular YO_{chroma} was significantly and moderately negatively correlated to chick body mass at 105 days (Table 1 & 2, Figure 4).

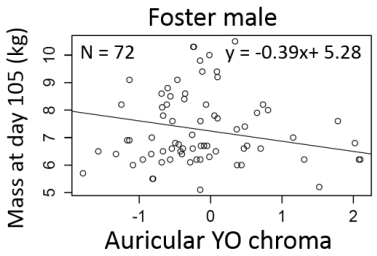


Figure 4. Relationship between foster male auricular YOchroma and chick body mass at 105 days. The equation is provide by the full-averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016), Sample size is given in the top left corner.

4.2.2 *Chick size*

At 10 days, biological female and foster male ornamental features were related to chick body size. In contrast, model selection for biological males and foster females included the intercept-only model in the subset of models with a ΔAIC < 2. For biological females, model selection retained auricular patch $YO_{brightness}$ beak $UV_{brightness}$, beak UV_{hue} and beak YO_{chroma}. Only auricular $YO_{brightness}$ had a RVI > 0.7 and was significantly moderately and negatively related to chick size at 10 days (Table 1, Figure 5). For foster males: beak $UV_{brightness}$, UV_{hue} and UV_{chroma}, auricular patch $YO_{brightness}$ and YO_{chroma}, auricular patch surface, and male structural size were retained by the model (table2). However, none was significantly related to chick size (CI_{95} overlapped zero; Table 1).

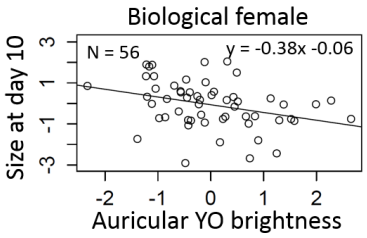


Figure 5. Relationship between foster male auricular $YO_{brightness}$ and chick size at ten days. The equation is provide by the full-averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016), Sample size is given in the top left corner.

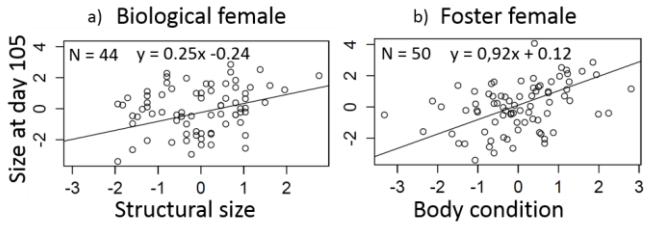


Figure 6. Relationship between a) genetic female's structural size and chick size, and b) foster female's body condition and chick size at 105 days. The equations are those provide by the full-averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016). Sample sizes are given on the top left corner of each panel.

At 105 days, no links were found between chick size and biological or foster males (intercept-only model in the subset of models with a ΔAIC < 2). For biological females, model selection

retained beak UV_{chroma}, female structural size and body condition. Female structural size was the only variable presenting a RVI > 0.7 (Appendix) and was strongly and positively related to chick size at 105 days (Table 1 & 2 and Figure 6a). For foster females, model selection retained beak $UV_{brightness}$, YO_{chroma}, auricular patch YO_{chroma} and female body condition. Female body condition had RVI = 1 other variable presenting RVI < 0.7 (Appendix). Body condition was significantly, strongly and positively related to chick size at 105 days (Table 1, Figure 6b).

4.2.3 Chick body condition

At 10 days, only biological male parameters were selected to explain chick body condition. Whereas model selection retained auricular patch surface, beak UV_{chroma}, beak YO_{chroma}, body condition, beak UV_{hue} and male structural, none of those variables were significantly related to chick body condition (CI_{95} overlapped zero; Table 1).

At 105 days, chick body condition was related to ornamental and morphometric parameters of the biological female and foster male. For biological females, model selection retained female body condition, beak $UV_{brightness}$, UV_{hue}, and UV_{chroma} as well auricular patch surface. Body condition of the biological mother at the beginning of the second incubation shift being the only variable presenting a RVI < 0.7 was also moderately and positively related to chick body condition at 105 days (Table 1 & 2 and Figure 7a). Model selection on foster males retained beak $UV_{brightness}$, UV_{hue}, and UV_{chroma}, beak YO_{chroma}, auricular patch $YO_{brightness}$, YO_{chroma} and surface, as well as structural size and male body condition. However, only auricular patch YO_{chroma} and beak beak UV_{hue} presented RVIs > 0.7 and were strongly and moderately, respectively, negatively related to chick body condition at day 105 (Table 1 & 2, Figure 7b and 7c).

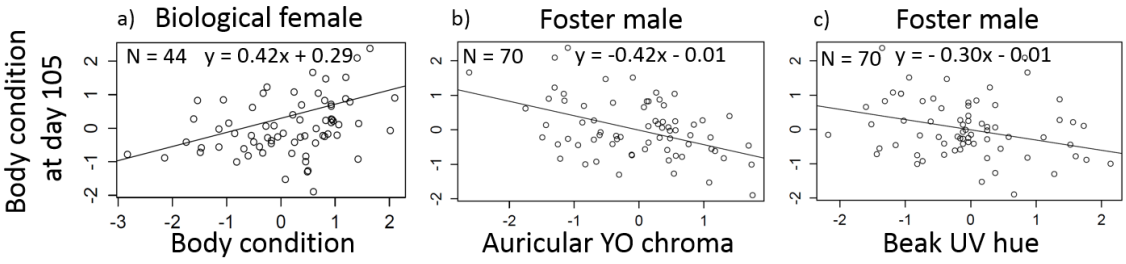


Figure 7. Relationships between a) body condition of the genetic females at the beginning of their first incubation shift and chick body condition b) auricular YO_{chroma} of the foster male and chick body condition c) Beak UV_{hue} of the foster males and chick body condition at 105 days. The equations are those provided by the full-averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016). Sample sizes are given on the top left corner of each panel.

4.2.4 Chick oxidative balance

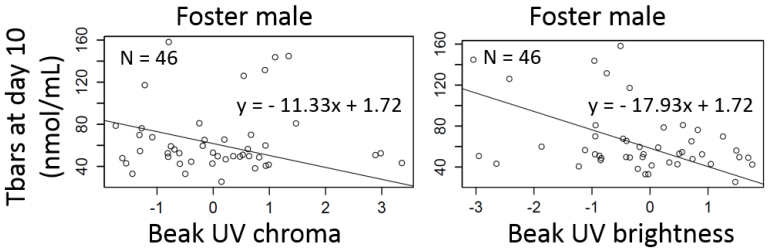


Figure 8. Relationship between beak UV brightness and chroma of the foster male and the TBARs plasmatic level of the chick at 10 days. The equation is provide by the averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016). Sample sizes are given on the top left corner of each panel.

Both at days 10 and 105, model selection did not reveal any important association between biological or foster parents parameters and chick D-ROMs or OXY plasma levels (intercept-only model included in the subset of models with a ΔAIC < 2).

In foster parents, we found a relationship linking ornamental traits to chick plasma TBARs level, both at day 10 and day 105. At day 10, model selection on foster males retained beak $UV_{brightness}$ and UV_{chroma}, and beak YO_{chroma}. Beak $UV_{brightness}$ and beak UV_{chroma} were strongly and moderately, respectively, negatively related to chick TBAR plasma levels (Table 1 & 2 and Figure 8). Model selection on biological female retained beak UV_{chroma}, and YO_{chroma} and body condition. However, none of those variables were significantly related to chick TBARs level (CI_{95} overlapped zero; Table 1)

At day 105, model selection on foster females retained beak $UV_{brightness}$ and UV_{chroma}, auricular patch YO_{chroma} and surface, as well as female structural size (Appendix). Beak UV_{chroma} showed a moderate positive link with chick plasma TBAR levels (Table 1, Figure 9).

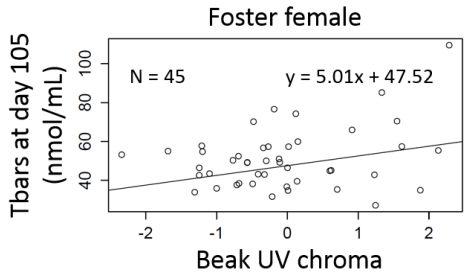


Figure 9. Relationship between beak UV_{chroma} of the foster female and the TBARs plasmatic level of the chick at 105 days. The equation is provided by the averaged selected models were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016). Sample size is given on the top left corner of each panel.

		Day 10										Day 105							
		EGG MASS		MASS		SIZE		BODY CONDITION		TBARs		MASS		SIZE		BODY CONDITION		TBARs	
		Zr	CI_{95}	Zr	CI_{95}	Zr	CI_{95}	Zr	CI_{95}	Zr	CI_{95}	Zr	CI_{95}	Zr	CI_{95}	Zr	CI_{95}	Zr	CI_{95}
Biological Male	Auricular YO brightness	0.323	0.111 ; 0.535																
	Auricular YO chroma			-0.225	-0.439 ; -0.010														
	Auricular patch size							0.127	-0.088 ; 0.341										
Biological Female	Beak UV chroma									0.189	-0.132 ; 0.511	0.179	-0.142 ; 0.501						
	Auricular YO brightness			-0.333	-0.608 ; -0.057	0.347	0.071 ; 0.622												
	Structural size													0.422	0.105 ; 0.739				
	Body condition															0.361	0.040 ; 0.682		
Foster Male	Beak UV brightness					0.165	-0.041 ; 0.371			-0.508	-0.816 ; -0.200								
	Beak UV hue					-0.141	-0.347 ; 0.065									-0.361	-0.605 ; -0.116		
	Beak UV chroma									-0.334	-0.642 ; -0.026								
	Auricular YO brightness			-0.229	-0.434 ; -0.024														
	Auricular YO chroma											-0.289	-0.530 ; -0.049			-0.517	-0.761 ; -0.272		
	Auricular patch size											0.172	-0.068 ; 0.413						
	Structural size											0.197	-0.044 ; 0.437						
Foster Female	Beak UV chroma																	0.334	0.022 ; 0.646
	Body condition													0.535	0.241 ; 0.830				

Table 1. Relationships between egg size or chick growth (day 10 and 105) and genetic or foster adult ornamental traits, structural size and body condition. Statistical analyses were done independently for each parents (genetic female, genetic male, foster female and foster male). Effect sizes (Zr, z-transformed r) and their associated 95% confidence intervals (CI_{95}) based on z-statistics of the averaged model using equations 11 and 19 from Nakagawa & Cuthill, 2007 are given. We present only explicative variables presenting a relatively high level of contribution in the averaged model (relative variable importance > 0.7). Results highlighted in blue indicate significant effects (CI_{95} do not overlapped 0). Obviously, we did not investigate the link between egg mass and foster parents, because it is biologically without meaning.

			DAY 10				DAY 105			
		EGG MASS	MASS	SIZE	BODY CONDITION	TBARs	MASS	SIZE	BODY CONDITION	TBARs
Biological Male	Beak UV brightness	0.37								
	Beak UV hue		0.22		0.20					
	Beak UV chroma	0.14								
	Beak YO chroma				0.57					
	Auricular YO brightness	1								
	Auricular YO chroma	0.19	1							
	Auricular patch size		0.15		0.83					
	Structural size	0.25			0.02					
	Body condition		0.14		0.49					
Biological Female	Beak UV brightness		0.08	0.60					0.22	
	Beak UV hue			0.15			0.08		0.62	
	Beak UV chroma		0.17			0.79	0.74	0.63	0.09	
	Beak YO chroma		0.21	0.25		0.50	0.08			
	Auricular YO brightness		1	1						
	Auricular patch size							0.31	0.09	
	Structural size						0.16	1		
	Body condition		0.34			0.42	0.14		1	
Foster Male	Beak UV brightness		0.61	0.84		1			0.08	
	Beak UV hue		0.23	0.83			0.05		1	
	Beak UV chroma		0.66	0.38		1			0.08	
	Beak YO chroma					0.30			0.47	
	Auricular YO brightness		1	0.67			0.08		0.04	
	Auricular YO chroma		0.20	0.13			1		1	
	Auricular patch size		0.08	0.05			0.83		0.15	
	Structural size		0.39	0.06			0.88		0.20	
	Body condition		0.07						0.31	
Foster Female	Beak UV brightness							0.57		0.21
	Beak UV chroma									1
	Beak YO chroma							0.52		
	Auricular YO chroma							0.11		0.15
	Auricular patch size									0.20
	Structural size									0.07
	Body condition							1		

Table 2: Relative Variable Importance levels obtained after model averaging were ΔAIC < 2 ('MuMIn' package in R; Barton, 2016). In blue are highlight the variable presenting a RVI > 0.7 for which we calculated the effect size and respective CI_{95} presented in table 1.

5 Discussion

Theoretical models point out that ornamental features should only evolve if they provide ultimate fitness benefits to their bearer (Darwin 1871; Harvey and Bradbury 1991; Kirkpatrick and Ryan 1991; Andersson 1994; Andersson and Iwasa 1996; Andersson and Simmons 2006), and numerous empirical studies have indeed reported positive associations between ornamentation and fitness (Johnstone 1995; Velando et al. 2001; Siefferman and Hill 2003; Jouventin et al. 2008; Vergara et al. 2015; Wiebe and Vitousek 2015). However, we still know little on the proximate mechanisms linking ornamentation to fitness and on the respective contributions of sexual and social selection in shaping the evolution of ornamentation. Indeed, ornaments might evolve because they facilitate access to reproduction (sexual selection; Darwin 1871; Harvey and Bradbury 1991; Kirkpatrick and Ryan 1991; Andersson 1994; Andersson and Iwasa 1996; Andersson and Simmons 2006), or because they provide advantages in a non-sexual context (e.g. defence of nest sites, parental care) and thus evolve via (non-sexual) social selection (West-Eberhard 1979; West-Eberhard 1983; Tanaka 1996; Lyon and Montgomerie 2012; Tobias et al. 2012). Because a single ornament may have multiple functions, for example for attracting mates during courtship and deterring rivals to engage in fights during territory defence outside courtship (Johnstone 1996; Tobias et al. 2011), trying to assign one ornament to one form of selection is often unproductive (Lyon and Montgomerie 2012; Tobias et al. 2012). Here, we used an alternative approach where we swapped eggs between breeding pairs of the monomorphic king penguin and investigated the links between ornamentation of biological and foster parents and fitness, using markers of chick quality as fitness proxies. This allows us to investigate direct and indirect relationships, with direct links being signalled by ornamentation of the biological parents (i.e. genetics and early maternal effects) and indirect links by ornamentation of the foster parents (i.e. parental care).

In king penguins, chicks entirely rely on parental care in terms of nourishment and thermoregulation until ca. 35 days. In this species, males are generally the first to return from sea to feed the newly hatched chick (Stonehouse 1960; Olsson 1996). Brighter auricular patches in males appear to predict poor parental care, as the $YO_{brightness}$ of foster males was negatively related to the body mass of the chick both at day 10 and day 105. In addition, auricular $YO_{brightness}$ and YO_{chroma} of biological parents (female and male, respectively) also showed negative relationships with chick body mass at day 10, suggesting poor maternal and genetic investments

Interestingly, our results show that egg mass was positively related to male auricular $YO_{brightness}$, suggesting that female investment into the egg was higher when mated with a brighter ornamented male. It has been shown for several species that females adjust the number, mass or

nutrient contents of the egg laid in response to the attractiveness of their partner (Reyer et al. 1999; Cunningham and Russell 2000; Uller et al. 2005; Horváthová et al. 2012). Two opposite hypotheses have been made: The *Reproductive Compensation Hypothesis* proposes that one of the parents attempts to make up for potential lowered offspring viability due to the poor quality of its partner by increasing reproductive investment and effort (Gowaty et al. 2007; but see Harris and Uller 2009), whereas the *Differential Allocation Hypothesis* predicts an increased investment into offspring produced with high-quality mates (Cunningham and Russell 2000; Sheldon 2000; Uller et al. 2005; Horváthová et al. 2012). In king penguin, the YO colour of male auricular patches being associated with chicks in poorer condition but larger maternal investments in the egg support the idea that female king penguins might adjust their investment by laying larger eggs when their partner's auricular ornamentation predicts poor genetic contribution and low parental care. Indeed, larger investments in eggs have been suggested to be associated with increased offspring development and fitness, especially in seabirds (Williams 1994; Krist 2011). The fact that ornamental colours of the ear patch were negatively related to chick growth in our study could suggest an underlying trade-off occurring for traits under social selection such as it has been shown for territory defences and parental care in songbirds where male with enlarged ornaments also showed reduced parental care (Burley 1988; de Lope and Møller 1993; Raouf et al. 1997). Interestingly, in king penguins auricular patch size has been shown to be related to territorial aggressiveness and is suggested to be under social selection (Viera et al. 2008; Keddar, Jouventin, et al. 2015). Whether auricular patch coloration is another modality of the signal used in social aggressiveness, however, remains to be determined.

Although several studies have considered the links between parental care and pigment based ornamentation (Møller 1993; Sundberg and Larsson 1994; Sætre et al. 1995; Wiehn 1997; Linville et al. 1998; Smiseth and Amundsen 2000; Senar et al. 2002; Voltura et al. 2002; Griggio et al. 2010b), only fewer have focused on the link between structural colouration and parental care (Keyser and Hill 2000; Smiseth et al. 2001; Siefferman and Hill 2003; Siefferman and Hill 2005; Limbourg et al. 2013) and results appear to be contrasted. Keyser and Hill (2000) found positive associations between the intensity of structural coloration in male blue grosbeaks (*Passerina caerulea)* and chick provisioning rates. In eastern bluebirds (*Sialia sialis*), brighter UV/blue males provisioned incubating females at higher rates, structural-based plumage appearing to be an honest signal of male parental care that might be assessed by potential mates or competitors (Siefferman and Hill 2003; Siefferman and Hill 2005) . In contrast, Smiseth et al. (2001) found no relationship between blue-UV plumage coloration and parental care in male bluethroats (*Luscinia svecica*), nor were structural colours related to parental care in either sex of the Blue tit (*Cyanistes caeruleus*) (Limbourg et al. 2013).

In our study, relationships between beak UV features and chick growth parameters were only found for foster parents. In opposition to the YO-ornaments, beak spots with brighter, purer (high chroma) and deeper (lower hue) UV colour appeared to reflect good parental care but only in males, leading to lower oxidative damage (lipid peroxidation) for chicks early in life and a higher body condition at the beginning of the winter. In contrast, female beak UV features showed a negative relationship with oxidative stress of the chick later in life. In king penguins, UV_{hue} in females has been negatively linked to their own plasmatic oxidative damages (ROMs) during breeding (Viblanc et al. 2016) and higher $UV_{brightness}$ of the beak spot has been experimentally associated with higher mating prospects (i.e. a greater likelihood to successfully pair; Nolan et al. 2010). TBARs have been shown to increase when the quality of the diet decreases (vitamin and anti-oxidant content; Monahan et al. 1994; Ahn et al. 1997; Dixon et al. 1998; Villar-patiño et al. 2002; Piršljin et al. 2008), as well as after a fasting period (Piršljin et al. 2008). Altogether, those results suggest that the quality of the diet the chick receives during the first days of life by the foster male (Stonehouse 1960; Olsson 1996) and during the entire first year of growth by both foster parents might have an impact on its ability to buffer oxidative stress, with consequences on plasmatic lipid peroxidation levels. Thus, the UV reflectance of the male beak might reflect male ability to provisioning the chick, which would be especially important early in life, explaining the link between $UV_{brightness}$, UV chroma and chick features at day 10. Finally, the body condition of the foster female being related to chick body condition later in life, suggests that high body condition may reflect generally high female quality and increased ability to forage at sea and provision the chick during its entire growth.

To conclude, we showed that chick early growth characteristics (at 10 days) where mostly explained by ornamental features of their biological parents, while later in life (at 105 days) foster parent ornaments became better predictors of the chick phenotype. Hence, our findings highlight that the links between ornaments and fitness can arise through cumulative effects of genetics and early maternal effects and high parental care throughout development. Auricular patch colours are mostly negatively associated with both initial investment and parental care, suggesting this ornament evolved mostly by social selection with likely trade-offs between social competition and parental care in both sexes. In contrast, UV beak features appeared to be reliable signals of father parental care. Those findings support the idea that in species living in social groups and with high levels of biparental care, ornamentation can evolve in both sexes in response to a blend of sexual and social selection (Tobias et al. 2012).

General discussion

1 A brief look back over the aims of my PhD and on my main achievements

During the course of my PhD I attempted to bring new insights on the evolution and function of ornaments using the king penguin as a study system. In this colonial bird species, both sexes display colourful ornaments (sexual monomorphism), which raises interesting questions about the evolutionary pressures favouring the expression of ornaments in both sexes, and notably whether ornaments signal the same qualities in male and females.

In a preliminary correlative study in which I took part at the start of my PhD, Viblanc et al. (2016) showed that monomorphic beak ornamentation reflects several aspects of physiological quality such as body condition, innate immune system, resting metabolic rate and stress response as well as oxidative balance, in breeding king penguins. Interestingly, the qualities signalled by mutual ornamentation may nonetheless differ (in fact be opposite) between the sexes, likely due to physiological differences and varying selection pressures. However, we collected measurement at one point in time at the beginning of the breeding season.

To better understand the signalling value of coloration of the beak spot, and notably its dynamicity, in the chapter 2, I collected repeated measures in breeding/fasting penguins and in penguins treated with an anti-parasitic solution. Results show that beak spot coloration is a dynamical trait, and notably that UV brightness declines during a natural 10-day fast and increases in individuals after the removal of their parasites. Those results indicate that beak coloration may serve as an honest signal of short-term changes in individual condition over the course of a single breeding season.

To gain a deeper understanding of the honesty of ornaments in the king penguin, in the chapters 1, using an experimental approach I paid a particular attention at changes in ornamentation during the moult. We found that some ornamental features show strong condition-dependence (yellow auricular feather chroma, yellow and UV chroma of the beak). This indicates that they are costly to produce and may be honest signals used in mate choice. Other features were condition-independent and remained highly correlated before and after the moult (auricular patch size and beak UV hue), which suggests that their honesty may require social mediation.

Parasites are ubiquitous, and Hamilton and Zuk (1982) have hypothesized an important role for parasites in the evolution of ornamentation. In the chapter 3a, I investigated this hypothesis by challenging males and females breeding king penguins with a novel antigen (New Castle Disease Vaccine) and link mutual ornamentation with the humoral immune response. The preliminary analysis of those results on males (females samples being still on the field) revealed that UV

brightness of the beak may reflect the efficiency of mounting an humoral immune response, at least in that sex.

Nowadays, the mechanisms that enforce the honesty of ornaments remain debated. Hill has recently shed light on the mitochondrion as a key organelle in ensuring the honesty of ornamentation (G. E. Hill 2014). In chapter 3b, I present the first study testing for links between coloration and mitochondrial function. We showed that the UV brightness of the beak spot is negatively linked to mitochondrial endogenous respiration measured in red blood cells, suggesting that this ornamental feature reflects the efficiency of energy management crucial to endure prolonged fasting periods

Finally, to address the links between ornamentation and fitness, in the chapter 4, I performed a cross-fostering experiment where I swapped eggs between breeding pairs. This designed allowed me to show that chick early growth characteristics where mostly explained by ornamental features of their biological parents, while later in life foster parent ornaments became better predictors of the chick phenotype. However results also highlight that the links between ornaments and fitness can arise through cumulative effects of genetics and early maternal effects and high parental care throughout development.

Merging correlative and experimental data, this thesis highlight that ornamentation in the King penguin honestly reflect individual quality. Since such relationships appear similarly in males and females but might also differ between sexes, it suggest that mutual ornamentation may evolved under mutual mate choice, allowing individual to access potential partners quality while parading. While some ornaments appear strongly condition dependent at the moment they are produce, some other seems to be cheap to produce and thus evolved mostly under social selection. Finally, the ornamented beak, being a dynamical trait convey rapid condition changes of the bearer along the breeding season, raising the question whether those dynamic changes may be perceived by conspecifics. To conclude, King penguin ornaments might evolve in both sexes in response to a blend of sexual and social selection.

2 Maintenance of the ornamentation in both sexes

2.1 Condition-dependency and the evolution of ornaments?

2.1.1 Signal honesty of structural colour: UV beak spots

In the king penguin, UV reflectance of the beak spot relies on the structural properties of the beak (Dresp et al. 2005; Dresp and Langley 2006), and depends on the thickness of the upper beak layer composed of the doubly folded membrane structures. Those result from the differentiation of basal cells into dead keratin (as is the case for skin renewal) (Dresp and Langley 2006). Although Nolan et al. (2010) demonstrate using an experimental approach that UV beak spot coloration may be used in mutual mate choice, the signalling value of the UV beak spot had received little attention. Specifically, we knew little about the links between UV reflectance and important physiological functions that appear as central mediators in life history trade-offs, i.e. metabolic rate, immunity, oxidative stress, activity of the hypothalamic-pituitary-adrenal axis (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003; Bize et al. 2008; Costantini 2008; Monaghan et al. 2009; Palacios et al. 2009; Schulenburg et al. 2009; Groscolas et al. 2010; Metcalfe and Alonso-Alvarez 2010; V. A. Viblanc et al. 2012; V. A. Viblanc et al. 2014; V. A. Viblanc et al. 2014). Thus, in our preliminary work with Viblanc and collaborators (2016), we investigated the links between ornament coloration and physiological functions. We found positive association between the UV brightness of beak spots and resting metabolic rate, and negative associations between beak UV hue and stress response. In addition, we found that beak UV brightness and UV hue were positively related to body condition and oxidative stress, respectively, in male but negatively in females (Viblanc et al. 2016). Whereas those results suggested that beak UV might be a condition-dependent factor signalling sex-specific physiological constraints in males and females, this study relied essentially on a correlative data set making it hard to establish causality links between UV production/maintenance and physiological mechanisms ensuring signal honesty. Hence, in my PhD work, I built upon those preliminary results by a mix of correlative and experimental studies. Specifically, I showed that (1) on a short time scale (days to weeks), beak spot colouration decreased in response to fasting and increased after an experimental parasite removal in both sexes (Chapter 2). Those rapid changes in a structural colour outside of the moulting period were surprising and highlight that structural colours are probably more costly to maintain than previously thought. In the king penguin, a likely candidate mechanism is cellular reorganisation of those structures and/or even cell division (Schull et al. 2016). However the exact cost of structural cell production were unknown. Thus (2) by experimentally stimulating the

immune response (LPS injection) during moult I highlighted the condition-dependence and the cost of production of UV chroma (Chapter 1). Two following correlative studies also showed that (3) UV brightness was positively related to humoral immune function and negatively to endogenous cellular respiration in male (Chapter 3 a&b), both suggesting that during mate choice female might access their potential partner ability in mounting an immune response necessary to deal with the important parasites loads on-land (Gauthier-Clerc et al. 2003; Mangin et al. 2003; Bize et al. unpublished data) and in managing energy reserves, crucial to endure prolonged fasting periods (Groscolas et al. 2000; Robin et al. 2001; Groscolas et al. 2008). However those relationships need to be investigated also in females in further experiments to investigate whether the reliability of those signals are maintain in both sexes. (4) Using and experimental cross-fostering design, I showed that chick growth parameters are predict by beak UV features for foster parents only. Those relationships were opposite between males and females. Beak UV chroma and beak UV brightness of the male were positively related to a better growth and lower oxidative stress of the chick later in life whereas foster female beak UV chroma showed a negative relationship with chick oxidative stress later in life. It highlights that UV beak features appear as a reliable signal of good father's parental care but poor parental investment by females (Chapter 4).

Mutual mate choice occurs in species showing ornamentation and choosiness in both sexes (Johnstone et al. 1996; Kokko and Johnstone 2002; Kraaijeveld et al. 2007; Nolan et al. 2010) and theory predicts that mutual choosiness can be adaptive when both sexes vary in quality (Parker 1983; Johnstone et al. 1996; Bergstrom and Real 2000; Kraaijeveld et al. 2007). Taken together, our results support the idea that the UV features of beak spots are important signals influencing mate choice in both sexes (Nolan et al. 2010), likely because it is a condition dependent signal that is costly both to produce and maintain. Using this ornamental feature when choosing a mate may provide individuals with important information both (1) on the efficiency of individual energy management processes (low metabolic rate, body condition, energy reserve depletion) and (2) individuals capacity mount humoral immune responses when facing parasites.

2.1.2 Signal honesty of endogenous and exogenous pigments

2.1.2.1 Yellow-orange beak spots

The yellow-orange reflectance of the beak spots of king penguins is likely caused by carotenoid pigments, assimilated through the diet (exogenous) (McGraw et al. 2007), that are only present in the deeper parts of the beak (Dresp et al. 2005; Dresp and Langley 2006). YO chroma has been shown to directly reflect carotenoid pigment concentration in several species (L Saks et al. 2003;

McGraw and Gregory 2004; McGraw et al. 2009; Butler and McGraw 2011). In our preliminary study (Viblanc et al. 2016), we found that the YO chroma of king penguin beak spots was positively related to body condition, but only in females (no link was found in males). One explanation might be that females YO colour of the beak depends on their foraging ability, where female of better foraging aptitude are also in higher condition and might afford to allocate more (or might acquire more) carotenoid pigments to their beak. Further investigation are required to establish weather a direct link might be find between carotenoid ornament and foraging abilities at sea.

The trade-off between current reproduction and survival is particularly important in this species (since breeding and foraging grounds are separated by long distances), and the efficient management of stored energy is critical to breeding success (adults generally abandon reproduction if stores are critically depleted; (Gauthier-Clerc et al., 2001; Groscolas et al., 2008; Olsson, 1997; Robin et al., 2001). Since the longest month-long fasting bout during reproduction is undertaken by the male (Stonehouse 1960; Weimerskirch et al. 1992) and success relies on the timely return of their female partner (else the egg being abandoned), this is consistent with the idea that the signal is selected in females and not necessarily in males. Notably, males facing a 24 day fasting period (see Chapter 2), the YO chroma of their beak significantly decreased. This observation is in line with the hypothesis of costly signalization by (limited) carotenoid-dependent structures (e.g. in birds: Alonso-Alvarez et al. 2004; in fish: Pike et al. 2010; but see Cote et al. 2010 in reptiles; and Costantini and Møller 2008 for a review), and highlights the cost of maintaining such ornament. This might be due to a re-allocation of beak carotenoid pigments to other important functions such as antioxidant defences. Indeed, because of their antioxidant propreties, carotenoid pigments have been suggested to act as central mediators lying at the cross-roads of oxidative stress and ornament expression (von Schantz et al. 1999; Mcgraw 2005; Simons et al. 2012). The decrease in beak YO chroma we observed is consistent with our recent finding showing that during long-term fasting, king penguins counteract an increase in oxidative stress by increasing enzymatic anti-oxidant defences and maintaining their overall anti-oxidant defences (including non-enzymatic) high (Schull et al. 2016b, appendix 1, Figure 1).

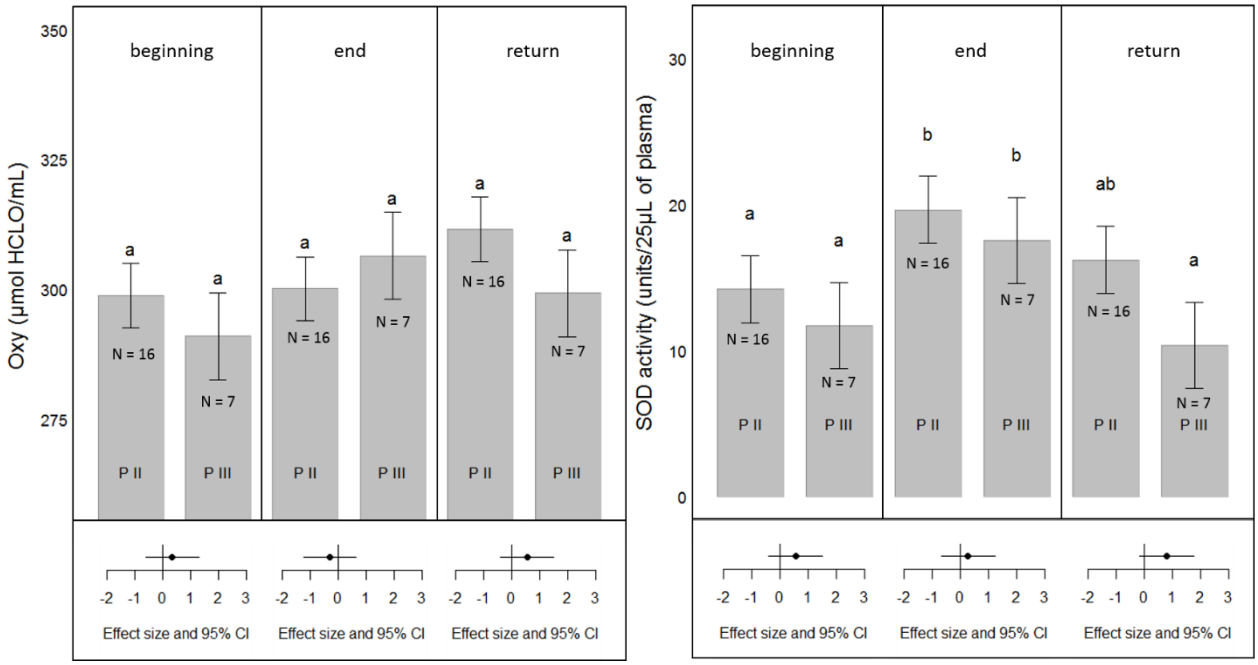


Figure 1. a) Plasmatic superoxide dismutase activity (SOD) and b) Total anti-oxidant plasmatic defences (OXY) at different fasting status (fast-beginning, fast-end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII) (n = 69; N = 23). Marginal means ± SE from the model are represented. Values not sharing a common letter are statistically different for $P < 0.05$ (Tukey HSD test). Effect sizes (Hedges' unbiased d) for differences between PII-PIII groups and 95% CI are provided below the figure.

Moreover, for genetic parents (see Chapter 4), the YO chroma of the beak and YO brightness of the auricular patch in females positively predicted the body condition and size of the chick later in life, while the same ornamental traits presented negative or no relationships in males.

Those results highlight that some ornamental features honestly reflect the quality of either males or the females. Such a different pattern of reliable traits in both sexes (though not necessarily based on the same mechanisms) strongly support the hypothesis that mutual mate choice might be a strong evolutionary pressure favouring the evolution of monormorphism in king penguin (Johnstone et al. 1996; Kokko and Johnstone 2002; Kokko et al. 2006; Wagner and Basolo 2007; Hooper and Miller 2008).

Based on unpublished results published by McGraw et al. (2007), it would appear that king penguin beak spots contain carotenoids pigments. However whether the yellow-orange coloration of beak spots involve other types of pigments and the contribution of different pigment types to producing the coloration measured on the beak spots is still unknown. During the course of my PhD, I attempted to test the importance of carotenoid limitation in the production of ornaments by implanting moulting birds with a carotenoid implant diffusing over 21 days. Although not presented in my thesis, results from this study suggest that carotenoid contribution might be limited. Moreover, studies from Dresp et al. (2005) may suggest that others pigment might be involved. Removing the upper layer of the beak spot revealed a spectrum relatively similar to that of the auricular patch (compare figure 2 and 3 below), highlighting that pigments present in the deeper layer of the beak absorb all wavelengths under 450 nm, while carotenoids usually absorb around ca. 450 nm, they reflect part of the light in the UV wavelength (under 400 nm; Fieser 1950). This suggests that other pigments (likely pterin) may be involved. With the aim to clarify that specific point, and with the contribution of chemist collaborators, I am currently identifying all pigments involved in producing the colour of auricular patches and beak spots in king penguins for which we previously measured reflectance spectra. This will allow to determine the type of pigments involved but also to investigate the relationship between pigment quantities and reflectance spectra measured in vivo.

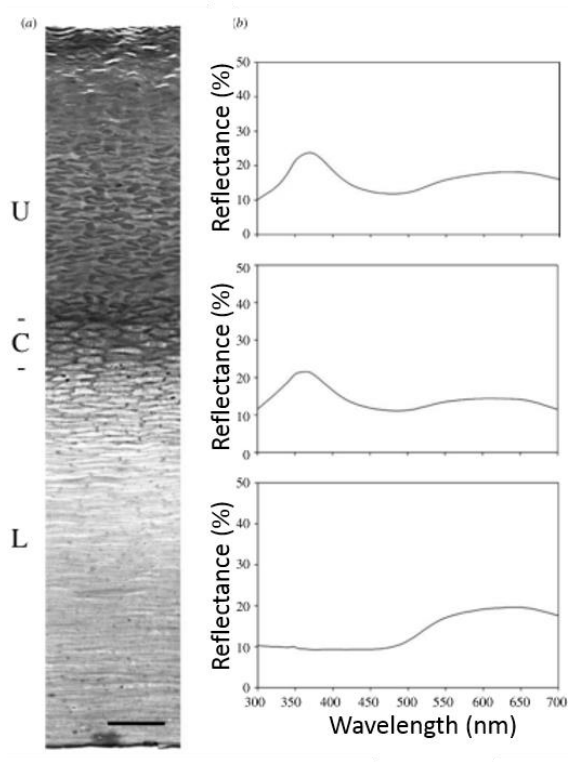


Figure 2. Histological transverse section of a king penguin's beak spot stained with Toluidine blue. U: upper layer, C: Central layer, L: lower layer. The joined spectrum have been measured after the removal of first the upper layer followed by the central layer.

2.1.2.2 Yellow-orange auricular patches

The yellow-orange colour of king penguins feathers results from the presence of a pterin-like pigment that has to be synthetized de novo by the organism (endogenous) (McGraw et al. 2007; Thomas et al. 2013). Here, I show (see Chapter 1) that experimental exposure to physiological stress generated by increasing corticosterone level or stimulating immunity during moult led to a diminished YO chroma of the auricular patches.

These results highlights the strong condition-dependence of the quantity of pterin-like pigment invested in this ornament. Being costly to produce, auricular patch colour might be a reliable feature of individual quality used for mate choice (Kodric-Brown and Brown 1984; Kokko et al. 2006; Hill 2011; Biernaskie et al. 2014). Although pterins are abundant in nature, occurring in the eyes of some birds (Oliphant 1987) and fruit flies (Ephrussi and Herold 1944), in the integuments of butterflies (Morehouse et al. 2007), amphibians and reptiles (Steffen and McGraw 2007; Steffen and McGraw 2009; Weiss et al. 2012), they have not been reported from feathers except in penguins (McGraw et al. 2007). Although, pterin pigment has been found in fish eyes (Bagnara 1966), none has been found in myctophids (de Busserolles 2013) known to be the principal prey on which King penguins feed (Cherel and Ridoux 1992; Olsson and North 1997), supporting the idea that they have to synthetized it de novo. Very little is known on pterin-based feathers ornament and the mechanisms associated with endogenous synthesis and allocation remain unclear. Since pterin derivates are known to present antioxidant functions (Oettl and Reibnegger 2002; Oettl et al. 2004; McGraw 2005; Weiss et al. 2011), this suggests that pterins could have been devoted to protection against CORT- (Costantini et al. 2011) or immune- (Oettl and Reibnegger 2002; Oettl et al. 2004) induced oxidative stress at the expense of auricular patch coloration.

Interestingly, our cross-fostering experiment shows that auricular ornaments negatively predict the morphometric measurements of the chick (mass, size and body condition) early and late in the development. This suggests that brighter auricular patches predict poor investment in the egg (maternal and genetic) but also poorer parental care. The results also suggest that female king penguins may use YO brightness of the auricular patch as a cue to assess their partner abilities and adjust their investment by laying bigger eggs when their partner's auricular ornamentation predict poor genetic contribution and low parental care involvement, suggesting that the cost of rearing brighter ornament might be not only at its production but also be maintained later during the breeding cycle suggesting those ornament parameters to be also constrain by social selection. Altogether, those results highlight that the fence between social-sexual selection and their relative implication on maintaining condition-dependent and -independent traits is rather complex (West-Eberhard 1979; Lyon and Montgomerie 2012).

2.2 Condition-independency, and the evolution of ornaments?

2.2.1 The UV hue of the beak spots

2.2.1.1 The social selection hypothesis

In addition to a certain condition-dependency, several aspects of UV coloration were not directly linked to individual condition. For instance, in Chapter 2, I found no effect of experimentally increasing physiological stresses or inducing an immune challenge during the moult on beak UV hue. This suggests that beak UV hue expression could either be largely determined by genetic or by developmental factors encountered early in life that have lifelong consequences (i.e. silver spoon effects; Metcalfe and Monaghan 2001; Tilgar et al. 2010; Minias et al. 2015), and thus might evolved under the "good genes hypotheses" of mate choice; Kokko et al. 2003; Mead and Arnold 2004; Neff and Pitcher 2005; Anderson 2006; but see Bonduriansky and Rowe 2005) or could be enforced by social mediation, e.g. reflecting aggressiveness and dominance. Yet, the fact that UV hue has previously been associated with individual features (the acute stress response, oxidative stress status; (Viblanc et al. 2016), suggests that this aspect of coloration is likely maintained by a blend of sexual and social pressure (Kokko et al. 2003; Mead and Arnold 2004; Neff and Pitcher 2005; Anderson 2006; but see Bonduriansky and Rowe 2005).

2.2.1.2 Some indication that genetic correlations might occur

The genetic correlation hypothesis suggests that the ornament is sexually selected in one sex and occurs in the other simply due to genetic correlation between the sexes (Lande 1980; Lande 1987; Mank 2009). It implies that one of the sexes gains no selective benefits or even might pay a cost from the expression of elaborate characters (Kraaijeveld et al. 2007; Bonduriansky et al. 2008). In king penguins, we observed opposite relationships between beak UV colour and physiological features in males and females. In males, UV brightness and UV hue were positively related with body condition and oxidative stress, respectively, whereas opposite relationships occurred in females. This suggests that females might pay a cost from the expression of elaborate characters and that the maintenance of monomorphic UV displays in king penguins might rely on genetic correlation between sexes (Lande 1980; Lande 1987; Kraaijeveld et al. 2007; Mank 2009).Alternatively, because ornamental traits likely reflect multiple individual characteristics which could be negatively intercorrelated, opposite relationships between the sexes could occur if males and females base their choice on different conveyed information.

2.2.2 The particular case of the auricular patches size

2.2.2.1 The social selection hypothesis

Several studies have shown that experimentally reducing auricular patch size delays pairing rate in courting king penguins (Jouventin et al. 2008; Pincemy et al. 2009), suggesting this ornament might be under sexual selection. A correlative study of Keddar et al. (2015b) pointed out that during a year of higher abundance of marine resources for king penguins, defined as a good year for the breeding season, males and females displayed larger auricular patches while courting. The authors suggested that the size of this ornament might depend on the available resources the birds found while foraging. However, this correlative study was only performed in 2 breeding season following different individuals each time. During the beginning of the first field season of this study, thousands of individuals were already incubating their egg, whereas during the beginning of the second field season only a few incubating individuals were observed and many birds were still returning ashore to start breeding (Keddar, Couchoux, et al. 2015). Hence, conclusions on those results are unclear, as an equally likely explanation is that differences in the proportion of individuals initiating a breeding event with large or small auricular patches differed between years independently of resource availability. Nonetheless, this approach is interesting and it would be interesting to re-conduct such study over several years, and if possible, using repeated measures of the same individuals. Hence whether the auricular patch may be condition-dependent to a certain extent, such as in response to large changes in food supply requires further investigation. Interestingly, we showed that the amount of pigment invested (colour reflectance but not size) in auricular patch is condition-dependent (Chapter 1) and negative association between patch size (both in males and females) and the innate immune function (Viblanc et al. 2016), suggest that individuals might pay a cost in term of immunity when expressing large ornaments. On the other hand, two correlative studies showed that individuals bearing larger auricular patches were more aggressive and therefore able to occupy more central breeding territories in the colony (Viera et al. 2008; Keddar, Jouventin, et al. 2015), often thought to be of higher quality in terms of breeding success (protection from predators, inclement weather conditions)(Côté 2000; Descamps et al. 2005; but see Descamps et al. 2009; or Viblanc et al. 2014a in term of individual stress). Also, the size of the auricular patch was strongly correlated before and after the moult in two independent studies (Chapter 1 and Chapter 2) while our experimental treatments on bird condition had no effect (Chapter 1). Taken together those latter results suggest that social selection may occur on auricular patch size. Moreover, our cross-fostering experiment showed that auricular patch colour features (not the size) were negatively related to offspring growth (Chapter 4), which might suggest to be due to more aggressive individuals spending

less time in parental care and more time in aggressive behavours. Finally, even if the particular role of auricular patches in mate choice seems strong, their particular role in social mediation out of a mating context is unclear. This is a question I am currently continuing to explore by using experimental reductions of the auricular patch to determine its use in non-breeding social contexts.

2.2.2.2 On the social cost of auricular patch size: and experimental approach

A substantial amount of experimental studies in avian species have manipulated individual condition to investigate its impact on ornament production and hence determine whether they might be condition-dependent (Møller, Christe, et al. 1999; McGraw and Hill 2000; Blount et al. 2003; Faivre, Grégoire, et al. 2003; Jacot and Kempenaers 2007; Doutrelant et al. 2008; Peters et al. 2008; Roulin et al. 2008; Mougeot, Martinez-Padilla, et al. 2010; Mougeot, Martínez-Padilla, et al. 2010; Sild et al. 2011; Doutrelant et al. 2012; Rosenthal et al. 2012; Weiss et al. 2012). However, whether the size or brightness of the ornament itself might inversely influence the physiological status of the bearer has been less studied (Burley 1988; Saino et al. 1997; Cornwallis and Birkhead 2007; Pariser et al. 2010), though such relationships might highlight costs that are defrayed to after trait production as might be expected for socially selected displays (West-Eberhard 1979; West-Eberhard 1983; Hill and Brawner 1998; Lyon and Montgomerie 2012). Given our current results in king penguins, the next step is to experimentally manipulate auricular patch size and integratively measure bird behaviour (aggression), condition, metabolic rate and stress response (Groscolas et al. 2010; V. A. Viblanc et al. 2012; V. A. Viblanc et al. 2014; V. A. Viblanc et al. 2014), oxidative balance (Costantini 2008; Monaghan et al. 2009; Metcalfe and Alonso-Alvarez 2010), immune function (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003; Bize et al. 2008; Palacios et al. 2009; Schulenburg et al. 2009) and cellular respiration (Hill 2011; G. E. Hill 2014; Koch, McGraw, et al. 2016) before and after manipulation (see Figure 2). This approach should help clarifying the social function of this ornament and the evolutionary pressures likely to favour it in king penguins (Figure 3).

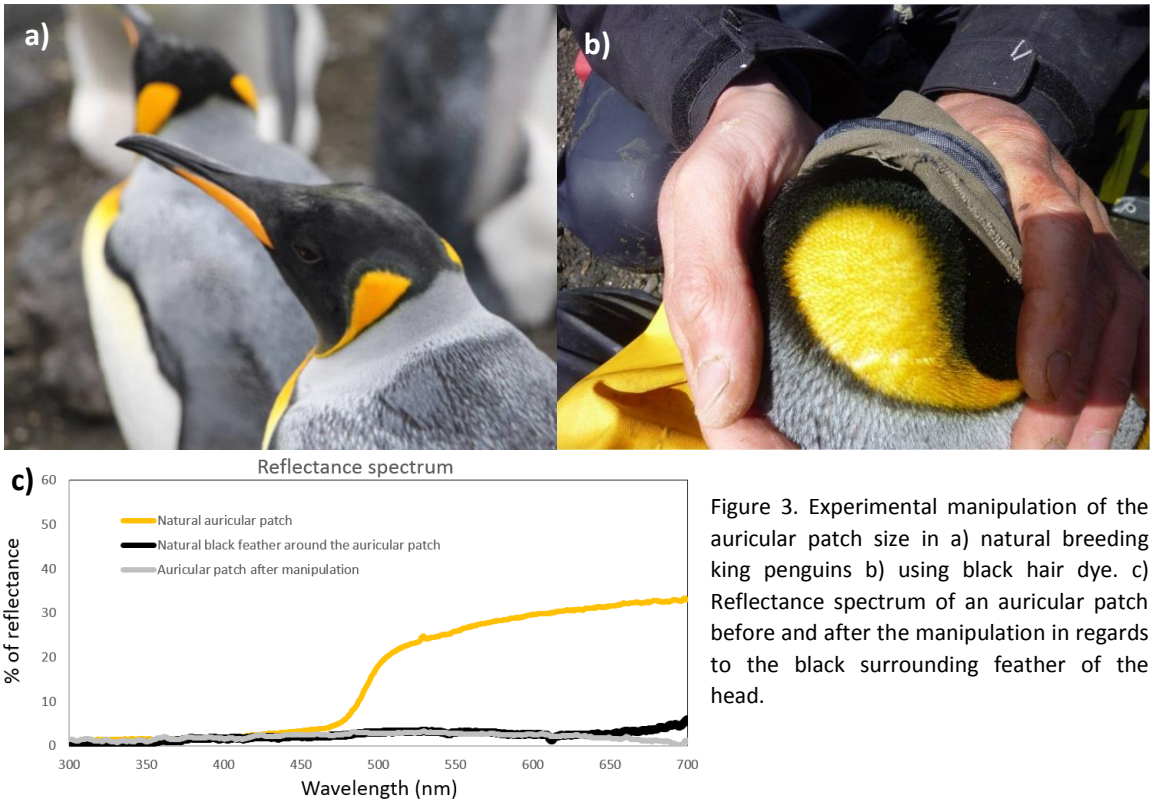


Figure 3. Experimental manipulation of the auricular patch size in a) natural breeding king penguins b) using black hair dye. c) Reflectance spectrum of an auricular patch before and after the manipulation in regards to the black surrounding feather of the head.

2.3 Conclusion: A patchwork of non-mutually exclusive theories

As described by Kraaijeveld et al. (2007), the evolution of exaggerated dimorphic ornaments may transition through a stage where both sexes display the ornament, before it subsequently regresses in one of them (see also introduction section 5). The evolutionary pressures maintaining the ornament at that intermediate stage may be diverse and non-mutually exclusive (mutual mate choice, genetic correlation, social selection, sexual mimicry, biases, reviewed in the introduction section 4.3). In the king penguin, the above results suggest that mutual ornamentation in king penguin might have been favoured mostly by mutual mate choice and social selection.

3 Consideration on mate choice

3.1 Assortative mating

One prediction of the mutual sexual selection hypothesis is that it should result in assortative mating (Trivers 1972). We found that king penguins pairs are assorted with respect to their beak UV hue a relatively condition-independent trait that might be mostly constrain by genetic or developmental factors encountered early in life having lifelong consequences (i.e. silver spoon effects; Metcalfe and Monaghan 2001; Tilgar et al. 2010; Minias et al. 2015). Interestingly, we found in an independent study on 73 king penguins pairs that assortative mating based on telomeres length occurs. (Figure 1, appendix 4).

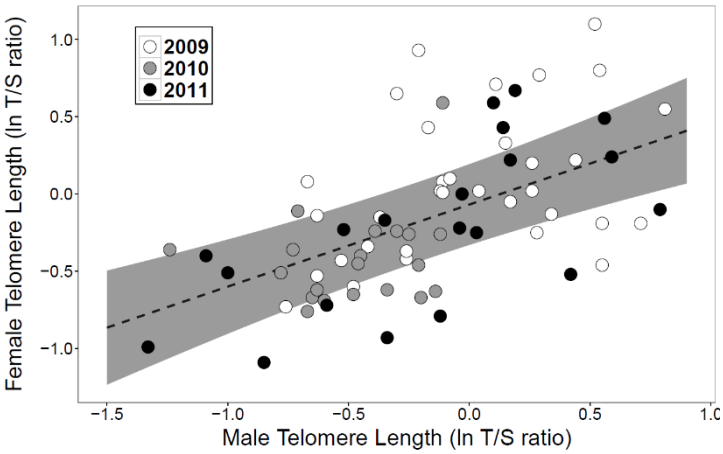


Figure 1. Assortative pairing by telomere length in 73 king penguin pairs followed over 2009-2011 in the Crozet archipelago. The relationship between male and female telomere lengths is tested using

Telomeres are DNA sequences that signal the real ends of linear chromosomes and differentiate them from DNA breaks in the double-stranded helix. They are implicated in the cell replication process and associated with cell and organism lifespan (Prowse and Greider 1995). Indeed, during each cell division, part of the telomere structure is not replicated and lost. Once critically shortened, telomere length trigger cell senescence (Harley et al. 1990). Although specific restoration mechanisms exist (for instance, the enzyme telomerase works to restore eroding telomeres; Haussmann et al. 2007), studies have shown that telomeres generally shorten as individuals age (Pauliny et al. 2006; Salomons et al. 2009; Heidinger et al. 2012; Barrett et al. 2013; Bauch et al. 2013; Boonekamp et al. 2013). In addition, even though telomere length is partly heritable (Reichert et al. 2015), the high inter-individual variability observed at birth (Okuda et al. 2002), and amongst individuals of the same age (Slagboom et al. 1994), suggests that telomere length is also determined by extrinsic environmental factors. Indeed, Telomeric DNA is much more susceptible to oxidative damage than non-telomeric DNA, at least partly due to the high guanine content (Von Zglinicki 2000; Richter and Von Zglinicki 2007) in both early life and adulthood (Epel et al. 2004; Valdes et al. 2005; Puterman et al. 2010; Blackburn and Epel 2012; Geiger et al. 2012; Boonekamp et al. 2014) and between-individual differences in telomere length as a possible consequence of early life stress can be conserved through age (Heidinger et al. 2012). In the last few years, telomeres have been studied in the context of life

history theory and thus proposed as an integrative proxy of individual quality (Bize et al. 2009; Geiger et al. 2012; Bauch et al. 2014; Le Vaillant et al. 2015). Interestingly, when investigating whether male/female assortative mating occurred based on body size or colour ornaments on my dataset, males and females showed a small assortative mating pattern by beak UV_{chroma} (LM; F = 4.45, P = 0.039; Figure1), but not by any other ornamental traits or body size (LMs; 0.01 < F < 2.67, 0.107 < P < 0.940).

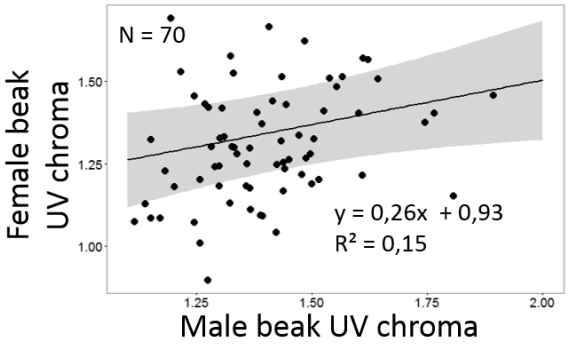


Figure 1. Assortative mating pattern in male and female king penguins based on the UV chroma of their beak spots. The equation and line for the best fit are provided along with the 95% confidence interval (LM including year as a fixed factor). R square coefficient (R^2) is given. N = 70 breeding pairs.

Therefore, it would be particularly interesting to investigate whether UV chroma might be linked to telomere length, allowing individuals to access not only the genetic content of potential mates but also the whole machinery maintaining/eroding telomeres and ultimately how individual deal with stress (Passos and von Zglinicki 2005; Haussmann et al. 2007; Houben et al. 2008; Monaghan 2010; Puterman et al. 2010; Boonekamp et al. 2014).

Those observations raise the intriguing question on the consequences of choosing its mates based on genetic features.

3.2 Mismatch as an evolutionary pressure on mate choice.

In 1974, Trivers already suggested that selection should not just favour the choice of a mate possessing “good genes” but also that of a mate with the most compatible set of genes in terms of producing adaptive gene combinations in the offspring (Trivers 1972; Mays and Hill 2004; Neff and Pitcher 2005; Puurtinen et al. 2009). Only recently, however, has growing empirical support for patterns of mate preference based on nuclear genotype compatibility led to a widespread consideration of “the compatible gene hypothesis” as an explanation for the evolution of ornamental traits (e.g., Zeh and Zeh 1996; Zeh and Zeh 1997; Group 2000; Jennions and Petrie 2000; Servedio 2001; Servedio and Noor 2003; Bernasconi et al. 2009). For instance, Dunn et al. (2013) found that in common yellowthroats (*Geothylpis trichas*), the black mask of male was related to greater major

histocompatibility complex (MHC) class II variation, which in turn signals better survival and disease resistance (Penn et al. 2002; Piertney and Oliver 2005). Therefore, female mate choice based on that ornament appears to provide information on the genetic allele variability of potential mates (Dunn et al. 2013).

Recently the concept has been pushed beyond nuclear genotype compatibility only, and the idea of mito-nuclear compatibility emerged (Hill and Johnson 2013). Mitochondria are cellular organelles responsible for the transduction of energy from food into the cell and whole organism during the oxidative phosphorylation process (OXPHOS) in aerobic organisms (Mitchell 1961). They are often defined as "the powerhouse of the cell" (Nicholls 2002). The electron transport chain is located in the inner membrane of the mitochondria and the mitochondrial genome includes genes that encode for proteins as well as for components of the translational machinery needed to create OXPHOS (Wallace 2007). However, over the course of evolution a number of mitochondrial genes have been transferred into the nuclear genome (Levin et al. 2014), and most of the proteins involved in mitochondrial processes (ca. 90%) are synthetized in the nucleus and imported into the mitochondria (Wolff et al. 2014). Thus, mitochondrial and nuclear gene products closely interact, a process known as mito-nuclear interactions (Woodson and Chory 2008; Lane 2011b). Mito-nuclear compatibility extends to how the match between mitochondrial and nuclear genes enables their functional potential (Burton et al. 2013; Meiklejohn et al. 2013). For instance, the imperative of choosing a mate that will provide compatible nuclear genes involved in the electron transport chain, leading to fully healthy offspring with efficient mitochondrial energy generation capacities is crucial (Levin et al. 2014). This has been proposed to be an important selective pressure in the evolution of female choice for ornamental traits, enabling females to select mates with nuclear genes that are compatible with maternal mitochondrial OXPHOS complexes genes (Hill and Johnson 2013; G. E. Hill 2014). This interesting theory regarding the evolution of ornamental traits and mate choice, urgently needs to be put to the tests, and requires experimental data investigating the relationship between mito-nuclear genetic diversity, how it is linked to ornament expression and how it ultimately influences mate choice and pairing. Moreover, this concept highlights the key role of mitochondria processes in maintenance of individual quality, setting mitochondrial functioning as the fundamental mechanism underlying mate choice (see below).

3.3 Multiple signals, one central mechanism?

In this thesis, I evidenced links between mitochondrial efficiency and an ornamental feature (beak UV brightness) in the king penguin. Further, as we also found for structural and endogenous based-ornamentation, several studies have highlighted tight correlations between ornament production and immune competence (Møller, Christe, et al. 1999; von Schantz et al. 1999; Faivre, Grégoire, et al. 2003; Doucet and Montgomerie 2003b; Anne Peters et al. 2004; Mougeot et al. 2005; Soler et al. 2007; Mougeot, Martinez-Padilla, et al. 2010; Sild et al. 2011; Rosenthal et al. 2012). Mounting an immune response leads to a massive production of reactive species directly involved either (1) in the defence processes of phagocytes (Barry and Bleackley 2002; Forman and Torres 2002; Schmid-Hempel 2003; Beutler 2004; Takeuchi and Akira 2010; reviewed in Fang 2004), or (2) in intracellular signalling, acting as secondary messengers triggering cellular proliferation, differentiation, migration and even apoptosis (Lander 1997; Kamata and Hirata 1999; Arsenijevic et al. 2000; Mates et al. 2000; Bai et al. 2005; Emre et al. 2007; Emre and Nübel 2010; Yun and Finkel 2014). The pleiotropic roles of pigments involved simultaneously in ornament colouration (McGraw 2005) and anti-oxidant defences (Edge et al. 1997; Oettl and Reibnegger 2002; Karl et al. 2004; Weiss et al. 2011; reviewed in McGraw 2005; but see Costantini and Møller 2008) necessary to counteract the deleterious effects of ROS increase during immune responses, has often being proposed as one of the underlying trade-offs sustaining the honesty of ornaments (Alonso-Alvarez et al. 2008; Mougeot, Martinez-Padilla, et al. 2010; Simons et al. 2012). In their seminal paper, Johnson et Hill (2013) pointed out that the oxidation of carotenoids necessary to produce colourful ornament occurs in the inner membrane of the mitochondria and is coupled with the efficiency of the Complexes I and III of the respiratory chain within ubiquinone biosynthesis (Johnson and Hill 2013). They suggested that carotenoid-based ornaments may also reflect the efficiency of the cellular respiratory system (Johnson and Hill 2013), thus proposing a direct link between carotenoid based ornament, immune function and mitochondrial efficiency. Since most studies focused on carotenoid based ornament (Jyonouchi et al. 1994; McGraw and Ardia 2003; Peters et al. 2004a; Peters et al. 2004b; Smith et al. 2007; Fitze et al. 2007; Mougeot 2008; Perez-Rodriguez et al. 2008; Biard et al. 2009; Leclaire et al. 2011; Kelly et al. 2012; Johnson and Hill 2013; reviewed in Simons et al. 2012), links between immune function and structural colours (Peters et al. 2004b; Soler et al. 2007; see also Chapter 3a), or non-carotenoid based ornament (Nolan et al. 2006; Martín et al. 2007; López et al. 2009; Viblanc et al. 2016) have also been highlight. Currently, whereas the anti-oxidant properties of the different pigments involved in bird coloration are known (Oettl and Reibnegger 2002; Karl et al. 2004; reviewed in McGraw 2005), their direct cost of production remains relatively unexplored. Our results suggest that the structural colour of the beak spot is strongly condition dependent (see Chapter 1

and Chapter 2) and reflects humoral immune function (Chapter 3a). Moreover, UV colour ornamentation in king penguins appears directly related to cellular respiration (Chapter 3 b). However, the question remains what functional links exist that enables ornaments to signal immune function in the first place. By merging the large expertise of behavioural ecologist on sexual selection with the knowledge on mitochondrial function provided by the biochemical and biomedical literature, (Koch, Josefson, et al. 2016) reviewed evidence placing mitochondria at the centre of the regulation of immune responsiveness (both innate and adaptive responses). Indeed, mitochondrial function might underly the associations between ornamentation, condition and immune systems for a broad range of traits across taxa (G. E. Hill 2014; Koch, Josefson, et al. 2016). They are the main site of ATP production in aerobic organisms, providing energy for immune function processes (Lochmiller and Deerenberg 2000; Demas 2004; Eraud et al. 2005; Demas and Nelson 2012), and are also involved in the innate immune response as well in cell immune function via ROS production as previously discussed (see above). Since damaged mitochondrial networks or mitochondria lacking membrane potential decrease functions of antiviral signalling pathways (Emre et al. 2007; Castanier et al. 2010; Koshiba et al. 2011; Weinberg et al. 2015), efficient mitochondria are required for a proper response against viruses. Altogether, 'Koch and collaborators' (2016a) synthesis of mitochondrial function and immunity, provides several hints that the production of ornamental features and immune function all converge to mitochondrial functions. The authors also offer a new mechanistic explanation for correlations between sexually selected traits and immunocompetence that is likely testable in king penguins. For instance, one could test this hypothesis in courting penguins by separately challenging each immune compartment using different experimental groups and simultaneously measuring red blood cell respiration to assess mitochondrial function (see Chapter 3b). One might use LPS immune stimulations to assess the efficiency of the innate immune response (Schmid-Hempel 2003; Alonso-Alvarez et al. 2004; Beutler 2004; Matson et al. 2005; Piault et al. 2008; Gasparini et al. 2009; Sild et al. 2011; Gray et al. 2013), the injection of repetitive PHA components to assess cellular immune function (Smits et al. 1999; Saks et al. 2003b; Ardia 2005; Costantini and Dell'Omo 2006; Tella et al. 2008; Perez-Rodriguez et al. 2008; Griggio et al. 2010; Simons et al. 2012; but see Biard et al. 2009), and the use of a NDV vaccination to assess humoral immune function (Matson et al. 2005; Bourgeon et al. 2007; Smith et al. 2007; Stier et al. 2009b; Stier et al. 2009a; Broggi et al. 2013; Velando et al. 2014; reviewed in Staszewski and Boulinier 2004).

4 Methodological consideration

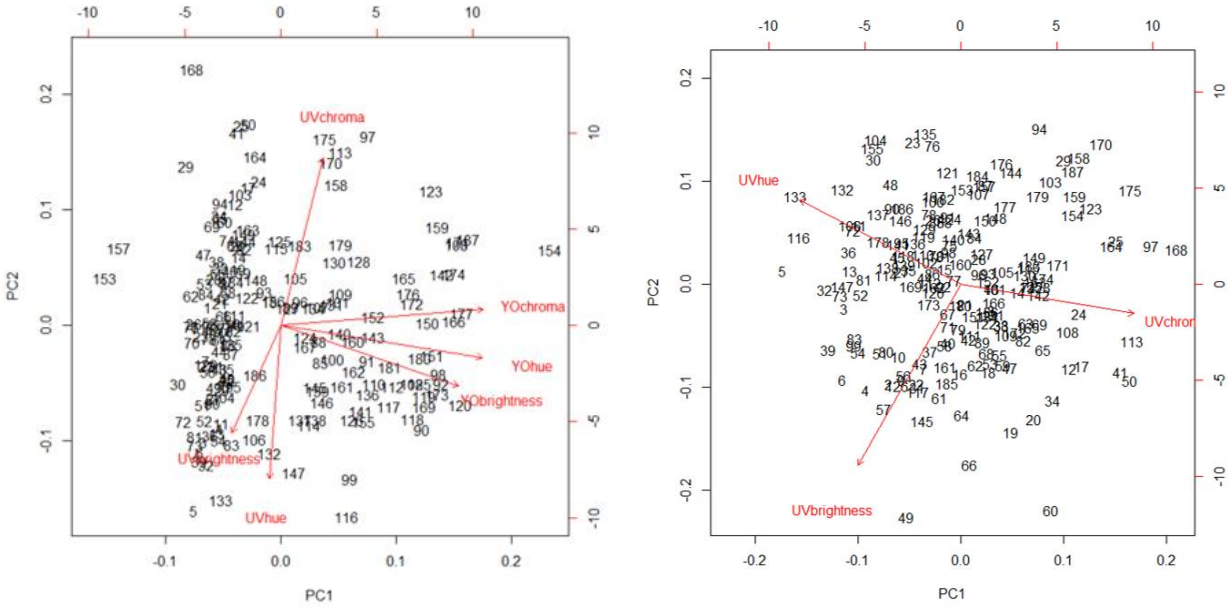
4.1 Measuring colour ornamentation in king penguins

4.1.1 Limitations of a Tristimulus approach

Being interested in investigating the underlying mechanisms linking structural and pigmentary colours to condition, we kept those structural and pigmentary-based measures separated in our different analyses (see introduction). We remain, however, aware that colour integration as perceived by congener occurs in the brain, and likely integrates both structural and pigmentary colours as one global signal. One possible way to deal with this is the use of multivariate analyses (such as Principal Components Analyses) to study colouration (Siefferman et al. 2005; Montgomerie 2006). However, PCA on beak colour ornaments in king penguins (see Figure 4) mainly dissociates structural UV from pigmentary signals along 2-3 axes. Moreover, when studying independently UV colour, parameters are not inter-related (Figure 3b,d), supporting the idea that those parameters reflect independent mechanisms (Dresp et al. 2005; Dresp and Langley 2006 and see introduction). A different approach may therefore be not only to focus on the colour signal itself, but on the visual system of the birds and what is at least likely perceived by the retina (if not integrated in the brain). This approach is known as the visual model approach (Montgomerie 2006; Maia et al. 2013).

a) Beak spot features

b) Focus on UV colour



c) Summary of a PCA analysis on beak spot features

Importance of components:						
	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
Standard deviation	1.6313	1.2377	0.9996	0.77142	0.38698	0.25092
Proportion of Variance	0.4435	0.2553	0.1665	0.09918	0.02496	0.01049
Cumulative Proportion	0.4435	0.6988	0.8654	0.96455	0.98951	1.00000

Rotation:						
	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
UVbrightness	-0.14377504	-0.41128025	-0.78163260	0.32021700	0.3094442	0.03054733
YObrightness	0.51778982	-0.23163706	-0.32759842	-0.16630255	-0.7315603	0.08988995
Uvhue	-0.03282579	-0.58806386	0.49414319	0.60489341	-0.1840690	0.09564750
YOhue	0.58801229	-0.12332641	0.07832360	0.05955250	0.3172833	-0.72710549
UVchroma	0.12206522	0.64228798	-0.15861135	0.70543158	-0.2140425	-0.06293464
YOchroma	0.59117387	0.06025341	0.07912088	0.05223305	0.4343956	0.67022015

d) Summary of a PCA analysis focused on UV colour

Importance of components:			
	PC1	**PC2**	**PC3**
Standard deviation	1.226	0.9585	0.7605
Proportion of Variance	0.501	0.3062	0.1928
Cumulative Proportion	0.501	0.8072	1.0000

Rotation:			
	PC1	**PC2**	**PC3**
UVbrightness	-0.3998891	-0.8970016	-0.1883529
UVhue	-0.6260072	0.4173941	-0.6587087
UVchroma	0.6694802	-0.1455001	-0.7284408

Figure 4. Separate plot and summary table for principal component analysis on (a, c) all beak colour features; UV brightness, UV hue, UV chroma, YO brightness, Yo hue and YO chroma and (b, d) focused UV colour parameters, UV brightness, UV hue and UV chroma. We compiled all colour measurements collected during the study period 2011 to 2014 for a total of N = 810 individuals.

4.1.2 The visual model approach: analysing photon catch

The main idea behind the visual model approach is to estimate the quantum or photon catch of each of the different types of cone receptors involved in bird colour vision (Montgomerie 2006). Indeed, colour vision of diurnal birds relies on the excitation of four different types of cones present in their retina, and each cone presents different wavebands of light sensitivity (Hart 2001; Cuthill 2006). The first type of cones is considered as ultraviolet- or violet-sensitive cones, the two following as shortwave-sensitive (blue) cones and medium (green) wave-sensitive cones respectively, and the last as long (red) wave-sensitive cones. Thus, the whole signal perceived by a bird relies on the relative stimulation of these four types of cones. Considering this, studies eager to investigate how ornamental features are perceived (received signal) either during mate choice or social conflicts might address to which extent each cone type is individual stimulated, providing a first assessment of what information might be transmitted to the brain. Analysing photon catch is important, especially considering the fact that most studies do not account for the fact that environmental light conditions may affect signal perception, which is crucial to the receiver (Endler 1987; Endler 1990; Endler and Thery 1996; Bradbury, J. W., Vehrencamp 1998; Moyen et al. 2006). On land, king penguins interact in open space (on the beach and without any vegetation) and the irradiance spectra of light at high latitudes, as well as the transmission properties of air is likely to affect photon catch and signal perception (Endler and Mielke 2005; Montgomerie 2006; Keddar, Altmeyer, et al. 2015). Analysing photon catch and modelling how a species might perceive colour signals requires to know about the transmission properties of the ocular media and photoreceptor sensitivities of that species (e.g. in blue tits (*Parus caeruleus)* Galván 2011; Peacocks (*Pavo cristatus)* Dakin and Montgomerie 2013; Pigeons (*Columba livia)* Leclaire et al. 2014). Up to date, the Humbolt penguin (*Spheniscus humboldti*) is the closest phylogenetic relative (same family; spheniscidae) for which the photoreceptor sensitivity has been determined (Bowmaker and Martin 1985; Hart 2001; but see Machovsky Capuska et al. 2011). Although analysing photon catch is complex, the recent R tool-package PAVO (*Perceptual Analysis, Visualization and Organization of Spectral Color Data*; Maia et al. 2013), may provide a good starting point for such analyses, and has recently been used for king penguins (Keddar, Altmeyer, et al. 2015). Such an approach would allow to model colour signals in a tetrahedral colour space (Goldsmith 1990; Endler and Mielke 2005; Stoddard and Prum 2008) considering bird cone sensitivity, allowing us to get closer to the photon information actually transmitted to the bird's brain. Note however, that this approach does not allow to make any inference on how the brain actually integrates the signal.

4.1.3 *The breast patch: a tricky ornament to measure.*

Just as the auricular patch and the beak spot, the breast patch of king penguins is very colourful. The breast patch shows a gradient of brown to light yellow that can be divided in four region (brown, orange, dark yellow and light yellow; Figure 5) which result from a pterin like pigment presence.

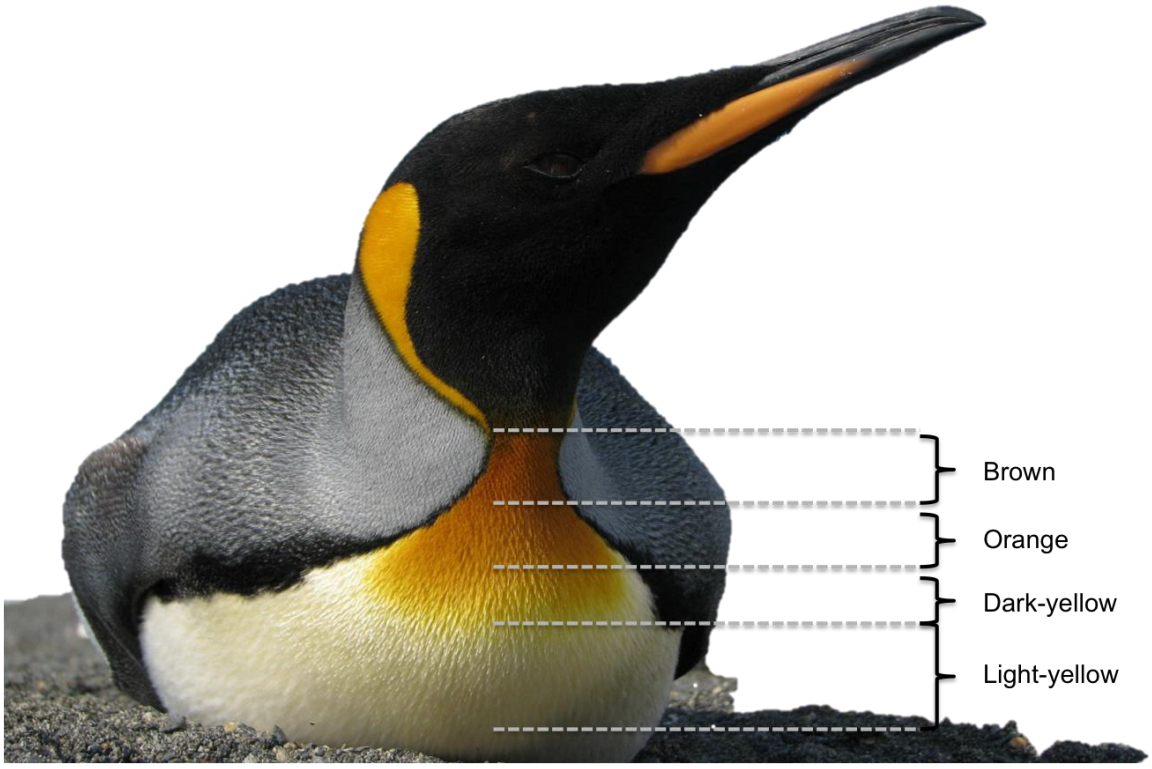


Figure 5: the different colour regions of a king penguin breast feather patch, ranging from brown to light-yellow.

The distances between those regions are variable among individuals (personal obs), suggesting that the size of the ornament and specific size of each region respective to the entire ornament might be relevant to consider. Moreover, distinguishing between colour transitions is rather subjective to the experimenter eyes. Because this ornament was complex to measure and overall lowly repeatable, we did not considered it in our previous experiment. However, since breast patch colouration was linked to immunocompetence in a previous study (Nolan et al. 2006), we included the unique measurement of the orange region when investigating links between ornament and humoral immune response in the Chapter 3a.

Nonetheless, progresses in the measure of the beast patch is needed as this ornament is likely to provide valuable information. One approach could be to make measurements every centimetres along the gradient using a flexible ruler, as already proposed by Keddar (Keddar 2013). Beside measures of coloration, this approach may also allow to estimate the total length of the breast patch.

Repeated measures of coloration along a gradient may also be useful to extract a 'slope' and an 'intercept' to better characterise variation in breast patch coloration. To do so however, the coloration gradient should follow a linear pattern, or the relevant parameter of the representative equation could change along the gradient. Yet, as a human eye is already able to perceive four distinct regions (Figure 5), this suggests that the gradient of coloration on the breast patch occurs by steps rather than continuously. The reason for those variations and their adaptive nature remains an open question and experimental data acquired during the coming will hopefully help us shed some light on this ornamental feature.

4.2 How to access individual quality

From a natural selection perspective, the concept of individual quality has typically been described by considering among-individual differences in traits (behavioural, morphological, physiological) associated with survival and reproduction, individuals of higher quality expressing an ensemble of traits of higher fitness (Cam et al. 2004; Wilson and Nussey 2010). Whereas the concept has flooded evolutionary biology literature over the past 20 years, there is yet no clear consensus at which traits we should be looking at when trying to assess individual quality (Bergeron et al. 2011). Because most traits characterising an individual at the level of the organism are polygenic and may be expressed differently in varying environmental conditions, a proper assessment of individual quality requires functional and evolutionary investigations of multiple traits (e.g. physiology, morphology, behaviour) over various life-history stages (Cam et al. 2004; Wilson and Nussey 2010). Understanding what makes an individual successful at transmitting its genes to the next generation is at the root of evolutionary biology. To do so, an individual has to go through different filters of selection, successfully developing and growing from a zygote to a complex multicellular organism, reaching maturity and finding a partner with whom to reproduce, and finally nurturing the next generation. Although life history theories posit that trade-offs between fitness components shape the evolution of organisms, with for instance investment into reproduction being made at the expense of survival, empirical research in natural populations indicate that fitness components often positively covary among individuals(Cam et al. 2002; Weladji et al. 2006). Wilson and Nussey (2010) suggested to use multivariate statistics, more specifically principal component analyses in which individual quality is treated as the "the axis of phenotypic variation that best explains variance in individual fitness".

Based on this hypothesis, I ran a principal component analysis on the large dataset obtained for the same individual during the same breeding season (Preface chapter 3; Viblanc et al. 2016). Thus

including body condition, Nab titer, Lysis titer, Resting metabolic rate, Corticosterone and Heart rate elevation in response to capture, and oxidative balance (ROM and OXY) (Figure 5).

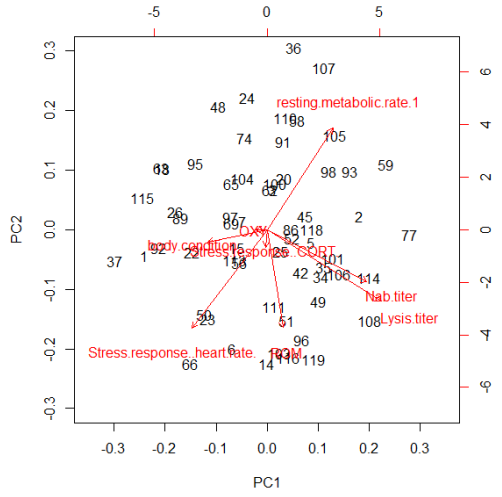
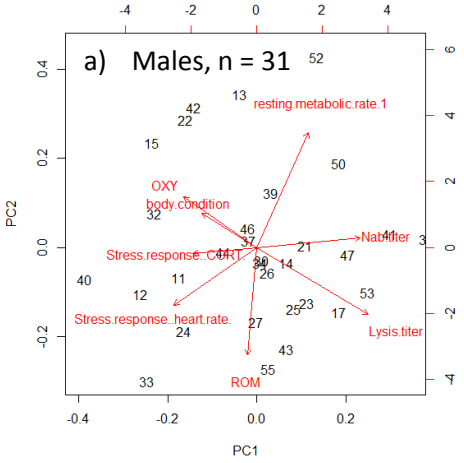
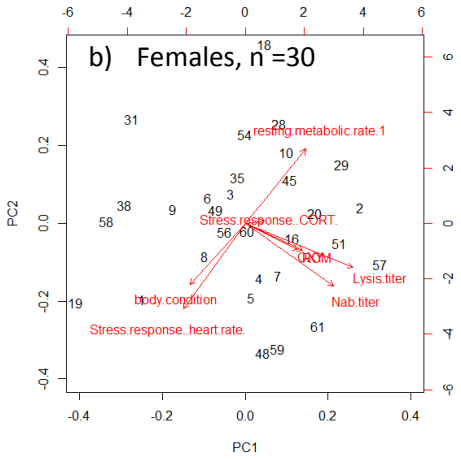


Figure 5. a) Plot and b) summary table of a principal component analysis on several physiological traits including body condition, Nab titer, Lysis titer, resting metabolic rate, Corticosterone and heart rate elevation in response to capture, and oxidative balance (ROM and OXY) for N = 61 breeding king penguins. Data provided by Viblanc et al. 2016.

Importance of components

	PC1	PC2	PC3	PC4	PC5
Standard deviation	1.3665	1.1856	1.0641	1.0396	0.9367
Proportion of Variance	0.2334	0.1757	0.1415	0.1351	0.1097
Cumulative Proportion	0.2334	0.4091	0.5507	0.6858	0.7954





c) Males n = 31
Importance of components:

	PC1	PC2	PC3	PC4	PC5
Standard deviation	1.4548	1.3015	1.1666	0.9163	0.85328
Proportion of Variance	0.2646	0.2117	0.1701	0.1050	0.09101
Cumulative Proportion	0.2646	0.4763	0.6464	0.7514	0.84237

d) Female n = 30
Importance of components:

	PC1	PC2	PC3	PC4	PC5
Standard deviation	1.4737	1.2699	1.0623	0.9934	0.8970
Proportion of Variance	0.2715	0.2016	0.1411	0.1234	0.1006
Cumulative Proportion	0.2715	0.4730	0.6141	0.7375	0.8380

Figure 6. Separate plot and summary table for males (a, c) and females (b, d) of principal component analysis on several physiological traits including body condition, Nab titer, Lysis titer, resting metabolic rate, Corticosterone and heart rate elevation in response to capture, and oxidative balance (ROM and OXY) breeding king penguins. Data provided by Viblanc et al. 2016. Sample size are provided in a) and b).

Unfortunately, no clear pattern emerged; no PC axis strongly related the different physiological traits together. However, as males and females often have differing physiological constraints opening the possibility that criteria of quality might differ between sexes. Unfortunately, running the principal component analysis on each sexes independently did not improve the relationships (proportion of variance explained by each PC axis remain relatively low; Figure 6). Accessing indices of individual quality remains an interesting challenge in our field (Wilson and Nussey 2010; Bergeron et al. 2011). This also raises one of the limits of this thesis and open new perspectives. Using free living king penguin population during punctual breeding season (never more than one year at a time) offers us the incredible opportunity to study the role and honesty of mutual ornamentation in a natural context. However it also implies strong one major drawback, i.e. we are unaware of age and past life history of the individuals under study.

4.2.1 A need for long-term monitoring studies

Understanding how sexual and social factors affect fitness by considering the individual over its entire life should allow us to better grasp the ultimate function and evolution of monomorphic ornaments. The use of Radio Frequency Identification (RFID) tags already allow to follow birds with minimal handicap (Saraux, Le Bohec, et al. 2011), providing information on past and present breeding events. Merging long term monitoring of breeding king penguin with correlative and experimental approaches on ornaments is urgently required. For instance, information on age or condition at first reproduction, at first breeding success, or the overall investment into seasonal and lifetime reproduction, as well as on seasonal and lifetime fitness (total number of offspring fledged and recruited in the population) should shed further light on the honesty of ornamentation. Moreover, ornamental changes along individual life (possibly associated with changes in social status or dominance) have yet to be studied and such monitoring technics would help in filling this gap in knowledge.

A final word

Today is the year 2016. It has been 146 years since Darwin first advanced the theory of sexual selection as an explanation to the evolution of extravagant morphological features that might, at first sight, appear detrimental to fitness. In over a century and a half, a plethora of authors have taken-up the challenge of testing and continuously refining this idea. From sexual selection to social selection in explaining the evolution of dimorphic to monomorphic ornaments, from behavioural to physiological attributes underlying signal honesty, from birds and mammals to invertebrates, one might wonder what is left to learn on the evolution of ornamentation. Yet, as our understanding of physiological processes, energy trade-offs, and evolution continuously improves, so too continuously increase our hypotheses on why ornaments exist and what mechanisms underlie their production and maintenance. This thesis has highlighted important physiological pathways mediating the honesty and evolution of condition-dependent ornaments in a monomorphic species, and has highlighted the existence of condition-independent ornaments likely used in non-sexual social contexts. It has further highlighted the importance of structural signals in mediating individual quality, and underlined that colours based on structure rather than pigments can nonetheless be highly dynamic. Finally, it has opened a first door in taking-up the challenge to link ornament production and energy production at the most fundamental unit, the power house of the cell. As our understanding of mitochondria function increases, studies on the links between mitochondrial energy production, immunity, mito-nuclear compatibility, and ornament production/maintenance, are likely to take the stage in behavioural ecology research on sexual/social selection. I anticipate those studies will keep us going for another many years… !

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Appendices

Appendix 1

The oxidative debt of fasting: evidence for short to medium-term costs of advanced fasting in adult king penguins

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In response to prolonged periods of fasting, animals have evolved metabolic adaptations helping to mobilize body reserves and/or reducing metabolic rate, to ensure a longer usage of reserves. Those metabolic changes can however be associated with higher exposure to oxidative stress, raising the question how species that naturally fast during their life cycle avoid an accumulation of oxidative damage over time. King penguins repeatedly cope with fasting periods up to several weeks. Here we investigated how adult male penguins deal with oxidative stress after an experimentally induced moderate fasting period (PII) or an advanced fasting period (PIII). After fasting in captivity, birds were released to forage at sea. We measured plasmatic oxidative stress on the same individuals at the start and end of the fasting period and when they returned from foraging at sea. We found an increase in activity of the antioxidant enzyme superoxide dismutase along with fasting. However, PIII individuals showed higher oxidative damage at the end of the fast compared to PII individuals. When they returned from re-feeding at sea, all birds had recovered their initial body mass and exhibited low levels of oxidative damage. Notably, levels of oxidative damage after the foraging trip were correlated to the rate of mass gain at sea in PIII individuals but not in PII individuals. Altogether, our results suggest that fasting induces a transitory exposure to oxidative stress and that effort to recover in body mass after an advanced fasting period may be a neglected carry-over cost of fasting.

The oxidative debt of fasting: evidence for short to medium-term costs of advanced fasting in adult king penguins

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Key words: fasting, oxidative stress, king penguin, foraging effort

ABSTRACT

In response to prolonged periods of fasting, animals have evolved metabolic adaptations helping to mobilize body reserves and/or reducing metabolic rate, to ensure a longer usage of reserves. Those metabolic changes can however be associated with higher exposure to oxidative stress, raising the question how species that naturally fast during their life cycle avoid an accumulation of oxidative damage over time. King penguins repeatedly cope with fasting periods up to several weeks. Here we investigated how adult male penguins deal with oxidative stress after an experimentally induced moderate fasting period (PII) or an advanced fasting period (PIII). After fasting in captivity, birds were released to forage at sea. We measured plasmatic oxidative stress on the same individuals at the start and end of the fasting period and when they returned from foraging at sea. We found an increase in activity of the antioxidant enzyme superoxide dismutase along with fasting. However, PIII individuals showed higher oxidative damage at the end of the fast compared to PII individuals. When they returned from re-feeding at sea, all birds had recovered their initial body mass and exhibited low levels of oxidative damage. Notably, levels of oxidative damage after the foraging trip were correlated to the rate of mass gain at sea in PIII individuals but not in PII individuals. Altogether, our results suggest that fasting induces a transitory exposure to oxidative stress and that effort to recover in body mass after an advanced fasting period may be a neglected carry-over cost of fasting.

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INTRODUCTION

Animals' ability to face prolonged periods of food shortage is under strong natural selection (Geiser and Stawski, 2011; Lindstedt and Boyce, 1985; McCue, 2012; Millar and Hickling, 1990; Staples, 2016). Body reserves (i.e. glycogen, lipids and proteins) play a key role in promoting survival under conditions of low energy intake or complete fasting (e.g. Cherel et al., 1994b; Phillips and Hamer, 1999; Secor and Carey, 2016). However, as storage energy is limited, vertebrates have evolved biochemical and physiological mechanisms allowing them to preserve body reserves while fasting, and to trigger re-feeding when energy reserves are critically depleted (Groscolas and Robin, 2001; Groscolas et al., 2008; McCue, 2010; Spée et al., 2010; for a review see Secor and Carey, 2016). The management of body reserves is tightly linked to changes in metabolic rates: reducing metabolism and physical activity helps extend the period during which energy stores sustain metabolism, while increased metabolism and physical activity promote food searching (Cherel et al., 1994b; Groscolas and Robin, 2001; Nordøy et al., 1990; Rey et al., 2008). Although such metabolic changes provide immediate lifesaving responses, medium to long-term costs associated with metabolic changes during fasting remain little investigated in wild species that typically cope with repeated and sometimes prolonged periods of food shortage (Vázquez-Medina et al., 2010). Since mitochondria are cornerstone organelles implicated in metabolic responses to fasting (Monternier et al., 2014), but also the first site of production of damaging reactive oxygen species (ROS) (Andreyev et al., 2005), direct oxidative costs to prolonged fasting may be expected (e.g. Chausse et al., 2015; Geiger et al., 2012; Sorensen et al., 2006; Wasselin et al., 2014).

In this study, we test for links between prolonged fasting and oxidative stress in a long-lived seabird, the king penguin (*Aptenodytes patagonicus*), for which fasting is a natural and important part of the life cycle. King penguins breed on land but forage for marine resources, mostly myctophid fish species (Bost et al., 1997), at the oceanic polar front several hundreds of kilometres away from their breeding site (Charrassin and Bost, 2001). Breeding partners must therefore alternate periods of prolonged fasting on land (caring for the single egg or chick) and foraging trips at sea (Olsson, 1996; Weimerskirch et al., 1992). While on land, adults rely entirely on energy reserves during fasting periods of up to 3-5 weeks (Groscolas and Robin, 2001). The longest fasting bout during reproduction is undertaken by the male and covers the month-long period of courtship and the first incubation shift, i.e. the male is the first to incubate the egg while the female replenishes her energy reserves at sea

(Stonehouse, 1960; Weimerskirch et al., 1992). The trade-off between current reproduction and survival is therefore particularly important in this species (since breeding and foraging grounds are separated by long distances), and the efficient management of stored energy is critical to breeding success (adults generally abandon reproduction if stores are critically depleted; (Gauthier-Clerc et al., 2001; Groscolas et al., 2008; Olsson, 1997; Robin et al., 2001).

Long-term fasting in penguins (and more generally in animals at large) is characterized by metabolic transitions that can be divided in 3 distinct phases (Cherel et al., 1994b; Groscolas, 1990; Groscolas and Robin, 2001; for a review see Secor and Carey, 2016). First, individual metabolism relies mostly on carbohydrates as the main energy substrate, and body mass loss per day rapidly drops during fasting phase I (hereafter referred to PI). At the same time glycogen stores are depleted, lipids become the principal energy resource. During this period, energy expenditure decreases to a minimal, and individuals enter a long energy-sparing period, the so-called fasting phase II (PII) (Cherel et al., 1994b; Nordøy et al., 1990). If fasting is prolonged still, for instance when the breeding partner's return from sea is delayed, individual body mass will decrease even further, and challenge an individual's investment in reproduction (Gauthier-Clerc et al., 2001; Groscolas and Robin, 2001; Groscolas et al., 2008). This final fasting phase (phase III, hereafter PIII) is accompanied by drastic metabolic changes, *i.e.* the close exhaustion of body fat reserves giving way to muscular proteolysis as a last extreme energy resource (Belkhou et al., 1991; Cherel et al., 1988a; Goodman et al., 1981; Le Maho et al., 1981; Robin et al., 1988). This critical state heralds a physiological limit beyond which adult survival may be compromised (Robin et al., 1998). During PIII, energy expenditure increases again (Le Maho et al., 1981; Cherel et al., 1994) along with glucocorticoid levels and non-reproductive behaviour (Kitaysky et al., 1999; Robin et al., 2001; Groscolas et al., 2008). This gradual reallocation of energy towards soma preservation rather than current reproduction results from a complex network of metabolic and (neuro-) hormonal changes called the "re-feeding signal" (Bertile et al., 2009; Groscolas and Robin, 2001; Minokoshi et al., 2004; Groscolas et al., 2008; Spée et al., 2010), and is expected to lead to the restoration of adult body condition at the expense of reproductive success.

In king penguins (*Aptenodytes patagonicus*), birds reaching fasting PIII are able to subsequently rebuild their energy reserves. However, PIII individuals may – but do not systematically – recover body mass as fast as PII individuals (Robin et al., 2001). Notably, for

birds breeding late in the season, PIII birds take longer to restore their body mass than PII birds, whereas this is not the case early in the breeding season (Robin et al., 2001). Such differences in recovering dynamics within a breeding season for early and late PIII birds suggest that costs of fasting up to PIII and/or of re-feeding are likely to exist, and may perhaps only be compensated (or worth paying) under specific environmental (or life history) circumstances. For instance, breeding birds may not be willing to increase their foraging effort (at a potential cost) to catch-up for lost body mass late in the season because of their extremely low likelihood of breeding success (Weimerskirch et al., 1992).

Because of its implications for life-history traits (Costantini, 2008; Costantini, 2014; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Selman et al., 2012; Stier et al., 2012), one important cost to assess for penguins at an advanced stage of fasting (i.e. PIII) is the production of ROS and the balance between ROS and antioxidant defences. Indeed, oxidative stress, *i.e.* an unbalance between ROS and counteracting defences, in PIII individuals may be associated (1) on the short-term with the transition from PII to PIII and associated increase in metabolic rate (Cherel et al., 1994b; Le Maho et al., 1981); and (2) on the medium-term with the intense foraging effort likely required by PIII birds to recoup their initial body condition (Andreyev et al., 2005; Hulbert et al., 2007; Isaksson et al., 2011; Speijer et al., 2014). Indeed, in addition to high metabolic rates that may result in the by-production of large quantities of ROS (Beckman and Ames, 1998; Stier et al., 2014a; Stier et al., 2014b; but see Speakman and Selman, 2011), king penguins forage in apnea during repetitive diving bouts (>1000 foraging dives per trip; Bost et al., 2007) carried out at high ambient pressure (>100-m diving depth; Kooyman et al., 1992). This temporarily exposes their tissues to critically low levels of oxygen (hypoxemia), before the transient re-perfusion of oxygen-rich blood when reaching the surface again – a situation known as ischemia-reperfusion (Meir and Ponganis, 2009) that may cause massive bursts of oxidative stress (Chouchani et al., 2016). Whereas deep-diving animals have typically evolved efficient anti-oxidant defences to deal with such a situation (Vázquez-Medina et al., 2011; Vázquez-Medina et al., 2012; Zenteno-Savin et al., 2010), penguins at an advanced stage of energy depletion may have compromised defence mechanisms making it harder to cope with oxidative stress.

We experimentally tested whether pre-reproductive king penguins forced to reach PIII (advanced fast) experienced short to medium-term oxidative costs compared to individuals leaving the colony in a higher body condition (PII, medium fast). We specifically focused on male king penguins as those naturally experience the longest fast of the breeding cycle in

natural conditions (Stonehouse, 1960; Weimerskirch et al., 1992). Thus, in a species repeatedly exposed to long-term periods of energy depletion and intense foraging effort, we questioned: (i) whether PIII was associated with the onset of oxidative stress (short-term cost), (ii) whether PIII individuals coped with increased oxidative stress while foraging at sea and (iii) whether, when returning from sea, oxidative homeostasis was re-established.

MATERIALS AND METHODS

General methods

This study was performed in the breeding colony of "La Baie du Marin" (approx. 20,000 breeding pairs), Possession Island, Crozet Archipelago (46° 26' S, 51° 52' E) over two field sessions: December 2011-February 2012 and December 2013-February 2014. Over the 2011-2012 and 2013-2014 summers, we identified 23 males based on their structural size and song during courtship (Stonehouse, 1960). They were caught and housed in open wooden pens of 3 × 4 m within 10-m from the breeding colony therefore being subjected to natural climatic conditions and colony sounds. Sixteen birds (5 birds in 2011-12 and 11 birds in 2013-14) were kept captive and released during fasting PII (mean fast duration ± SE = 20.78 ± 1.66 days), and seven birds (3 birds in 2011-12 and 4 birds in 2013-14) were kept captive until they entered fasting PIII (mean fast duration ± SE = 28.7 ± 1.88 days). The fasting status of birds was determined by changes in mass specific daily body mass loss (see below). Birds were left undisturbed except for mass measurements and blood sampling. We ensured that all birds departed to sea upon release, usually within a couple of hours after release.

Body mass measurements

Birds were left undisturbed except for weighing once a day (±1 g), for the first 8 days of the fast, and every second day until the end of the phase II. Weighing occurred every morning between 9:00 and 9:30 AM. We determined transitions between fasting phases (PI – PII – PIII) based on changes in the rate of mass specific daily body mass loss (dm/mdt), which decreases from PI to PII, is low and stable in PII, and increases in PIII (Cherel et al., 1988). King penguins typically enter PIII at a critical mass threshold of around 9.3 kg (Cherel et al., 1994a; Gauthier-Clerc et al., 2001; Viblanc et al., 2012). We used both the critical mass threshold and changes in dm/mdt to determine the entry to PIII in the field. Those results were

later validated by determining plasma uric acid concentrations (see Supplementary Materials Fig 1), an index of protein catabolism in birds (Robin et al., 1988). During fasting in PIII, birds were weighed daily until their release. Birds in the PII or PIII groups had similar body mass when first captured and put in the enclosure (see Results). A final mass measurement was taken when birds came back from their foraging trip.

Blood sampling

To control for possible effects of season, daytime and stress (Dawson et al., 2001; Romero and Romero, 2002), we standardized captures. Blood sampling was performed before weighing the birds at the same period of the year and at the same hour of the day (every day at 9:00 AM ± 30 min). The bird's head was covered with a hood to minimize stress and agitation and blood samples (1 mL) were taken from the brachial vein using a G22-1 ½ needle fitted to a 2.5 mL a heparinized syringe. All blood samples were obtained within 3 min of handling. After centrifugation (3000 g for 10 min), plasma was kept frozen at –20 °C and moved to a -80°C ultra-cold freezer at the end of the day until assayed.

Laboratory measures of oxidative stress, uric acid and protein content

Total plasma antioxidant capacity

Total plasma antioxidant capacity was measured using the OXY Adsorbent test (5 µL of 1:100 diluted plasma) (Diacron International©, Grosseto, Italy) in accordance with methods reported in previous studies (e.g. Costantini and Dell'Omo 2006; Stier et al. 2013). The OXY adsorbent test quantifies the ability of the plasma to buffer massive oxidation through hydroperoxyde acid. All sample measurements were duplicates. Intra-individual variation was 3.35% and inter-plate variation based on a standard sample repeated over plates was 4.25%.

Antioxidant enzymatic activity

The enzymatic activity of superoxide dismutase in plasma was measured using a commercial SOD activity kit following the manufacturer protocol (25 µL of 1:6 diluted plasma) (Enzo Life Sciences© Villeurbanne, France). All sample measurements were

duplicates. Intra-individual variation based on duplicates was 3.11% and inter-plate variation based on a standard sample repeated over plates was 6.92%.

Reactive oxygen metabolites (ROMs)

Plasma ROMs levels were measured using the d-ROMs test (8 µL of plasma) (Diacron International©, Grosseto, Italy), in accordance with methods reported for previous studies (e.g. Costantini and Dell'Omo, 2006; Stier et al., 2013). The d-ROMs test measures hydroperoxydes which are the main compounds contributing to the oxidant ability of the plasma (Costantini, 2016) and is expressed as mg of H_2O_2 equivalent/dL. All sample measurements were duplicates. Intra-individual variation was 5.29% and inter-plate variation based on a standard sample repeated over all plates was 5.39%.

Uric acid measurements

OXY measurements may be influenced by the concentration of uric acid and proteins in plasma (Costantini, 2011). Hence, the plasma concentration of uric acid (mg/dL) was determined using an enzymatic method (10µL of 1:25 diluted plasma) (Uric acid assay, ©Randox Laboratories Ltd. Roissy, France). All sample measurements were duplicates. Intra-individual variation was 3.23% and inter-plate variation based on a standard sample repeated over all plates was 3.55%.

Total protein content

We also measured plasma total protein concentration (g/L) using a colorimetric assay (10µL of 1:10 diluted plasma) (Bradford Reagent #B6916 ©Sigma Aldrich). All runs were duplicates. Intra-individual variation was 3.12 %. Plasma protein levels and uric acid level did not significantly explain variation in plasma OXY levels (LMM with individual ID as a random factor; F = 2.41, P = 0.124 and F = -0.01, P = 0.938, respectively) and thus we did not control for these variables in our statistical analyses.

Statistical analyses

All analyses were run in the statistical computing software R (v.3.1.1; R development Core Team 2013). To investigate effects of our experimental fasting experiment on bird variation in body mass and exposure to oxidative stress, we divided our statistical analyses into three parts.

First, for each individual, we characterized the exact day of transition between each phase (PI; PII; PIII) using segmented regression models with the R package 'segmented' (Muggeo 2008). We searched for break points in uric acid levels in relation to the number of days of fasting (see Supplementary Materials Fig 1). From a starting value for the linear predictor (fasting days) describing the response (uric acid concentration), an iterative algorithm was used to fit a new linear regression model at each iteration, identifying marked changes in slope coefficients as breakpoints (for details on the procedure, see Muggeo 2008). Break-point estimates confirmed the transition stages obtained from segmented relationships with mass specific daily body mass loss (dm/mdt).

Second, we ran Linear Mixed Models (LMMs) to compare variation in body mass, plasma oxidative damage (ROMs) and anti-oxidant capacity (OXY and SOD) for birds released in PII or PIII at the 3 following periods: (1) beginning of the fast, (2) end of the fast, and (3) when birds returned from their post-fast foraging trip at sea. Body mass, oxidative and antioxidant markers were entered as dependent variables in separate LMMs. A six-level (PII-beginning, PIII-beginning, PII-end, PIII-end, PII-returned, and PIII-returned) fixed factor was considered in the models. Individual ID and year of sampling were considered as random effects to control for intra-individual and yearly variation in response variables. Tukey HSD post-hoc comparisons ('glht' function from the 'multcomp' R package; Bretz et al. 2010) were used to compare responses of PII and PIII individuals. Considering a set of statistical inferences simultaneously and to limit multiple testing issues (Type I errors), only biological relevant comparisons were investigated: (1) PII *vs.* PIII separately at each period, and (2) within each bird group (PII or PIII) the differences between values at the beginning of the fast, end of the fast, and return from the post-fast foraging trip. Effect sizes (Hedges' unbiased d) for differences between PII-PIII groups and 95% CI were calculated following Nakagawa and Cuthill (2007) and are provided in the figures.

Third, we used LMMs to investigate whether PII and PIII individuals paid a different oxidative cost in terms of the foraging effort required to rebuild energetic resources at sea

(defined as birds' daily mass gain during their foraging trip in g/day). We specified ROMs, SOD and OXY levels measured at birds' return from their foraging trip as the dependent variable under scrutiny and tested for an interaction between fasting phase and foraging effort (daily body mass gain). Individual ID and year were treated as random effects in the models. All results are reported as means ± SE. For each model, the number of observations (*n*) and of individuals (*N*) is given.

RESULTS

Bird fasting duration and variation in body mass

PIII birds fasted significantly longer (28.7 ± 1.9 days) than PII birds (20.8 ± 1.8 days) (Linear Mixed Model (LMM); $F = 30.01$, $P < 0.001$, $n = 23$, $N = 23$). In both groups, body mass showed significant differences according to metabolic status (Fig 1, LMM; $F = 67.68$, $P < 0.001$, $n = 69$, $N = 23$). At the start of the fast, PII and PIII birds did not differ significantly in body mass (13.52 ± 0.23 kg vs. 13.26 ± 0.32 kg, for PII and PIII birds respectively; Tukey's HSD; $z = 0.67$, $P = 0.979$), but as expected, at the end of the fast PIII birds had a significantly lower body mass than PII birds 10.29 ± 0.23 kg vs. 9.06 ± 0.32 kg, for PII and PIII birds respectively; Tukey's HSD; $z = 3.25$, $P = 0.010$). When returning from their foraging trip at sea, both PII and PIII birds had restored their body reserves. Body mass was no longer different between the two groups and was not different to that at the beginning of the fast (Tukey's HSD; $-0.68 < z < 0.54$, $0.978 < P < 0.996$).

Re-feeding period at sea in relation to fasting

The mean time individuals spent at sea rebuilding their energy stores following fasting was similar for PII and PIII individuals (LMM; $F = 0.51$, $P = 0.48$, $n = 23$, $N = 23$) (Fig 2A). Birds released in PIII gained significantly more mass than PII birds during the time they spent foraging at sea (LMM; $F = 7.38$, $P = 0.013$, $n = 23$, $N = 23$) (Fig 2B). Daily mass gain was 176.3 ± 36.0 g/day for PII birds and 239.2 ± 43.9 g/day for PII birds, though the difference was not significantly different (LMM; $F = 1.98$, $P = 0.17$, $n = 23$, $N = 23$) (Fig 2C).

Variation of oxidative stress measurements in relation to fasting

Plasmatic variation in antioxidant superoxide dismutase activity (SOD) and pro-oxidant markers (reactive oxygen metabolites; ROMs) were significantly different between PII and PII groups (SOD: LMM; $F = 6.08$, $P < 0.001$, $n = 69$, $N = 23$; ROMs: LMM; $F = 3.01$, $P = 0.017$, $n = 69$, $N = 23$). Post-hoc tests showed that SOD levels significantly increased between the beginning and end of their fast in both PII and PIII individuals (Fig. 3). In PIII individuals however, SOD significantly decreased between the end of fast and the subsequent return from the foraging trip. In contrast, the decrease in PII birds was not significant (Fig. 3). There was nonetheless no difference in SOD levels between the start of the fast and the return from sea in both PII and PIII individuals (Fig. 3). ROMs levels significantly increased between the start and end of the fast in PIII individuals only (Fig. 4). After foraging, ROMs levels were similar to those observed at the beginning of the fast both in PII and PIII individuals (see Fig. 4). Plasma total antioxidant capacity (OXY) levels did not differ throughout the fasting period independent of the fasting stage (LMM; $F = 1.7$, $P = 0.15$, $n = 69$, $N = 23$) (Fig. 5).

Re-feeding effort and oxidative stress

Re-feeding effort (calculated as the mass gain/day during foraging) may come at the cost of maintaining oxidative balance, especially in individuals enduring long fasting periods. Accordingly, re-feeding effort was significantly positively related with ROMs levels measured at the return from sea in PIII individuals only (Table 1; LMM; interaction: *group x mass gain/day*; $F = 5.02$, $P = 0.038$, $n = 23$, $N = 23$) (Fig 6). No relationship was found between re-feeding effort and OXY ($F = 0.88$, $P = 0.36$, $n = 23$, $N = 23$) or SOD levels ($F = 0.73$, $P = 0.40$, $n = 23$, $N = 23$).

DISCUSSION

In this study, we tested for a potential oxidative cost of long-term fasting in king penguins, for which fasting is a natural and important part of its life cycle (Groscolas, 1990; Stonehouse, 1960). Our results suggest that birds fasting up to PIII (advanced fast) paid an additional cost of recovering from that fast compared to birds fasting up to PII (medium fast), *i.e.* a debt paid in terms of oxidative imbalance to restore body reserves. Our results complement previous findings in captive ducks and rats (Geiger et al., 2012; Wasselin et al., 2014) describing an oxidative cost of entering phase III of fasting. Birds that fast up to PIII utilize more energy reserves than birds that stop fasting in PII, PIII being characterized by the onset of protein (muscle) catabolism once fat stores are close to exhaustion (Cherel et al., 1994b; Groscolas and Robin, 2001). Our study shows that all individuals (PII and PIII birds), had fully recovered their body mass when returning from their foraging trip at sea (Fig. 1), both PII and PIII individuals spending a similar amount of time at sea. These results suggest that PIII individuals either exhibited greater foraging effort (e.g. in terms of prospection of the water column while diving), or were more efficient at processing/assimilating, caught food resources. Apparently, fasting up to PIII was achieved at an oxidative cost, since we observed higher oxidative damage in PIII birds both at the end of the fast (higher plasma levels of ROMs) and after the re-feeding trip (decreased enzymatic antioxidant defences, SOD). In addition, foraging effort was positively related to ROMs levels when returning from the foraging trip in PIII but not PII birds.

Whereas fasting PII has previously been characterized by a decrease in energy expenditure along with a slow decrease in plasmatic antioxidant defences and oxidative damage (e.g. in ducks; Geiger et al., 2012), reaching the critical stage of PIII enhances oxidative respiration allowing individual to mobilize its last resources necessary to undertake foraging activities (Goodman et al., 1981; Groscolas and Robin, 2001). Increases in metabolic rate with the onset of PIII have indeed been observed in penguins (Cherel et al., 1994b; Groscolas and Robin, 2001; Groscolas et al., 2000) and rats (Koubi et al., 1991), and the transition of PII to PIII fasting appears to be accompanied by an increase in oxidative damage (in ducks; Geiger et al., 2012; in rats; Wasselin et al., 2014).

Several mechanisms may be suggested to explain the onset of oxidative stress in fasting PIII. First, the energetic reserves mobilized during fasting might also include exogenous antioxidants (Mårtensson, 1986) (antioxidant compounds not produced by the

organism but acquired from the diet, such, carotenoids, vitamin E, etc.). However, this seems unlikely in king penguins since total plasma antioxidant capacity (OXY) in our study did not appear to vary with fasting duration. Second, shifting from a lipid to a protein oxidative pathway has been suggested to increase amino-acid input into the Krebs cycle leading to the generation of large amounts of NADH, thereby enhancing oxidative respiration (Wasselin et al., 2014). Third, plasmatic concentrations of glucocorticoid (GC) hormones (corticosterone in birds) known to increase rapidly at the onset of PIII, promote gluconeogenesis and enhance energy resource mobilization (Cherel et al., 1988a; Robin et al., 1998). The corticosterone increase (and a concurrent decrease in prolactin levels) has been associated with a "re-feeding signal" in various bird species, including king and other penguins, resulting in a decrease of current reproduction to the benefit of self-maintenance (Angelier and Chastel, 2009; Criscuolo et al., 2002; Groscolas and Robin, 2001; Groscolas et al., 2008; Spée et al., 2011). GC-related beneficial effects on adult survival are then likely to be counter-balanced by detrimental impacts on the oxidative balance when exposure to high levels of GCs is sustained in time (Costantini et al., 2011; Lin et al., 2004). Together, these processes likely add-up to explain the oxidative status reached at advanced fasting stages (Lin et al., 2004; Morales et al., 2004; Sorensen et al., 2006; Wasselin et al., 2014).

One alternative explanation for the oxidative rise in phase III could be that it is adaptive. Indeed, ROS/RNS (specially H_2O_2 and NO) are involved in many transduction signalling pathways as secondary messengers (tyrosine kinase membrane receptors, MAP kinases, nuclear factor kB or Ras; Kamata and Hirata 1999; Allen and Tresini 2000), raising the question of whether oxidative stress in itself during PIII could play a role in the re-feeding signal (Geiger et al., 2012) by modulating cellular hormesis (Costantini, 2014; Ristow and Zarse, 2010). This hypothesis support the idea that oxidative stress may act as an important mediator of life-history trade-offs (Costantini, 2008; Costantini, 2014; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Selman et al., 2012). Experimentally increasing oxidative stress level (*e.g.* using a prooxidant such as paraquat; Isaksson and Andersson 2008) or decreasing antioxidant activity (e.g. using buthionine sulfoximine-BSO; Koch and Hill 2016) in birds experiencing a moderate fast (PII) may allow testing whether high oxidative stress leads to energy reallocations between breeding and foraging.

The oxidative cost of reaching fasting PIII might appear surprising given the life-history and breeding cycle of long-term fasters such as king penguins (Cherel et al. 1988a; Cherel et al. 1988b). These birds indeed fast repeatedly throughout the life cycle (Cherel et al.

1987; Cherel et al. 1988a and b). As seems to be the case, adaptation to fasting might actually prevent the occurrence of oxidative stress during advanced fasting. Penguins seem to be able to maintain high antioxidant defences (*e.g.* OXY levels) regardless of their status of energy depletion (actually SOD activity increases with advancing fasting), and those defences appear to shield the organism from oxidative stress during phase II. In contrast, in humans, rats and mice antioxidant defences decrease during fasting, which may trigger a pro-oxidative cascade increasing mitochondrial oxidant generation (Ceriello and Motz, 2004; Sorensen et al., 2006; Souza Rocha et al., 2008; Szkudelski et al., 2004). However, once in PIII, the increase in antioxidant defences is no longer sufficient to counteract the damages caused by reactive oxygen (ROS) or nitrogen (RNS) species, leading to short-term oxidative stress. Thus, the adaptation of those long-term fasters to oxidative stress relies on the fact that PIII birds seem to be able to recover rapidly from short-term oxidative stress. In fact, they recover to similar ROMs levels as PII individuals over the same duration of foraging at sea. In addition, the oxidative cost of long-term fasting does not seem to affect body mass recovery (PIII and PII). Both PII and PIII birds appear to recover to similar body mass, and PIII birds appear to be more efficient at assimilating energy resources (body mass was recovered within a similar amount of time in PII and PIII birds without increasing re-feeding effort) as has been shown for rats (Robin et al., 2008). The oxidative cost of reaching PIII also likely did not affect future reproduction, as PIII penguins were observed returning to the colony to court and breed (QS; pers. obs., Robin et al. 2001). Nonetheless, since oxidative stress may result in either deleterious effects (Bize et al., 2008), or adaptive (hormetic) responses (Costantini, 2014; Ristow and Zarse, 2010; Yun and Finkel, 2014), cumulative long-term effects of chronic exposure to oxidative stress are harder to predict. Notably, oxidative stress is known to be an important predictor of health and biological ageing through cumulative detrimental effects on DNA telomere length (Richter and Von Zglinicki, 2007; Von Zglinicki, 2000; Von Zglinicki, 2002), and telomere length has recently been shown to be a good proxy to individual quality (breeding performance and immunity) in king penguins (Le Vaillant et al., 2015). Thus, it would be interesting to further consider whether birds repeatedly entering advanced fasting stages pay a long-term cost in terms of telomere attrition rates. This could be achieved using long-term monitored individuals followed through multiple breeding cycles (e.g. Le Vaillant et al., 2015).

Similarly to our findings in king penguins, an increase in antioxidant defences to face increasing oxidative stress with advanced fasting has also been observed in seals (Vázquez-

Medina et al., 2011). Interestingly, one common life history feature of penguins and seals is the alternation of long deep-diving events with short surface events for breathing. Those animals have to cope with prolonged apnoea exposing tissues to high levels of hypoxemia due to high pressure when diving, followed by rapid tissue re-perfusion and transiently high oxygen concentration in tissues during brief surface episodes (Meir and Ponganis, 2009). This situation, known as ischemia-reperfusion, induces the mass activation of nitric oxide synthase (Huang et al., 1994; Iadecola et al., 1997) and xantine oxidase (Granger, 1988) enzymes known to promote ROS. Coping with repeated exposure to high levels of ROS during repeated diving has been suggested to explain the higher levels and activity of anti-oxidant enzymes in the muscles and livers of diving birds (Zenteno-Savin et al., 2010), and to protect seals against oxidative damage during prolonged fasting (Zenteno-Savín et al., 2002). Land-based marine predators forage at sea but generally experience long-term fasts while breeding/moulting on land. It is likely that evolutionary pressures acting both on diving and fasting simultaneously selected for high anti-oxidant defence mechanisms in diving animals (Vázquez-Medina et al., 2012).

Nonetheless, our results suggest a limit to this adaptation. Indeed, although PII and P III birds had similar ROMs levels when returned from sea, it appeared that an increase in foraging effort (mass gain/day) lead to an increase in oxidative stress in PIII birds but not in PII birds. Whereas those results suggest a cost for PIII birds to rapidly restore their energy reserves, our limited sample size warrants some caution. Increased foraging effort implies higher energy expenditure (Froget et al., 2004), which likely increases oxidative stress (Finaud et al., 2006). Thus, PIII birds recovering from an oxidative debt at the end of their fast are apparently not able to cope with the additional oxidative load imposed by high foraging effort. This might explain previous differences observed in the time taken by early and late breeding PIII birds to replenish their energy stores (Robin et al., 2001). Indeed, early in the season (as birds in our study) PIII birds appeared to recover from a foraging fast as rapidly as PII birds: the total duration of post-fast foraging trips are similar and they do not differ in terms of body mass upon return to the colony (Robin et al., 2001). In contrast, late-breeding PIII birds spend a significantly longer time at sea to recover their body mass. Given that late-breeding breeding success is virtually zero (Olsson, 1996; Weimerskirch et al., 1992), late-breeding PIII birds may take longer to replenish energy reserves to avoid the oxidative costs of foraging.

To conclude, we highlight a short-term cost of prolonged (PIII) fasting in long-term fasting seabirds, which may play a role in the re-feeding signal promoting individual survival over current reproduction. This cost appears to be compensated to a great extent (but not entirely) during the subsequent foraging trip at sea. The consequences of short-term costs may be carried-over in the form of mid-term costs if birds increase their foraging effort at sea. Whether those short to medium costs of fasting may accumulate over a longer time scale (individuals fast repeatedly during reproduction) to affect subsequent reproductions and adult fitness remains to be determined.

Ethical statement

All experiments described in this study were approved by the Ethics Committee of the French Polar Institute (IPEV). Authorizations to enter the colony were obtained from Terres Australes et Antarctiques Françaises (TAAF). The experiments comply with the current laws of France.

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COMPETING INTERESTS

No competing interests declared

AUTHOR CONTRIBUTIONS

QS contributed to study design, data collection and analyses, and writing the manuscript. VAV contributed to data analyses and writing the manuscript. HS contributed to data collection and laboratory analyses. AS, EL contributed to data collection. FC contributed to data analyses and writing the manuscript. PB contributed to study design, data analyses and writing the manuscript. JPR contributed to study design, data collection and writing the manuscript.

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TABLE

Factors	Estimates	Std. Error	Df	F-value	P-value
Fasting group (PII)	12.188	8.626	18.1	2.00	0.175
Daily mass gain	0.082	0.032	18.2	5.65	0.029*
Fasting group (PII)* Daily mass gain	-0.078	0.035	18.1	5.02	0.038*

Table 1. Linear mixed model estimates for the effects of foraging effort (daily body mass gain; g/day) in king penguins (*Aptenodytes patagonicus*) on total oxidative damage levels in plasma measured at the end of a post-fast foraging trip at sea (n = 23; N = 23). Birds had either undergone a previous fasting period up to fasting phase II (PII) or fasting phase III (PIII). Estimates for the fasting group and interaction are considered against the reference level PIII. Year was specified as random factor.

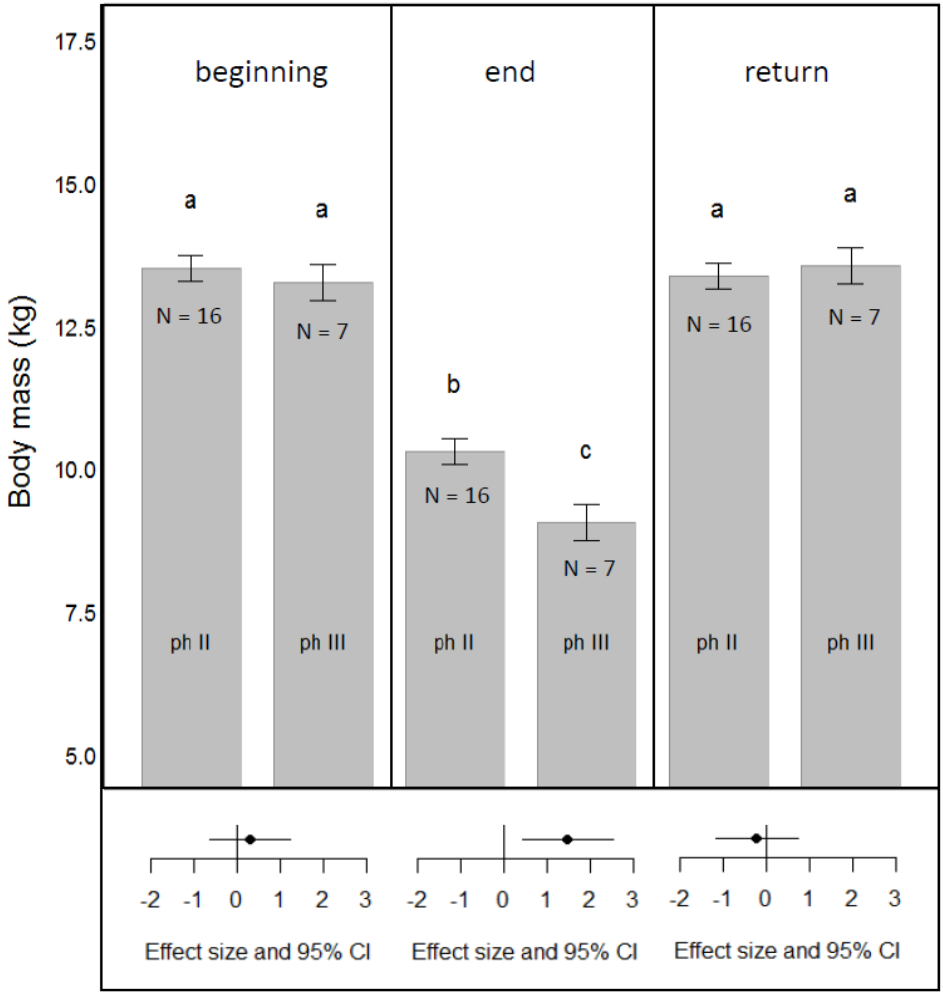

Figure 1. Body mass at different fasting status (fast-beginning, fast-end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII) (n = 69; N = 23). Marginal means ± SE from the model are represented. Values not sharing a common letter are statistically different for $P < 0.05$ (Tukey HSD test). Effect sizes (Hedges' unbiased d) for differences between PII-PIII groups and 95% CI are provided below the figure.

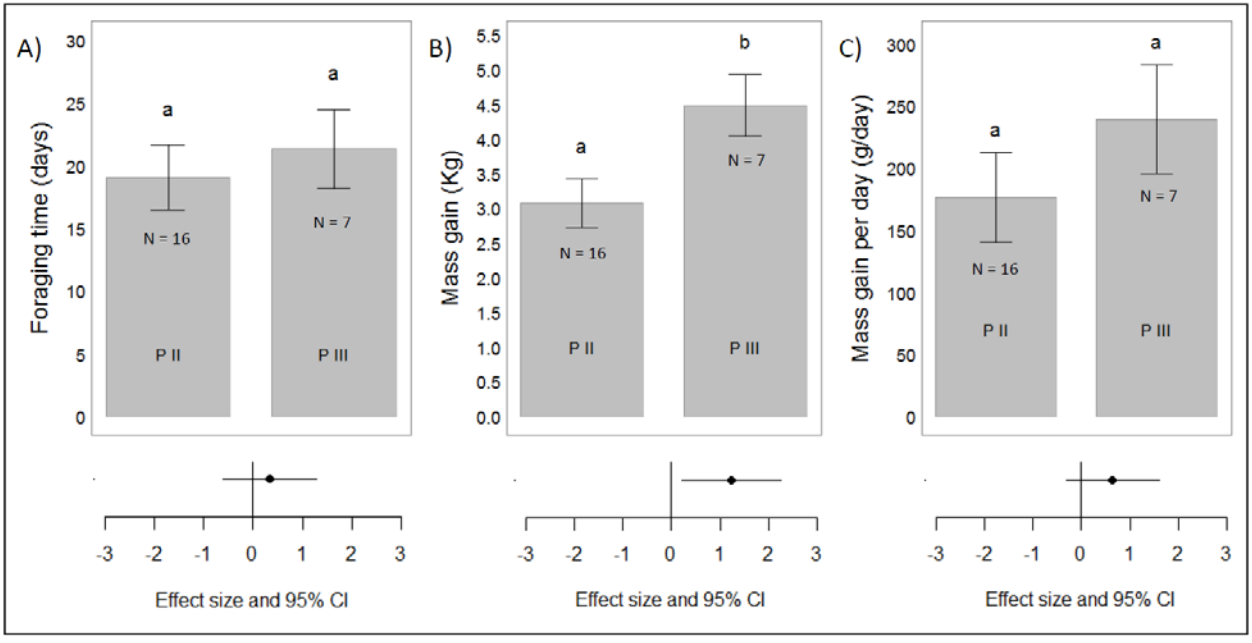

Figure 2. (A) Mean time spent (in days) at sea , and (B) mean mass gain (in kg) of foraging king penguins (*Aptenodytes patagonicus*) during their re-feeding trip following release from an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII). (C) Foraging effort in term of body mass gain per day (g/day) by birds during their foraging trip whether they were released in PII or PIII (n = 23; N = 23). Marginal means ± SE estimated by LMM models are presented. Values not sharing a common letter are statistically different for $P < 0.05$ (LMMs with year as random factor. Effect sizes (Hedges' unbiased d) for differences between PII-PIII groups and 95% CI are provided below the figure.

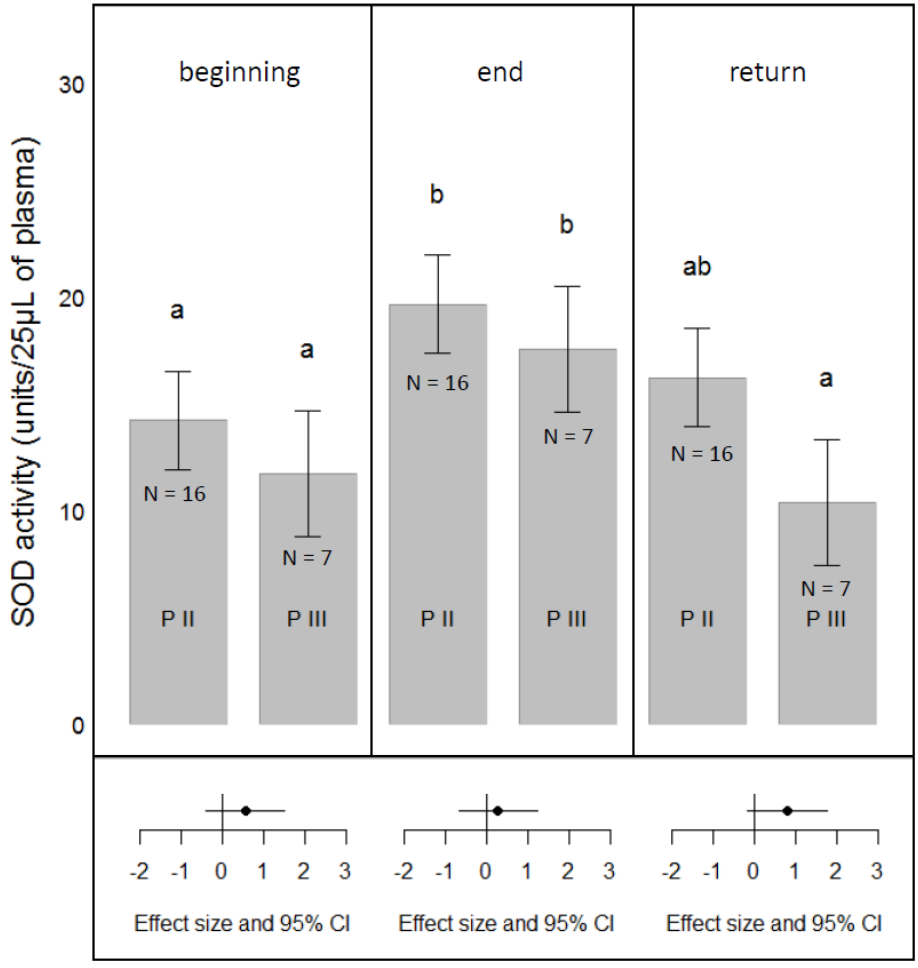

Figure 3. Plasmatic superoxide dismutase activity (SOD) at different fasting status (fast-beginning, fast-end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII) (n = 69; N = 23). Marginal means ± SE from the model are represented. Values not sharing a common letter are statistically different for $P < 0.05$ (Tukey HSD test). Effect sizes (Hedges' unbiased d) for differences between PII-PIII groups and 95% CI are provided below the figure.

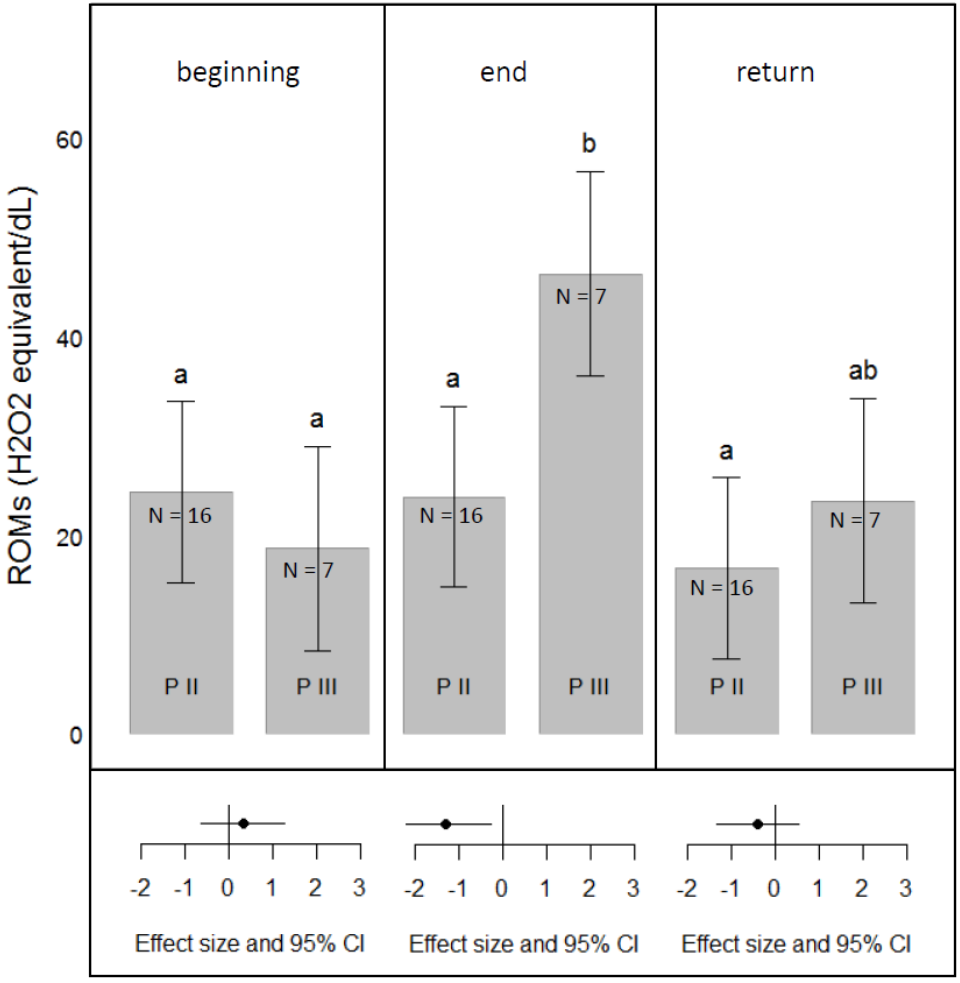

Figure 4. Total oxidative plasmatic damages (ROMs) at different fasting status (fast-beginning, fast-end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII) (n = 69; N = 23). Marginal means ± SE from the model are represented. Values not sharing a common letter are statistically different for $P < 0.05$ (Tukey HSD test). Effect sizes (Hedges' unbiased d) for differences between PII-PIII groups and 95% CI are provided below the figure.

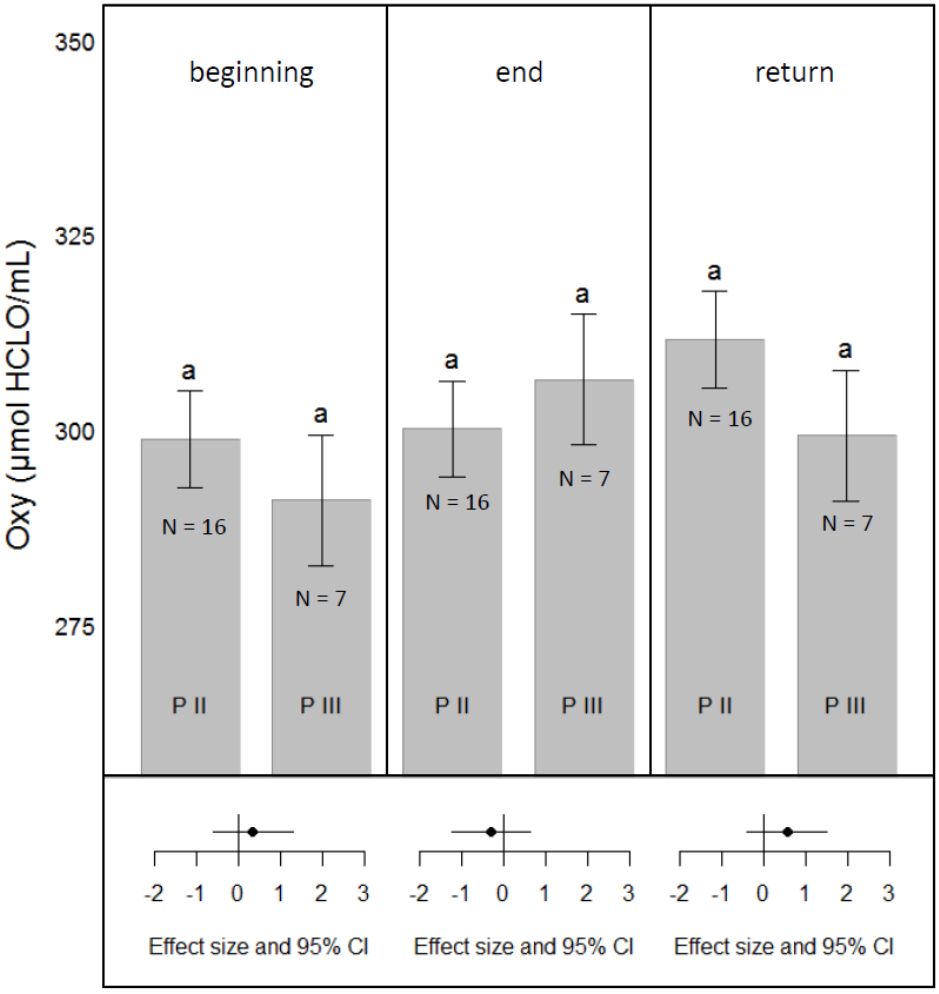

Figure 5. Total anti-oxidant plasmatic defences (OXY) at different fasting status (fast-beginning, fast-end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII) (n = 69; N = 23). Marginal means ± SE from the model are represented. Values not sharing a common letter are statistically different for $P < 0.05$ (Tukey HSD test). Effect sizes (Hedges' unbiased d) for differences between PII-PIII groups and 95% CI are provided below the figure.

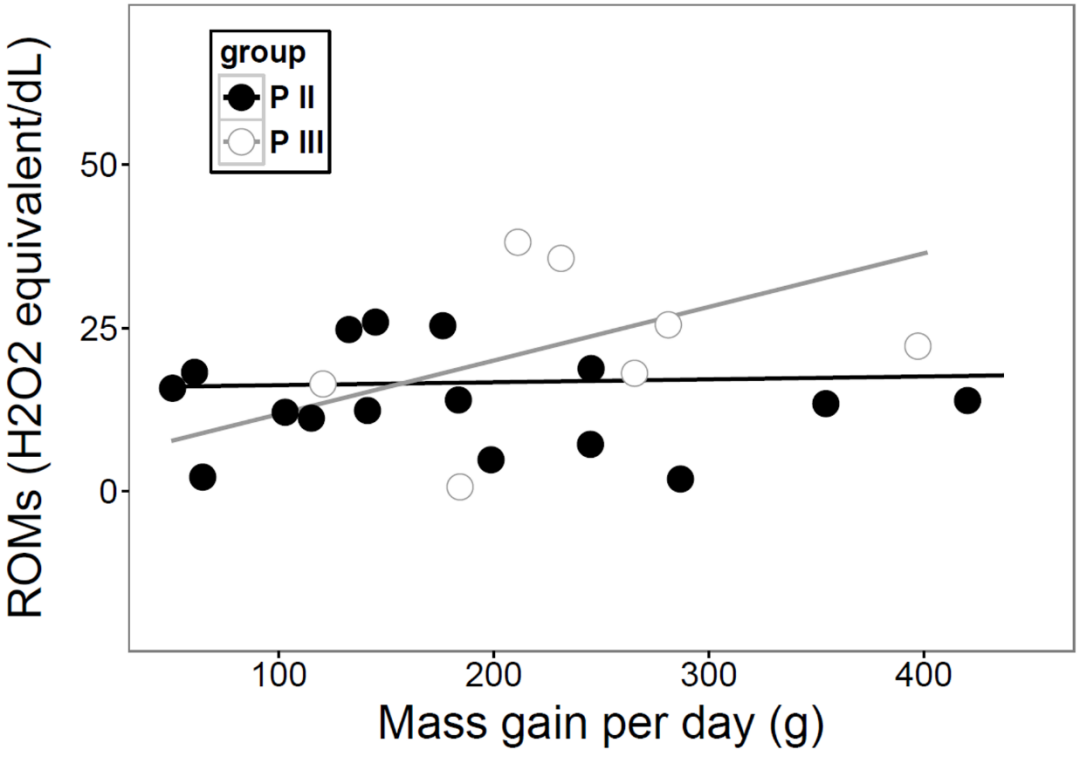

Figure 6. Interactive effect of the effort invested during the re-feeding trip (body mass gain per day) on the total plasmatic oxidative damage of birds returning from their foraging trip depending on whether they were released in PII or PIII of fasting (n = 23; N = 23).

Appendix 2

Mutually honest? Physiological 'qualities' signalled by colour ornaments in monomorphic king penguins

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In species for which successful reproduction relies strongly on shared and substantial parental investment by males and females, mate choice is expected to be important to the fitness of both sexes. Reciprocal selection may then favor the evolution of morphological signals providing mutual information on the condition/quality of tentative partners. However, because males and females often have differing physiological constraints, it is unclear which proximate physiological pathways guarantee the honesty of male and female signals in similarly ornamented species. We used the monomorphic king penguin (*Aptenodytes patagonicus*) as a model to investigate the physiological qualities signaled by color and morphological ornaments known to be under sexual selection (coloration of the beak spots and size of auricular feather patches). In both sexes of this slow-breeding seabird, we investigated the links between ornaments and multiple indices of individual quality; including body condition, immunity, stress and energy status. In both sexes, individual innate immunity, resting metabolic rate, and the ability to mount a stress response in answer to an acute disturbance (capture) were similarly signaled by various aspects of beak coloration or auricular patch size. However, we also reveal interesting and contrasting relationships between males and females in how ornaments may signal individual quality. Body condition and oxidative stress status were signaled by beak coloration, though in opposite directions for the sexes. Over an exhaustive set of physiological variables, several suggestive patterns indicated conveyance of honest information about mate quality in this monomorphic species. However, sex-specific patterns suggest that monomorphic ornaments may signal different information concerning body mass and oxidative balance of males and females, at least in king penguins.

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Mutually honest? Physiological 'qualities' signalled by colour ornaments in monomorphic king penguins

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Mate choice is expected to be important for the fitness of both sexes for species in which successful reproduction relies strongly on shared and substantial parental investment by males and females. Reciprocal selection may then favour the evolution of morphological signals providing mutual information on the condition/quality of tentative partners. However, because males and females often have differing physiological constraints, it is unclear which proximate physiological pathways guarantee the honesty of male and female signals in similarly ornamented species. We used the monomorphic king penguin (*Aptenodytes patagonicus*) as a model to investigate the physiological qualities signalled by colour and morphological ornaments known to be under sexual selection (coloration of the beak spots and size of auricular feather patches). In both sexes of this slow-breeding seabird, we investigated the links between ornaments and multiple indices of individual quality; including body condition, immunity, stress and energy status. In both sexes, individual innate immunity, resting metabolic rate, and the ability to mount a stress response in answer to an acute disturbance (capture) were similarly signalled by various aspects of beak coloration or auricular patch size. However, we also reveal interesting and contrasting relationships between males and females in how ornaments may signal individual quality. Body condition and oxidative stress status were signalled by beak coloration, although in opposite directions for the sexes. Over an exhaustive set of physiological variables, several suggestive patterns indicated the conveyance of honest information about mate quality in this monomorphic species. However, sex-specific patterns suggested that monomorphic ornaments may signal different information concerning body mass and oxidative balance of males and females, at least in king penguins. © 2015 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **118**, 200–214.

KEYWORDS: body condition – king penguin – monomorphic seabird – mutual mate choice – ornament – oxidative stress – sexual selection – ultra-violet signals.

INTRODUCTION

The evolutionary explanation for conspicuous and similar ornaments in both sexes (i.e. in sexually monomorphic ornamented species) has been a long-standing quandary in evolutionary biology (reviewed by Kraaijeveld, Kraaijeveld-Smit & Komdeur, 2007). Two main hypotheses have been proposed to explain mutual ornamentation. The first suggests that female ornaments are non-functional, but arise as a

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by-product of genetic correlations between the sexes (Lande, 1980; Price, 1996). The second, mutual selection, suggests that functional ornaments may result from selection on their expression in both sexes. Processes that may select for both male and female ornaments include mimicry to conceal sexual identity (Burley, 1981), mutual sexual selection for high quality partners (Hooper & Miller, 2008), or social competition over non-mate resources in both sexes (West-Eberhard, 1979; Tobias, Montgomerie & Lyon, 2012). As pointed out by Kraaijeveld *et al.* (2007), these processes are not mutually exclusive, as traits may be used in several contexts, for instance both in contests over resources (either mates or non-mate resources) and mate choice (Berglund, Bisazza & Pilastro, 1996).

Mutual sexual selection is expected when variance in reproductive success is similar between males and females, and when mate quality is an important predictor of variation in male and female success (Trivers, 1972; Clutton-Brock & Vincent, 1991), such as in slow-breeding seabirds (e.g. Velando, Lessells & Márquez, 2001). Where both sexes should be choosy in their pairing preferences, ornaments may be favoured because they assist the individual expressing them in acquiring a high quality mate, whereas preferences for ornaments may do the same for receivers (Johnstone, Reynolds & Deutsch, 1996; Kokko & Johnstone, 2002; Hooper & Miller, 2008). Furthermore, mating systems with extended mate-sampling periods are expected to lead to reduced mutual ornamentation ('dull monomorphism'; Badyaev & Qvarnström, 2002; Badyaev & Hill, 2003), whereas mating systems with short mate-sampling periods should favour extravagant 'bright' monomorphism (Fitzpatrick, 1994). However, because males and females often differ in physiological constraints, the aspects of individual quality signalled and of interest to receivers may differ between the sexes (Alvarez, Sanchez & Angulo, 2005; Lopez, Figuerola & Soriguer, 2008). For instance, in goldfinches (*Spinus tristis*), monomorphic bill coloration is correlated with acquired immunity in females but not males, probably linked to the different functional roles of beak coloration in male and female social communication (Kelly *et al.*, 2012).

King penguins (*Aptenodytes patagonicus*) are monomorphic seabirds, where both sexes experience a highly energy demanding breeding cycle (Groscolas & Robin, 2001) and cooperate for as long as 14 months to successfully raise a single chick (Stonehouse, 1960). Both males and females display conspicuous colour ornaments including auricular feather patches that only reflect yellow-orange colours, a breast feather patch that reflects yellow to rusty-brown colours (Pincemy, Dobson & Jouventin, 2009), and keratin beak spots on their lower mandibles that reflect yellow-orange *and* UV colour (Jouventin *et al.*, 2005). Although it has been previously demonstrated that feather and beak spot colorations are used in mate choice (Pincemy *et al.*, 2009; Nolan *et al.*, 2010), few facts are known on the information carried by those ornaments. We tested whether the ornaments of king penguins convey similar information in both sexes in order to determine whether the condition dependence of ornamental features occurs only in one sex, suggesting that selection operates primarily in that sex and that monomorphism is the outcome of genetic correlation between the sexes; or whether condition dependence occurs in both sexes (though not necessarily on the same ornaments nor related to the same qualities) supporting the idea of mutual sexual selection. We aimed at providing an extensive list of quality measures choosing key mediators of vertebrate life histories expected to exhibit important associations with fitness. Those included body condition, immune status, energy expenditure, hormonal stress status, hormonal and heart rate stress responsiveness, and oxidative status (e.g. Norris & Evans, 2000; Monaghan, Metcalfe & Torres, 2009).

Because beak UV is important to pairing decisions for both male and female king penguins (Nolan *et al.*, 2010), we expected it to reflect information on individual quality in both sexes. In contrast, larger auricular patches are more important to females during mate choice (Pincemy *et al.*, 2009; Dobson, Couchoux & Jouventin, 2011), but have also been positively linked to social aggressiveness in both sexes (Viera *et al.*, 2008). Thus, we expected auricular patch size to yield information on male quality, or non-exclusively to signal male and female abilities to cope with their aggressive colonial environment, including via physiological stress responses (e.g. Parker, Knapp & Rosenfield, 2002; Bortolotti *et al.*, 2009). Social competition has been suggested to favour the evolution of ornaments as 'badges of status' that are used in alternative contexts to mate choice (West-Eberhard, 1979; Kraaijeveld *et al.*, 2007). King penguins are known to aggressively compete over breeding sites, and thus coloured ornaments might convey information about social dominance or aggressiveness (Viera *et al.*, 2008; Keddar, Jouventin & Dobson, 2015a). Specifically, given that males perform the first and longest reproductive fast of the breeding cycle (typically 1-month including courtship and incubation; Stonehouse, 1960), information on body condition should be more important to females. We predicted that ornamental features should be associated with body condition, especially in males. In contrast, information relating to immunity should be particularly relevant to both sexes in

this species, as ticks (*Ixodes uriae*) are prevalent in king penguin colonies and detrimentally affect adult and offspring fitness (Mangin *et al.*, 2003; P. Bize, Q. Schull, S. Pardonnet, Y. Handrich, F. Criscuolo, V.A. Viblanc, J.P. Robin, unpubl. data). Finally, stress status (including oxidative stress; von Schantz *et al.*, 1999) in relation to mate choice (e.g. parental breeding quality; Angelier & Chastel, 2009) or social territory acquisition should be mutually important to males and females, and associated with ornamental traits in both sexes.

METHODS

Field site and study species

This study was conducted in the king penguin colony of *La Baie du Marin* (Possession Island, Crozet Archipelago; 46°25′S, 51°45′E) during the 2011–2012 breeding season (Dec.–Mar.). After an initial courtship period (~15 days), male and female penguins alternate periods fasting on land and foraging at sea during incubation and chick-brooding (Stonehouse, 1960). Hatching occurs after approximately 54 days and both parents alternate feeding and guarding duties on land during most of the austral summer.

In early November (breeding onset), we captured 31 penguin pairs and marked them with non-permanent animal dye (Porcimark; Kruuse, Langeskov, Denmark) and plastic flipper-bands. Because of logistical constraints, all birds were caught after courtship, and had already undergone the mate choice and the pairing processes. We assumed that ornaments at mate choice were correlated with the moment at which we measured them, after birds had paired (see below). Accordingly, the size of the ear patch is determined at molt and beak measures at the start of breeding showed little within-individual variation compared with between individual variation (Q. Schull, V.A. Viblanc, F.S. Dobson, P. Bize, unpubl. data). Males ($N = 31$) were tagged during the first incubation shift, shortly after the female had departed to feed at sea. Females ($N = 30$) were tagged upon return from their foraging trip. Birds were observed daily from a distance, during the entire breeding season (November–March) to monitor their breeding status and determine sex-specific breeding shifts. All plastic flipper-bands were removed at the end of the study.

Morphometric measures

Flipper (± 1 mm) and beak length (± 0.1 mm) were measured using a solid metal ruler and dial calipers (Stonehouse, 1960). Body girth (thoracic circumference) was measured (± 1 mm) with a flexible tape-ruler just below the upper articulation of the flippers to the body (Viblanc *et al.*, 2012a). Birds were measured at the onset of incubation shift 2 for females and incubation shift 3 for males, to insure that both males and females had experienced similar minimal fasting durations (2–3 days) on land.

Ornament measures

Standardized measures of the width and height of the right and left auricular feather patches were performed using a flexible tape-ruler (see online Fig. S1). Left and right distances were averaged and the surface of the patch was calculated as $width \times height$ (mm^2).

Reflectance measurements of the beak spot were obtained using a portable JAZ spectrophotometer (Ocean Optics Inc., Dunedin, FL, USA) with a spectral resolution of 0.3 nm across the spectral range 320–700 nm. The spectrophotometer contained a pulsed-xenon light module and was calibrated against a white Spectralon standard. All measures were performed using a 200 μm fiber probe with a 90° angle window. Measures were repeated three times across each bill plate (in the orange region from bill tip to base) and spectra were averaged using an R script adapted from Montgomerie (2008). From spectral data, we calculated tri-stimulus colour variables: mean brightness, hue and chroma. We considered the spectral range 320–700 nm, given the range of spectral sensitivity in birds (Cuthill, 2006). The reflectance of king penguin beak spots is characterized by a bi-modal pattern including a reflectance peak in UV and a peak/plateau in the yellow-orange (YO) portion of the spectrum (see Fig. 1). Thus, we calculated colour variables over wavelength sub-regions of interest. For yellow-orange colours, we focused on the 500–700 nm portion of the spectrum. For the UV peak, we focused on the range 320–450 nm. Although this region extends beyond UV coloration *per se*, the choice was deliberate to account for the UV peak of king penguin beak spots in its entirety (Jouventin *et al.*, 2005). Mean brightness is a measure of spectral intensity of the ornament, and yellow-orange and UV mean brightness were calculated by averaging reflectance over wavelengths 500–700 nm and 320–450 nm, respectively (Montgomerie, 2006). Hue is a measure of colour appearance (e.g. 'blue', 'yellow', etc.). For the YO plateau portion of the spectrum, it was calculated as the wavelength at which the reflectance was halfway between its maximum and minimum (Keddar *et al.*, 2013). For the UV peak, hue was calculated as the wavelength of maximum reflectance between 320 and 450 nm. Finally, chroma is a measure of colour purity and was calculated as the difference between maximum and

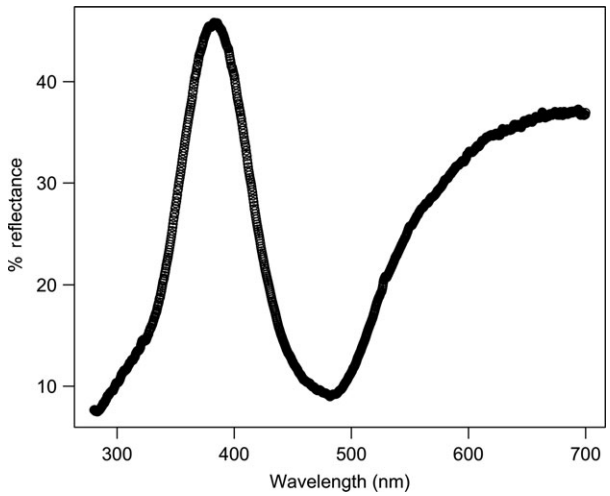

Figure 1. Reflectance curve obtained from the beak spot of a breeding king penguin (*Aptenodytes patagonicus*). Note the typical bi-modal pattern with a UV peak around 380–390 nm and a yellow-orange plateau from 500 to 700 nm.

minimum reflectance over the mean reflectance for that particular region (formula S_8; Montgomerie, 2006).

Body condition

We used a principal component analysis to calculate a structural size index (SSI), which explained 86% of the variation in beak size and flipper length (SSI = 0.95 × flipper + 0.31 × beak). We then regressed body girth on this SSI ($F_{1,59} = 18.87$, $P < 0.001$, $R^2 = 0.24$) and used the residuals as an index of body condition. This method yields condition indices very similar to classical mass/size regressions (correlation, $r = 0.92$; Viblanc *et al.*, 2012a), but is more practical than weighing birds within the breeding colony.

Immunity measures

Immune status was assessed from blood samples collected during the second incubation shift of males and females. Blood (1 mL) was collected within 3 min of capture (see stress protocol below) from the marginal flipper vein using a 0.7*40 mm, 22G needle fitted to a 5 mL heparinized syringe. Within 10 min of sampling, blood was centrifuged at 3000 *g* for 5 min separating plasma and blood cells. Samples were kept at −18°C until the end of the day before being transferred at −80°C until lab-analyses. Constitutive innate humoral immunity was determined using the hemolysis-hemagglutination assay described for birds (including seabirds) by (Matson, Ricklefs & Klasing, 2005). This assay evaluates natural antibody (NAb) levels and associated complement activation potential in plasma. Briefly, NAbs are innate non-specific antibodies encoded by the germ line that react with virtually any antigen. They are naturally present in antigen-naïve individuals, form a large portion of serum immunoglobulin, and initiate the complement enzyme cascade that ends in cell lysis (Matson *et al.*, 2005). We exposed 25 μL of penguin plasma (serially diluted from 1 to 1/1024) to 25 μL of a 1% rabbit blood cell suspension and scored lysis (lysis titres) and agglutination (NAb titres) for each sample. All assays were run on the same day and scored by the same observer (AS). Within and among-assay variation was 2.4 and 7.5% for lysis, and 3.0 and 4.1% for agglutination titres, respectively.

Resting metabolic rate

An estimate of bird's resting metabolic rate was obtained by measuring their daily resting heart rate (rHR). The conversion of HR to VO_2 (the classic measure of metabolic rate) using previously established calibrations is complicated by various issues including error measurement (for a discussion see Green, 2011). Thus, we used raw HR data as a qualitative rather than quantitative index of metabolic rate in king penguins (Viblanc *et al.*, 2014). We attached external HR-loggers (Polar® RS800 and RS800CX, Polar Electro Oy, Kempele, Finland) to breeding birds on the 6th day of their second incubation shift (shift 3 for males, $N = 26$; shift 4 for females, $N = 24$). Details on logger attachment, technology and accuracy of HR measurement are provided elsewhere (Groscolas *et al.*, 2010). Birds' HR was recorded for 48 h (until day 8 of their incubation shift) at a rate of 1 value every 5 or 2 s (depending on the logger model and memory). HR typically recovered to resting levels within 30 min of the initial capture stress (Viblanc *et al.*, 2012b). We thus systematically discarded the first 60 min of each recording to avoid confounding our calculations with handling stress. We calculated daily rHR using moving averages to determine the 10 consecutive minutes where HR was lowest over 12-h periods. Daily rHR values were highly repeatable ($r = 0.95$; Lessels & Boag 1987) and were averaged (Viblanc *et al.*, 2014).

Stress status

We assessed penguins' stress status by measuring plasma total corticosterone (CORT), the main glucocorticoid stress hormone in birds. We determined both basal total CORT levels and acute total CORT increase to a standardized capture stress on the 8th day of second incubation shift, at the same time that

HR-loggers were removed. The capture stress was a standardized approach starting > 25 m away from the bird, before hooding and capturing it. At the start of the approach, the experimenter insured that the bird was resting. The time at which it became vigilant to the approaching experimenter was considered T_0 and a first blood sample (as previously described) was made within the following 3–5 min. In king penguins, plasma CORT levels do not significantly increase due to a capture-handling stress within this time period (Ménard, 1998). After initial blood sampling, the experimenter loosely maintained the bird captive for 30 min and performed a second blood sample at T_{30}. Concentrations of plasma CORT were measured in duplicate using a quantitative competitive sandwich enzyme immunoassay technique according to guidelines provided by the manufacturer (ELISA Corsticosterone kit, Enzo Life Sciences, Farmingdale, NY, USA). Kit sensitivity was 27.0 pg mL^{-1}, intra- and inter-assay variation were 7.6 and 13.3%, respectively. The CORT response to acute stress was calculated as $100*(CORT_{30} - CORT_0)/CORT_0$.

During the standardized capture protocol we also measured HR response. We defined the initial resting HR (HR_i) as the HR at the moment preceding a rapid constant increase in HR due to the approaching experimenter (Viblanc *et al.*, 2012b). Maximal HR (HR_{max}) in response to the capture corresponded to the maximal HR achieved in the 3 min following the onset of the stress. The maximum increase in HR was then calculated as $100*(HR_{max} - HR_i)/HR_i$. HR-loggers were removed at the end of the stress.

Oxidative status

On the 8th day of the second incubation shift, we determined plasma oxidative status as previously described for king penguins (Geiger *et al.*, 2012). The anti-oxidant capacity of penguin's plasma (OXY) and its concentration of reactive oxygen metabolites (ROM; a measure of exposure to oxidative stress) were respectively measured using commercially available OXY adsorbent and dROM kits (Diacron International srl, Grosseto, Italy). Intra- and inter-assay variation was 7.4 and 7.0% for OXY, and 6.4 and 7.9% for ROM.

Data analyses

Analyses were performed using R v.3.0.2. All individuals only appeared once in the data set and we had no repeated measures. First, we investigated male and female dimorphism by considering the effect of sex on structural size, beak colour variables and auricular patch surface in linear models. For auricular patch surface, we also considered sexual dimorphism controlling for structural size (specified as a covariate in the analysis). We then investigated whether ornaments reflected physiological variables (i.e. could the birds 'predict' physiological quality from the ornaments) by running separate models for each physiological trait and specifying beak colour traits (hue, chroma and brightness) and auricular patch size as predictor variables in our models. Sex was included as a cofactor in the analyses and its interactions with beak coloration variables and auricular patch size were considered. The area of the colony in which the bird was sampled (close to the beach or further up the valley) was fixed as a cofactor in all analyses to account for known colony-related differences in parasites and stress responses (Viblanc *et al.*, 2012b). Independent variables were standardized prior to analyses, so that model estimates were comparable (Schielzeth, 2010). We used multi-model inference with Akaike's Information Criterion corrected for small sample size to identify the best model (AICc and AIC weights) for each physiological parameter considered ('dredge' package in R; Barton, 2015). We retained the most parsimonious model within potential candidates ($\Delta AICc < 2$). Models were compared using Maximum Likelihood. Because most colour variables were correlated to some extent (see Fig. S2), we insured collinearity was not an issue before performing model selection in our analyses. We checked for variance inflation factors (VIFs) in the full model (suggested cut-off = 5; Zuur, Ieno & Smith, 2007). Yellow hue was the only variable which appeared problematic in all models, with $7.2 < VIF < 9.4$. Thus, we removed it from all analyses, and subsequent collinearity was low ($1.2 < VIFs < 5.2$). Due to sampling and slight variations in success of laboratory analyses, sample sizes varied across physiological measures. Diagnostic plots and the Shapiro–Wilk normality test were used to inspect model residuals for normality and potential outliers. When necessary (i.e. for resting HR and the acute CORT response), data were transformed prior to analyses using Box-Cox power transformations (Viblanc *et al.*, 2012b) to insure residual normality. For each model, we calculated effect sizes (ES, Hedges' unbiased d and z-transformed r) and their associated 95% confidence intervals based on respective t-statistics using equations 10, 11, 14, 15, 17 and 19 from (Nakagawa & Cuthill, 2007). We use the benchmarks $r = 0.1$, 0.3, 0.5 and $d = 0.2$, 0.5, 0.8, to discuss small, medium and large effect sizes (Nakagawa & Cuthill, 2007).

RESULTS

Male and female dimorphism in sexual ornaments

Males were slightly but significantly larger than females (3–4% for flipper and beak, respectively;

Fig. 2; Table S1), and had significantly larger auricular patches (14%), even when accounting for structural size as a covariate in the model (Fig. 2). Sexes did not differ significantly in terms of ornamental colours, except for UV chroma, which was slightly higher in males (Fig. 2).

Body condition and ornaments

The most parsimonious model explaining body condition in breeding birds with the lowest AICc and highest AIC weight retained beak UV brightness, yellow-orange chroma, and their interactions with sex as important factors (Table 1, see Table S2). Patterns of association between beak UV brightness, yellow-orange chroma, and body condition were different in males and females (Fig. 3, Table 1). Beak UV brightness was weakly positively (Zr = + 0.29; CI_{95} = [+ 0.00, 0.59]) related to body condition in males, but moderately negatively in females (Zr = −0.51; CI_{95} = [−0.22, −0.80]) (Fig. 3A). Beak yellow-orange chroma was moderately positively related to body condition in females (Zr = + 0.53; CI_{95} = [0.24, 0.82]), but not in males (Zr = −0.06; CI_{95} = [−0.35, 0.23]) (Fig. 3B).

Oxidative status and ornaments

UV hue, sex and their interaction were selected by AICc as important variables related to ROM levels (Table 2, Table S3). In females, beak UV hue was strongly negatively related to ROM levels (Zr = −0.59; CI_{95} = [−0.20, −0.99]), whereas the association was positive in males, though the effect was weak as CI barely overlapped zero (Zr = + 0.37; CI_{95} = [−0.02, 0.77]) (Fig. 4). In contrast, OXY levels were not related to beak coloration or auricular patch surface, i.e. only the intercept was retained in the best model (Table S4).

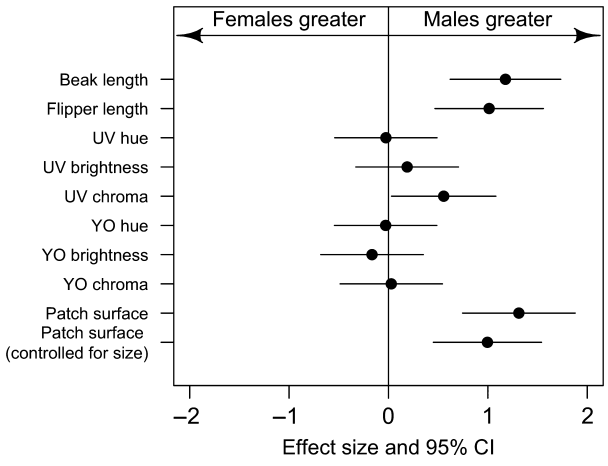

Figure 2. Effect sizes and 95% confidence intervals for ornamental and structural size dimorphism between king penguin males and females. Effect sizes and 95% CI were calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero.

Table1. Model estimates for the influence of beak colour variables on body condition in breeding king penguin (*Aptenodytes patagonicus*). The sex effect is given in reference to the female level [F]. The colony area effect is given in reference to area [A2]. See Fig. 3 for effect sizes with 95% CI

	Estimate	SE	*t*-value
Intercept	−2.00	0.53	−3.73
Sex [M]	2.46	0.55	4.45
UV brightness	−1.59	0.45	−3.51
YO chroma	1.71	0.47	3.61
Colony area [A1]	0.82	0.58	1.41
Sex[M]*UV brightness	2.34	0.59	3.99
Sex[M]*YO chroma	−1.85	0.59	−3.11

Immunity and ornaments

The most parsimonious model retained YO beak chroma as a feature explaining variation in lysis scores in both sexes, but no sex interaction (Table 3, Table S5). YO chroma was weakly negatively (Zr = −0.24; CI_{95} = [−0.54, 0.05]) related to lysis titres (Fig. 5A). NAb titres were moderately negatively (Zr = −0.42; CI_{95} = [−0.72, −0.12]) related to patch surface in both sexes (again, no sex interaction) (Table 4, Table S6) (see Fig. 5B).

Resting metabolic rate and ornaments

Model selection retained UV brightness as a variable related to daily resting HR, but no sex interaction (Tables 5 and S7). UV brightness was moderately positively (Zr = + 0.35; CI_{95} = [0.05, 0.66]) associated with daily resting HR levels (Fig. 6).

Stress and ornaments

Beak and patch ornaments did not relate to basal total CORT levels, as the best and most parsimonious model only retained colony area as an important factor explaining CORT levels ($d_{unbiased}$ = + 0.94; CI_{95} = [0.29, 1.59], see Table S8). Birds breeding further up the valley had significantly higher basal CORT (3.56 ± 0.35 ng mL^{-1}) levels than birds breeding close to the beaches (2.15 ± 0.23 ng mL^{-1}).

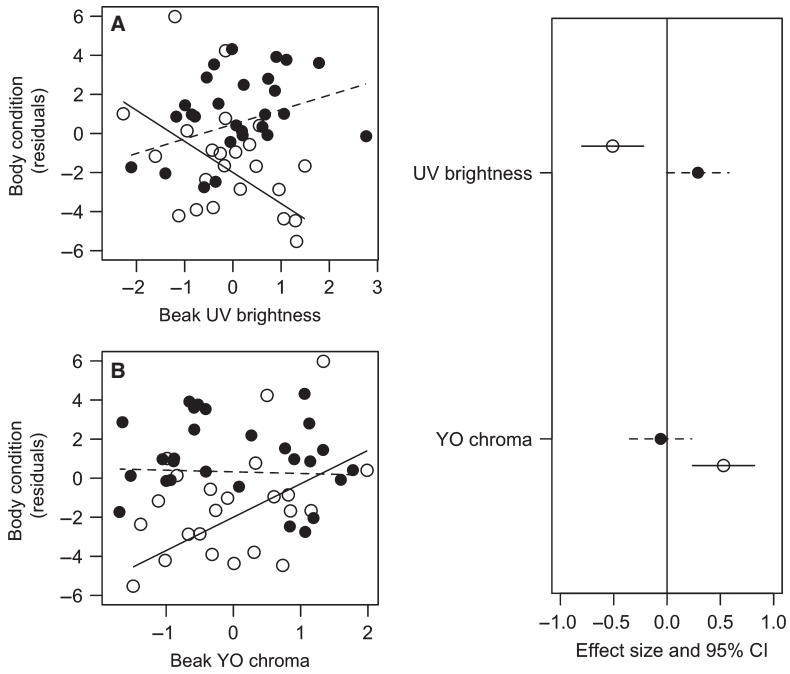

Figure 3. Relationships between beak coloration and body condition in breeding king penguins. Relationships are given for (A) beak UV brightness, and (B) beak yellow-orange chroma. Females are depicted by open circles and a full line, males by filled circles and a dashed line. The right panel provides effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero.

Table 2. Model estimates for the influence of beak UV hue on plasma reactive oxygen metabolite levels in breeding king penguin (*Aptenodytes patagonicus*). The sex effect is given in reference to the female level [F]. See Fig. 4 for effect sizes with 95% CI

	Estimate	SE	*t*-value
Intercept	2.43	0.20	12.10
Sex[M]	0.18	0.27	0.66
UV hue	−0.50	0.16	−3.20
Sex[M]*UV hue	0.93	0.27	3.48

For the birds' acute CORT response to a standardized 30-min capture, model selection retained UV hue as a variable explaining variation in the CORT response, but no sex interaction (Table 6; see Table S9). UV hue ($Zr = -0.37$; $CI_{95} = [-0.69, -0.06]$) was moderately negatively related to the acute CORT response (Fig. 7). Finally, birds' HR response to capture did not appear to be related to beak or auricular patch ornaments. Indeed, the best and most parsimonious model only retained colony area as an important factor explaining variation in birds' acute HR response to stress ($d_{\text{unbiased}} = +0.59$; $CI_{95} = [-0.09, 1.26]$; see Table S10). Birds breeding up the valley had slightly higher HR responses to captures (132.6 ± 8.1%) than birds breeding close to the beaches (113.8 ± 11.6%).

DISCUSSION

The two main hypotheses proposed to explain the evolution of elaborate ornamentation in males and females are the 'genetic correlation' and the 'mutual selection' hypotheses (Kraaijeveld *et al.*, 2007). The former proposes that showy ornaments are functional in males, but evolve as non-functional by-products of genetic correlations between the sexes in females (Lande, 1980). Selection then operates in males and the condition-dependence of ornaments should be primarily related to the male sex. The latter proposes that ornaments are functional in both sexes, evolving as honest signals of individual quality related to sexual or other, not mutually exclusive, forms of social selection (e.g. social competitiveness for breeding sites) (Johnstone *et al.*, 1996; Kokko & Johnstone, 2002; Hooper & Miller, 2008; Tobias *et al.*, 2012). Although the genetic correlation hypothesis predicts that ornaments should convey information mostly in males, the mutual selection hypothesis predicts that ornaments should convey information in both sexes.

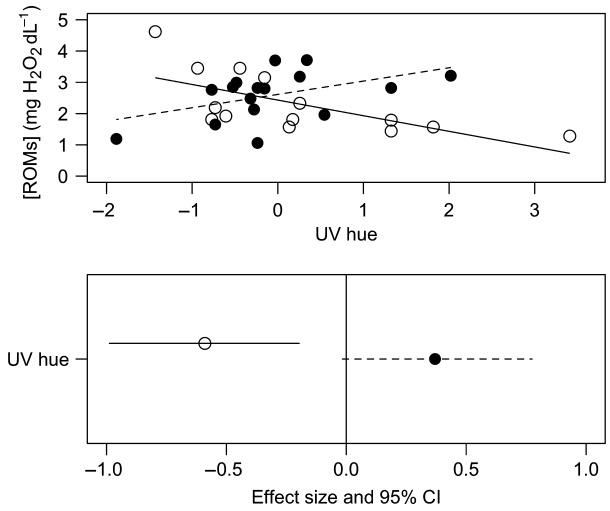

Figure 4. Relationship between beak coloration and standardized plasma concentration of reactive oxygen metabolites [ROM] in breeding king penguins. Females are depicted by open circles and a full line, males by filled circles and a dashed line. The lower panel provides effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero.

Table 3. Model estimates for the influence of beak YO chroma on plasma lysis titres in breeding king penguin (*Aptenodytes patagonicus*). The colony area effect is given in reference to area [A2]. See Fig. 5A for effect sizes with 95% CI

	Estimate	SE	*t*-value
Intercept	3.33	0.14	23.13
YO chroma	−0.15	0.09	−1.70
Colony area [A1]	−0.65	0.18	−3.55

In agreement with the mutual selection hypothesis, in king penguins we found that the showy ornaments used in mate choice were related to various aspects of physiological quality in both sexes. Successful breeding in this species involves obligate biparental care over an extended 14-month. period (Stonehouse, 1960). Adults experience high annual divorce rates (up to 81%; Olsson, 1998) and courting birds encounter prospective mates at a high rate. Such conditions provide scope for mutual choosiness (Johnstone *et al.*, 1996; Kokko & Johnstone, 2002) and are indeed expected to favour the evolution of ornamental signals reflecting individual quality in both sexes (Kraaijeveld, 2003; Kraaijeveld *et al.*, 2007). However, we also found that not all facets of physiological quality were similarly related to ornamentation in both sexes, suggesting that mutual ornamentation may be maintained by varying selective pressures in males and females (e.g. Murphy, 2007).

Mutual ornamentation and immunity

One important cost of colonial breeding is parasitism (Mangin *et al.*, 2003). The immunocompetence hypothesis predicts that, given limited resources (energy, nutrients, protein), trade-offs occur between energy allocations to immunity or to the production and maintenance of ornamentation (Saino, Bolzern & Møller, 1997; Verhulst, Dieleman & Parmentier, 1999). Consistently, we found weak to moderate negative associations between measures of innate immunity and ornamental features in both sexes. Lysis and NAb titres were negatively related to YO beak chroma and auricular patch surface respectively suggesting that investing into larger auricular patches and more YO beaks may incur a cost in terms of immunity. Interestingly, Nolan *et al.* (2006) previously documented a link between the PHA skin test and breast coloration in males, although they failed to detect an association with beak coloration or auricular patch size. Unlike the PHA test that measures a wide range of immune responses involving both innate and acquired immunity (Tella *et al.*, 2008), NAb titres reflect a well defined component of the innate immune response not induced by an experimental infection (Matson *et al.*, 2005). These findings support the notion that different ornaments may signal different components of immunity in breeding birds (Kelly *et al.*, 2012).

Mutual ornamentation and body condition

Acquiring information on body condition should be especially important to mate choice in breeding seabirds that undergo extended periods of fasting while caring for the egg or chick (Groscolas & Robin, 2001). Surprisingly, we found that body condition was related to beak spot coloration differently in males and females. Better body condition was associated with lower UV brightness and higher YO chroma (both strong effects) in females, but higher UV brightness (moderate effect) in males. These results are consistent with previous findings of lower UV brightness for females in better body condition (Dobson *et al.*, 2008), but at odds with the idea that mutual selection for high UV reflectance occurs in both sexes (Nolan *et al.*, 2010; Keddar *et al.*, 2015b). One explanation is that males and females use beak spot signals differently. As males have to endure the longest reproductive fast (Stonehouse, 1960), including courtship and the first incubation shift,

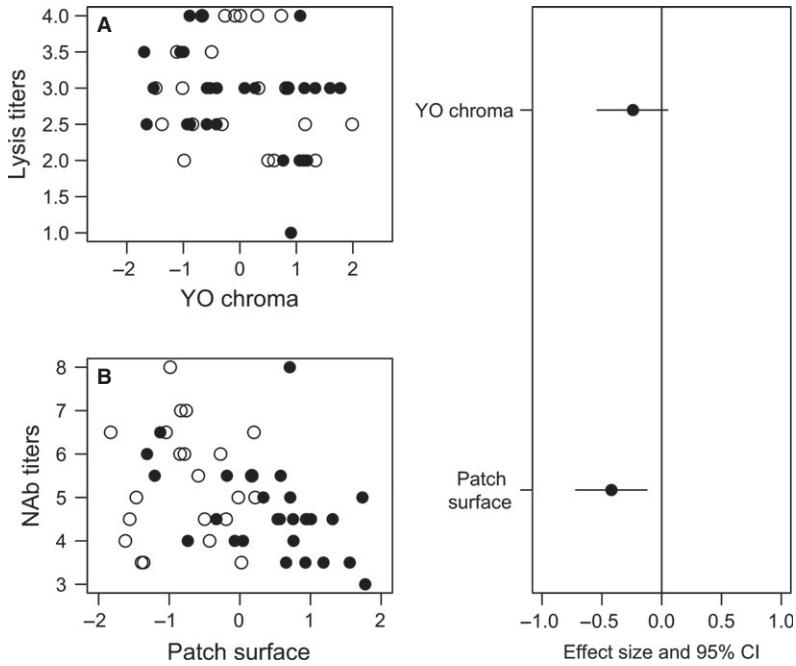

Figure 5. Relationship between beak coloration, auricular patch surface and innate immunity in breeding king penguins. Relationships are given for (A) plasma lysis titres and yellow-orange chroma, and (B) plasma NAb titres and auricular patch surface. On the left panel, males are depicted by filled circles, females by open circles. The right panel provides effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero.

Table 4. Model estimates for the influence of auricular patch surface on plasma NAb titres in breeding king penguin (*Aptenodytes patagonicus*). The colony area effect is given in reference to area [A2]. See Fig. 5B for effect sizes with 95% CI

	Estimate	SE	*t*-value
Intercept	5.46	0.24	22.32
Patch surface	−0.47	0.16	−2.94
Colony area [A1]	−0.85	0.31	−2.70

Table 5. Model estimates for the influence of UV brightness on daily resting heart rate in breeding king penguin (*Aptenodytes patagonicus*). The colony area effect is given in reference to area [A2]. See Fig. 6 for effect sizes with 95% CI

	Estimate	SE	*t*-value
Intercept	5.97	0.07	83.36
UV brightness	0.11	0.05	2.40
Colony area [A1]	−0.07	0.09	−0.72

choosing mates of high body condition should be especially important for females. In females, poor body condition to an extent could reflect greater investments into reproduction to the detriment of self-maintenance, which should be favoured by males. In females, body condition was negatively associated with increasing UV brightness but positively associated with increasing YO chroma, raising questions about the interactions between carotenoid and structural signals (Shawkey & Hill, 2005; Mougeot *et al.*, 2007; Dugas & McGraw, 2011). For instance, in red grouse (Mougeot *et al.*, 2007) and nestling house sparrows (Dugas & McGraw, 2011), carotenoid pigments appear to act as a mask, decreasing UV reflectance in soft structures. There is some suggestion that carotenoid pigments are also found in the beak of king penguins (see McGraw *et al.*, 2007), and similar interactions might explain the opposite relationships we find for beak YO chroma and UV brightness. Further, only high condition females may have been able to allocate carotenoid pigments to their beak spots to function as signals (Blount *et al.*, 2003; Mougeot *et al.*, 2010).

Mutual ornamentation and metabolic rate

We found that beak UV brightness was positively (medium effect size) associated with resting HR levels

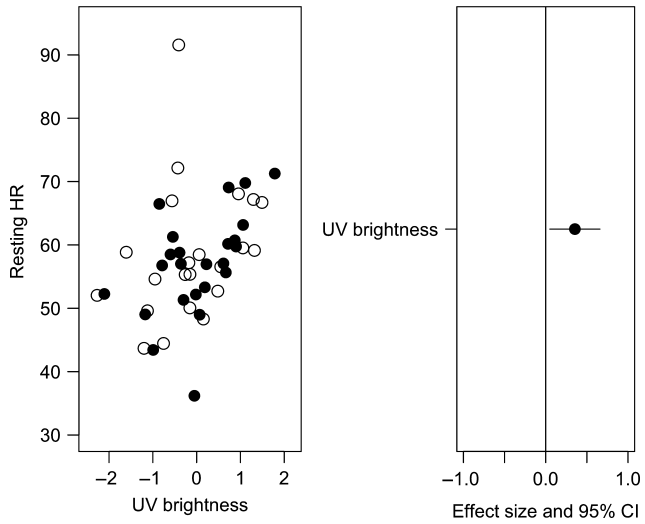

Figure 6. Relationship between beak UV brightness and daily resting HR levels (bpm) in breeding king penguins. On the left panel, males are depicted by filled circles, females by open circles. The right panel provides effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero.

Table 6. Model estimates for the influence of beak UV hue on the acute relative increase in plasma total corticosterone levels in response to a standardized 30-min capture in breeding king penguin (*Aptenodytes patagonicus*). The colony area effect is given in reference to area [A2]. See Fig. 7 for effect sizes with 95% CI

	Estimate	SE	*t*-value
Intercept	2.42	0.24	10.25
UV hue	−0.36	0.15	−2.45
Colony area [A1]	−1.16	0.31	−3.74

(a proxy for resting metabolic rate; Viblanc *et al.*, 2014) in both sexes. High resting metabolic rates may reflect increased capacities to engage in a suite of challenging activities such as foraging, caring for the young or competing for resources, and might be honestly reflected by colour ornaments (Biro & Stamps, 2010; Kelly *et al.*, 2012). The links between UV coloration and metabolic rate may lie within the energy costs of producing/maintaining structural colours (Siefferman & Hill, 2005; Doutrelant *et al.*, 2012). For example, Siefferman & Hill (2005) showed that experimentally reducing the energy cost of reproduction by reducing brood size in bluebirds (*Sialia sialis*) allowed males to increase their investment into plumage UV in the subsequent year. Rather than a long-term energy trade-off between competing functions (conserving energy for ornament production vs. expanding it for current reproduction), our results suggest possible indirect metabolic costs, such as keeping the beak clean, for UV maintenance.

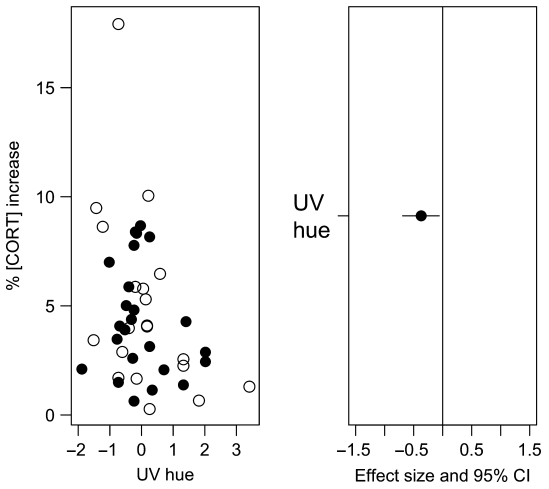

Figure 7. Relationship between the relative corticosterone increase in response to a standardized 30 minute capture and beak UV hue in breeding king penguins. On the left panels, males are depicted by filled circles, females by open circles. The right panel provides effect sizes and 95% CI calculated after Nakagawa & Cuthill, 2007. Effects are considered significant if their 95% CI does not overlap zero.

Mutual ornamentation and stress

Glucocorticoid hormones (GC) play key roles in mediating physiological trade-offs and energy allocation, and baseline GC levels have been suggested to ensure signal honesty (Husak & Moore, 2008; Weiss *et al.*, 2013). Whereas we found no link between baseline CORT and ornaments in our study, UV hue

was moderately and negatively associated with the birds' CORT response to acute stress ($Zr = -0.37$; $CI_{95} = [-0.69, -0.06]$). Birds with more UV hued beaks mounted a greater stress response to capture. Because stress responses are energy costly, this is consistent with the idea that the ability to mount stress responses while fasting is reflected in ornamentation, which may be particularly relevant in the context of colonial breeding during exposure to overt social aggressiveness (Côté, 2000). In contrast, we did not observe a link between ornaments and the acute HR response to stress, suggesting that HPA and sympathetic stress pathways may be modulated and signalled independently in breeding birds (e.g. Nephew, Kahn & Romero, 2003). We found that birds up the valley mounted slightly higher HR responses to capture, and had higher baseline CORT levels than birds breeding close to the beach. These results suggest two alternatives: that birds breeding close to the beach might have habituated to chronic human disturbance (Viblanc *et al.*, 2012b), and that birds up the valley may have been more exposed to parasites (P. Bize, Q. Schull, S. Pardonnet, Y. Handrich, F. Criscuolo, V.A. Viblanc, J.P. Robin, unpubl. data), Manipulating circulating CORT levels in breeding birds may allow further exploration of the interplay between ornamentation, glucocorticoids, and cardiovascular function. For instance, chronic experimental increases in baseline stress levels (via CORT implants) have been shown to negatively affect UV and orange-red reflectance in female striped plateau lizards (*Sceloporus virgatus*) (Weiss *et al.*, 2013).

Mutual Ornamentation and Oxidative Stress

We observed sex-related differences in UV advertising for oxidative stress. In females, lower UV hue (i.e., hue more strongly embedded in the peak UV wavelengths) was strongly and positively associated with higher pro-oxidant levels (higher ROM but not higher OXY levels), whereas the opposite occurred in males (a moderate effect and the CI overlapped zero). This result was surprising for a structural colour, as links between ornamentation and oxidative status are expected for yellow-orange colours, because of the allocation trade-off of carotenoid pigments to either anti-oxidant or ornamental functions (von Schantz *et al.*, 1999; Mougeot *et al.*, 2010). However, the interplay between UV and yellow-orange colour reflectance might also convey information on carotenoid availability (Jacot *et al.*, 2010). Carotenoids absorb wavelengths of short to medium wavelengths (400–515 nm), and greater deposition of carotenoids in feathers has been experimentally shown to cause a shift in the UV peak to shorter wavelengths in great tits (Jacot *et al.*, 2010). The precise link between carotenoid concentration and beak reflectance both in UV and YO wavelengths remains to be determined in king penguins. But our result may suggest that females depositing more carotenoids in their beak suffered from greater oxidative stress, highlighting a trade-off between pigment allocation to anti-oxidant defences or beak coloration. The exhaustive measurement of oxidative status of breeding birds requires supplementary markers of oxidative damage and anti-oxidant defence (e.g. lipid peroxidation, anti-oxidant enzymatic activity), and preferentially in different tissues (Selman *et al.*, 2012). However, our results add to the evidence that condition-dependent UV signals indeed occur in many bird species (Keyser & Hill, 2000; Bize *et al.*, 2006; Mougeot *et al.*, 2010), likely in interaction with carotenoid signalling.

CONCLUSION

Taken together our results suggest that monomorphic ornamentation reflects several aspects of physiological quality in king penguins, supporting the mutual selection hypothesis. Interestingly, the qualities signalled by mutual ornamentation may nonetheless differ (in fact be opposite) between the sexes, likely due to physiological differences and varying selection pressures. Because we collected the physiological and ornamental measures only at only one point in time, it remains to be explored if some of those traits are dynamic (e.g. beak coloration: Faivre *et al.*, 2003; Pham *et al.*, 2014) and whether birds may use them for short-term behavioural decisions. The further study of monomorphic species should shed new insights on the maintenance, information and costs of sexual signals.

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AUTHOR CONTRIBUTIONS

Designed the study: VAV, FSD and PB. Did the fieldwork: BG, MK, SP, JPR and PB. Did the lab work: VAV, AS, QS. Analysed the data: VAV, CS and PB. Wrote the paper: VAV. All authors contributed to its revision.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Standardized measures of the auricular patches of breeding king penguin (*Aptenodytes patagonicus*).

Figure S2. Correlation matrix for the ultraviolet (UV) and yellow-orange (YO) beak coloration measures (hue, brightness and chroma), and auricular patch surface, of breeding king penguins (*Aptenodytes patagonicus*).

Table S1. Summary statistics of the structural size and ornamental data of breeding king penguin (*Aptenodytes patagonicus*) used in the study.

Table S2. Model selection for the effects of beak coloration and auricular patch surface on body condition (residuals, see Methods) in breeding king penguin (*Aptenodytes patagonicus*).

Table S3. Model selection for the effects of beak coloration and auricular patch surface on plasma reactive oxygen metabolite (ROM) levels in breeding king penguin (*Aptenodytes patagonicus*).

Table S4. Model selection for the effects beak coloration and auricular patch surface on plasma anti-oxidant capacity (OXY) in breeding king penguin (*Aptenodytes patagonicus*).

Table S5. Model selection for the effects of beak coloration and auricular patch surface on plasma lysis titres in breeding king penguin (*Aptenodytes patagonicus*).

Table S6. Model selection for the effects of beak coloration and auricular patch surface on plasma NAb titres in breeding king penguin (*Aptenodytes patagonicus*).

Table S7. Model selection for the effects of beak coloration and auricular patch surface on daily resting heart rate in breeding king penguin (*Aptenodytes patagonicus*).

Table S8. Model selection for the effects of beak coloration and auricular patch surface on baseline plasma total corticosterone levels in breeding king penguin (*Aptenodytes patagonicus*).

Table S9. Model selection for the effects of beak coloration and auricular patch surface on the relative corticosterone increase in response to a standardized 30 min capture in breeding king penguin (*Aptenodytes patagonicus*).

Table S10. Model selection for the effects of beak coloration and auricular patch surface on the relative heart rate increase in response to a standardized capture in breeding king penguin (*Aptenodytes patagonicus*).

Appendix 3

How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution

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Mitochondria are the powerhouse of animal cells. They produce through oxidative phosphorylation more than 90% of the cellular energy (ATP) required for organism's growth, reproduction and maintenance. Hence, information on mitochondrial function is expected to bring important insights in animal ecology and evolution. Unfortunately, the invasiveness of the procedures required to measure mitochondrial function (e.g. sampling of liver or muscles) has limited its study in wild vertebrate populations so far. Here, we capitalize on the fact that bird red blood cells (RBCs) possess functional mitochondria to describe a minimally-invasive approach to study mitochondrial function using blood samples. In the king penguin, we present a protocol using a high-resolution respirometry system and specific agonists and antagonists enabling to assess mitochondrial function in RBCs. We evaluated the inter-assay repeatability of our measures of mitochondrial function, and tested the influence of sample storage and bird handling time on these measures. We also compared measures of mitochondrial function in RBCs and in the pectoral muscle obtained from the same individuals. Mitochondria from RBCs showed the expected responses to mitochondrial agonists and antagonists, and therefore the presented protocol allows computing effective measures of mitochondrial function. The different measures of RBCs mitochondrial function were significantly repeatable and were overall not affected by the handling time of the bird prior to blood sampling (*i.e.* stress response) or the storage time of the sample at 4°C up to 24h. Most notably, we showed that mitochondrial parameters measured in RBCs moderately correlated to those measured in the pectoral muscle. The present study sheds light on the use of RBCs in birds as a valuable and minimally-invasive source of information on mitochondrial function. This approach opens new opportunities to study mitochondrial function in free-living animals and could bring knowledge gains in ecology and evolution. Fish, amphibians and reptiles also possess mitochondria in their RBCs, and the presented approach could also be applicable to these taxa.

How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution

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Summary

1. Mitochondria are the powerhouse of animal cells. They produce through oxidative phosphorylation more than 90% of the cellular energy (ATP) required for organism's growth, reproduction and maintenance. Hence, information on mitochondrial function is expected to bring important insights in animal ecology and evolution. Unfortunately, the invasiveness of the procedures required to measure mitochondrial function (e.g. sampling of liver or muscles) has limited its study in wild vertebrate populations so far. Here, we capitalize on the fact that bird red blood cells (RBCs) possess functional mitochondria to describe a minimally-invasive approach to study mitochondrial function using blood samples.

2. In the king penguin, we present a protocol using a high-resolution respirometry system and specific agonists and antagonists enabling to assess mitochondrial function in RBCs. We evaluated the inter-assay repeatability of our measures of mitochondrial function, and tested the influence of sample storage and bird handling time on these measures. We also compared measures of mitochondrial function in RBCs and in the pectoral muscle obtained from the same individuals.

3. Mitochondria from RBCs showed the expected responses to mitochondrial agonists and antagonists, and therefore the presented protocol allows computing effective measures of mitochondrial function. The different measures of RBCs mitochondrial function were significantly repeatable and were overall not affected by the handling time of the bird prior to blood sampling (*i.e.* stress response) or the storage time of the sample at 4°C up to 24h. Most notably, we showed that mitochondrial parameters measured in RBCs moderately correlated to those measured in the pectoral muscle.

4. The present study sheds light on the use of RBCs in birds as a valuable and minimally-invasive source of information on mitochondrial function. This approach opens new

opportunities to study mitochondrial function in free-living animals and could bring knowledge gains in ecology and evolution. Fish, amphibians and reptiles also possess mitochondria in their RBCs, and the presented approach could also be applicable to these taxa.

Keywords: mitochondria, erythrocyte, non-invasive methodology, high-resolution respirometry, metabolism

Introduction

Life history theory (Roff 1992) and metabolic theory of ecology (Brown *et al.* 2004) suggest that metabolic rate – the rate at which organisms take up, transform and allocate energy to growth, reproduction and maintenance – is at the heart of adaptation and success of organisms to particular environments. In animals more than 90% of the cellular energy is produced as adenosine triphosphate (ATP) during mitochondrial respiration (Nicholls & Ferguson 2002). Hence, our understanding of the evolutionary success of particular individuals requires insights about the factors that shape mitochondrial function (defined here as the ability to use O_2 to oxidize substrate and produce ATP and heat) and the downstream effects that mitochondrial function can exert on life histories (Salin *et al.* 2012; Toews *et al.* 2013; Hill 2014; Stier *et al.* 2014; Salin *et al.* 2015; Schwartz *et al.* 2015; Bar-Yaacov *et al.* 2015; Koch *et al.* 2016; Delhaye *et al.* 2016).

The mitochondrion consists of an outer- and inner- phospholipids membranes separated by an intermembrane space, and contain mtDNA and ribosome in the mitochondrial matrix (Fig. 1). ATP is produced by the mitochondria through a process called oxidative phosphorylation (hereafter referred as OXPHOS; (Nicholls & Ferguson 2002)). The inner-membrane has a controlled permeability to protons and contains the five OXPHOS complexes responsible for the coupling of substrate oxidation to ATP production (Fig. 1). Complexes I to IV transport electrons from the substrates (NADH, succinate and FAD-linked substrates) toward molecular oxygen while pumping protons from the mitochondrial matrix into the inter-membrane space at the same time. This process builds up an electrochemical gradient across the mitochondrial inner-membrane, and the energy released by the backflow of protons to the matrix through complex V (i.e. the ATP synthase) is used for the phosphorylation of ADP into ATP. Protons can also backflow in some extent to the matrix

without passing by the complex V, leading to an energy released mostly as heat. This phenomenon is referred as the mitochondrial proton leak (Divakaruni & Brand 2011). The level of mitochondrial coupling between substrate oxidation and ATP production is not constant, and is one parameter of biological interest since it determines the amount of ATP and heat generated for a given amount of O_2/substrate consumed (Brand 2005). This mitochondrial coupling between respiration and ATP production is usually estimated by the ratio between the overall mitochondrial O_2 consumption and the residual O_2 consumption linked to proton leak, a parameter also known as the respiratory control ratio (RCR). Finally, some electrons can also escape during their transport among the different complexes (especially in complex I and III), which leads to the production of reactive oxygen species (ROS) that are implicated, at least to some extent, in the ageing process (Speakman *et al.* 2015).

Given that studying mitochondrial function could provide important insights in ecology and evolution it might look surprising that only very few ecologists and evolutionary biologists have embarked on this path (e.g. Salin *et al.* 2012; Toews *et al.* 2013; Monternier *et al.* 2014). A lack of communication and transfer of knowledge between mitochondrial biologists and ecologists/evolutionary biologists can probably explain in part this phenomenon. However, we strongly believe that methodological considerations have been a main limiting factor for the study of mitochondrial function in natural populations. Indeed, the classical approach to investigate mitochondrial biology is to obtain a tissue sample (typically from the liver or muscles) and then work with isolated mitochondria, permeabilized cells or homogenate samples (Brand & Nicholls 2011). Consequently, studying mitochondrial function usually involves terminal sampling in small animals (e.g. Toews *et al.* 2013) or laborious surgical procedures in larger animals (e.g. Monternier *et al.* 2014). Those

104 invasive procedures are nevertheless rarely compatibles with the research aims of most
105 ecologists and evolutionary biologists, eager to collect information in natural populations
106 while keeping the disturbance to their study system as low as possible and/or to perform
107 repeated measurements of the same individual over time (*i.e.* longitudinal design; Stier *et al.*
108 2015). In this context, our aim was to develop a minimally-invasive method to study
109 mitochondrial function in non-mammalian vertebrates, and in particular bird species. Blood
110 sampling is frequently performed in natural populations of birds and well accepted as a
111 minimally invasive procedure (Sheldon *et al.* 2008). RBCs are by far the most abundant cell
112 type in the blood, and interestingly RBCs of birds (as well as other non-mammalian
113 vertebrate species) possess not only a nucleus but also functional mitochondria (Stier *et al.*
114 2013; Stier *et al.* 2015). In the present study, our aim is to validate the use of RBCs to study
115 mitochondrial function in birds.

116 We describe a standard protocol that allows measuring mitochondrial function in bird
117 RBCs using a high-resolution respirometry system. We investigated the response of intact
118 RBCs to well-known mitochondrial agonists and antagonists that allow dissecting
119 mitochondrial function (Brand & Nicholls 2011). We conducted our study in a natural
120 population of king penguins (*Aptenodytes patagonica*), which is a large bird species
121 frequently used to assess mitochondrial function in the wild using pectoral muscle biopsies
122 (e.g. Rey *et al.* 2008; Monternier *et al.* 2014). We used this opportunity to measure
123 mitochondrial function in RBCs and to compare our results with findings from the skeletal
124 muscle, which is a tissue commonly used to assess mitochondrial function. To evaluate the
125 robustness of measures of mitochondrial function in RBCs, we tested the sensitivity of our
126 mitochondrial parameters to the effect of handling stress (*i.e.* the time elapsed between
127 capture and blood sampling) and of storage time (i.e. the time elapsed between blood

sampling in the field and mitochondrial analysis in the laboratory; 4h to 24h). We also report on the repeatability of our measures and on two different ways of normalizing mitochondrial respiration.

Material and Methods

STUDY SITE AND ANIMALS

This study took place in the king penguin colony of "La Grande Manchotière" (*ca.* 24,000 breeding pairs) on Possession Island in the Crozet Archipelago (46° 25'S; 51° 52'E). Adult king penguins were caught either during courtship on the beach near the research facility (N = 9 females and 9 males) or during incubation 3 days after the start of their incubation shift (N = 46 females in incubation shift 2 and 29 males in incubation shift 3).

SAMPLING PROCEDURES

Birds caught during courtship were immediately transferred to the nearby research facility (< 2min walking distance). A blood sample (c.a. 2mL) was then collected from the marginal flipper vein using a heparinised syringe and stored on crushed ice until further processing. A 200 mg muscle biopsy was taken under isoflurane-induced anesthesia from the superficial pectoralis muscle as described previously (Rey *et al.* 2008). Fifty mg of muscle were immediately immersed in ice-cold BIOPS solution (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1) until further processing.

Birds caught during incubation were blood sampled in the colony within 4 min after capture. For 23 of these birds, a second blood sample was taken after 30min of standardized

handling (see Viblanc *et al.* 2015 for details on capture and handling protocol) to test the effect of handling time (i.e. stress response) on mitochondrial measurements.

All blood samples were kept on crushed ice (< 2 hours) prior to centrifugation at 3000*g* for 10min to separate plasma from RBCs. Plasma fraction was then removed and 100µL of RBCs was transferred into a new tube containing 1mL of ice-cold phosphate buffer saline (PBS). After gentle homogenisation, RBCs were washed a first time by centrifuging the samples at 600*g* for 5 min to pellet the cells and discarding the supernatant. RBCs were then re-suspended in 1mL of ice-cold PBS and stored at 4°C until being used for mitochondria measurements.

MITOCHONDRIAL MEASUREMENTS IN INTACT RBCs

We choose to work with intact RBCs, since our preliminary observations revealed some difficulties to permeabilize properly avian RBCs. Immediately before the start of the mitochondrial measurements, samples were washed a second time as described above and re-suspended in 1mL of respiratory buffer MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L), pH 7.1). We then added 1mL of RBC suspension to 1mL of MiR05 buffer already equilibrated at 38°C in the respirometry chamber of one Oxygraph-2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). This system allows measuring small changes in O_2 concentration in a closed chamber, and thereby provides a good opportunity to measure mitochondrial respiration using minimum amount of biological samples.

We applied a protocol involving serial additions of various mitochondrial agonists/antagonists to our RBC suspension in order to get a comprehensive assessment of

mitochondrial function, as illustrated in Fig. 2A, and in the corresponding stepwise description below:

1. Baseline O_2 consumption is recorded after approximately 5-10min of stabilization following the addition of the sample to the chamber.
2. ATP-dependent O_2 consumption is inhibited by adding oligomycin (1 $\mu g.mL^{-1}$), an inhibitor of ATP synthase. The residual oxygen consumption at this stage is mostly linked to mitochondrial proton leak.
3. Maximal uncoupled O_2 consumption is then obtained by the addition of the mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone) at a final concentration of 1µM. At this concentration, FCCP abolish the proton gradient, thereby forcing the OXPHOS system to work at its maximum capacity to compensate for proton leakage. The maximal uncoupled respiration is limited by the capacity of the electron transport chain to oxidize the available substrate.
4. Mitochondrial O_2 consumption is then abolished by adding antimycin A (5 µM), an inhibitor of mitochondrial complex III. The residual oxygen consumption after antimycin A inhibition reflects non-mitochondrial oxygen consumption.
5. The maximal capacity of the electron transport chain to reduce O_2 is then evaluated by the addition of 2.5 mM of ascorbate and 0.5mM TMPD (N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride). Those chemicals provide electrons directly to complex IV (cytochrome c oxidase: COX) that uses them to reduce O_2 to H_2O. This measure estimates the maximal mitochondrial O_2 consumption without being limited by substrate availability/oxidation. Due to a quick and massive release of O_2 during this last step (probably resulting from changes in haemoglobin-O_2 affinity within

RBCs), we opened transiently the chamber during 5 min before measuring O_2 consumption (see Fig. 2A).

We determined mitochondrial O_2 consumption by subtracting residual non-mitochondrial O_2 consumption (measured after antimycin A inhibition) from O_2 consumption measured in response to the other conditions. We computed five measures of mitochondrial respirations and two different respiratory control ratios (RCR) to evaluate the degree of mitochondrial coupling between O_2 consumption and ATP synthesis (Brand & Nicholls 2011). The seven measures of mitochondrial function derived from our protocol are described in Table 1.

NORMALIZATION OF MITOCHONDRIAL RESPIRATION

Pipetting an exact volume of RBCs (*i.e.* 100µL) might be challenging considering the viscosity of the cell pellet after centrifugation. Consequently, the volume of cells might not be as accurate as desired and biased our estimates of mitochondrial parameters. Hence, we tested two methods of *post-measurement* normalization using 18 samples collected in courtship birds. We either weighted the amount of RBCs pipetted before the start of the analyses using a high-precision electronic balance (± 0.1mg, Sartorius AC211S®), or we quantified the total protein content in the remaining RBCs samples at the end of the analyses using the Pierce BCA protein assay (ThermoScientific). We calculated standardised respiration rates by dividing respiration rates either by the fresh mass of RBCs or by their protein content.

REPEATABILITY OF RBC MITOCHONDRIAL MEASUREMENTS

We evaluated the repeatability of our mitochondrial measurements by assaying 14 samples in duplicate, coming from both courtship and incubating birds. We evaluated the repeatability both on raw data and on data normalized by the fresh mass of RBCs.

EFFECTS OF STORAGE TIME

To evaluate the effect of storage time on mitochondrial measurements, we used two approaches. First, we measured 8 samples twice, a first time after 4h of storage at 4°C and a second time after 24h of storage. Second, we used single measurements collected from the 75 incubating birds, in which the time elapsed between blood sampling and mitochondrial measurements varied between 2 and 10 hours.

MITOCHONDRIAL MEASUREMENTS IN SKELETAL MUSCLE

Mitochondrial respiratory function of pectoral muscle was determined in permeabilized muscle fibers using a method described previously by Pesta & Gnaiger (2011). Structurally sound fiber bundles were selected from biopsies maintained in ice-cold BIOPS, and mechanically separated, removing any visible adipose and connective tissue. Fiber bundles were transferred in BIOPS solution containing saponin (50µg/ml) for permeabilization and mixed gently at 4°C for 30 min. Then, permeabilized fibers were washed 10 min at 4°C in the Mir05 buffer. Permeabilized fibers were carefully blotted on Whatman filter paper for 2-3s, weighed and placed in the Oxygraph chamber containing 2mL of MiR05 at 38°C. Respiration was fuelled using either pyruvate/malate (5/2.5mM) or succinate (5mM) as respiratory substrates. Phosphorylating state of respiration (*i.e.* state III) was determined in the presence of ADP (1 mM). Then, cytochrome-c (10 µM) was added in order to check the integrity of mitochondria within permeabilized fibers by the absence of

stimulation of respiration. Mitochondrial preparations exhibiting an increase in O_2 uptake greater than 15% in response to cytochrome-c were excluded from subsequent analysis (Kuznetsov *et al.* 2008). Basal non-phosphorylating respiration (*i.e.* state IV) was obtained by addition of oligomycin (1 µg/ml). Thereafter, the maximal uncoupled respiration rate was induced by sequential addition of 1µM of FCCP. Finally, antimycin A (20 µM) was added to allow the measurement of non-mitochondrial oxygen consumption rate. To determine mitochondrial O_2 consumption, we subtracted residual O_2 consumption measured after antimycin A inhibition, from O_2 consumption measured in response to the other conditions. Mitochondrial respiration rates of permeabilized fibers were expressed as pmol $O_2.s^{-1}.mg^{-1}$ wet weight.

STATISTICS

We used Generalized Estimating Equations (GEE) with *bird identity* as individual factor and *state* as the repeated effect to evaluate 1) differences in O_2 consumption in response to the different experimental conditions (i.e. baseline, oligomycin, FCCP, antimycin A, ascorbate, and TMPD) and 2) differences between mitochondrial parameters (i.e. endogenous, ATP-dependent, leak, maximal uncoupled and maximal COX respirations). Paired-comparisons involving two groups (*i.e.* RCRs, effects of storage time, effects of sampling time) were performed using non-parametric exact Wilcoxon paired-tests considering the relatively small sample sizes ($N \leq 23$). To evaluate the relevance of normalizing RBC mitochondrial respiration, we ran multivariate analyses (MANOVAs) with either fresh RBC mass or total protein content as explanatory factors of mitochondrial O_2 consumption rates. To evaluate the repeatability of mitochondrial parameters, we calculated the intraclass coefficients of correlations (ICC), but also the coefficients of variation (CV)

271 expressed in % for the 14 samples assessed in duplicates. Correlation tests were performed
272 using either non-parametric Spearman correlations (for $N \leq 18$) or parametric Pearson
273 correlations (for $N \geq 40$). Means are always quoted ± SE and p-values ≤ 0.05 were considered
274 as significant.
275
276 ETHICAL STATEMENT
277 The present study used samples collected as part of two on-going scientific programs
278 of the French Polar Institute (IPEV 119 ECONERGY and IPEV 131 PHYSIONERGY). All
279 experiments were approved by an independent ethics committee (Comité d'éthique Midi-
280 Pyrénées pour l'expérimentation animale) commissioned by the French Polar Institute, and
281 comply with the current laws of France. Authorizations to enter the breeding colony and
282 handle the birds were provided by the "Terres Australes and Antarctiques Françaises"
283 (permit n°2013-72 issued on 29 October 2013).
284
285 ## Results
286 OXYGEN CONSUMPTION IN RESPONSE TO MITOCHONDRIAL AGONISTS AND ANTAGONISTS
287 We found a significant effect of mitochondrial agonists/antagonists on O_2
288 consumption (Fig 2A & 2B; GEE model: $\chi^2 = 1524.8$, $p < 0.001$). Post-hoc comparisons
289 revealed that inhibition of ATP synthase significantly decreased O_2 consumption by 62.7% (p
290 < 0.001), while mitochondrial uncoupling with FCCP increased significantly O_2 consumption
291 by 17.9% compared to baseline ($p = 0.012$). Inhibition of mitochondrial respiration with
292 antimycin A decreased O_2 consumption by 86.2% compared to baseline ($p < 0.001$), while
293 maximal stimulation of mitochondrial respiration with ascorbate + TMPD resulted in a
294 significant increase of 521.9 % ($p < 0.001$).

Seven measures of mitochondrial function (see Table 1) were computed from the changes in mitochondrial respiration rates in response to mitochondrial agonists/antagonists (Fig 2C).

NORMALIZATION OF RBC MITOCHONDRIAL RESPIRATION RATES

Multivariate analyses revealed that mitochondrial respiration rates were significantly influenced by the fresh mass of cells used (MANOVA: $F_{4,13} = 7.7$, $p = 0.002$, effect size (partial η^2) = 0.70), or in a slightly lesser extent by the total protein content of RBC samples (MANOVA: $F_{4,13} = 5.7$, $p = 0.007$, effect size (partial η^2) = 0.64).

REPEATABILITY OF RBC MITOCHONDRIAL MEASUREMENTS

Mitochondrial parameters were overall significantly repeatable as shown in Table 2. Maximal COX respiration and $RCR_{uncoupled/leak}$ were the less repeatable parameters, with an ICC inferior to 0.80. Normalizing mitochondrial parameters by the fresh mass of RBCs increased the repeatability of mitochondrial measurements in almost all cases.

EFFECT OF STORAGE TIME ON RBC MITOCHONDRIAL PARAMETERS

The comparison of mitochondrial parameters measured 4h after sampling or 24h after sampling did not reveal any significant differences (Wilcoxon paired exact tests, all $p > 0.10$, Fig. 3). In addition, we found no significant correlations between storage time and mitochondrial parameters using the 75 samples collected from incubating birds (Pearson correlations, all $p > 0.38$), except for a slight but significant negative correlation with $RCR_{uncoupled/leak}$ ($r = -0.23$, $p = 0.045$).

EFFECT OF HANDLING TIME ON RBC MITOCHONDRIAL PARAMETERS

We found overall no significant impact of handling time on mitochondrial parameters (Wilcoxon paired exact tests, all $p > 0.21$, Fig 4).

RELATIONSHIPS BETWEEN MITOCHONDRIAL PARAMETERS IN RBCs AND SKELETAL MUSCLE

Muscle state III vs. RBC endogenous and ATP-dependent respiration rates

We found significant correlations between muscle state III respiration rate fuelled by pyruvate-malate and RBC endogenous (Fig 5A; Spearman correlation: $\rho = 0.71$, $p = 0.007$), and ATP-dependent (Fig 5C; Spearman correlation: $\rho = 0.71$, $p = 0.006$) respiration rates. While muscle state III fuelled with succinate was not significantly correlated to RBC endogenous respiration (Fig 5B; Spearman correlation: $\rho = 0.25$, $p = 0.39$), such relationship was significant when using RBC ATP-dependent respiration (Fig 5D; Spearman correlation: $\rho = 0.53$, $p = 0.049$).

Muscle state IV vs. RBC leak respiration rate

RBC leak respiration was not significantly correlated to muscle state IV respiration fuelled either with pyruvate-malate (Spearman correlation: $\rho = -0.13$, $p = 0.67$) or succinate as substrates (Spearman correlation: $\rho = 0.19$, $p = 0.52$).

Muscle vs. RBC maximal uncoupled respiration rates

Maximal uncoupled respiration rates were significantly correlated between RBC and muscle tissues when succinate was used a substrate (Fig 6B; Spearman correlation: $\rho = 0.73$, $p = 0.003$) but not when pyruvate-malate was used as a substrate (Fig 6A; Spearman correlation: $\rho = 0.07$, $p = 0.82$).

Discussion

In this study, we present and validate the use of a novel approach where intact RBCs are used to measure mitochondrial function in a minimally invasive manner. We illustrate this approach in birds, but such methodology could likely be adapted to other non-mammalian vertebrates (i.e. fish, amphibians and reptiles) since they also have functional mitochondria in their RBCs (Stier *et al.* 2015). Despite mammalian RBCs are lacking mitochondria, blood sampling might nonetheless also be used in this taxon to measure mitochondrial function, though using other blood cell types (e.g. platelets or white blood cells), as recently demonstrated in humans (Sjövall *et al.* 2013; Pecina *et al.* 2014). However, larger blood volume and isolation of specific blood cells will be required in mammals to have access to sufficient amount of mitochondria. The use of blood samples to assess mitochondrial function should open new opportunities to study mitochondrial function in free-living vertebrates and address fundamental roles of mitochondria in ecology and evolution (Dowling *et al.* 2008; Ballard & Pichaud 2013; Hill 2014; 2015; Salin *et al.* 2015; Koch *et al.* 2016).

CHARACTERIZING MITOCHONDRIAL FUNCTION IN INTACT RBCs

Mitochondria from intact RBCs of king penguins exhibited the expected responses to classical mitochondrial agonists and antagonists. It confirms previous finding in zebra finches about the presence of functional mitochondria in bird RBCs (Stier *et al.* 2013). We show how mitochondrial drugs can be used to extract 7 parameters of interests reflecting various aspects of mitochondrial function (Table 1). Endogenous respiration reflects the natural activity of mitochondria under the current physiological and cellular state (*i.e.* substrate and ADP availability, ATP turnover, proton leak) and is an intermediary state between the

367 classical state III (unlimited availability of substrate and ADP) and state IV (unlimited
368 availability of substrate but zero ATP synthesis) classically measured in isolated mitochondria
369 or permeabilized tissues/cells (Brand & Nicholls 2011). We decomposed RBCs' endogenous
370 mitochondrial respiration in two components, the ATP-dependent respiration and the leak
371 respiration. The first one reflects the ability of mitochondria to produce ATP via OXPHOS and
372 the second one reflects the proton leakiness of the mitochondria. Both components have
373 importantly different biological implications. ATP is important to fulfil most cellular activities,
374 and therefore variation in ATP-dependent respiration may account for variation in cell
375 replication and growth, and by extension for variation in organismal growth, maturation and
376 reproduction. Alternatively, proton leak is known to be important to produce heat and/or
377 regulate ROS production (Brand 2000) and thus variation in proton leak may account for
378 variation in cell (and organismal) maintenance and lifespan. The values we observe here for
379 RBCs (72.7% of mitochondrial respiration linked to ATP synthesis *vs.* 27.3% linked to proton
380 leak) are close to those found in various cell types of mammals and birds, since in those cells
381 approximately 60-80% of the mitochondrial respiration is used to synthesize ATP, and 20-
382 40% is linked to the proton leak (Porter & Brand 1995; Else *et al.* 2004; Jimenez *et al.* 2014).
383 Maximal uncoupled respiration rate represents the maximal mitochondrial activity when
384 ATP turnover is not a limiting factor, but while substrate availability and oxidation might be.
385 Comparing maximal uncoupled respiration to endogenous respiration can help to identify
386 the parameters limiting endogenous respiration, namely ATP turnover or substrate
387 oxidation. Indeed, if ATP turnover is the limiting factor, maximal uncoupled respiration will
388 be markedly higher than endogenous respiration, while the difference between these two
389 parameters will be minimal if substrate oxidation is the limiting factor. However, some
390 caution is needed when interpreting this parameter since the use of uncoupling agents in

intact cells has deleterious side effects, such as an intra-cellular acidification (see Brand & Nicholls 2011 for details). Finally, the maximal COX respiration indicates the capacity of the complex IV (COX) to use O_2 as a final electron acceptor, which is used to assess the maximal oxidative capacity of the electron transport chain, but might also been used as a proxy of the mitochondrial content/density (Larsen *et al.* 2012).

Cell respiratory control ratio (RCR) is believed to be the single most useful general test of mitochondrial functioning using intact cells (Brand & Nicholls 2011). Moreover, since RCRs are ratios, they are internally normalized, which facilitate their interpretation (see below for a discussion on normalization). RCRs provide indirect information on the coupling efficiency of the mitochondria between O_2 consumption and ATP production, either under the current physiological state ($RCR_{endogenous/leak}$) or under stimulated conditions ($RCR_{uncoupled/leak}$). RCR values are usually characteristic of a specific method in a specific tissue and in a specific group of organisms. Consequently, we do not have currently standard RCR values in RBCs to compare to our data. Still, $RCR_{endogenous/leak}$ in king penguin RBCs are close to those found in cultured myoblasts/fibroblasts of Japanese quails (≈ 3-4; Jimenez *et al.* 2014).

ROBUSTNESS OF RBC MITOCHONDRIAL MEASURES

Our results indicate that our different measures of mitochondrial function were significantly repeatable, that RBCs could be stored at 4°C up to 24h without effects on measures of mitochondrial function (at the exception of $RCR_{uncoupled/leak}$), and that handling time did not alter measures of mitochondrial function. Those findings are in agreement with a recent study in human platelets pointing out that mitochondrial function in those blood cells is relatively well conserved at 4°C up to 48h after blood collection (Sjövall *et al.* 2013).

The possibility to conserve samples at 4°C for several hours before measurement would undoubtedly be a real asset for field studies since field sites and laboratory facilities are often not located at the same place. The absence of effect of handling time on mitochondrial parameters is another asset for field studies, since capturing and blood sampling wild animals under controlled time condition can be very laborious. It is nonetheless important that future studies test for repeatability and investigates the importance of storage time and handling time on measures of mitochondrial parameters before generalities on the robustness of RBC measures can be drawn.

CORRELATIONS BETWEEN MITOCHONDRIAL PARAMETERS IN RBCS AND PECTORAL MUSCLES

Although demonstrating the presence of functional mitochondria in RBCs is an important first step, the next logical question is whether measures of mitochondrial function in RBCs do reflect what is happening in other tissues of the same individual, and as such can provide general information at the scale of the organism. Here, we showed that mitochondrial parameters measured in RBCs moderately correlated to those measured in the pectoral muscle (i.e. ATP-dependent respiration and uncoupled respiration fuelled with succinate). Interestingly, similar findings have been found in humans between blood mononuclear cells and organs such as kidney and heart (Karamercan *et al.* 2013). Altogether, it indicates that mitochondrial parameters are to some extent correlated among tissues, including blood. Consequently, RBCs can be used as a valuable source of information on mitochondrial function. This opens new opportunities to ecologists and evolutionary biologists eager to investigate links between mitochondrial function and organismal performance using minimally invasive sampling techniques (i.e. blood sampling). Having access to such minimally-invasive methodology is a pre-requisite when it comes to make

links with fitness traits such as reproductive success and survival, but also when working with protected species.

FURTHER IMPROVEMENTS IN CHARACTERIZING MITOCHONDRIAL FUNCTION USING RBCS

We see at least three methodological improvements to be addressed in future studies. First, while working with intact cells has several advantages (e.g. working in an undisturbed cellular environment and lack of artefacts due to mitochondrial preparation; Brand & Nicholls 2011), it may be beneficial for some studies to better control the environment in which mitochondrial function is measured (i.e. substrate and ADP availability). This could be achieved either by isolating mitochondria or by being able to permeabilize properly RBCs and artificially providing substrates and ADP (Brand & Nicholls 2011). Such methodological development will undoubtedly broaden the scope of questions that could be answered using mitochondria coming from non-mammalian RBCs.

Second, normalizing mitochondrial respiration is not an easy task, and has several implications for data interpretation (Brand & Nicholls 2011). We have shown that normalizing measurement by the fresh mass of cells used or by their protein content improve the repeatability of the measurement. However, it would also be possible to normalize mitochondrial respiration by the number of cells (e.g. Sjövall *et al.* 2013) or by the mitochondrial content of these cells (e.g. Salin *et al.* 2016).

Finally, as stated in the introduction, mitochondrial function is not only reflected in terms of O_2 consumed and ATP produced, but also in terms of ROS produced. Assessing ROS production is challenging, but has already being done using fluorescent probes in non-mammalian vertebrate RBCs (e.g. Olsson *et al.* 2008; Stier *et al.* 2014; Delhaye *et al.* 2016). Interestingly, it is now possible to simultaneously record O_2 consumption and fluorescence

463 signal using the O2k-fluorescence module (Oroboros Instruments, Innsbruck, Austria) that
464 could be added to the O2k-Oroboros device that we used in this study. Other fluorescent
465 probes may also help to collect additional information on mitochondrial function, such as
466 mitochondrial membrane potential, ATP synthesis, or calcium flux, and in turn help to
467 broaden the scope of questions that can be addressed in ecology and evolution.
468
469 PERSPECTIVES IN ECOLOGY & EVOLUTION
470 The applications of our methodology in ecology and evolution are likely to be broad
471 in terms of scientific questions that could be addressed. Indeed, subtle variations at the
472 cellular level in mitochondrial function are likely to have profound consequences at the
473 organismal level (Salin *et al.* 2015), and we believe that the links between mitochondrial
474 function and organismal phenotype deserves now more attention than ever. Hereafter, we
475 highlight four promising avenues where measures of mitochondrial function in RBCs could
476 help to gain knowledge.
477 First of all, whole organism metabolic rate, which is the result of oxygen consumption
478 by mitochondria at the cellular level, has been a trait under great scrutiny in ecology and
479 evolution in the last decades (Brown *et al.* 2004). However, metabolic rate is in the vast
480 majority of cases measured in terms of O_2 consumption, while the true energetic currency is
481 ATP, and that the relationships between O_2 consumption and ATP production are not
482 constant (Brand 2005; Salin *et al.* 2015). Since the fractions of O_2 consumption used for ATP
483 synthesis and mitochondrial proton leak have very different biological implications, gaining
484 insight about mitochondrial function at the cellular level should further improve our
485 understanding of metabolic rate acting as a factor driving ecological and evolutionary
486 processes.

Secondly, mitochondrial function requires a close collaboration between the nuclear and the mitochondrial genomes (*i.e.* named mito-nuclear interactions) since more than 90% of the proteins required for mitochondrial function are encoded in the nucleus and imported into the mitochondria (Wolff *et al.* 2014). While mitochondrial genome was thought to be an evolutionary bystander for a long time, we have now evidence arguing for the existence of evolutionary adaptations at the mtDNA level (e.g. Pavlova *et al.* 2013; Ballard & Pichaud 2013). Such phenomenon might also give rise to mito-nuclear incompatibilities between individuals/populations (i.e. decreased fitness of hybrids), and such incompatibilities are believed to be one potential driver of reproductive isolation and speciation (Bar-Yaacov *et al.* 2015). Characterizing mitochondrial function of mtDNA variants appears essential to evaluate their adaptive value, and characterizing mitochondrial function of potential mito-nuclear hybrids appears essential to shed light on the mechanisms underlying mito-nuclear incompatibilities. However, characterizing mitochondrial function in these two contexts has rarely been done to date (but see Toews *et al.* 2013).

Thirdly, mitochondria are only inherited from the mother, even if inheritance patterns could be slightly more complex in some cases (White *et al.* 2008). This gives rise to evolutionary constraints for males, since mitochondrial mutations benefitting females could spread even if they harm males, a phenomenon known as the “mother’s curse” (Gemmell *et al.* 2004). In vertebrate species, we often lack information about differences in mitochondrial function arising form such constraints, and even more surprisingly, we have no information to date about the inheritance and heritability patterns of mitochondrial function *per se*.

509 Finally, mitochondrial function undoubtedly contributes to determine animal
510 performance and fitness, probably in an environment-dependent manner (Stier *et al.* 2014;
511 Salin *et al.* 2015; Conley 2016). Indeed, mitochondrial function will condition the amount of
512 nutrients and O_2 used, as well as the amount of ATP and ROS produced. Decreasing
513 mitochondrial efficiency to produce ATP might seem counter-productive at a first glance.
514 However, such mitochondrial "uncoupling" between O_2 and ATP production could be useful
515 for endotherms to produce heat such as in the brown fat of mammals (Cannon &
516 Nedergaard 2004), but also to slow-down ageing by reducing ROS production (Brand 2000).
517 In contrast, increasing mitochondrial efficiency might be beneficial when resources are
518 limited or to optimize physical performances (Monternier *et al.* 2014; Conley 2016), while it
519 might incur some costs in terms of ROS production.

520

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527

528 **Author's contribution**

529 A.S. designed the study, did the fieldwork, conducted laboratory analyses on RBCs, analyzed
530 the data and wrote the paper. P.B. provided guidance on data analysis, and wrote the paper.
531 D.R. provided guidance on the experiments and helped to draft the manuscript. Q.S., E.L.
532 and JP.R. contributed to the realization of the project, the collection of samples in the field,

533 and commented on the manuscript. C.R. provided invaluable technical advice on
534 mitochondrial measurements, and conducted laboratory analyses on muscle samples.
535
536 **Data accessibility**
537 Data will be loaded on Dryad after the manuscript has been accepted.
538
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652

Table 1. Calculation and meaning of mitochondrial parameters measured in intact RBCs

Parameter	Calculation	Information
Endogenous respiration	Baseline O_2 consumption subtracted from non-mitochondrial O_2 consumption	Mitochondrial O_2 consumption under cellular physiological conditions
ATP-dependent respiration	Decrease in O_2 consumption due to ATP synthase inhibition by oligomycin.	Fraction of O_2 used for ATP synthesis: ability of mitochondria to produce ATP via OXPHOS
Leak respiration	O_2 consumption insensitive to ATP synthase inhibition subtracted from non-mitochondrial O_2 consumption	Fraction of O_2 consumed by mitochondrial proton leak: proton leakiness of the mitochondria
Maximal uncoupled respiration	O_2 consumption in response to the uncoupler FCCP subtracted from non-mitochondrial O_2 consumption	Maximal respiration under the current cellular state (i.e. availability/oxidation of substrates)
Maximal COX respiration	O_2 consumption in response to Ascorbate+TMPD subtracted from residual O_2 consumption obtained in the presence of ascorbate only	Maximal potential of the Electron Transport Chain to use O_2 as a final acceptor of e-
$RCR_{endogenous/leak}$	Ratio between Endogenous and Leak respiration measurements	Coupling efficiency of the mitochondria between O_2 consumption and ATP production under endogenous conditions
$RCR_{uncoupled/leak}$	Ratio between Maximal uncoupled and Leak respiration measurements	Potential coupling efficiency of the mitochondria between O_2 consumption and ATP production under stimulated conditions

Table 2. Repeatability of measures of mitochondrial function based on 14 samples ran in duplicates. Two indicators of repeatability are shown: the intra-class coefficients of correlations (ICC) and the coefficient of variation (CV). P-values associated with ICC are given between brackets. Repeatability estimates are reported both for uncorrected parameters and for parameters corrected by the fresh mass of RBCs, except for RCRs since they are ratios.

Parameter	*Raw value ICC*	CV ± SE (%)	Mass corrected ICC	CV ± SE (%)
Endogenous	***0.884*** (< 0.001)	6.6 ± 1.1	***0.934*** (< 0.001)	6.2 ±1.0
ATP-dependent	***0.915*** (< 0.001)	6.3 ±1.1	***0.947*** (< 0.001)	6.3 ± 1.2
Leak	***0.873*** (< 0.001)	10.4 ± 1.9	***0.912*** (< 0.001)	9.3 ± 1.5
Max. Uncoupled	***0.876*** (< 0.001)	9.2 ± 2.1	***0.930*** (< 0.001)	7.4 ± 1.3
Max. COX	***0.777*** (0.009)	6.6 ±1.5	***0.761*** (0.012)	8.8 ± 1.3
RCR1 (Endo./Leak)	***0.891*** (< 0.001)	5.8 ± 1.2	*NA*	*NA*
RCR2 (Unc./Leak)	***0.785*** (0.004)	7.6 ± 1.6	*NA*	*NA*

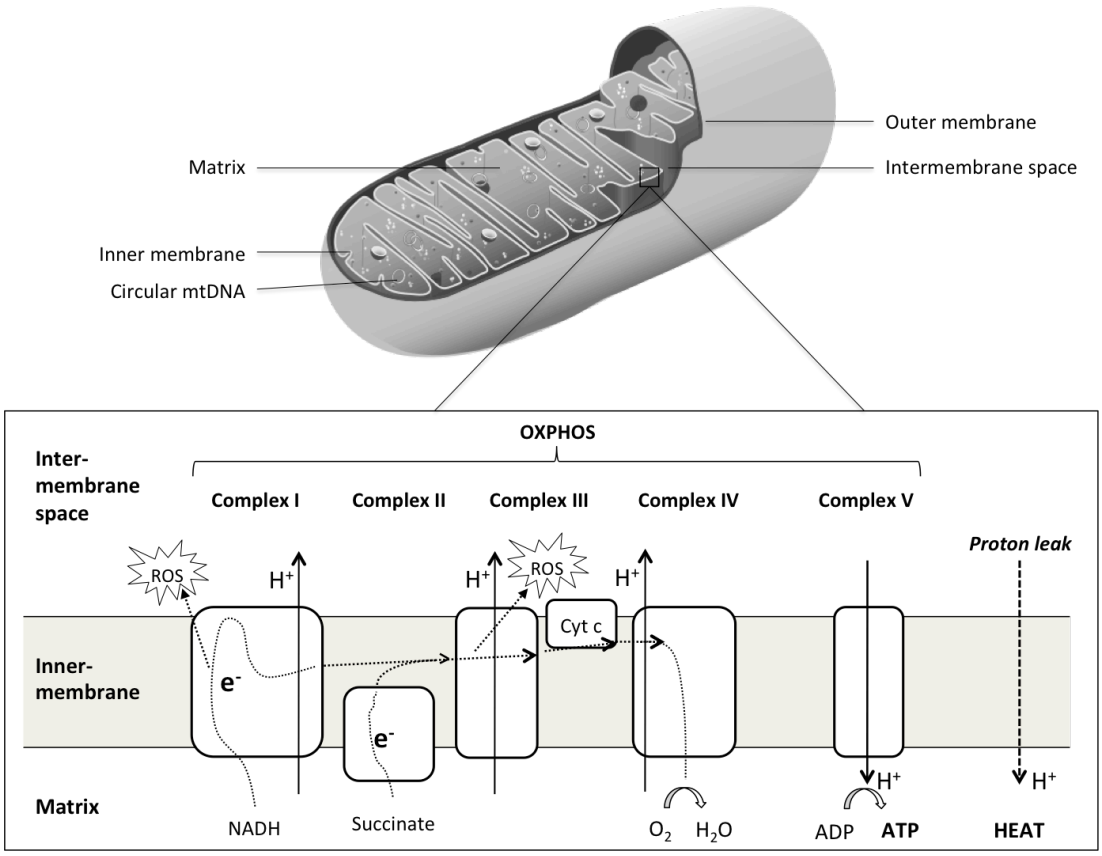

Fig. 1. The mitochondrion and OXPHOS system.

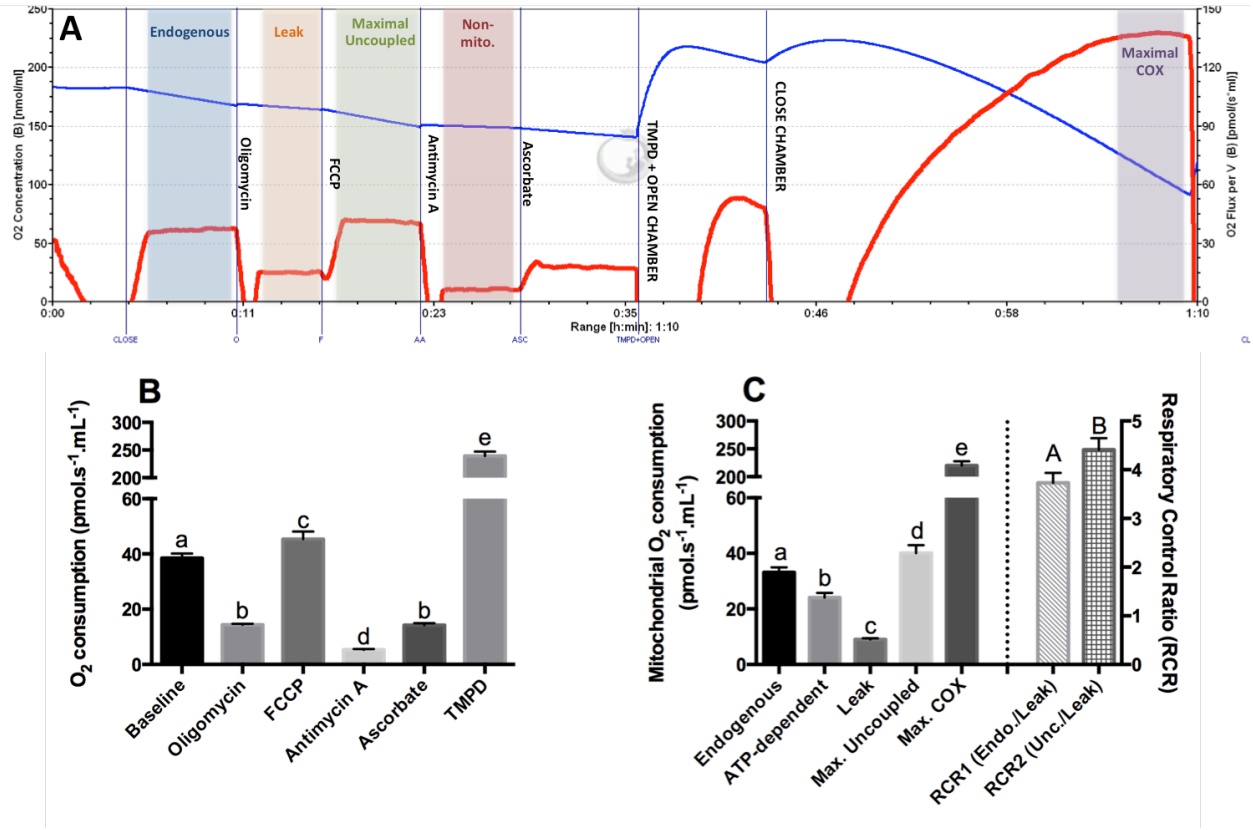

Fig. 2. Bioenergetics assessment of intact red blood cells: (A) Typical mitochondrial measurement run (O_2 concentration: blue line and O_2 consumption: red line), (B) Average response to the mitochondrial agonists/antagonists in terms of O_2 consumption, (C) Mitochondrial parameters of interest. Means are quoted ± SE and different letters indicate significant differences according to GEE models and associated post-hoc tests, or an exact Wilcoxon paired test in the case of RCRs (N = 18).

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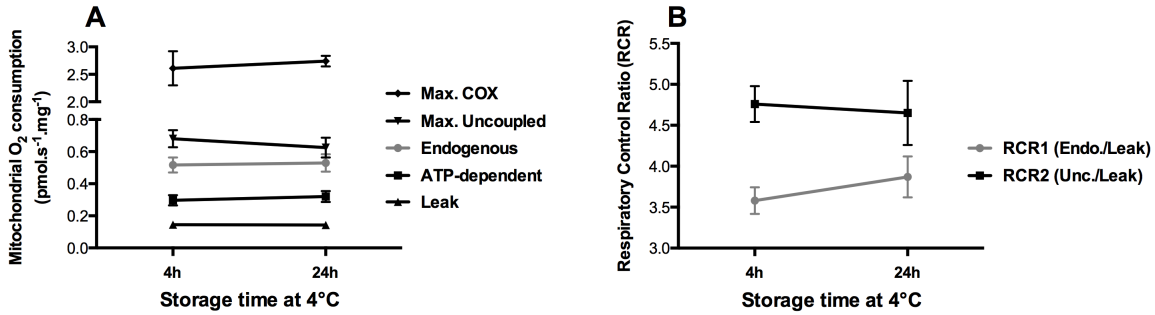

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677

678 **Fig 3.** Mitochondrial parameters of the same samples measured 4 or 24h after collection: (A)

679 mitochondrial respiration rates, and (B) mitochondrial respiratory control ratios. Means are

680 quoted ± SE (N = 8).

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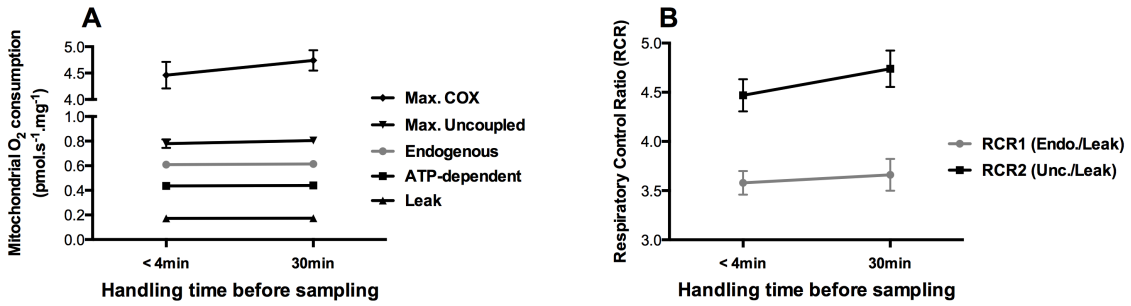

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684

685 **Fig. 4:** Mitochondrial parameters of the same individuals sampled once before 4 minutes of

686 handling, and once after 30 minutes of standardized handling: mitochondrial respiration

687 rates (A) and mitochondrial respiratory control ratios (B). Means are quoted ± SE (N = 23).

688

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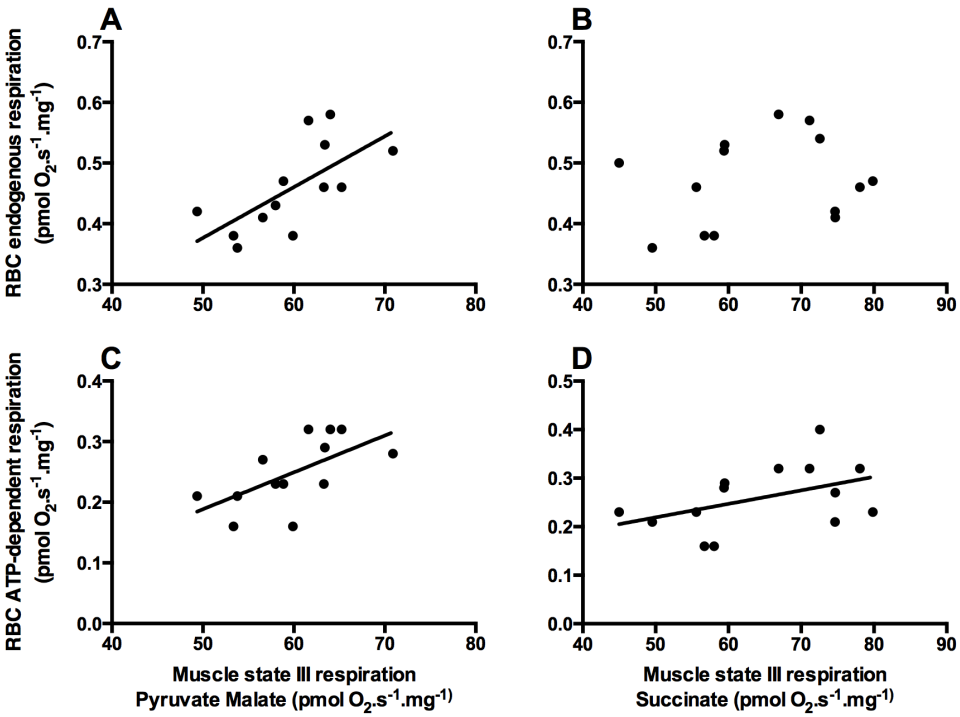

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691 **Fig 5.** Relationships between muscle state III respiration fuelled with pyruvate-malate (A,C)

692 or succinate (B,D) and RBC endogenous (A,B) and ATP-dependent (B,D) respiration rates (N =

693 13 for pyruvate-malate and N = 14 for succinate).

694

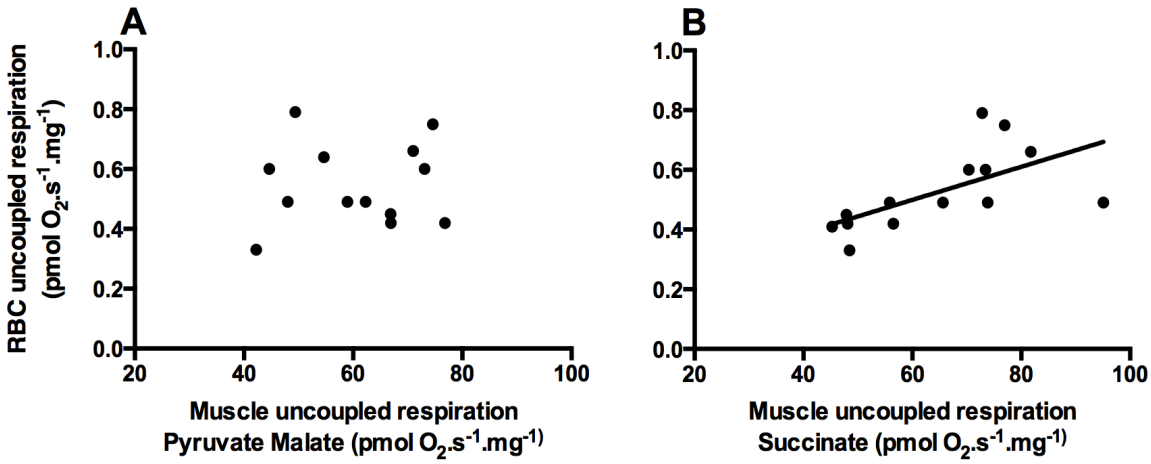

Fig. 6. Relationships between muscle uncoupled respiration fuelled with pyruvate-malate (A) or succinate (B) and RBC uncoupled respiration rate (N = 13 for pyruvate-malate and N = 14 for succinate).

Appendix 4

King penguins' telomere length: assortative mating and relationship with breeding success

Manuscript submitted to Naturwissenschaften

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[#]Shared seniorship

Telomeres are non-coding genetic repeats protecting the ends of linear chromosomes. Because long telomeres are often associated with higher survival, inter-individual variation in telomere length has been proposed as a proxy of fitness and, by extension, of individual quality. Sexual selection often leads to the pairing of high quality individuals, especially in species with mutual mate choice. Then, if telomere length and individual quality are positively linked, individuals with similar telomere length should breed together. We investigated assortative pairing by an association of relative telomere length (RTL) and links with breeding success in wild king penguins. Monomorphic king penguins display mutual mate choice. Both parents have to finely cooperate to raise their single chick over an entire year (summer and winter). We followed 73 penguin pairs over the entire breeding season in three consecutive years of contrasting environmental conditions, known to strongly influence breeding success. We found an assortative pairing by RTL. However, only females' RTL was positively associated to chick survival up to fledging. This relationship was only significant in 2009, when environmental conditions were neither particularly bad nor good compared to other years. The positive link between RTL and breeding success confirmed that telomere length is somehow related with individual biological state at a given time, and the observed assortative pairing by RTL suggests that this may have ultimate consequences for individual fitness.

King penguins' telomere length: assortative mating and relationship with breeding success

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Keywords: telomere, penguins, assortative mating, reproduction, sexual selection

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Abstract

Telomeres are non-coding genetic repeats protecting the ends of linear chromosomes. Because long telomeres are often associated with higher survival, inter-individual variation in telomere length has been proposed as a proxy of fitness and, by extension, of individual quality. Sexual selection often leads to the pairing of high quality individuals, especially in species with mutual mate choice. Then, if telomere length and individual quality are positively linked, individuals with similar telomere length should breed together. We investigated assortative pairing by an association of relative telomere length (RTL) and links with breeding success in wild king penguins. Monomorphic king penguins display mutual mate choice. Both parents have to finely cooperate to raise their single chick over an entire year (summer and winter). We followed 73 penguin pairs over the entire breeding season in three consecutive years of contrasting environmental conditions, known to strongly influence breeding success. We found an assortative pairing by RTL. However, only females' RTL was positively associated to chick survival up to fledging. This relationship was only significant in 2009, when environmental conditions were neither particularly bad nor good compared to other years. The positive link between RTL and breeding success confirmed that telomere length is somehow related with individual biological state at a given time, and the observed assortative pairing by RTL suggests that this may have ultimate consequences for individual fitness.

Introduction

Telomeres are non-coding genetic repeats protecting the ends of linear chromosomes. They are not fully replicated during cell division and shorten with time though not linearly and regularly. This results in high TL variability early in life (partially through inheritance (Reichert et al. 2015) and adulthood (Hall et al. 2004). Several studies highlighted accelerated reduction in TL in response to environmental stressors, under poor environmental conditions during growth (Tarry-Adkins et al. 2009), higher investment in reproduction (Reichert et al. 2014), or exposure to glucocorticoids (Haussmann et al. 2012). Finally, variation in adult TL both reflects life stress and predicts fitness-related traits such as future survival and reproductive success (Bize et al. 2009). Thus, TL could be what Wilson and Nussey called a scalar abstraction of multiple phenotypic traits related to individual fitness, a variable that should be informative on the overall quality of individuals (Wilson and Nussey 2010). Previous studies supported this idea, showing that TL is linked to physiological or fitness components of individual quality (in terms of variation of longevity but also performances) (Bauch et al. 2014; Le Vaillant et al. 2015). If under selection, individual quality should be targeted by potential breeding partners. Thus in species with mutual mate choice, assortative mating by quality might ensue and be reflected by paired male and female TL. We tested this hypothesis in breeding king penguins (*Aptenodytes patagonicus*), a monomorphic species with mutual mate choice (Nolan et al. 2010). Partner quality is crucial for breeding success as both parents cooperate for 14 months to successfully raise the single chick until its departure to sea (Weimerskirch et al. 1992). During this long period, both parents face strong energy constrains that require fasting resistance on land and high foraging capacities at sea (Stonehouse 1960). Mutual mate choice in king penguins should thus particularly favour pairs of individuals showing similar TL (Kraaijeveld et al. 2007).

57 **Material and Methods**

58 We worked at Possession Island within a king penguin colony of ca. 20,000 breeding pairs in
59 2009-2011. Marked differences in environmental conditions (sea surface temperatures)
60 known to be strongly related to the breeding success occurred (Electronic Supplementary
61 Material (ESM 1; Bost et al. 2015): 2010 had favourable breeding conditions, 2011 was harsh
62 and 2009 intermediate. The breeding biology of king penguins and general procedures of
63 individual monitoring are described elsewhere (Stier et al. 2014). King penguins raise a single
64 chick over a period of 14 months. Breeding starts in November with courtship and mate choice
65 (Jouventin et al. 2008), and egg-laying spans from late-November to early March (ESM1). Both
66 partners alternate egg/chick attendance and foraging at sea, with the male initiating
67 incubation. Over winter (May-September), chicks are left mostly unattended and gather in
68 "crèches", surviving mostly on fat reserves gathered during the previous summer (Cherel and
69 Le Maho 1985). Chick rearing resumes in September for 2-3 months of intense parental
70 feeding, before a subsequent moult and departure at sea.

71 We followed breeding pairs over their entire season (33 pairs in 2009, 20 pairs in 2010, and
72 20 pairs in 2011; ESM1) from hatching to fledging of the chick. Ten days after hatching, the
73 male and chick of each pair were caught and body size and mass (missing for some adults)
74 were recorded to the nearest ± 4g using a platform balance (Kem IT60K2LIP). Flipper (±1mm)
75 and bill length (±0.1mm) were measured using a solid metal ruler. Adult blood (1mL) was
76 collected from a flipper vein using a heparinized syringe (2.5mL, G22- 1 ½ needle), centrifuged
77 (4000 rpm for 5 min), and plasma and red blood cells were separated and stored at -80°C.
78 Females were measured and sampled following the same protocol during their first brooding
79 shift. Breeding pairs and their chicks were followed by daily observations, allowing chick death
80 date to be recorded. Relative telomere length (RTL) was obtained from red blood cells DNA

using the qPCR method as described elsewhere (Criscuolo et al. 2009) and adapted to king penguins (ESM2).

We used Pearson correlations to evaluate associations between RTL (ln transformed to reach normality and homoscedasticity), body mass and size proxies of mated pairs over the study period. In addition, a linear mixed model (LMM) with female RTL as a response variable, with male RTL as fixed factor and years as random factor was run to control for annual variation in pairs' RTL. We assessed whether breeding partners' RTL and breeding success were associated using independent linear mixed models on males and females for (a) chick body mass at day 10, and using a GLM with a logistic binary distribution for (b) chick survival at fledging (survival/death = 1/0). Fixed effects included female or male RTL, sampling year, and the year x RTL. Chick body mass (for b) was included as a covariate. Post-hoc Tukey HSD tests were conducted using the 'multcomp' R package (Bretz et al. 2010). We used R3.1.3 (R Development Core Team 2008). Tests were two-tailed with $P< 0.05$ considered significant.

Results

Over 2009-2011, there were no evidences of RTL correlations with body size (wing or beak length) nor body mass in both sexes, neither were birds in pairs assorted by body mass or size (ESM3). However, males and females were assorted by RTL after controlling for years (Table 1, see Pearson correlation in Figure 1).

Controlling for year, chick body mass at ten days was not influenced by male or female RTL (Table 1). Chick survival at fledging was positively associated with chick body mass at ten days independently of the year. The interaction of female RTL and year predicted chick survival. In 2009 females with longer RTL were more successful at raising their chick but no

104 such pattern was evident in 2010/2011 (Figure 2, Table 1). Male RTL had no effect on chick
105 body mass or fledging success.
106
107 **Discussion**
108 In species where sexes express similar phenotypes and mate choice is mutual, assortative
109 pairing by individual quality is expected (Kraaijeveld et al. 2007). The assortative pairing by
110 RTL in king penguins supports this hypothesis and suggests that individuals with long
111 telomeres are of better body / physiological condition (i.e. of higher quality). Accordingly,
112 previous studies found that long telomeres were positively associated with individual fitness
113 proxies in the wild (e.g. reproduction, survival) in wild swifts (Bize et al. 2009), corvids
114 (Salomons et al. 2009), or common terns (Bauch et al. 2014). In king penguins, measures of
115 RTL using qPCR lead to similar observations on reproductive success, but also underlines a
116 RTL–physiological quality proxy relationship (i.e. natural antibody levels, Le Vaillant et al.
117 2015).
118 Preferential choice for a similar aged partner could lead to assortative mating by RTL, either
119 directly (because of an RTL – age negative relationship) or indirectly (because of the co-
120 selection with age of high quality individuals also having long telomeres). Even if king
121 penguin's RTL and age were unrelated in 5-8 years old adults (Le Vaillant et al. 2015; see also
122 Monaghan 2010 for a review on RTL and individual state), it is known that phenotypic traits
123 like foraging behavior, potentially of great impact on adult penguin body condition and
124 reproductive success, are changing with age (Le Vaillant et al. 2012). Therefore, we need to
125 gather more data on how RTL and individual performances are changing with age in our
126 species, to definitely conclude whether, as previously found (Bauch et al. 2012), individual
127 quality and RTL are correlated independently of age.

Interestingly, the association between RTL and reproductive success was only significant in females and depended on years. Females with long telomeres had higher breeding success in 2009, when environmental conditions were intermediate to 2010 (+) and 2011 (-). These results are suggestive of a difference between years in the telomere–fitness links, maybe because they are not easy to detect under harsh or favorable conditions, when reproductive constraints may be overwhelming even for high quality individuals, or universally absent. More data are need to better infer the actual impact of environmental conditions on RTL-fitness links. Male RTL was unrelated to breeding success in our study, leaving the question open of why females choose males of similar RTL. Male king penguins take charge of the first incubation shift, fasting for a period of *ca* 30 days (including courtship) (Weimerskirch et al. 1992). Our current investigations suggest a potential oxidative debt to prolonged fasting (Schull et al.; unpublished data), so female mate choice for male capacity to buffer oxidative stress, known to strongly affect TL (Von Zglinicki 2002), is possible.

Female king penguin's RTL is positively associated with chick survival. In a species using mutual mate choice for pairing, it plaids in favor of a non-random assortative mating by RTL. Those observations raise intriguing questions about the "how" (mechanisms) and the "why" (fitness consequences) of the breeding pair RTL association.

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Figures

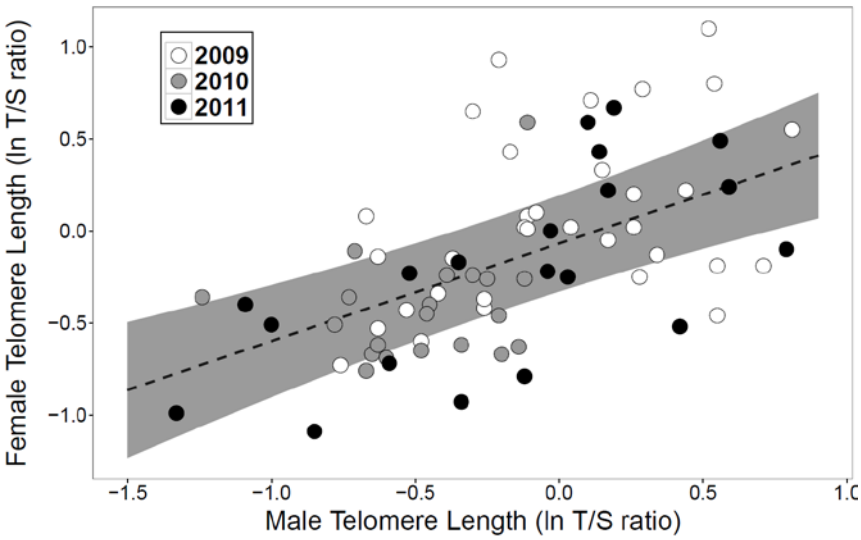
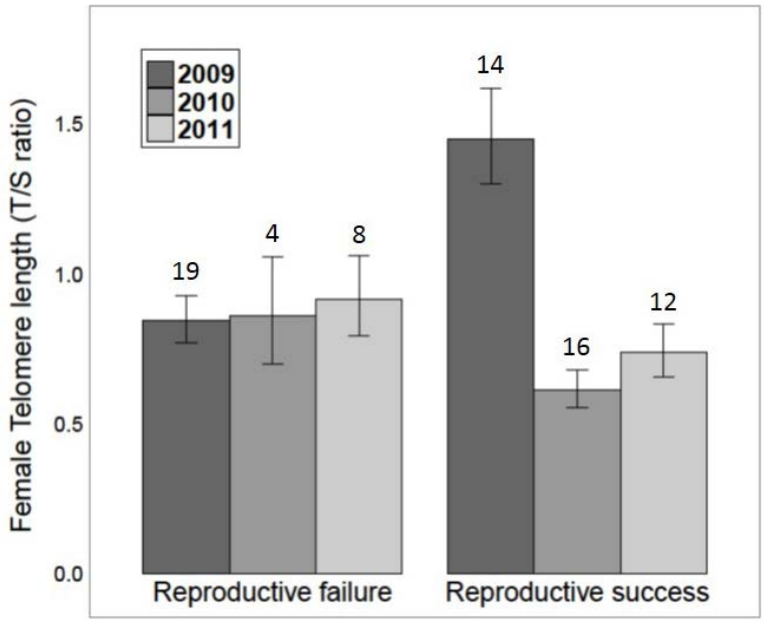


Figure 1. Assortative pairing by telomere length in 73 king penguin pairs followed over 2009-2011 in the Crozet archipelago. The relationship between male and female telomere lengths is tested using Pearson's correlation (y = 0.53x - 0.068; r= 0.57, F= 35.108, P< 0.001).



158 **Figure 2.** Mean (±SE) telomere length of female king penguins that failed reproduction or
159 successfully raised their single chick up to fledging in years 2009-2011.There was a significant
160 *TL x year* interaction (Table 1), showing that females with longer telomeres had higher success
161 fledging their chick, but only in 2009 (see text for statistics). Numbers indicate sample size.
162

Table 1. Linear mixed model estimates for the male and female relationship relative telomere length (RTL) of king penguins breeding pairs (A). Linear (a) and binomial (b) model estimates for the effects of adults' relative telomere length (RTL) of king penguins breeding females (B) and males (C) on chick body mass, and fledging success.

A) Females Variables (N=73)	Estimates	± SE	*t*-value (or z)	*P*-value
(a) Laying-date				
Female RTL	-14.01	2.49	-2.62	0.011*
Years 2010	-22.16	5.35	-3.68	< 0.001*
2011	-14.99	6.03	-3.55	< 0.001*
Female RTL x Years				**(0.011)***
2010	14.36	11.99	1.198	0.235
2011	24.87	8.07	3.08	0.003*
(b) Chick body mass at day 10				
Female RTL	92.28	55.83	1.71	0.093
Years 2010	-79.97	65.75	-1.22	0.228
2011	25.08	45.83	0.55	0.586
Laying Date	2.39	1.21	1.97	0.053*
Female RTL x Years				**(0.206)**
2010	-154.73	120.51	-1.28	0.204
2011	-137.87	85.78	-1.61	0.113
(c) Fledging success				
Female RTL	3.06	1.28	2.38	0.017*
Years 2010	0.96	1.36	0.70	0.482
2011	0.57	0.79	0.73	0.469
Laying Date	-0.03	0.03	-1.14	0.254*
Chick body mass at day 10	< 0.01	< 0.01	2.49	0.013*
Female RTL x Years				
2010	-6.66	2.95	-2.26	0.024*
2011	-3.55	1.66	-2.15	0.032*
B) Males Variables (N=73)	**Estimates**	**± SE**	***t*-value (or z)**	***P*-value**
(a) Laying-date				
Male RTL	**-3.20**	**2.62**	**-0.52**	**0.604**
Years 2010	-21.26	6.15	-2.97	0.004*
2011	-15.46	7.15	-3.54	<0.001*
Male RTL x Years				**(0.559)**
2010	3.58	13.62	0.26	0.794
2011	9.17	8.49	1.08	0.284
(b) Chick body mass at day 10				
Male RTL	-39.69	58.66	-0.68	0.50
Years 2010	-125.53	72.51	-1.73	0.088
2011	16.22	45.33	0.36	0.722
Laying Date	1.55	1.16	1.33	0.188
Male RTL x Years				**(0.654)**
2010	-58.72	129.78	-0.45	0.652
2011	50.03	84.62	0.61	0.542
(c) Fledging success				
Male RTL	-0.09	1.06	-0.09	0.931
Years 2010	2.12	1.30	1.63	0.103
2011	-0.02	0.74	-0.03	0.974
Layingdate	-0.05	0.02	-2.11	0.035*
Chick body mass at day 10	0.01	0.01	2.92	0.003*
Male RTL x Years				
2010	1.08	2.30	0.47	0.639
2011	-0.31	1.37	-0.22	0.824

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Quentin SCHULL

Sexual selection, social selection and individual quality: underlying mechanisms and ultimate consequences of the ornamentation in a monomorphic species, the King penguin (*Aptenodytes patagonicus*)

Résumé

Depuis 157 ans et la publication originelle de la théorie de l'évolution par sélection naturelle de Charles Darwin, ce concept n'a cessé d'évoluer. Notamment, comprendre l'existence de traits morphologiques handicapants chez de nombreuses espèces, qui à première vue peuvent paraître détrimentaire en termes de survie et de reproduction, fascine les biologistes évolutifs depuis des décennies car ces traits semblent en contradiction directe avec la théorie de Darwin. Sous les concepts de la sélection sexuelle et de la sélection sociale, les théories permettant d'expliquer l'existence et le maintien d'ornements extravagants sont aujourd'hui multiples et chacune trouvent des supports empiriques. Un concept fondamental suggère que des traits bien qu'handicapants aient pu évoluer, dans la mesure où ils sont employés dans un contexte sexuel et/ou social, informant de manière honnête les congénères soit sur la qualité intrinsèque (seuls des individus à même de supporter leur coûts peuvent les produire), soit sur la qualité sociale (dominance, capacité à monopoliser des ressources) des individus les exprimant. Le manchot royal (*Aptenodytes patagonicus*) est un modèle exceptionnel permettant de tester la valeur sélective de ce signal dans un contexte sexuel et social au sens large (non-reproductif). Il s'agit d'une espèce monomorphique chez lequel les deux sexes présentent des ornements colorés au niveau du plumage et du bec. La coloration de ces ornements repose sur trois mécanismes différents : i) la coloration du plumage ornemental est due à la présence de pigments endogènes, tandis que la coloration du bec est due à la fois à la présence ii) de pigments exogènes, et (iii) l'agencement fin de structures cellulaires superficielles (couleurs structurelles). Au travers de cette thèse j'ai pu déterminer le coût associé à la production et la maintenance de ces traits ornementaux, démontrant ainsi leur caractère honnête quant aux signaux qu'ils transmettent, ce qui en fait donc de bons supports pour évaluer la qualité de l'individu dans un contexte sexuel. Mes résultats suggèrent donc que l'apparition et le maintien de ces ornements au cours de l'évolution se sont opérés en partie sous l'influence de la sélection sexuelle et d'un choix mutuel du partenaire chez les deux sexes. Par ailleurs, mes résultats montrent que certains aspects de l'ornementation sont relativement constants dans le temps et insensibles à la condition de l'individu, suggérant que le coût associé à l'expression de ces traits puisse être différé dans le temps et de ce fait être contraint par la sélection sociale. Ce travail de recherche participe à la compréhension des mécanismes impliqués dans l'évolution de signaux coûteux, et à la nature des bénéfices ultimes que ces traits procurent, questionnements qui attisent la curiosité de nombreux biologistes depuis toujours, et qui amènent à de nouvelles hypothèses qui ne cesseront sans doute jamais d'émerger.

Mots clé : sélection sociale, sélection sexuelle, évolution, signal honnête, espèce monomorphique, Manchot royal

Summary

Darwin's seminal theory of evolution by means of natural selection, first published 157 years ago, has been in constant refinement ever since. Specifically, evolutionary biologists have been fascinated by the existence of animal armaments and ornaments, as at first glance, such morphological features might appear detrimental to individual survival and reproduction, and thus in contradiction with Darwin's original idea. However, as already pointed out by Darwin in 1871, handicapping traits in several species might evolve if they provide benefits in the acquisition of mating partners. The production and maintenance of extravagant ornaments was more widely suggested to evolve by conspecific preference providing information on individual intrinsic quality in sexual contexts (sexual selection) or on individual social quality in non-reproductive contexts (social selection). Under those respective frameworks, several hypotheses have been proposed and empirical support has been provided for most. The king penguin (*Aptenodytes patagonicus*) is an outstanding model allowing to investigate several of those hypotheses simultaneously. The king penguin is a monomorphic bird species, for which both males and females display similar colourful ornaments, both on the plumage and the beak. Plumage ornament coloration is produced by i) endogenous pigments, whereas beak ornament coloration is produced by both ii) exogenous pigments and iii) structural cellular features. Throughout this thesis, I identified the costs associated with the production and maintenance of those ornamental features highlighting their honest character in signalling the quality of their bearer. My results show that those ornaments are partly condition-dependent, and reliable traits that may be used to assess the quality of a potential sexual partner in both sexes, implying that their evolution and maintenance is partly determined by mutual mate choice and sexual selection. On the other hand, some traits remained condition-independent in their production, suggesting that the cost associated with their expression was deferred over time and the evolution of those ornaments likely shaped by non-sexual social selection. This research work aimed at improving our comprehension of the mechanisms implicated in the evolution of extravagant traits and the ultimate fitness benefits of such traits, questions that have stirred the curiosity of evolutionary biologists for decades. In the process, it has empirically shed first lights on the fundamental energy mechanism likely underlying the evolution of animal ornamentation.

Keywords: social selection, sexual selection, evolution, honest signal, monomorphic species, king penguins