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Evaluation en laboratoire du parasitoïde *Gryon gonikopalense* (Hym : Scelionidae) pour le contrôle biologique *de Bagrada hilaris* (Hem : Pentatomidae), une punaise invasive aux Etats-Unis

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"Yes there are two path you can go by, but in the long run, there's still time to change the road you're on"

Stairway to heaven, Led Zeppelin

"Le doute est une force, une vraie et belle force, veille simplement qu'elle te pousse toujours en avant »

Pierre Bottero

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Introduction générale

Dans le contexte actuel de mondialisation des échanges commerciaux et de changement climatique global, l'impact des ravageurs de cultures est en augmentation (Hulme 2009 ; Bradshaw et al. 2016). Parmi eux, les insectes herbivores invasifs sont responsables de 8.7 Mds de dollars de pertes annuelles en moyenne pour un total de plus de 400 Mds de dollars entre 1970 et 2017 (Diagne et al. 2021). Les principaux pays producteurs de ces ressources comme les Etats-Unis et la Chine sont les plus impactés, mais sont également les principales sources de ravageurs invasifs (Paini et al. 2016 ; Tuberlin et al. 2017).

Parmi ces nouveaux envahisseurs, les punaises phytophages (Hemiptera., Pentatomidae) attaquant les cultures vivrières dans le sud de l'Asie, l'Afrique et l'Amérique du sud bénéficient de ces changements globaux. Au cours des cent dernières années, certaines d'entre elles sont devenues de nouvelles menaces pour l'agriculture en Europe et en Amérique du Nord, où leurs ennemis naturels (prédateurs ou parasitoïdes) sont absents (Panizzi et al. 2000 ; McPherson 2018). A ce jour, leur régulation est principalement basée sur l'application de pesticides de synthèse à large spectre, efficaces à court-terme mais problématiques d'un point de vue économique, écologique et sanitaire. Pourtant, de nombreuses alternatives pourraient être développées, notamment au profit de l'agriculture biologique.

La plus récente de ces punaises invasives est *Bagrada hilaris* (Burmeister). Originaire d'Afrique et d'Asie, elle a été introduite accidentellement en 2008 dans les sud-ouest des Etats-Unis. Cette espèce polyphage est rapidement devenue une peste majeure des cultures de brassicacées de la Californie au Texas. Aujourd'hui, elle est présente au Mexique et au Chili, et seule son absence du continent Européen semble empêcher sa distribution presque mondiale. Aux Etats-Unis, les populations de ce ravageur sont régulées par l'application régulière de pyréthrinoïdes et de produits organophosphatés, incompatibles avec la filière agricole biologique qui subit fortement l'impact négatif de la punaise.

Afin de répondre à ce besoin, l'USDA-ARS (United State Department of Agriculture, Agricultural Research Service) a financé un programme de lutte biologique impliquant deux principaux acteurs sur le sol américain et européen : l'ISPH (Invasive Species and Pollinator Health, Albany, Californie) et l'EBCL (European Biological Control Laboratory, Montpellier, France). Le projet a débuté en 2015 avec la recherche d'agents de lutte biologique dans une des aires natives de la punaise bagrada (Pakistan). C'est à travers une collaboration avec le CABI Pakistan (Centre for Agricultural Bioscience International) que trois hyménoptères parasitoïdes (Scelionidae), candidats pour la lutte biologique, ont été collectés. Ceux-ci ont été répartis entre trois unités de recherche : l'université de Californie Riverside, l'ISPH et l'EBCL. C'est donc en Février 2016 que l'EBCL met en place dans sa quarantaine agréée les élevages de la punaise bagrada et deux de ses parasitoïdes : *Trissolcus hyalinipennis* Rajmohana & Narendran et *Gryon gonikopalense* Sharma. Le troisième parasitoïde collecté au Pakistan, *Ooencyrtus mirus* Triapitsyn & Power, est quant à lui mis en élevage à l'Université

de Californie Riverside. En parallèle, de multiples collaborations sont mises en place entre l'EBCL et l'Afrique du Sud, le Kenya, le Pakistan et les Etats-Unis pour l'obtention de souches natives et invasives de bagrada.

C'est dans ce contexte, et grâce à un financement nord-américain, que mon projet de thèse a vu le jour en 2018 avec un partenariat entre l'EBCL et l'Institut Agro - Montpellier SupAgro. Les principaux enjeux de cette thèse sont de 1) valoriser l'approche de la lutte biologique classique dans la gestion intégrée des punaises invasives aux Etats-Unis et 2) contribuer à la connaissance des données biologiques d'auxiliaires pour réguler la punaise bagrada en Californie. L'objectif principal de ce travail de thèse est une évaluation générale du parasitoïde *G. gonikopalense* comme agent de lutte biologique en décrivant sa biologie et son comportement de recherche d'hôte. Dans le cadre de ma thèse, j'ai également voulu promouvoir une démarche pro-active en considérant deux problématiques : 1) optimiser la production de l'auxiliaire dans un projet de lutte biologique et 2) poser les bases d'un programme de lutte biologique européen si bagrada venait à coloniser le continent. L'issue de mes différents travaux de recherche est de pouvoir proposer des éléments de discussion en faveur ou en défaveur de l'utilisation de *G. gonikopalense* comme auxiliaire contre bagrada.

Pour se faire, ce manuscrit de thèse se décline en six chapitres :

Le **premier chapitre** fera dans un premier temps un état des lieux des conséquences économiques des insectes invasifs dans le monde avec une emphase particulière sur les Etats-Unis. Je présenterai ensuite les enjeux d'un programme de lutte biologique classique contre un insecte invasif et les critères qui permettent de sélectionner un auxiliaire. Les réussites et les limites de la lutte biologique classique seront abordées en continuant d'apporter un éclairage particulier sur les Etats-Unis, pays pionnier et principal acteur de cette pratique à l'échelle mondiale. Dans une troisième partie, je présenterai les punaises pentatomidés invasives aux Etats-Unis, les enjeux économiques qu'elles soulèvent et les moyens potentiels ou mis en œuvre pour réguler leurs populations, notamment la lutte biologique. Dans une quatrième partie, le modèle biologique *Bagrada hilaris* sera présenté à travers sa biologie, son impact sur les productions de brassicacées et les méthodes de gestion existantes pour la réguler. La dernière partie de ce chapitre sera quant à elle consacrée aux parasitoïdes oophages de la famille des Scelionidae. Je présenterai leur biologie et leur potentiel comme agents de lutte biologique, en particulier contre les punaises invasives, ainsi que le candidat auquel cette thèse est consacrée : *Gryon gonikopalense*.

Le **deuxième chapitre** se focalisera sur la biologie du parasitoïde candidat *G. gonikopalense* et comportera deux articles (**Articles 1 et 2**). Le premier vise d'une part à statuer sur l'identité taxonomique du parasitoïde d'autre part à présenter une première description de sa biologie. Plusieurs traits d'histoire de vie en lien avec son potentiel rôle d'auxiliaire (développement, reproduction, longévité, taux de parasitisme) sont détaillés. L'article 2 a pour objectif d'estimer la capacité de *G. gonikopalense* à

s'établir dans la zone d'introduction de bagrada (*i.e*. la Californie). L'acclimatation à un nouvel environnement thermique est l'un des principaux facteurs explicatifs du succès ou de l'échec de l'introduction d'un agent de lutte biologique. C'est pourquoi nous avons exploré l'effet d'une large gamme de températures sur les principaux traits d'histoire de vie du parasitoïde.

Le **troisième chapitre** portera sur la capacité de *G. gonikopalense* à parasiter les œufs que bagrada pond sous la surface du sol, et comporte deux articles (**Articles 3 et 4**). Dans l'article 3, nous décrivons de quelle manière *G. gonikopalense* détecte et parasite ces œufs enterrés supposément protégés par la barrière physique du sol. Dans la continuité de ces résultats, l'article 4 a pour objectif de déterminer si la capacité de *G. gonikopalense* à parasiter les œufs enterrés de bagrada est un atout significatif pour réguler la punaise. Cette étude vise donc à la fois à décrire la distribution spatiale des pontes de *B. hilaris* et la localisation du parasitisme de *G. gonikopalense.* Nous questionnons notamment l'influence de la plante hôte et de la densité de punaise sur ces distributions.

Dans le **quatrième chapitre** (**Article 5**), nous aborderons la possibilité d'optimiser la production de *G. gonikopalense* en laboratoire. L'un des défis d'un programme de lutte biologique est en effet de pouvoir produire en masse l'auxiliaire et le lâcher au moment voulu dans les cultures à protéger. La possibilité d'optimiser et d'organiser sa production en laboratoire est donc primordiale. Dans cet article nous explorons la possibilité de stocker les œufs de bagrada à différentes conditions thermiques et sur des durées variables. Nous nous intéressons en particulier à l'effet de ce stockage sur le taux de parasitisme de *G. gonikopalense* et de possibles effets létaux ou sub-létaux sur sa descendance. Parallèlement, nous évaluons les conséquences d'une privation d'hôte sur les capacités de reproduction et la descendance de *G. gonikopalense*.

Le **cinquième chapitre** fera l'objet d'un élargissement de la problématique de *B. hilaris* au continent Européen. Présente au Maroc, en Sicile et à Malte, l'expansion bagrada dans le Sud de l'Europe est à envisager dans les années à venir, notamment sous l'effet du réchauffement climatique. Ce chapitre présentera les tests de spécificité conduits sur des punaises non-cibles euro-méditerranéennes effectués en parallèle de ceux menés aux Etats-Unis depuis 2017. En supposant l'efficacité de *G. gonikopalense* à contrôler bagrada ces tests représentent une démarche pro-active à l'encontre de la punaise ailleurs qu'aux Etats-Unis, tout en fournissant des résultats complémentaires.

Enfin, **le sixième et dernier chapitre** sera une discussion générale des résultats obtenus au cours de ma thèse. Je discuterai des critères d'évaluation de *G. gonikopalense* qui permettent de le valider comme agent de lutte biologique contre *B. hilaris*, et des aspects de sa biologie qui pourraient limiter son utilisation. Les performances de *G. gonikopalense* seront notamment discutées en comparaison de deux autres parasitoïdes collectés au Pakistan et étudiés en Californie. Les résultats obtenus soulèvent de nombreuses questions, liées aux capacités de recherche d'hôte de *G. gonikopalense*, la dynamique spatiale et temporelle de sa population ou encore sa spécificité vis-à-vis de bagrada. Ces différents éléments seront abordés en questionnant notamment la faisabilité du programme de lutte biologique initial sur le terrain. Je terminerai ce manuscrit de thèse par un ensemble de perspectives de recherche

qui pourraient découler du travail effectué, en incluant des premières réflexions sur la possibilité d'introduire *G. gonikopalense* sur l'île de Pantelleria (Sicile).

Chapitre 1

Etat des connaissances

I. Les Etats-Unis au cœur de la problématique mondiale des insectes invasifs

1. Les réseaux d'invasion : un phénomène polarisé

Une invasion biologique se caractérise par l'introduction, l'établissement et l'expansion d'une espèce exogène (exotique) dans un écosystème dont elle était absente (Richardson et al. 2000 ; Blackburn et al. 2011). On parle alors d'une espèce exotique envahissante.

Il existe une importante asymétrie entre les pays émetteurs d'organismes invasifs et les pays qui les reçoivent (Fig. 1). Les pays d'Asie du sud-est sont par exemple la source de près de 40% des d'espèces invasives à travers le monde tandis que la majorité des pays d'Afrique sont la source de moins de 4% des invasions (Paini et al. 2016 ; Tuberlin et al. 2017). Il existe également une asymétrie entre les milieux continentaux et insulaires puisque les territoires contenant le plus d'espèces invasives sont principalement insulaires comme Hawaii, la Nouvelle-Zélande, la Polynésie Française ou les îles Fiji (Tuberlin et al. 2017).

Concernant plus particulièrement les arthropodes, les cinq espèces les plus invasives (présents dans le plus grand nombre de pays) sont des insectes, et trois d'entre eux sont des phytophages ravageurs de cultures : *Icerya purchasi* Maskell (Hem., Margarodidae), *Aphis spiraecola* Patch (Hem., Aphididae) et *Frankliniella occidentalis* Pergande (Thys., Thripidae) (Tuberlin et al. 2017). Ces insectes circulent à travers deux voies d'invasion principales : le commerce de denrées alimentaires et celui de plantes ornementales.

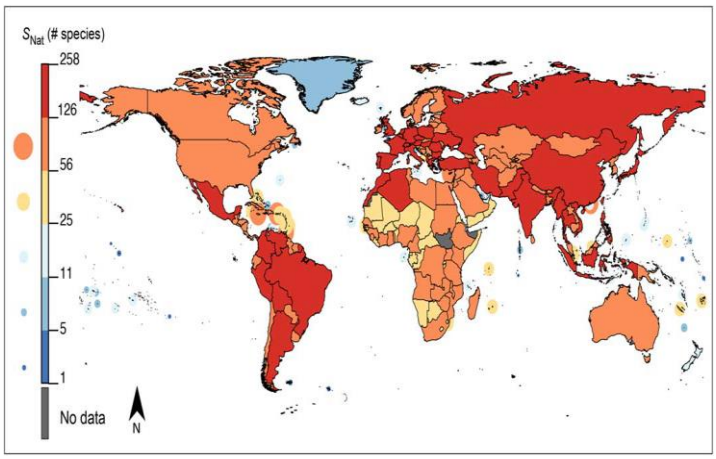


Figure 1 : Planisphère représentant le nombre d'espèces natives dans un pays mais invasives ailleurs dans le monde. Les données sont issues de the Global Invasive Species Database (GISD 2016) et the CABI Invasive Species Compendium (CABI ISC 2016). Les pays en rouge sont les plus importantes sources d'espèces invasives dans les autres pays du monde (Figure tirée de Tuberlin et al. 2017)

1.1. Les Etats-Unis, acteurs majeurs du réseau d'invasion

Sur 1517 espèces invasives recensées dans le monde, plus de 200 sont originaires des Etats-Unis, et un plus grand nombre encore sont devenues invasives sur ce territoire, notamment à cause de l'intensification et de la globalisation des échanges commerciaux internationaux (Work et al. 2005 ; Countryman et al. 2018). Ainsi, les Etats-Unis est le pays qui enregistre le plus d'espèces invasives (Fig.

2) (toutes espèces confondues) avec 1071 espèces recensées en 2016, la plupart d'entre elles (51,1%) étant concentrées dans les milieux insulaires tels que Hawaii (Tuberlin et al. 2017).

L'augmentation des invasions est notamment due à l'intensification des échanges commerciaux entre l'Asie et l'Amérique du Nord (Countryman et al. 2018). Ainsi, l'Asie est l'aire native de beaucoup d'espèces devenues nuisibles dans les agro-systèmes nord-américains comme la punaise marbrée *Halyomorpha halys* Stal (Hem., Pentatomidae), le longicorne asiatique *Anoplophora glabripennis* Motschulsky (Col., Cerambycidae), ou la drosophile à ailes tachetées *Drosophila suzukii* Matsumura (Dipt., Drosophilidae) (Wermelinger et al. 2008 ; Dodds & Orwing 2011 ; Rota-Stabelli et al. 2013) Dans ce contexte, la Chine représente 17% des espèces invasives totales aux USA (Tuberlin et al. 2017).

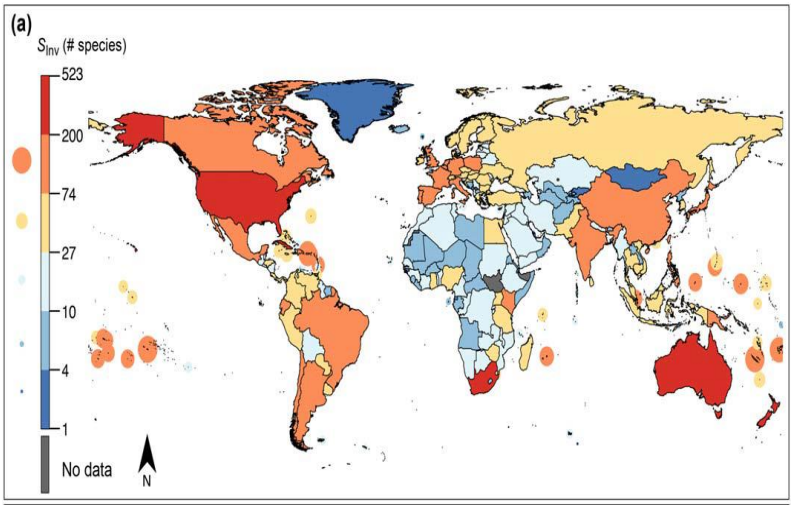


Figure 2 : Planisphère représentant le nombre d'espèces invasives par pays. Les données sont issues de the Global Invasive Species Database (GISD 2016) et the CABI Invasive Species Compendium (CABI ISC 2016) Les pays en rouge sont ceux qui comptent le plus d'espèces invasives (d'après Tuberlin et al. 2017).

Tableau 1 : Liste non exhaustive des principaux insectes invasifs et nuisibles aux Etats-Unis parmi les espèces ayant le plus fort impact économique ou écologique. Les espèces sont classées par Ordre, et sont accompagnées de leur aire d'origine et du type d'impact qu'elles ont aux Etats-Unis.

Espèce	**Ordre**	**Origine**	**Impact**	**Référence**
Coptotermes formosanus	Isoptera	Chine, Taïwan, Japon	Dégâts habitations humaines	Chouvenc et al. 2016
Harmonia axyridis	Coleoptera	Asie orientale	Compétitive avec les espèces natives	Roy et al. 2006
Anoplophora glabripennis	Coleoptera	Asie (Chine, Corée)	Ravageur de feuillus (Erables)	Dodds et Orwing 2011
Agrilus planipennis	Coleoptera	Asie orientale	Ravageur du frêne	Bauer et al. 2008
Popillia japonica	Coleoptera	Japon	Dégâts sur plantes ornementales et cultivées	Bragard et al. 2018
Aedes albopictus	Diptera	Asie orientale	Vecteur de pathogènes	Bonizzoni et al. 2013
Bactrocera dorsalis	Diptera	Asie	Ravageur de fruits	Qin et al. 2018
Drosophila suzukii	Diptera	Asie du Sud-Est	Dégâts sur fruits	Rota-Stabelli et al. 2013
Adelges tsugae	Hemiptera	Japon	Ravageur de conifères	Havill et al. 2016
Bemisia tabaci	Hemiptera	Inde et Moyen-Orient	Dégâts sur cultures variées	Goolsby et al. 2005
Diaphorina citri	Hemiptera	Asie	Dégâts sur agrumes	Qureshi et al. 2009
Halyomorpha halys	Hemiptera	Chine, Taiwan, Japon	Dégâts sur fruits et légumes	Wermelinger et al. 2008
Solenopsis invicta	Hymenoptera	Amérique du Sud	Santé humaine	Caldera et al. 2008
Iridomyrmex humilis	Hymenoptera	Argentine	Compétitive avec les espèces natives	Human & Gordon, 1997
Lymantria dispar	Lepidoptera	Europe / Asie	Ravageur de conifères	Brown & Cameron 1982
Scirtothrips dorsalis	Thysanoptera	Asie du Sud	Dégâts sur une très grande variété de cultures	Dickey et al. 2015

2. Les insectes invasifs : conséquences économiques

2.1. Bilan mondial

Les coûts économiques engendrés par l'ensemble des espèces invasives dans le monde sont considérables, bien que leur estimation soit difficile et donc peu documentée (Hoffmann & Broadhurst 2016). La très récente étude de Diagne et al. (2021) estime par exemple le coût annuel moyen des espèces invasives dans le monde à 26.8 Mds de dollars entre 1970 et 2017 mais Xu et al. (2006) recensait plus de 14 Mds de dollars de pertes annuelles rien qu'en Chine tandis que l'Europe compterait près de 20 Mds d'euros de pertes annuelles (Kettunen et al. 2009).

Les insectes invasifs seraient quant à eux responsables de 8.7 Mds de dollars de pertes annuelles en moyenne, soit près d'un tiers des coûts totaux (Diagne et al. 2021). D'après Bradshaw et al. (2016) plus d'un tiers des pertes engendrées par les insectes provient des systèmes agricoles, et ces pertes économiques sont croissantes (Hulme 2009 ; Diagne et al. 2021), notamment à cause du changement climatique et de la mondialisation des échanges commerciaux (Bebber et al. 2013 ; Bradshaw et al. 2016), ces derniers étant la principale voie d'invasion des insectes ravageurs (Tuberlin et al. 2017).

2.2. Le cas des Etats-Unis

Les Etats-Unis concentrent la majeure partie des coûts liés aux espèces invasives dans le monde (Fig. 3), qui, d'après Pimentel (2005), représenteraient annuellement 120 Mds de dollars dont 13.5 Mds liés aux insectes, incluant les pertes de production et les coûts liés à leur régulation par les pesticides. Ces valeurs apparaissent cependant surestimées en comparaison de l'étude de Diagne et al. (2021) qui estime les coûts totaux entre 350Mds et 550 Mds de dollars pour la période 1970-2017 (Fig. 3).

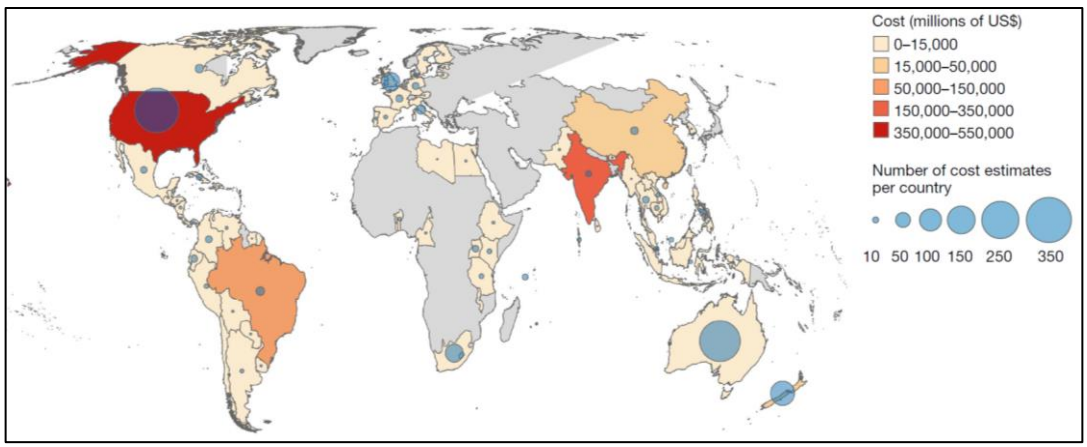


Figure 3 : Distribution géographique du coût total lié aux espèces invasives dans le monde entre 1970 et 2017 (d'après Diagne et al. 2021)

Parmi les plus grosses sources de pertes économiques, on peut citer par exemple le bombyx disparate (*Lymantria dispar* L.), un ravageur majeur des forêts de conifères, de chênes et d'espèces ornementales aux Etats-Unis (Keena & Richards 2020) : sa régulation impliquerait des coûts annuels de près de 11 millions de dollars (Campbell & Schlarbaum 1994 ; Pimentel et al. 2005). Dans un autre registre, les dégâts occasionnés aux habitations par le termite *Coptotermes formosanus* Shiraki sont

estimés à près d'1 Mds de dollars par an, ce qui en fait l'une des espèces invasives les plus coûteuses pour les Etats-Unis (Lax & Osbrink 2003). L'état de Californie, en tant que principal producteur de fruits et légumes du pays, est quant à lui particulièrement vulnérable à ces ravageurs de cultures toujours plus nombreux tels que le psylle *Diaphorina citri* Kuwayama, ou la mineuse des agrumes *Phyllocnistis citrella* (Ferguson & Grafton-Cardwell 2014).

II. La lutte biologique : contrôler durablement les espèces invasives

La lutte biologique consiste en l'utilisation volontaire par l'homme d'organismes vivants appelés agents de lutte biologique ou auxiliaires de cultures, pour limiter ou empêcher les pertes et/ou les dégâts causés par des organismes nuisibles sur les productions humaines (Eilenberg et al. 2001). Il existe trois méthodes de lutte biologique (Eilenberg et al. 2001 ; Heimpel & Mills 2017) :

- La lutte biologique **inondative** est basée sur l'élevage et le lâcher en masse d'agents de lutte biologique de manière répétée dans les milieux naturels où les populations naturelles sont limitées et ne parviennent pas à réguler les populations d'organismes nuisibles.
- La lutte biologique **par conservation** consiste à promouvoir l'installation, la survie et la reproduction des auxiliaires de culture naturellement présents dans l'écosystème où se trouve la ressource à protéger contre les organismes nuisibles (Landis et al. 2000).

Dans cette thèse nous nous focaliserons sur la troisième forme de lutte biologique : **la lutte biologique classique**, ou **par importation,** qui est au cœur de mon projet de recherche.

1. Fondement de la lutte biologique classique contre les insectes invasifs

Le principe fondamental de la lutte biologique classique (= LBC) repose sur l'hypothèse que le succès invasif d'une espèce introduite est lié à l'absence de ses ennemis naturels dans la zone d'introduction (Keane & Crawley 2002 ; Paula et al. 2021). Pour rétablir l'équilibre perdu, il s'agit donc de compenser ce « relâchement des ennemis » (« enemy release hypothesis ») par l'introduction d'ennemis naturels spécialistes (agents pathogènes, insectes, acariens, etc.) dans l'aire d'invasion (Eilenberg et al. 2001). Leur rôle est de réduire la densité des populations de l'espèce invasive sous un seuil de nuisibilité acceptable d'un point de vue économique et / ou écologique (Hajek & Eilenberg 2018). Dans le cas de la LBC, l'objectif est atteint lorsque les auxiliaires introduits parviennent à durablement contrôler l'espèce cible, sans intervention humaine supplémentaire (Fig. 4). Cela repose donc sur des interactions naturelles de prédation (*lato sensu*) entre différents organismes.

La naissance de la LBC telle qu'elle est pratiquée aujourd'hui est généralement attribuée à Charles Valentine Riley et Albert Koebele (Sorensen et al. 2019). Ce cas emblématique de la fin des années 1880 illustre en effet comment l'importation de la coccinelle Australienne *Rodolia cardinalis*

(Mulsant) (Col., Coccinellidae) en Californie a permis la régulation durable de la cochenille invasive *I. purchasi* qui attaquait les vergers d'agrumes de la région (Sorensen et al. 2019).

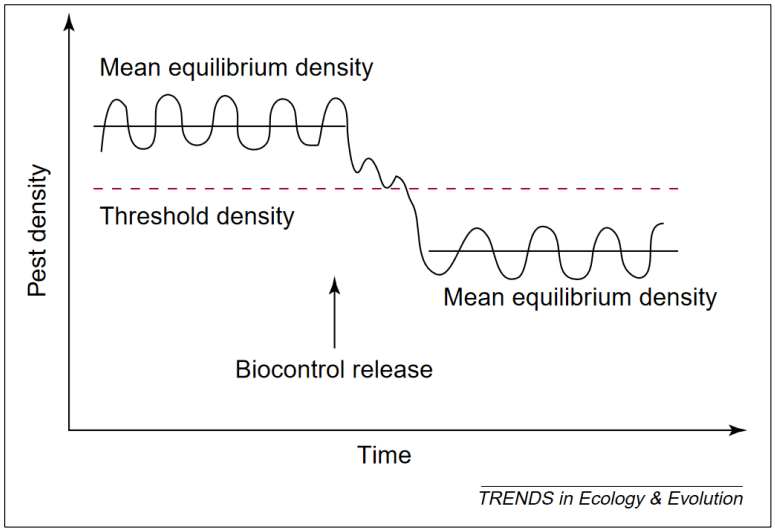


Figure 4 : évolution théorique de la dynamique de population d'une espèce nuisible au cours d'un programme de lutte biologique classique (d'après Pearson & Callaway (2003))

2. Quelle place pour la lutte biologique aux Etats-Unis ?

Les Etats-Unis font partie des pays pionniers dans l'application de la LBC. Depuis le succès de la régulation de *I. purchasi*, le pays est resté leader dans le domaine, avec 33.6% des lâchers d'auxiliaires du monde (1956 introductions) dont 206 succès (Cock et al., 2016). Entre 1985 et 2016, près de 70 % des agents de lutte biologique (prédateurs et parasitoïdes) contre des insectes nuisibles ont été lâchés sur le territoire américain (Driesche & Hoddle 2016). Cependant, la place de la LBC dans les pratiques agricoles a fortement évolué en plus d'un siècle (Fig. 5). Après un « âge d'or » entre 1880 et 1940, le développement et le succès des pesticides de synthèse à partir de la seconde guerre mondiale a relégué la LBC à une stratégie marginale (Cock et al. 2016 ; Barratt et al. 2018). Ce sont les premières études pointant du doigt les effets néfastes de la lutte chimique qui ont permis à nouveau à la LBC de retrouver une place dans les pratiques agricoles à partir des années 1960 (Barratt et al. 2010 ; Gay 2012). D'un autre côté, l'émergence de critiques à l'encontre des effets non-intentionnels de la LBC a mené à une reconsidération de cette pratique par la communauté scientifique (Clarke et al.1984 ; Heimpel & Cock 2018). Cela a mené à une forte diminution de son utilisation à travers le monde à partir des années 1990 (Cock et al. 2016). En ce qui concerne la lutte biologique contre les insectes nuisibles, Driesche & Hoddle (2016) ne dénombraient par exemple que 178 espèces de parasitoïdes et prédateurs introduits à l'échelle mondiale entre 1985 et 2015, ce qui représente moins de 3% des introductions recensées par Cock et al. (2016) depuis 1880.

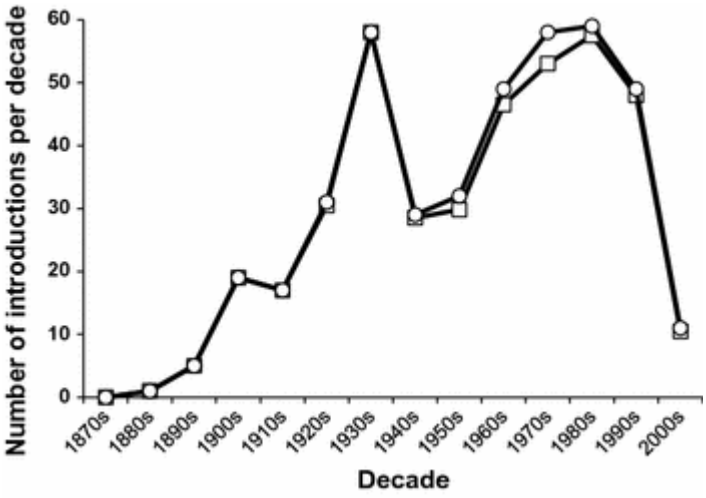


Figure 5 : évolution du nombre d'introduction d'agents de lutte biologique depuis 1870 à l'échelle mondiale (d'après Cock et al. 2016)

3. Méthodologie et processus de recherche

Le processus de recherche qui permet d'aboutir à la sélection d'un ou plusieurs agents de lutte biologique se déroule généralement en six étapes clés (Fig. 6). De la recherche de l'espèce nuisible au suivi des agents auxiliaires sur le terrain, chaque étape peut remettre en question le programme de LBC, comme le soulignent Hokkanen & Sailer (1985) : « In fact, each project is like a chain of events and choices, and the end result is probably determined by the weakest link in the chain ».

- **L'exploration** de terrain vise à rechercher le ravageur dans son aire native (la plupart du temps) ou invasive afin d'échantillonner ses ennemis naturels (Borowiec & Sforza, 2020). La détection du ravageur passe souvent par la recherche de sa plante hôte, car elle est plus facile à localiser. Des outils comme le « climate matching » permettent d'estimer en amont la présence de cette plante hôte et du ravageur en se basant sur leurs niches écologiques climatiques (Jung et al. 2017). La recherche de l'espèce cible est cependant simplifiée si sa plante hôte est une espèce cultivée.
- **Le piégeage** et ses différentes techniques permet de capturer un cortège d'espèces. Par exemple, l'échantillonnage des parasitoïdes oophages passe généralement par l'utilisation d'œufs sentinelles. Ces derniers sont les œufs du ravageur exposés en plein champ pour attirer les parasitoïdes présents dans l'environnement. L'intérêt de cette technique réside dans le fait que les parasitoïdes collectés sont assurés d'attaquer l'espèce cible et donc d'être de potentiels auxiliaires.
- **L'identification** des spécimens collectés peut ensuite être une étape complexe lorsqu'il s'agit d'organismes peu étudiés ou mal décrits taxonomiquement et génétiquement. La taxonomie intégrative permet d'enrichir les observations morphologiques par une approche moléculaire, principalement via un barcoding moléculaire (DNA-barcoding) (Hebert & Gregory 2005).
- **L'élevage** et l'évaluation de l'efficacité et de la spécificité des ennemis naturels collectés constituent le cœur de la recherche en lutte biologique puisqu'elle conditionne la possibilité d'un lâcher de

l'auxiliaire retenu. L'évaluation de sa gamme d'hôte en absence (test de non-choix) ou en présence de l'espèce cible (test de choix) a notamment pour but d'anticiper autant que possible des effets non-intentionnels sur des espèces non-cibles natives, qui peuvent être d'intérêt écologique, économique ou patrimonial. Les critères de sélection de l'agent auxiliaire seront détaillés dans la section suivante.

- Ces connaissances sur la biologie et la spécificité de l'agent de lutte biologique sont ensuite utilisées pour rédiger une **pétition**, évaluée par les autorités sanitaires du pays d'introduction, qui autorisent ou non l'utilisation de l'agent. Aux Etats-Unis, ce document prend la forme d'une demande d'autorisation de lâcher validée par des experts mandatés par l'APHIS (Animal and Plant Health Inspection Service).
- **La Production de masse et le lâcher** de l'auxiliaire sur le terrain implique ensuite une optimisation de sa production en laboratoire et un contrôle de son développement et sa reproduction. Cela permet de déclencher son émergence au moment voulu, et de maximiser son impact sur l'espèce cible.
- **Le suivi de la population** d'auxiliaire introduite est l'ultime phase d'un programme de lutte biologique. Par des échantillonnages réguliers d'individus, ce suivi vise à s'assurer que la population se maintient correctement dans son environnement, que ce soit en termes d'effectif (survie, reproduction des individus et risques d'effet Allee (Bompard et al. 2013)), de diversité génétique (présence de consanguinité, dérive génétique) ou de sex-ratio.

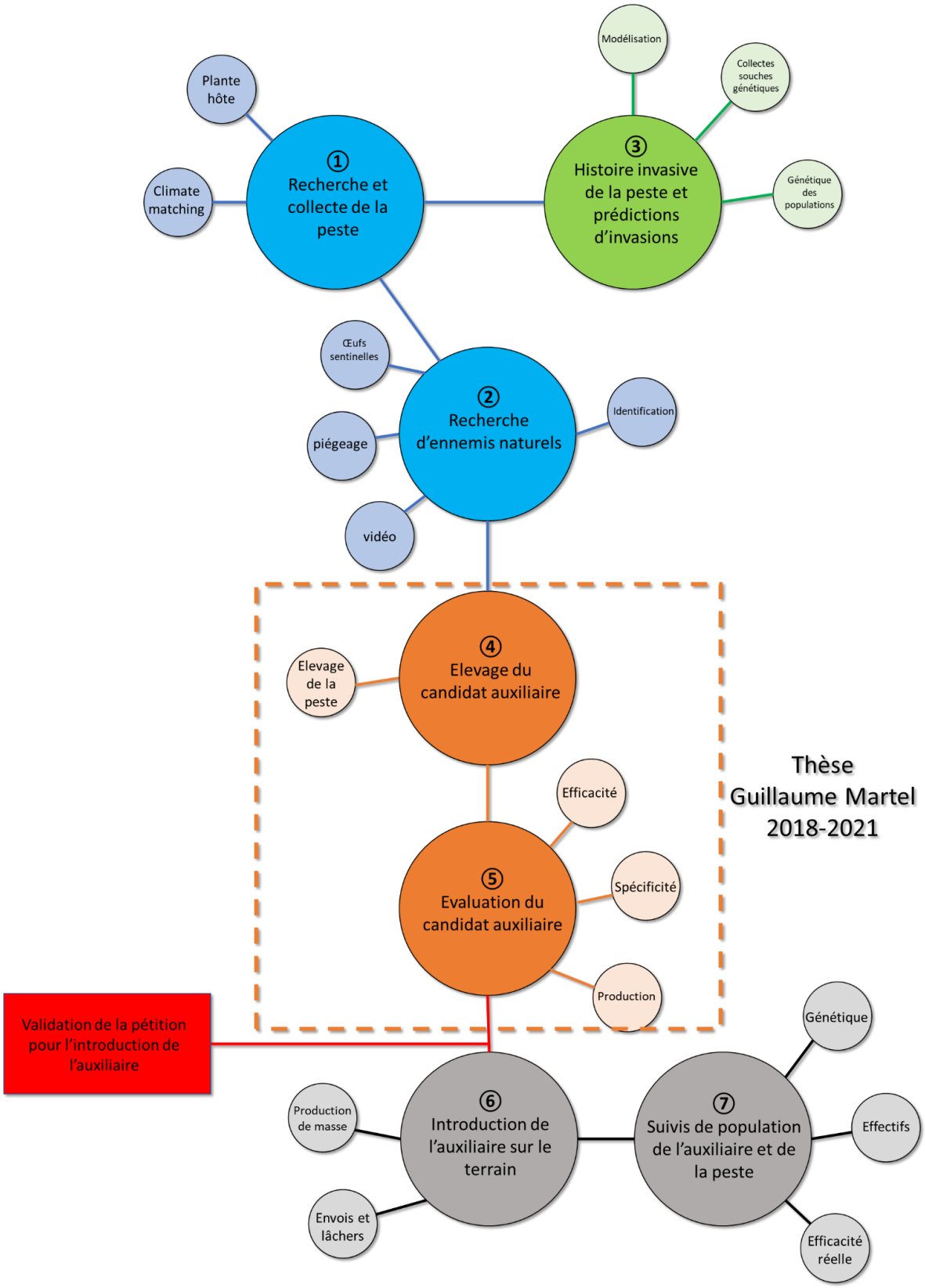


Figure 6 : Principales étapes d'un programme de lutte biologique classique

4. Choisir l'auxiliaire : quels critères de sélection ?

La recherche d'ennemis naturels dans l'aire native d'un insecte ravageur conduit souvent à l'identification de plusieurs espèces. La sélection des meilleurs candidats est donc la première étape avant un lâcher en plein champ. Dans cette section, nous aborderons les principaux critères qui permettent cette sélection, en évaluant à la fois l'efficacité de l'auxiliaire, et les risques potentiels liés à son introduction.

4.1. Reproduction et développement de l'auxiliaire

Comme nous l'avons vu, l'efficacité d'un auxiliaire est déterminée par sa capacité à abaisser l'effectif du ravageur ciblé sous un seuil économique ou écologique déterminé. Pour assurer ce contrôle, l'auxiliaire doit consommer le ravageur plus vite que celui-ci ne se multiplie (Hokkanen 1983). Autrement dit, la population de l'auxiliaire doit s'accroître plus vite que celle du ravageur. D'après Hokkanen & Sailer (1985), les organismes impliqués dans des succès en LBC font en moyenne deux générations pour une génération du ravageur.

Dans une population où les classes d'âges sont à l'équilibre, le taux d'accroissement r est donné par la **relation** $\mathbf{r = \ln(\sum l_x m_x) / T}$ (Birch, 1948) où **x** est l'âge des individus en jours, $\mathbf{l_x}$ le taux de survie de chaque classe d'âge, $\mathbf{m_x}$ le nombre de femelles produites par chaque classe d'âge et **T** le temps de génération. Le taux d'accroissement dépend donc de quatre traits d'histoire de vie : 1) l**e temps de développement** des individus ; 2) le **taux de survie** ; 3) la **fécondité** et **4) la sex-ratio** des individus produits à chaque génération.

Par conséquent, les organismes privilégiés en LBC sont ceux qui maximisent leur fécondité, leur sex-ratio et leur survie (adulte et larvaire) tout en ayant un temps de développement le plus court possible (Hokkanen & Sailer 1985). Les hyménoptères parasitoïdes répondent généralement à ces critères : par exemple, *Trichogramma pretiosum* Riley (Hym., Trichogrammatidae) complète son développement en neuf jours à 25°C tandis que son hôte *Anticarsia gemmatalis* Hübner (Lepidoptera : Erebidae) se développe en 25 jours dans les mêmes conditions (Foerster & Foerster 2009 ; da Silva et al. 2012). *Trissolcus basalis* (Wollaston) (Hym., Scelionidae) utilisé contre *Nezara viridula* L. (Hem., Pentatomidae) a quant à lui une sex-ratio de plus de 60% de femelles (Colazza & Wajnberg 1998).

4.2. Acclimatation et établissement de l'auxiliaire

L'objectif principal d'un programme de LBC est l'établissement de l'auxiliaire dans sa zone d'introduction. Cependant, les nouvelles caractéristiques environnementales abiotiques auxquelles il est confronté (température, humidité, photopériode) peuvent différer de son aire native. Généralement ectothermes, les auxilaires utilisés en LBC subissent particulièrement les variations thermiques de leur environnement qui peuvent avoir des effets importants sur leur survie ou leur reproduction notamment (Régnière et al. 2012). Leur capacité d'acclimatation est donc généralement considérée comme le plus

important facteur de succès des programmes de LBC (Hokkanen & Sailer 1985 ; Stiling 1993 ; Hoelmer & Kirk 2005). Ceci est particulièrement le cas dans les régions tempérées du globe où la capacité de l'auxiliaire à survivre à des températures basses en hiver est un élément décisif de leur établissement sur le long-terme. On comprend alors l'intérêt pour la LBC de collecter l'auxiliaire dans l'aire native du ravageur : soumises à des conditions environnementales identiques, leurs périodes d'activité journalière ou saisonnière sont généralement synchronisées, ce qui maximise leur rencontre spatiale et temporelle. De plus, les chances que l'auxiliaire survive là où le ravageur a réussi à s'établir sont maximisées.

4.3. Efficacité VS spécificité

Depuis les années 1990, la LBC a privilégié l'utilisation d'organismes spécialistes (comme des parasitoïdes) plutôt que des prédateurs généralistes car ils présentent intrinsèquement moins de risques de s'attaquer à des espèces non-cibles (Driesche & Hoddle 2016 ; Taylor & Snyder 2020). La spécificité de l'auxiliaire vis-à-vis du ravageur est donc l'un, si ce n'est le critère ayant le plus de poids dans la validation d'un auxiliaire avant son utilisation, et fait l'objet de tests de spécificités systématiques comme nous l'avons vu dans la section **II.2**.

D'autres part, un auxiliaire doit être capable de localiser son hôte, en particulier lorsque la densité de ce dernier est faible (Hokkanen & Sailer 1985 ; Fernandez-Arhex & Corley 2003). Il doit donc présenter des capacités adaptatives (morphologiques ou comportementales) qui lui permettent de détecter, atteindre et exploiter l'espèce cible efficacement. Du fait d'une coévolution souvent étroite avec leur proie (ou leur hôte pour les parasitoïdes), les auxiliaires spécialistes sont plus adaptés à contrôler un ravageur à faible densité de population tout en maintenant leur propre effectif (Hokkanen & Sailer 1985).

La nécessité qu'un auxiliaire soit spécifique est cependant questionnable du point de vue de son efficacité (Taylor & Snyder 2020). En effet, les prédateurs généralistes diminuent significativement l'abondance d'une peste dans 75% des cas (Symondson et al. 2002 ; Stiling & Cornelissen 2005), ce qui s'explique par leur capacité à se nourrir de proies alternatives, et donc de moins subir les variations démographiques de leur proie principale. Ils peuvent par conséquent se maintenir plus facilement dans l'environnement, ce qui peut amener à des extinctions locales du ravageur sans forcément une diminution de l'auxiliaire.

4.4. Dynamiques populationnelles : viser l'équilibre

Comme nous l'avons vu précédemment, la dynamique populationnelle de l'auxiliaire est une des clés du succès d'un programme de lutte biologique. Pour atteindre à nouveau l'équilibre perdu par le « relâchement des ennemis naturels » sur le long-terme, il est toutefois nécessaire que l'auxiliaire et le ravageur puissent tous les deux se maintenir dans l'environnement. Cela est théoriquement possible

si les deux espèces atteignent un point d'équilibre dans leur relation proie-prédateur ou hôte-parasitoïde (Mills & Getz 1996). A l'équilibre, la densité de population des deux espèces doit pouvoir osciller entre deux extrêmes : 1) une densité forte de l'auxiliaire qui entraîne une réduction de la densité de population du ravageur jusqu'à 2) une densité faible du ravageur qui entraîne une diminution de la reproduction de l'auxiliaire. Ce sont ces oscillations qui sont représentées sur la figure 4.

La modélisation de ces relations proie-prédateur ou hôte-parasitoïde a fait l'objet de nombreuses mises à jour, sur la base de deux principaux modèles : Lotka-Volterra et Nicholson-Bailey (Mills & Getz 1996 ; Fernandez-arhex & Corley 2003). Ces modèles (que nous ne présenterons pas ici) intègrent notamment le temps de recherche du ravageur par l'auxiliaire. Certains facteurs peuvent favoriser l'atteinte du point d'équilibre recherché, et peuvent donc être des critères de sélection d'un auxiliaire. On peut notamment citer :

- Une réponse fonctionnelle densité-dépendante (de type III) : ce type de relation implique notamment qu'à faible densité du ravageur, ce dernier est plus difficile à trouver pour l'auxiliaire, qui exerce donc une pression de prédation moins importante.
- La capacité du ravageur et de l'auxiliaire à se maintenir à faible densité
- L'existence de refuges pour le ravageur qui le rendent « invulnérables » à l'auxiliaire : par exemple, une barrière physique limitant la prédation / le parasitisme (Weis et al. 1985).
- Une sex-ratio densité dépendante chez l'auxiliaire, qui contribue à limiter la croissance de sa population et une éradication du ravageur.

4.5. Vers une production de masse

La production d'un auxiliaire en grandes quantités est souvent un challenge et peut impacter la faisabilité de la LBC (Fauvergue et al. 2020). Par exemple, la micro-guêpe *Torymus sinensis* Kamijo (Hym., Torymidae), n'a jamais pu être totalement produite au laboratoire et a dû être collectée massivement sur le terrain pour pouvoir être utilisée avec succès contre le cynips du châtaignier en Europe de l'Ouest (Borowiec et al. 2018 ; Ferracini et al. 2019). De plus, les auxiliaires prédateurs ou parasitoïdes nécessitent d'assurer la production de trois niveaux trophiques : une ressource végétale ou un milieu nutritif pour un insecte phytophage, l'insecte phytophage, et l'auxiliaire.

Bien que la mise en élevage d'un auxiliaire soit l'une des premières étapes d'un programme de LBC, l'optimisation de sa production en masse est souvent considérée plus tardivement. Par exemple, les premiers travaux pour optimiser l'élevage et la production de *Trissolcus japonicus* Ashmead (Hym., Scelionidae) sur *H. halys* datent de 2019 (McIntosh et al. 2019) tandis que le parasitoïde est considéré comme le candidat auxiliaire privilégié contre cette punaise depuis 2015. Pourtant, l'impossibilité de produire l'auxiliaire efficacement est un frein à son utilisation autant qu'un auxiliaire inefficace ou non-spécifique (Fauvergue et al. 2020). Plusieurs points sont donc à valider :

- L'hôte doit pouvoir être produit en grandes quantités en laboratoire
- Dans le cas contraire, un hôte de substitution doit pouvoir être utilisé. Par exemple, *Psyttalia lounsbury* Silvestri (Hym., Braconidae) produite en masse à l'EBCL pour des lâchers contre *Bactrocera oleae* Rossi (Dipt., Tephritidae) en Californie est élevée sur *Ceratitis capitata* Wiedemann (Dipt., Tephritidae) (Chardonnet et al. 2019).
- L'auxiliaire doit bien entendu pouvoir être produit en grande quantité. Par exemple, plus de 100 000 individus de *P. lounsbury* ont été produits à l'EBCL entre juillet et Novembre 2017 pour des lâchers réguliers en Californie sur cette période (Chardonnet et al. 2019).

La production en masse de l'auxiliaire n'est toutefois pas suffisante. La possibilité de le produire et l'utiliser au moment voulu, en synchronisant leur émergence au champ par exemple, est un des principaux défis à relever (Fauvergue et al. 2020). Cela nécessite notamment de pouvoir contrôler la vitesse de développement des individus, ou de pouvoir stocker (au froid par exemple) l'hôte et/ou l'auxiliaire jusqu'à leur utilisation (Wong et al. 2021). Une bonne connaissance de la biologie de l'hôte et de l'auxiliaire sont donc nécessaire en amont.

5. Succès en lutte biologique : quel bilan ?

5.1. Le succès des auxiliaires sélectionnés : quelle réalité sur le terrain ?

Une grande partie des auxiliaires introduits ne parvient pas à s'établir durablement, et ceux qui y parviennent ne garantissent pas un contrôle de l'espèce invasive ciblée (Fig. 7). Parmi les 6158 introductions d'insectes auxiliaires (2384 espèces) entre 1880 et 2010, seulement 10% a permis un contrôle du ravageur ciblé (Cock et al. 2016). Ce constat s'observe aussi à l'échelle d'un seul programme de LBC : contre le Carpocapse du pommier *Cydia pomonella* par exemple, de nombreuses espèces de parasitoïdes attaquant différents stades de développement du papillon ont été introduits dans de nombreuses régions du monde. Mais seulement deux ont réussi à s'établir en Australie (*Trichogramma minutum* Riley (Hym., Trichogrammatidae) et aux Etats-Unis (*Ascogaster quadridentatus* Wesm. (Hym., Braconidae)) (Cross et al. 1999). Toutefois, la proportion d'introductions menant à des succès a augmenté alors que le nombre d'introductions a diminué depuis les années 1990, ce qui reflète une meilleure sélection des auxiliaire (Cock et al. 2016).

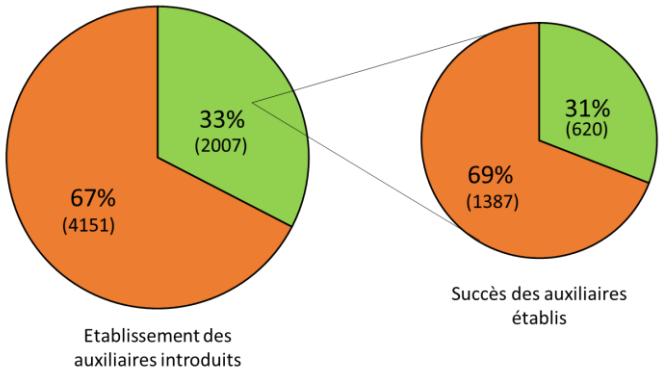


Figure 7 : Proportion d'introductions d'insectes auxiliaires menant à un contrôle efficace d'insectes nuisibles parmi toutes les introductions réalisées entre 1880 et 2010 (d'après Cock et al. 2016). Les nombres entre parenthèses correspondent aux cas comptabilisés d'introductions ayant abouti (en vert) ou échoué (en orange) à l'établissement de l'auxiliaire (graphique de gauche) ou son succès (graphique de droite).

En outre, la faible proportion de succès en LBC a tout de même permis un contrôle efficace d'au moins 220 espèces d'insectes invasifs depuis les années 1880 (Wyckhuys et al. 2018). De nombreux exemples de programmes ayant abouti à une régulation à long-terme d'insectes invasifs peuvent être cités (Greathead 1995), parmi lesquels le contrôle de la cochenille du manioc *Phenacoccus manihoti* Matile-Ferrero (Hem., Pseudococcidae) par le parasitoïde *Anagyrus lopezi* De Santis (Hym., Encyrtidae) en Afrique (Gutierrez et al. 1998 ; Neuenschwander et al. 1988) ou plus récemment le contrôle complet de la cicadelle *Homalodisca vitripennis* Germar à Tahiti (Grandgirard et al. 2008) ou le contrôle de *Dryocosmus kuriphilus* Yasumatsu (Hym., Cynipidae) par *Torymus sinensis* au Japon (Moriya et al. 2003) qui a motivé son introduction récente en Italie et en France (Borowiec et al. 2018).

A défaut de permettre systématiquement le contrôle d'un ravageur, la LBC peut *a minima* permettre de déstabiliser sa dynamique populationnelle. Par conséquent, cette stratégie peut être intégrée à d'autres outils de gestion des insectes nuisibles telles que l'utilisation de pièges à phéromones ou de cultures pièges (Cook et al. 2007), comme nous le verrons dans la partie **III.3**.

5.2. Bénéfices économiques de la lutte biologique contre les insectes

Les contrôles permanents d'insectes nuisibles obtenus avec la LBC ont eu des retombées économiques positives à travers le monde (Cock et al. 2016 ; Naranjo et al. 2015). Le ratio moyen bénéfices/coûts des programmes de lutte biologique à travers le monde est de plus de 61 pour 1, avec des bénéfices nets allant de 150 000 à plus de 9 Mds de dollars (Oleke et al. 2013).

Par exemple, le contrôle d'*Erionota thrax* L. (Lep., Hesperiidae) par *Cotesia erionotae* Wilkinson (Hym., Braconidae) entre 1990 et 1998 dans les cultures de banane en Papouasie Nouvelle Guinée a permis de multiplier les rendements par dix (Waterhouse et al.1998 ; Wyckhuys et al. 2020). De même, le contrôle de *P. manihoti* avec *A. lopezi* en Thaïlande a permis de plus que tripler la production de manioc (Thancharoen et al. 2018 ; Wyckhuys et al. 2018, 2020).

Aux Etats-Unis, le contrôle biologique d'insectes herbivores ou vecteurs de maladies pour les plantes représente un gain annuel évalué à 4.5 Mds de dollars pour l'agriculture (Losey and Vaughan 2006). En Californie par exemple, Le parasitoïde *Encarsia inaron* Walker (Hym., Aphelinidae) a été introduit contre l'aleurode *Siphoninus phillyreae* Haliday (Hem., Aleyrodidae) responsable de la défoliation d'espèces ornementales et d'arbres fruitiers (Bellows et al. 1992 ; Paine et al. 2003). Ce programme de lutte biologique a été estimé à 1.2 M de dollars, mais en une dizaine d'années, les bénéfices ont été estimés entre 324 et 412 M de dollars et continuent de s'accroître (Jetter et al. 1997 ; Paine et al. 2003). Un exemple parmi les plus spectaculaires est celui de l'introduction du parasitoïde *Coccophagus gurneyi* Compere (Hym., Aphelinidae) contre la cochenille des agrumes *Pseudococcus fragilis* (Hem., Pseudococcidae) en Californie, ce qui a permis d'épargner plus de 172 M de dollars pour un coût initial estimé à 24 000 dollars (Gutierrez et al. 1999).

Il peut également être pertinent de mettre les ratios bénéfices / coûts des programmes de lutte biologique classique en perspective des performances de l'industrie agro-chimique qui ne sont pas forcément plus enviables. En effet, la découverte et la commercialisation de nouveaux insecticides peuvent avoisiner les 300 M de dollars pour une durée de développement de près de 10 ans (Sparks & Lorsbach 2017 ; Sparks et al. 2019). Cependant, il faut considérer un possible biais dans l'estimation des bénéfices attribuables à des programmes de lutte biologique puisque moins de 1% d'entre eux ont fait l'objet d'une étude économique, comme le soulignent Naranjo et al. (2019).

III. Gestion des punaises pentatomidés invasives aux Etats-Unis

La gestion des punaises invasives, des plus anciennes aux plus récentes fait l'objet de recherches très actives à travers le monde. Le bilan de ces recherches est rapporté dans **l'Article 7 (Annexe 1)** qui traite notamment des travaux menés en Amérique du sud et en Europe. La présente section se focalisera uniquement sur le cas des Etats-Unis qui nous intéresse dans le cadre de cette thèse.

1. De nouvelles invasions

Les Pentatomidae représentent la plus grande famille des Pentatomoidea (Super-Famille) avec près de 5000 espèces. Les Pentatominae constituent la principale sous-famille avec 660 genres et près de 3500 espèces présentes sur tous les continents (McPherson 2018), dont celles qui nous intéressent dans cette partie.

Aux Etats-Unis, cinq punaises pentatomidés sont considérées comme invasives et ravageurs d'une grande variété d'agrosystèmes : *Halyomorpha halys*, *Murgantia histrionica* Stal, *Nezara viridula*, *Piezodorus guildinii* (Westwood) et *Bagrada hilaris* (McPherson 2018) (Tableau 2). Il existe toutefois d'autres espèces natives considérées comme nuisibles, comme *Chinava hilaris* (Say) ou *Euschistus servus* (Say), des ravageurs du soja (Pezzini et al. 2019). Le succès invasif de ces punaises repose principalement sur leur régime polyphage, leur capacité à survivre dans des conditions

environnementales défavorables, le réchauffement climatique et la réduction de la fréquence d'application des pesticides à large spectre (McPherson 2018).

Tableau 2 : Punaises pentatomidés invasives aux Etats-Unis, d'après McPherson 2018

Espèce		Aire d'origine	1er signalement	Plante hôte
Murgantia histrionica		Amérique centrale, Mexique	1864, Comté de Whashington (Texas)	Brassicacées
Nezara viridula		Asie, Afrique	1880, Texas	Légumineuses
Halyomorpha halys		Asie orientale	1996, Allentown (Pennsylvanie)	Légumineuses, arbres fruitiers
Bagrada hilaris		Asie, Afrique	2008, Los Angeles (Californie)	Brassicacées
Piezodorus guildinii		Amérique centrale, Caraïbes, Brésil	1960, Everglades (Floride)	Légumineuses

2. Biologie des pentatomidés et conséquences agronomiques

La très grande majorité des pentatomidés sont des phytophages de la guilde des punaises piqueurs-suceurs de sève. Elles se nourrissent de plantes hôtes variées comme le coton, les haricots ou la tomate pour *N. viridula*, les arbres fruitiers (pommiers, poiriers, pêchers, etc.) pour *H. halys* ou bien les cultures de chou et de brocoli pour *M. histrionica* (McPherson 2018). Ces espèces sont actives toute l'année, mais peuvent marquer une diapause hivernale. Au cours de leur période d'activité, elles restent le plus souvent sur leur plante hôte pour se nourrir, s'accoupler et pondre. Leurs œufs, de forme cylindrique, sont pondus sur diverses parties de la plante hôte (feuilles, siliques, tige, boutons floraux, etc.) et agencés en ooplaques de diverses tailles et formes (Bundy & McPherson 2000 ; Matesco et al. 2009) : par exemple, *Eurydema ornata* L. dépose ses œufs en deux rangées de six (Fig. 8B) tandis que *Graphosoma italicum* Muller forme des ooplaques subhexagonales d'environ quatorze œufs (Fig. 8D). Cinq stades larvaires se succèdent de l'œuf à l'adulte, et la plupart des espèces sont multivoltines (McPherson 2018).

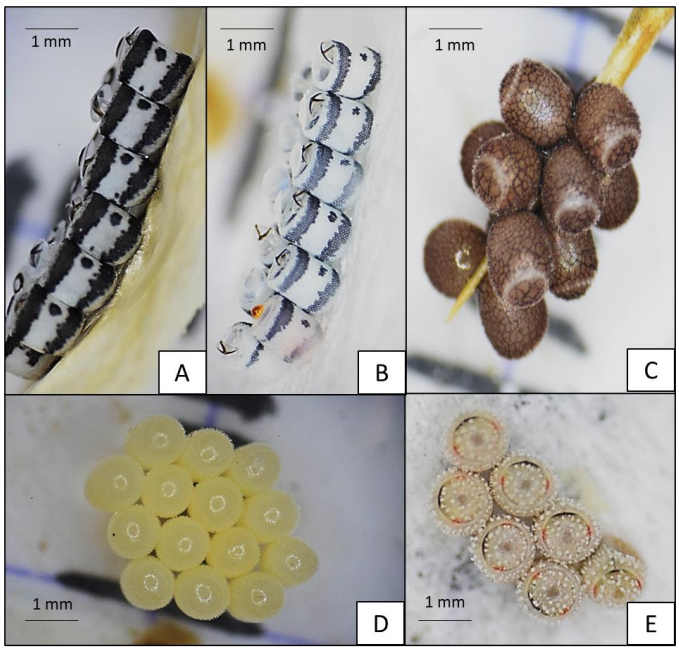


Figure 8 : Ooplaques de cinq espèces de pentatomidés. (A) *Eurydema ventralis* ; (B) *Eurydema oleracea* ; (C) *Carpocoris mediterraneus* ; (D) *Graphosoma italicum* ; (E) *Ancyrosoma leucogrammes*. Photos par Martel G.

Presque aucun agrosystème n'échappe à ces ravageurs et les enjeux économiques sont considérables. En 2010 par exemple, les dégâts occasionnés par *H. halys* sur la production de pommes dans les états de la côte Atlantique étaient estimés à 37 M de dollars et plus de 90% de pertes ont été enregistrées dans certains vergers de pêches (Leskey et al. 2014 ; McPherson 2018). Dans la partie Sud des Etats-Unis, les coûts attribués aux punaises dans la culture du coton représentaient près de 70 M de dollars en 2014 (Williams 2015). En plus des dégâts occasionnés par leur piqûre, les punaises peuvent également être vectrices de phytopathogènes comme la bactérie *Pantoea agglomerans*, responsable du « feu bactérien » qui entraîne la flétrissure des bouquets floraux et empêche la formation de fruits (Medrano et al. 2009).

3. Quelles méthodes de gestion ?

3.1. Un usage généralisé des pesticides

Aux Etats-Unis, les populations de punaises invasives sont principalement régulées par des insecticides à large spectre (Temple et al. 2013 ; Sosa-Gomez et al. 2020). Par exemple, L'utilisation de pyréthrinoïdes et néonicotinoïdes depuis les années 1950 a permis de contrôler la punaise *M. histrionica* dans les cultures de brassicacées (Kuhar & Doughty 2009 ; McPherson 2018). D'autres composés comme les organophosphates restent aujourd'hui la méthode la plus efficace pour réguler *N. viridula* (Tillman 2006) et *P. guildinii* dans les cultures de soja (Temple 2011). Il est pourtant souhaitable que l'application de ces insecticides soit réduite ou supprimée pour des raisons de santé humaine, économiques et écologiques. De plus, l'application régulière d'insecticides est la source d'une pression de sélection très importante sur les populations d'arthropodes en général (Hajek & Eilenberg 2018).

Cela peut donc être un moteur de l'apparition de résistances comme été observé chez *Euschistus heros* (F.) ou *Dichelops melacanthus* Dallas au Brésil (Sosa-Gomez et al. 2001 ; Sosa-Gomez et al. 2020).

3.2. Principales stratégies de gestion intégrées

Plusieurs approches peuvent être envisagées pour limiter l'utilisation de pesticides de synthèse. Souvent complémentaires, elles reposent sur 1) la manipulation du comportement du ravageur, 2) la lutte biologique et 3) la conservation d'organismes entomophages dans l'écosystème (Fig. 10).

3.2.1. Les cultures pièges

Le principe des « cultures pièges » est d'utiliser une plante attractive pour le ravageur dans le but de le détourner de la culture que l'on souhaite protéger (Hokkanen 1991). Cet hôte alternatif peut être intercalé au sein de la culture (on parle alors de culture intercalée, ou « inter-cropping ») ou en périphérie. Dans les cultures biologiques de soja du sud des Etats-Unis par exemple, l'impact de *N. viridula* et *E. servus* peut être diminué grâce à la plantation de triticale (*Triticum secale* Link) ou de vesce velue (*Vicia villosa* Roth) au printemps, puis du sorgho ou du sarrasin (*Fagopyrum esculentum* Moench) en été et à l'automne (Mizell et al. 2008). Des stades immatures de la plante cultivée, parfois plus attractifs que les plantes matures, peuvent aussi être utilisés comme culture piège. C'est le cas des jeunes pousses de soja, qui peuvent être plantées à proximité de plantes plus âgées pour piéger *N. viridula* (Todd & Schumann 1988 ; McPherson 2018). Cependant, l'efficacité des plantes pièges ne garantit pas forcément une diminution de la densité du ravageur au sein de la parcelle cultivée, et la période d'attractivité est limitée comme cela a été montré avec *M. histrionica* ou *H. halys* (Ludwig & Kok 1998 ; Mathews et al. 2017). Hormis des essais concluants contre *N. viridula*, l'utilisation de cultures pièges demeure donc marginale aux Etats-Unis.

3.2.2. Méthode push-pull

Les stratégies « push-pull » ont pour objectif de modifier la distribution et l'abondance d'un ravageur pour l'écarter d'une ressource à protéger. Cette méthode peut également être utilisée pour attirer un auxiliaire dans une culture attaquée par un ravageur. Les stratégies push-pull impliquent simultanément plusieurs stimuli répulsifs (effet « push ») et attractifs (effet « pull ») qui vont modifier le comportement des insectes (Cook et al. 2007). Ces stimuli sont essentiellement visuels ou sémiochimiques, comme des phéromones sexuelles et d'agrégation, ou répulsives comme des phéromones d'alerte (Fig. 9).

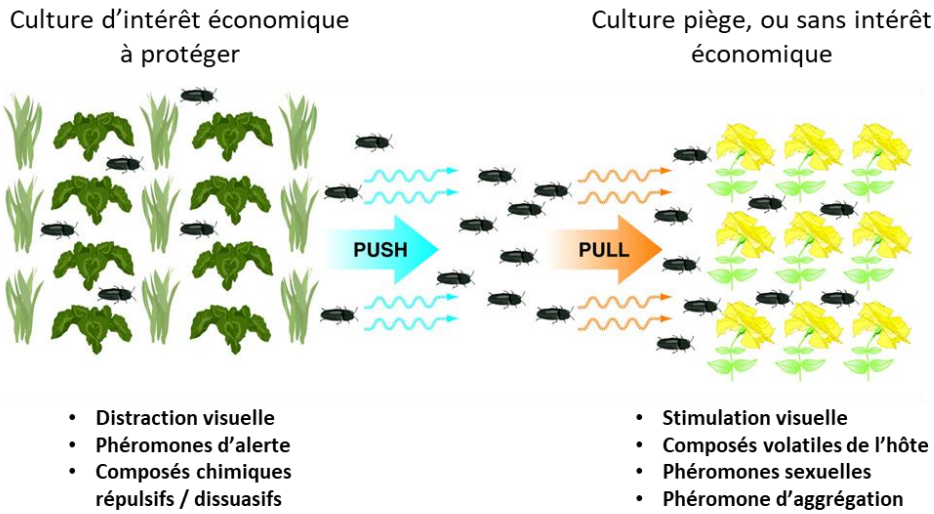


Figure 9 : Fonctionnement général de la stratégie push-pull (adapté de Cook et al. 2007)

L'utilisation de cultures pièges peut être une composante d'une stratégie push-pull : couplée à des pièges à phéromones attractifs, la plantation de sorgho (*Sorghum bicolor* (L.)) permet par exemple de réduire la densité et la dispersion *E. servus* dans les champs de coton. (Cook et al. 2007). La plupart des combinaisons de méthodes push-pull restent cependant au stade expérimental et aucun pentatomidé invasif n'est efficacement régulé de cette manière pour le moment (McPherson 2018).

Les pièges à phéromones seuls s'avèrent quant à eux globalement inutilisés pour réguler les populations de pentatomidés dans les cultures : cette méthode est par exemple inefficace contre *N. viridula* (McPherson 2018) et n'a pas encore été utilisée sur *H. halys* dont les phéromones d'agrégation n'ont été caractérisées que récemment (Khrimian et al. 2014 ; Rice et al. 2018). L'utilisation de murgantiol (phéromone d'agrégation) pour attirer *M. histriconica* s'est avérée concluante (Zahn et al. 2008 ; Wallingford 2012 ; Weber et al. 2014) mais pas au point d'être intégrée aux pratiques de gestion déjà existantes.

3.2.3. Quelle place pour la LBC contre les pentatomidés invasifs ?

L'utilisation de la LBC a été principalement envisagée pour réguler deux pentatomides invasives au cours du 20e siècle : *N. viridula* et *M. histrionica*.

Entre 1987 et 1989 *Trissolcus basalis* a été introduit à Hawaï pour réguler *N. viridula*, ainsi que des mouches tachinaires du genre *Trichopoda*. Ces deux parasitoïdes ont globalement été un succès en réduisant les populations de la punaise, mais ce succès s'avère finalement mitigé à long-terme puisque les dégâts occasionnés par *N. viridula* sont toujours au-dessus du seuil économique à Hawaii (Abram et al. 2020). Contre *M. histrionica*, la LBC, n'a pas été particulièrement efficace et marginalement utilisée au cours du 20e siècle. *Trissolcus murgantiae* Ashmead (Hym., Scelionidae) a été introduit en Californie (De Bach 1942) et *Ooencyrtu. johnsoni* et *Trissolcus brochymenae* Ashmead (Hym., Scelionidae) ont

été introduits dans les années 1940 à Hawaii. Ces derniers se sont établis mais leur efficacité à contrôler *M. histrionica* n'a jamais été démontrée (Abram et al. 2020). Contrairement à *N. viridula*, *M. histrionica* s'est avérée peu impactée par l'utilisation de mouches tachinaires, et les parasitoïdes natifs comme *Telenomus podisi* Ashmead (Hym., Scelionidae) et *T. murgantiae*, ont quant à eux montré des taux de parasitisme trop faibles et donc aucune chance de contrôle de la punaise (Koppel et al. 2009 ; McPherson 2018).Aujourd'hui, les efforts en lutte biologique classique sont tournés vers le contrôle de *H. halys* qui est devenu une peste mondiale au cours des années 2010. Le scélionide *T. japonicus* est le principal ennemi naturel de *H. halys* en Asie et est donc considéré comme un agent de lutte biologique prometteur (Haye et al. 2020), d'autant plus que ce parasitoïde est désormais établi aux Etats-Unis (Talamas et al. 2015)

Finalement, la LBC s'est avérée généralement inefficace pour maintenir les dégâts subis par les cultures en dessous du seuil économique sur le long-terme (McPherson 2018 ; Abram et al. 2020). La raison principale est le manque d'établissement des auxiliaires utilisés et le maintien d'une lutte chimique très importante, efficace et incompatible avec une régulation biologique des punaises. C'est pourquoi l'utilisation de parasitoïdes comme agents de lutte biologique doit être intégrée à d'autres méthodes de gestion des agrosystèmes tels que ceux qui ont été présentés précédemment

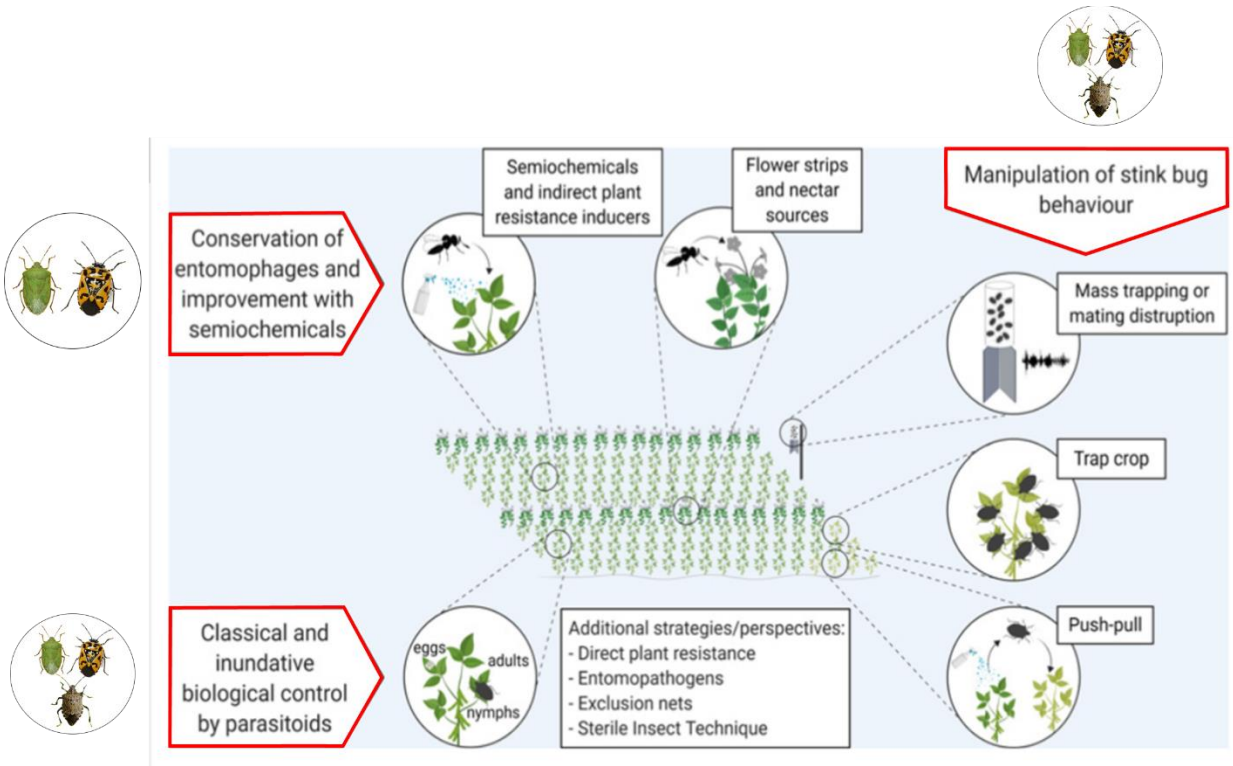


Figure 10 : Principales méthodes de gestion intégrée des punaises pentatomidés aux Etats-Unis et espèces associées : *Nezara viridula* ; *Halyomorpha halys* ; *Murgantia histrionica* (adapté de Conti et al. 2021)

IV. *Bagrada hilaris* : un problème ancien dans le Nouveau Monde

1. Taxonomie

1.1. Phylogénie

Bagrada hilaris (Burmeister) est un hémiptère Pentatomidae de la tribu des Strachinii, qui contient 20 genres et 142 espèces (McPherson 2018). Le genre *Bagrada* comprend 16 espèces, distribuées exclusivement en zones paléarctique, afrotropicale et indomalaise, allant de l'Inde à l'Afrique du Sud. Cette espèce est à ce jour la seule représentante de son genre à avoir colonisé les Amériques (cf. **IV. 2**).

1.2. Nomenclature

Sa dénomination binomiale a été révisée de nombreuses fois entre 1835 (décrite initialement comme *Cimex hilaris* Burmeister) et 1865, Dans la littérature scientifique et de vulgarisation, *Bagrada hilaris* (Burmeister) peut encore être trouvée sous d'anciennes appellations, comme *Bagrada picta* F. ou *Bagrada cruciferarum* Kirkaldy notamment dans les pays d'Asie du Sud comme l'Inde et le Pakistan et généralement avant les années 2000 (McPherson 2018). Aujourd'hui, *Bagrada hilaris* (Burmeister) est la nomenclature officielle. Dans le langage courant, une grande variabilité existe également : « mustard painted bug » en Inde, « caper bug » en Italie, « bagrada bug » ou « painted bug » aux Etats-Unis.

Du fait de son absence du territoire Français, aucune appellation vernaculaire n'existe à ce jour. Ainsi, dans ce manuscrit, « bagrada bug » a été choisi pour les articles en anglais et « bagrada » ou « punaise bagrada » pour la partie française.

1.3. Morphologie

Au stade adulte, bagrada mesure entre 6 et 8 mm de long, et présente un dimorphisme sexuel de taille qui permet généralement de distinguer les femelles qui sont plus grosses que les mâles (Reed et al. 2013). Les hémélytres, le pronotum et le scutellum sont de couleur noir et présentent des motifs rouges et orange bordés de blanc tandis que le corps est entièrement noir (Fig. 11A, B). Des variations peuvent toutefois être observées, certains individus présentant parfois des coloris plus ternes, voire presque entièrement noir. Ce sont les motifs qu'elle arbore qui lui ont valu son nom de « painted bug », mais qui sont également à l'origine de confusion aux Etats-Unis avec *M. histrionica* notamment, qui arbore des couleurs semblables (Fig. 10C) et partage le même habitat. En France, on pourrait confondre facilement bagrada avec les espèces *Eurydema ornata*, *E. oleracea* ou *E. ventralis*, très semblables en taille et en coloris (Fig. 10C).

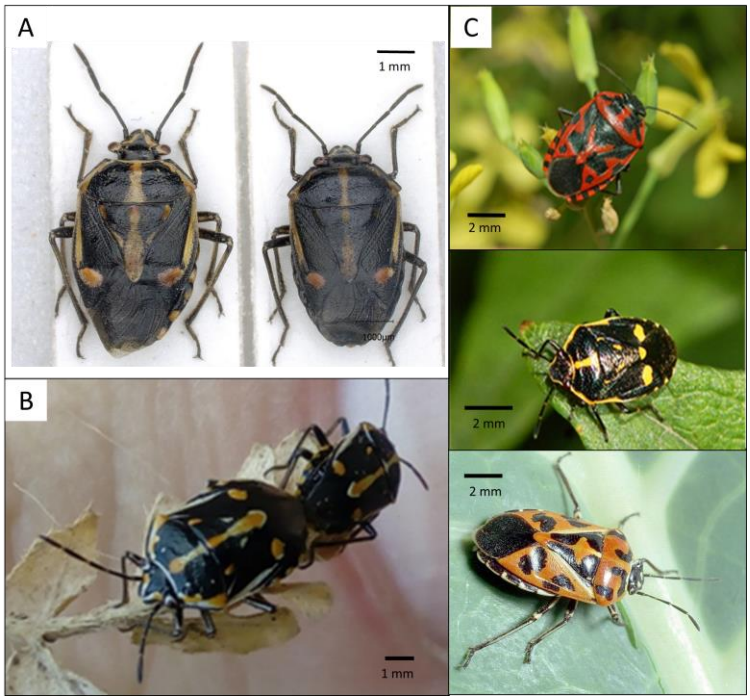


Figure 11 : Photos de *Bagrada hilaris* et comparaison avec d'autres punaises de la tribu Strachinii sur chou. (A) *B. hilaris* mâle (à droite) et femelle (à gauche). (B) copula de *B. hilaris*. (C) punaises pentatomides de la tribu Strachinii, de haut en bas : *Eurydema ventralis, Eurydema oleracea, Murgantia histrionica*. Photographies : (A) Simonot O., (B) Martel G., (C) photographies libres de droit.

2. Origine et distribution

Comme nous l'avons vu dans la section **I.1.**, la plupart des espèces invasives aux USA sont originaires d'Asie, ce qui est également le cas de bagrada. L'aire native de cette punaise couvre une large portion de l'Afrique de l'Est et du Sud, et de l'Asie du sud-ouest y compris le proche et moyen orient (Fig. 12). En Europe, deux populations invasives sont issues d'une colonisation des îles de Malte et Pantelleria (Sicile, Italie) à la fin des années 1970 (CAB 1981 ; Palumbo et al. 2016 ; Arif et al. 2020).

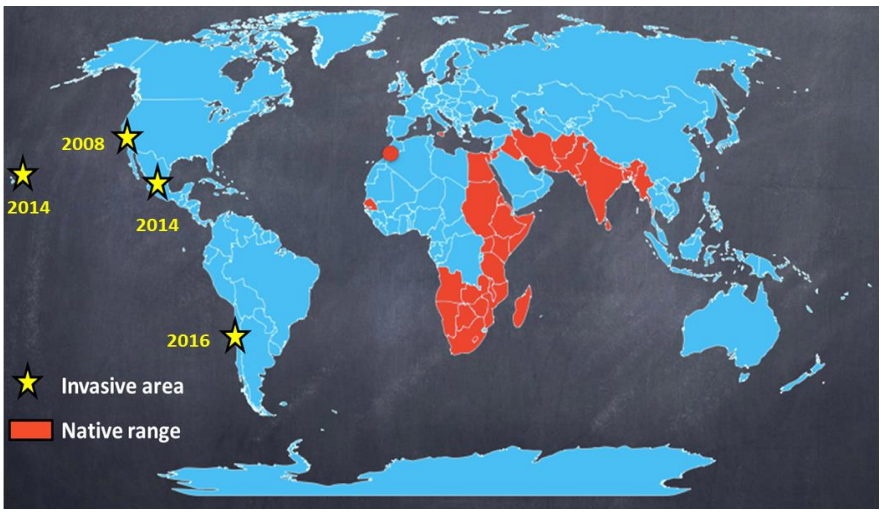


Figure 12 : Distribution de *Bagrada hilaris* dans son aire native (en rouge) et invasive (étoiles) (Sforza et al. 2017)

2.1. De l'aire native aux Amériques : histoire d'une invasion

2.2.1. Chronologie

La punaise bagrada a été détectée pour la première fois en Amérique du Nord à Los Angeles (Californie) en 2008 (Palumbo & Natwick 2010). Dès 2010, son invasion s'étend aux états voisins de l'est (Fig. 13) (Bundy et al. 2012 ; Reed et al. 2013 ; Palumbo et al. 2016). Le Texas s'ajoute à la liste en 2012, suivi par Hawaii en 2014 (McPherson 2018). Cette même année, bagrada est détectée au Mexique où elle poursuit son invasion (Sánchez-Peña 2014). C'est en 2016 à Santiago au Chili (Faundez 2016) qu'elle est détectée en Amérique du Sud pour la première fois et se répand rapidement à travers le pays en étendant son territoire de près de 650 km en 2 ans (Faundez et al. 2018).

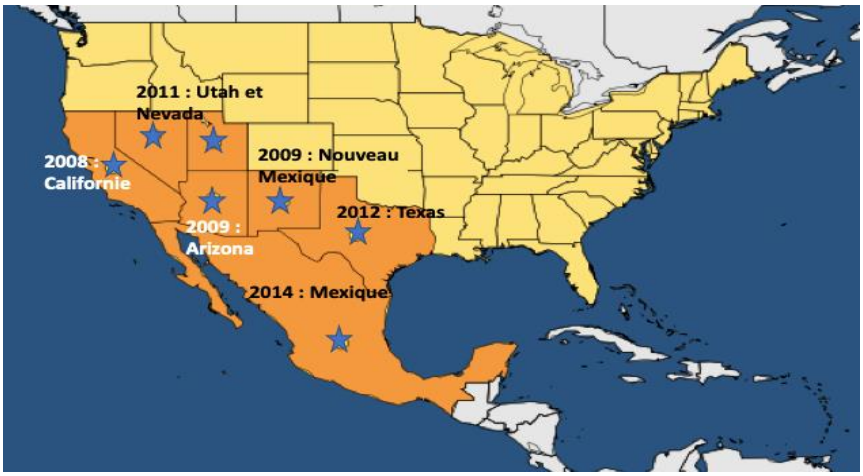


Figure 13 : Chronologie de l'invasion de *Bagrada hilaris* (Hem : Pentatomidae) aux États-Unis, depuis sa signalisation en Californie en 2008. Fond de carte extrait de l'EPPO, (https://gd.eppo.int/taxon/BAGRHI/distribution)

2.2.2. Origine de l'invasion

La voie d'introduction de bagrada en Californie n'a pas été déterminée avec certitude, mais il est très probable qu'elle soit arrivée via des conteneurs de marchandises, puis disséminée par les routes de transports dans les états voisins (McPherson 2018). En revanche, son origine phylogéographique a pu être établie par une analyse du locus CO1 (Sforza et al. 2017). Le réseau d'haplotypes constitué à partir de spécimens appartenant à différentes populations montre que ceux collectés aux Etats-Unis font partie du même haplotype (Fig. 14). Ce dernier est plus proche de l'un des haplotypes les plus prévalents du Pakistan qui serait donc l'origine géographique de l'invasion Californienne (Fig. 14). Les spécimens provenant du Kenya, de l'Afrique du Sud et du Maroc sont quant à eux génétiquement plus distants des spécimens américains.

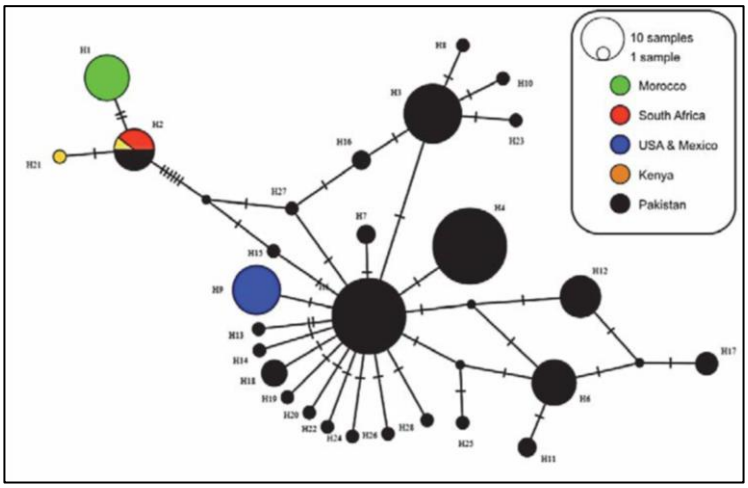


Figure 14 : Réseau d'haplotypes illustrant la distance génétique entre différentes populations de *Bagrada hilaris*. Chaque cercle représente un haplotype différent, la couleur correspond au pays, le diamètre des cercles est proportionnel à l'abondance et les hachures croisées indiquent le nombre de mutations (changements de nucléotides) entre les haplotypes (Sforza et al. 2017)

2.2. Invasions futures

Bagrada est aujourd'hui régulièrement interceptée en Floride (Etats-Unis) par les autorités de protection des plantes, et son établissement dans le Sud-Est du pays est donc un risque imminent (McPherson 2018 ; Pinkerton et al. 2020). Sur le continent Européen où elle est encore absente, bagrada serait susceptible de s'établir dans les régions côtières du pourtour méditerranéen qui partage avec la Californie un climat chaud et sec (voire aride) la plupart de l'année, et des hivers doux propices au développement de bagrada. Ainsi, le Portugal, l'Espagne, l'Italie et la côte Turque sont des régions qui pourraient voir arriver bagrada dans les années à venir (Carvajal et al. 2019).

3. Biologie et niche écologique : entre classicisme et originalité

3.2. Cycle de développement

Bagrada a un développement hétérométabole avec cinq stades larvaires (L1 à L5) (Fig. 15) qui durent en moyenne 35 jours à 25°C de l'œuf à l'imago (Reed et al. 2017). Bien que son développement soit possible entre 16 et 40°C, l'optimum thermique se situe à 33°C, température à laquelle le taux d'émergence est le plus élevé (86%) pour un développement complet de 16 jours (Reed et al. 2017).

Après éclosion, les larves L1 restent généralement à proximité de l'oeuf (Atwal 1959) et ne se nourrissent pas (Reed et al. 2013 ; Grettenberger & Joseph, 2019). C'est à partir du stade L2 que les larves commencent à chercher une source de nourriture (Grettenberger & Joseph 2019). D'abord entièrement rouge à l'éclosion, la mélanisation de certaines parties du corps (pattes et antennes, pronotum, abdomen) s'effectue durant les mues jusqu'au stade L5 caractérisé par l'apparition de fourreaux alaires et de marques blanches le long de l'abdomen. Bagrada est une espèce multivoltine, mais le nombre de générations *in natura* n'a jamais été clairement déterminé, notamment à cause du chevauchement total des stades larvaires et adultes. Ainsi, les observations de terrain varient de quatre

à dix générations en Inde (Gunn 1918 ; McPherson 2018) à seulement trois aux Etats-Unis (Nouveau-Mexique) (Taylor et al. 2015).

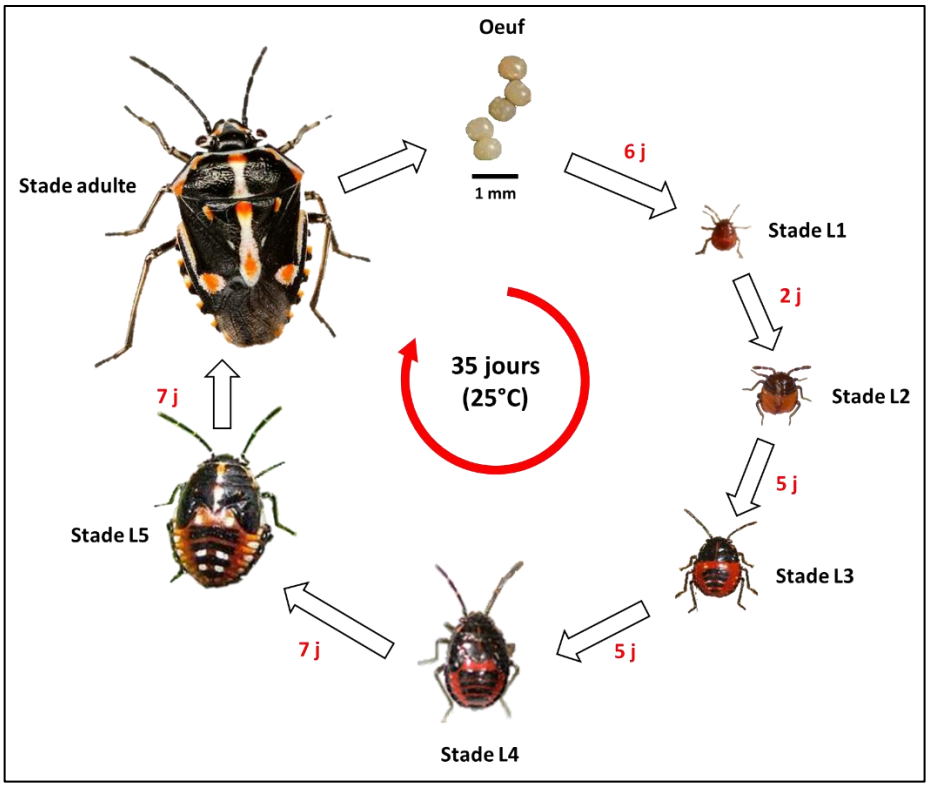


Figure 15 : Cycle de développement de *Bagrada hilaris* de l'œuf à l'adulte à 25°C. L'échelle indiquée s'applique à tous les stades de développement.

3.3. Dynamiques populationnelles

Bagrada est une punaise grégaire, et les stades larvaires et adultes sont généralement trouvés agrégés sur leur plante hôte pendant la journée (Huang et al. 2013 ; Reed et al. 2013). L'activité d'alimentation et de reproduction (accouplement et ponte) est maximale durant les heures les plus chaudes de la journée oscillant entre 25 et 41°C dans l'aire invasive (Huang et al. 2013 ; Reed et al. 2013). En dehors de cet optimum thermique, bagrada reste peu active à la surface du sol (Reed et al. 2013).

A l'échelle des saisons, les populations de bagrada présentent généralement deux pics d'abondance annuels dans l'aire native et invasive, qui coïncident avec le calendrier des cultures qu'elles attaquent. En Inde par exemple, un premier pic arrive à l'automne (Octobre-Novembre), et correspond à la période de semis du colza (*Brassica napus* L.) et de la moutarde indienne (*Brassica juncea* L.), dont bagrada attaque les jeunes plants. Les températures d'automne sont encore comprises dans l'optimum de bagrada, ce qui permet le développement des œufs et larves. Au printemps (Mars-Avril), le deuxième pic populationnel correspond à la période où les plantes sont complètement développées et représentent une source importante de nourriture, couplée à une augmentation de la température moyenne journalière

(Singh & Malik 1993 ; Patel et al. 2017). En Californie, sur les cultures de chou (*Brassica oleracea* var. *capitata* L.) et de brocoli, (*Brassica oleracea* var. *italica* Plenck) les mêmes cycles d'abondance saisonniers ont été observés (Reed et al. 2013)

Ces pics populationnels s'alternent avec deux périodes où les populations de bagrada sont peu actives : en hiver, les températures moyennes journalières inférieures à 15°C ne permettent pas - ou difficilement - le développement des embryons et des larves dont le taux de survie est inférieur à 40% (Reed et al. 2017). Bagrada hiverne au stade adulte, généralement dans la litière ou les anfractuosités du sol. En été, la température n'est pas un facteur limitant puisque bagrada se développe parfaitement jusqu'à des températures avoisinant les 40°C (Reed et al. 2017). C'est toutefois la nourriture qui vient à manquer, la récolte des brassicacées ayant généralement lieu en Mai-Juin (Le Strange et al. 2010 ; Reed et al. 2013). La punaise subsiste alors jusqu'à l'automne sur des plantes hôtes alternatives telles que la moutarde méditerranéenne (*Hirschfeldia incana* L.), La roquette jaune (*Sisymbrium irio* L.), l'alyssum maritime (*Lobularia maritima* (L.) Desvaux), l'Euphorbe hérissée (*Euphorbia hirta* L.) ou encore le laiteron des champs (*Sonchus arvensis* L.) (Singh & Malik 1993 ; Reed et al. 2013).

3.4. Comportement de ponte

Nous avons vu dans la **section III** que les pentatomidés pondent leurs œufs sur leur plante hôte sous forme d'ooplaques de tailles et de formes variables. Bien loin de cette convention, l'oviposition de bagrada est unique au sein de sa famille et se distingue par deux points :

- Les œufs sont pondus individuellement. De petits agrégats (entre 2 et 11 œufs) ont déjà été observés mais ils sont rares et ne constituent jamais une ooplaque régulière et structurée (Taylor et al. 2014, 2015)
- Les œufs sont déposés sur divers supports dans le milieu naturel : feuilles et tiges de sa plante hôte, débris végétaux au sol, mais également dans les anfractuosités du sol et sous sa surface (Taylor et al. 2014, 2015 ; Bundy et al. 2019). Loin d'être une observation marginale, Taylor et al. (2014) ont montré que les femelles enfouissaient activement leurs œufs à l'aide de leurs pattes arrière (Fig. 16 A, B). Les œufs pondus de cette manière sont alors recouverts de particules de sol plus ou moins fines (Fig. 16 C, D). Bagrada préfère des sols secs pour pondre, (Bundy et al. 2019) et il est possible que par des temps humides, les pontes soient davantage localisées sur les plantes hôtes. Même si la caractérisation précise de ce comportement est récente, les premières observations qui en font mention sont anciennes en Inde et au Pakistan (Gunn 1918 ; Samuel 1949). Par ailleurs, il est peu probable que l'oviposition de bagrada relève d'adaptation(s) locale(s) puisque ce comportement a été observé dans de très nombreuses populations de l'aire native (Pakistan, Inde, Afrique du Sud) et invasive ancienne (Italie dans les années 1970 (CAB 1981)) ou récente (des Etats-Unis au Chili depuis 2008).

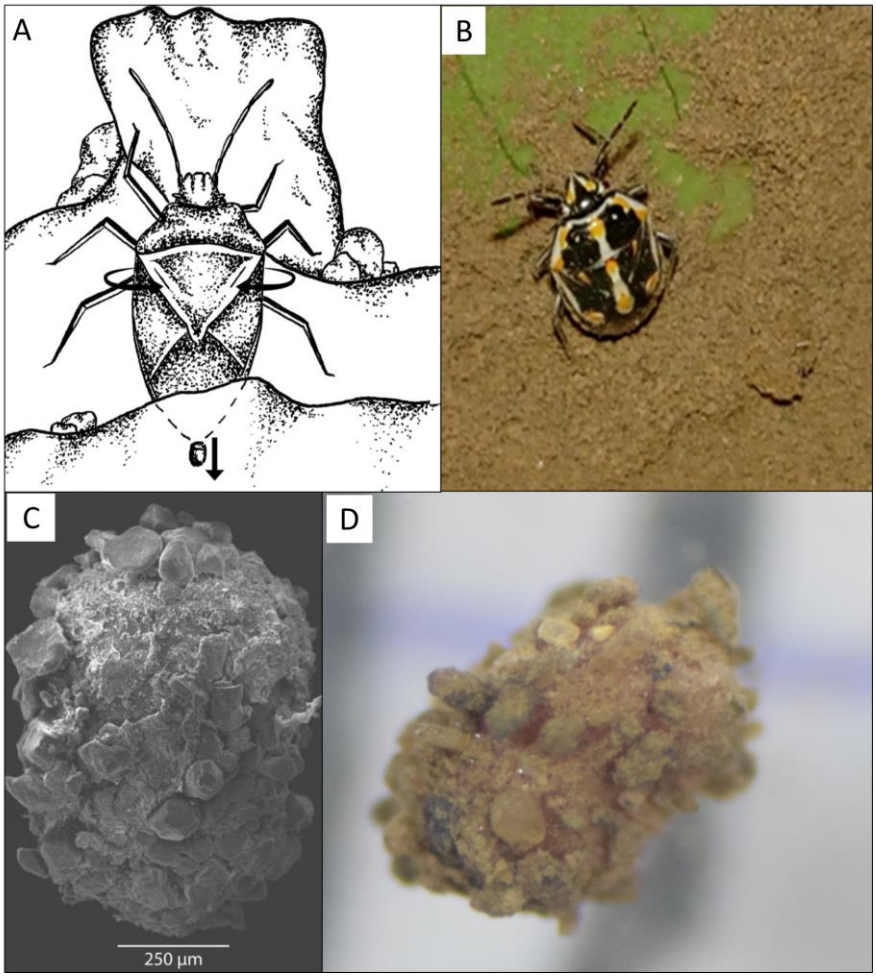


Figure 16 : Comportement d'oviposition de *Bagrada hilaris* dans du sable. (A) illustration de l'oviposition de bagrada dans un substrat (issu de Taylor et al. 2014) ; (B) Femelle *B. hilaris* d'une population naturelle de Pantelleria (Sicile, Italie) en train de pondre (vue dorsale) ; (C) photographie en microscopie électronique d'un œuf de bagrada recouvert de particules de sol (issu de Taylor et al. 2015) ; (D) photographie d'un œuf de bagrada collecté à Pantelleria et couvert de particules de sol. Photographies (B) et (D) : Martel G.

3.5. Plantes hôtes

Le spectre d'hôtes de bagrada est très large avec 24 familles recensées et près d'une centaine d'espèces végétales cultivées (75%), adventices (18%) ou ornementales (7%) (Tableau 2) (Palumbo et al. 2016 ; McPherson 2018). Toutefois, bagrada s'attaque principalement aux Brassicaceae, qui représentent 32% de son spectre d'hôtes (Fig. 17) (McPherson 2018). C'est également sur ces espèces que sont enregistrées les principales pertes agronomiques (brocoli et chou en Californie et Arizona, moutarde en Inde et au Pakistan). Parmi les brassicacées, certaines espèces ou variétés sont préférées comme le radis (*Raphanus sativus* L.), le chou rouge (*Brassica oleracea* var capitata) ou l'alyssum maritime (*Lobularia maritima* L.) (Huang et al. 2014).

Tableau 3 : Liste non exhaustive des plantes hôtes de Bagrada hilaris cultivées aux Etats-Unis

Famille	**Nom scientifique**	**Nom vernaculaire**	**Localité**
Amaranthaceae	*Spinacia oleracea* L.	Epinards	Etats-Unis (E-U) (Arizona et Californie)
Brassicaceae	*Brassica juncea* (L.) Czern	Moutarde Indienne	Inde, Sri Lanka, E-U (Arizona et Californie)
	Brassica oleracea var. *italica* Plenck	Brocoli	Inde, E-U (Arizona et Californie)
	Brassica oleracea var. *botrytis* L.	Chou-fleur	Inde, Sri Lanka, E-U (Arizona et Californie)
	Brassica oleracea var. *capitata* L.	Chou	Botswana, Inde, Afrique du Sud, E-U (Arizona et Californie)
	Brassica oleracea var. *gemmifera* L.	Chou de Bruxelles	E-U (Arizona)
	Raphanus sativus L.	Radis	Inde, Pakistan, Afrique du Sud, E-U (Arizona)
	Lobularia maritima (L.) Desvaux	Alyssum maritime	Afrique du Sud, E-U (Arizona, Californie, Nouveau-Mexique)
	Eruca sativa Miller	Roquette	Inde, Pakistan, E-U (Arizona, Californie)
Cucurbitaceae	*Citrullus lanatus* (Thunberg)	Pastèque	E-U (Arizona et Californie)
Fabaceae	*Phaseolus vulgaris* L.	Haricot vert	E-U (Californie)
Malvaceae	*Gossypium hirsutum* L.	Coton	Tanzanie, E-U (Californie)
Poaceae	*Sorghum bicolor var. sudanense* Piper	Sorgho	E-U (Californie)
	Zea mays L.	Maïs	E-U (Californie)
Solanaceae	*Capsicum annuum* L.	Poivron	E-U (Californie)

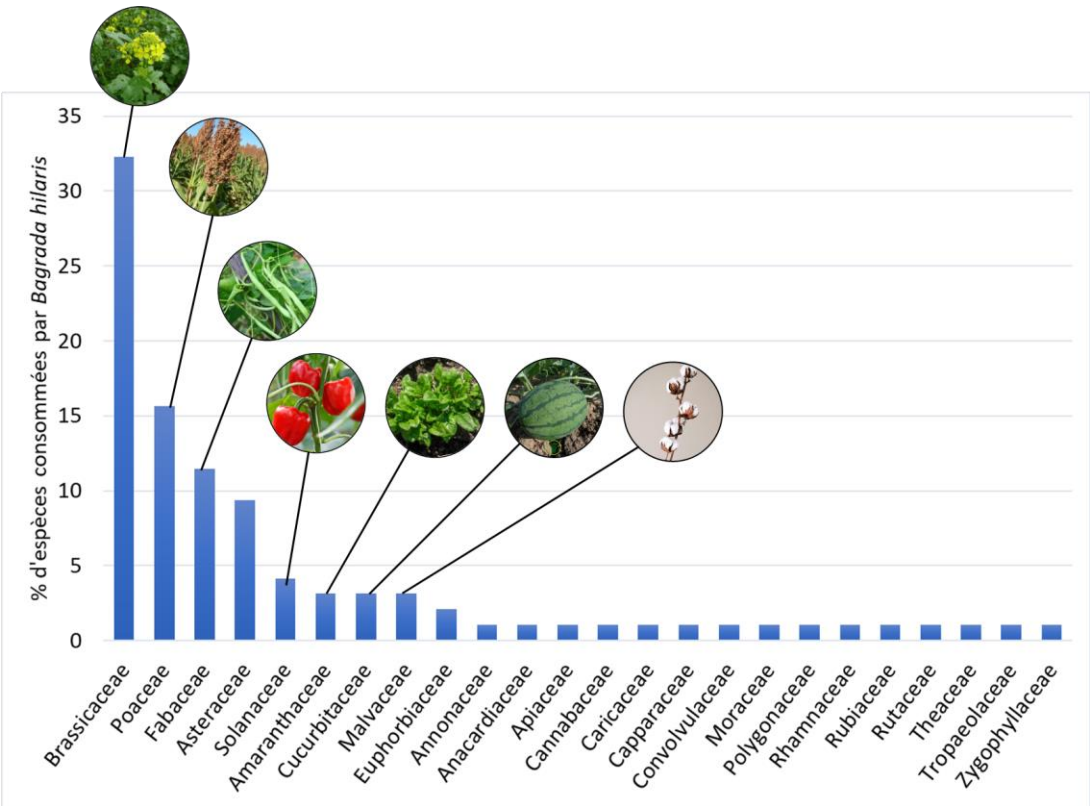


Figure 17 : Principales familles de plantes attaquées par *Bagrada hilaris* dans son aire native et invasive (96 espèces et 24 familles) (d'après McPherson 2018).

4. Impact agronomique

4.1. Dégâts sur les plantes cultivées

Bagrada est un insecte piqueur-suceur, et s'attaque aux structures aériennes de ses plantes hôtes : feuilles, tiges, siliques, gousses, boutons floraux. Les principaux dégâts agronomiques sur les brassicacées sont toutefois observés sur les parties foliaires (cotylédons ou feuilles). La piqûre de bagrada provoque des tâches chlorotiques qui peuvent entraîner une nécrose des tissus (Fig. 18A, D). Le dessèchement de ces derniers provoque leur mort et celle de la plante entière si les lésions se multiplient (Fig. 18B).

Bagrada préfère généralement s'attaquer aux jeunes stades végétatifs dont les tissus foliaires sont plus tendres et plus sensibles aux dégâts occasionnés (Huang et al. 2014). Sur des plantes plus âgées, bagrada s'attaque aux feuilles matures et à celles entourant le méristème apical. Cela conduit à une déformation des feuilles en développement et un retard de maturité de la plante, une croissance foliaire ralentie ou la croissance de structures non désirées comme les tiges chez le chou (Reed et al. 2013) ou des couronnes surnuméraires chez le brocoli (Fig. 18C) (Palumbo & Natwick 2010).

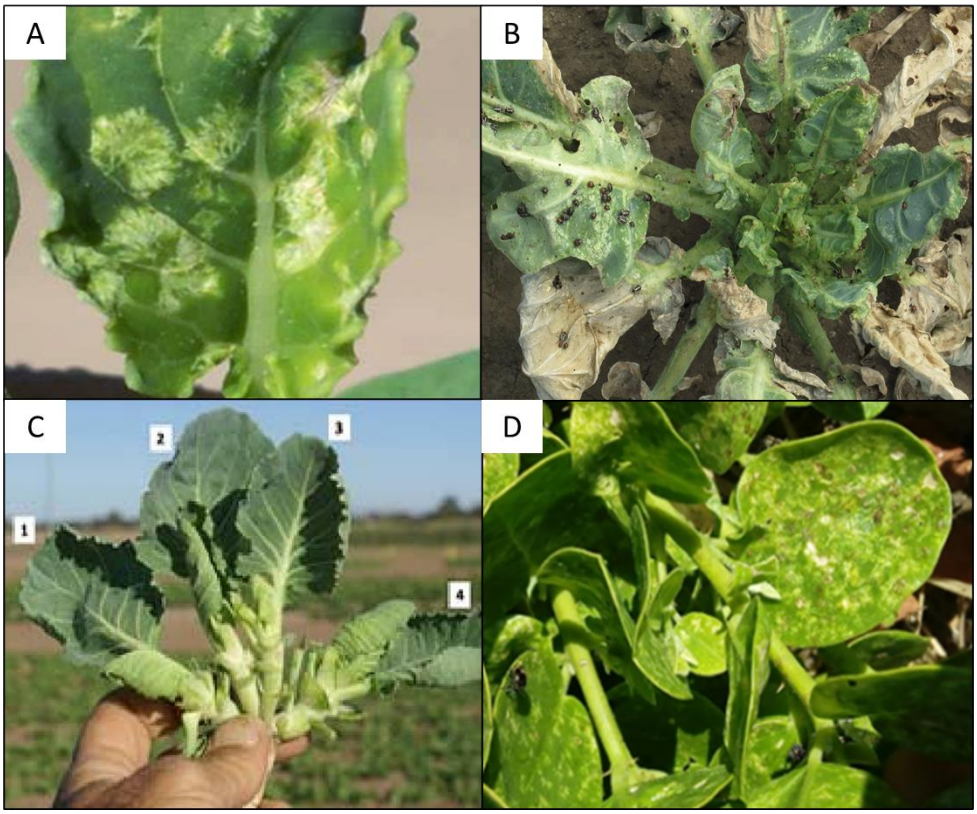


Figure 18 : Dégâts occasionnés par *Bagrada hilaris* sur ses plantes hôtes. (A) tâches chlorotiques sur une feuille de brocoli en Californie ; (B) plant de brocoli nécrosé après attaque de bagrada en Californie ; (C) têtes surnuméraires d'un brocoli attaqué par bagrada en Californie ; (D) tâches chlorotiques sur feuille de câprier à Pantelleria (Italie). Photos A-C tirées de Reed et al. 2013 ; Photo D par Martel G.

4.2. Pertes de production et risques économiques

4.2.1. La culture de brassicacées dans le monde et aux Etats-Unis

C'est une des productions agricoles les plus importantes au monde, essentiellement concentrée en Asie, en Europe et en Amérique du Nord. En 2019, près de 2,5 M d'hectares ont été cultivés pour une production de plus de 70 M de tonnes (Food and Agriculture Organization of the United Nations, fao.org). Aux Etats-Unis, 90% de la production maraîchère des brassicacées est effectuée dans les vallées agricoles de Californie (Vallée Centrale et Vallée Impériale notamment) et d'Arizona. En 2019, la culture de brocoli en Californie représentait 89,3% de la surface nationale (40 347 ha de récoltes) pour une production estimée à 786 millions de dollars (90% de la production nationale) (nass.usda.gov). La même année, la Californie seule représentait 24.5% des surfaces nationales de chou récoltées (5 868 ha) pour une production estimée à 212 millions de dollars (41% de la production nationale) (nass.usda.gov).

4.2.2. Les ravageurs de brassicacées : quelle place pour bagrada ?

La culture de brassicacées aux Etats-Unis est la cible de nombreux organismes nuisibles (Fig. 19). Parmi eux, on trouve principalement des insectes phytophages tels que *Plutella xylostella* L. (Lep., : Plutellidae), l'un des principaux ravageurs de chou à travers le monde (Sarfraz et al. 2005), des piérides, des pucerons, des punaises comme *M. histrionica* comme nous l'avons vu dans la partie III, mais également des champignons responsables du Mildiou ou de la Hernie du chou. La présence de bagrada dans les cultures de brassicacées des Etats-Unis est donc une nouvelle menace et sa gestion doit prendre en compte les méthodes utilisées pour réguler les autres ravageurs de brassicacées.

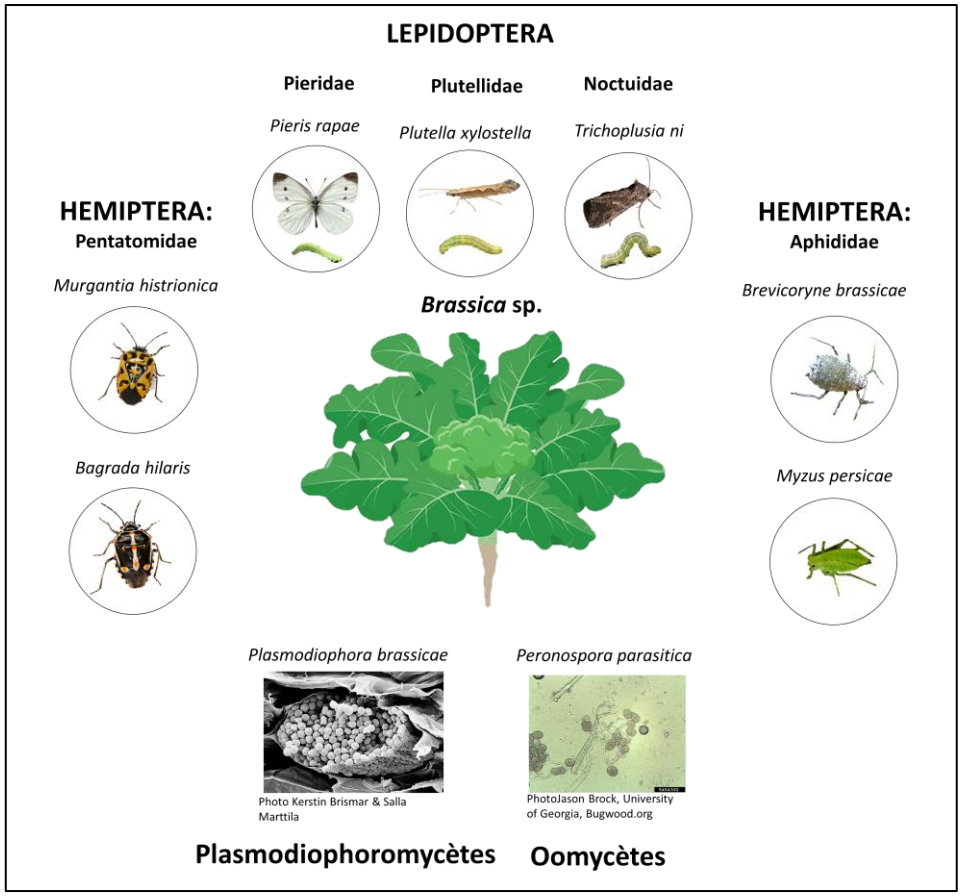


Figure 19 : Principaux ravageurs de brassicacées aux Etats-Unis

4.2.3. Impact agronomique dans l'aire native et invasive.

Depuis le début du 20^{e} siècle, peu d'études ont quantifié les dégâts et les pertes économiques engendrés par bagrada. Dans son aire native (Inde et Pakistan principalement), la punaise sévit dans les cultures de brassicacées oléagineuses telles que le colza (*Brassica napus* L.) ou la moutarde indienne (*Brassica juncea*). Sur les plantes matures, elle se nourrit notamment des graines, ce qui entraîne leur dessiccation et engendre des pertes de rendements entre 27 et 70% dans les champs de moutarde non protégés du Rajhastan (Inde) (Joshi et al. 1989) et 30% en Haryana (Inde) (Singh et al. 1980).

Dans son aire invasive, en 2010 et 2011 à Yuma (Arizona, Etats-Unis) et dans la Vallée Impériale (Californie, Etats-Unis), une enquête menée auprès des agriculteurs a estimé que 90% des

cultures de brocoli étaient infestées par bagrada. Les dégâts occasionnés dans les cultures de chou-fleur et brocoli ont ainsi engendré respectivement un minimum de 5 et 10% de pertes (Huang et al. 2013 ; Palumbo et al. 2016), mettant ainsi en péril plusieurs millions de dollars de récolte. La densité moyenne de *B. hilaris* n'est généralement pas mentionnée dans les études, mais une première estimation réalisée par Palumbo & Carrière (2015) sur de jeunes plants de brocoli en Arizona a montré qu'elle serait d'une quinzaine d'individus par plante. Toutefois, cette densité dépend fortement du stade de la plante, des traitements insecticides utilisés et leur fréquence, ainsi que l'année de production.

5. Méthodes de gestion de Bagrada

5.1. Insecticides et pesticides de synthèse

Comme pour les autres pentatomidés invasives, l'usage régulier de pyréthrinoïdes ou des produits organophosphatés est le principal moyen de lutte contre bagrada dans son aire native et aux Etats-Unis (Chauhan & Yadav 2007 ; Joseph et al. 2016). De nouveaux pesticides et bio-pesticides sont à l'étude depuis 2010 mais restent inutilisés pour le moment, soit parce qu'ils ne sont pas assez efficaces, soit parce qu'ils se dégradent trop vite dans l'environnement (Joseph 2018).

5.2. Stratégies intégrées

5.2.1. Méthodes de gestion par les pratiques culturales

Plusieurs pratiques culturales préventives sont utilisées dans l'aire native de bagrada depuis de nombreuses décennies, bien qu'elles soient peu considérées aux Etats-Unis. Elles ont principalement pour objectif de prévenir les pics de population en limitant les ressources alimentaires en amont, ou en modifiant l'habitat de la punaise. Celles-ci sont :

- **L'arrachage** des plantes hôtes de substitution de bagrada avant un nouveau semis, pour éliminer les individus qui s'y seraient réfugiés et qui pourraient recoloniser la parcelle,
- **La destruction** du précédent cultural s'il s'agissait de brassicacées, avec le même objectif que précédemment (Sachan et Purwar 2007),
- **Le contournement** de la dynamique saisonnière des populations en décalant la période des semis : un semis tardif pour une variété qui se développe rapidement ou un semis précoce pour une variété qui se développe lentement (comme pour *Piezodorus guildinii* sur soja),
- **La mise en terre** de jeunes plants, plus robustes contre les piqûres de bagrada que les germinations obtenues par des semis directs (Reed et al. 2013),
- **Le secouage** des denrées récoltées pour éviter que bagrada y subsiste et occasionne des dégâts post-récoltes (McPherson 2018),
- **La rotation culturale** pour éviter la persistance de bagrada sur une parcelle d'une année à l'autre.

Il est à noter que même si ces pratiques culturales semblent efficaces et répandues en Asie (Inde et Pakistan notamment) et en Afrique (Kenya), les articles souvent cités ne permettent pas de rendre compte d'études convaincantes par leur méthodologie ou leurs résultats (cf. Patel et al. 2017).

5.2.2. Lutte biologique

Bien que la biologie de bagrada en Asie et en Afrique soit étudiée depuis plus d'un siècle, les connaissances sur ses ennemis naturels dans son aire native restent parcellaires. Ce sont principalement des parasitoïdes (diptères ou hyménoptères) qui s'attaquent au stade adulte comme *Alophora pusilla* Meigen (Diptera : Tachinidae) ou à ses œufs comme *Telenomus samueli* Mani (Hym., Scelionidae) ou *Trissolcus hyalinipennis* Rajmohana & Narendran (Hym., Scelionidae) et *Ooencyrtus mirus* (Hym. Encyrtidae) découverts au Pakistan (Mahmood et al. 2015) (Tableau 3). Dans l'aire invasive de bagrada, l'identification de nouveaux prédateurs et parasitoïdes est extrêmement récente du fait de l'absence de la punaise aux Etats-Unis jusqu'en 2008. Ce sont essentiellement des parasitoïdes oophages qui ont été recensés (Encyrtidae et Scelionidae), bien que des prédateurs comme la punaise *Podisus maculiventris* Say (Hem : Pentatomidae) aient également été observés (Grasswitz 2016) (Tableau 1). Cependant, aucun des différents ennemis naturels de bagrada ne semble être suffisamment efficace pour permettre une régulation de la punaise dans les cultures (McPherson 2018).

Le contrôle de bagrada aux Etats-Unis par LBC est donc un projet extrêmement récent et sans équivalence dans l'aire native de la punaise. L'évaluation d'insectes candidats pour la LBC débute seulement, tandis que la récolte de nouveaux candidats se poursuit dans l'aire native (Kenya, Afrique du Sud).

Tableau 4 :Ennemis naturels de *Bagrada hilaris* dans son aire native et invasive (rangés par Ordre et Famille)

Classification	Ennemi naturel	Localisation	Référence
Coleoptera Melyridae	*Collops vittatus* Say	USA (Nouveau-Mexique)	Bundy et al. 2012 ; Grasswitz 2016
Diptera : Sarcophagidae	*Sarcophaga kempi* Senior-White	Inde	Thompson 1950
Diptera : Tachinidae	*Phasia pusilla* Meigen	Pakistan	Cheema et al. 1973
	Phasia indica Mesnil	Pakistan	Cheema et al. 1973
	Phasia sp.	Pakistan	Herting and Simmonds 1971
Hemiptera : Reduviidae	*Sinea diadema* Fabricius	USA (Nouveau-Mexique)	Grasswitz 2016
Hemiptera : Pentatomidae	*Podisus maculiventris* Say	USA (Nouveau-Mexique)	Grasswitz 2016
Hymenoptera : Scelionidae	*Trissolcus basalis* Wollaston	USA (Californie) ; Mexique (Coahuila)	Ganjisaffar et al. 2018 ; Felipe-Victoriano et al. 2019
	Trissolcus hyalinipennis Rajmohana & Narendran	USA (Californie) ; Pakistan	Ganjisaffar et al. 2018 ; Mahmood et al. 2015 ; Subba Rao and Chacko 1961
	Gryon myrmecophilum Ashmead	Mexique (Coahuila)	Felipe-Victoriano et al. 2019
	Telenomus podisi Ashmead	Mexique (Coahuila)	Felipe-Victoriano et al. 2019
	Idris elba Talamas	Mexique (Guanajuato)	Lomeli-Flores et al. 2019
	Gryon gonikopalense Sharma	Pakistan	Mahmood et al. 2015
	Telenomus samueli Mani	Inde	Samuel 1949 ; Gunn 1918
	Gryon karnalensis Chacko and Katiyar	Pakistan	Cheema et al. 1973 ; Narayanan et al. 1959
Hymenoptera: Encyrtidae	*Ooencyrtus mirus* Triapitsyn & Power	Pakistan	Power 2020 ; Mahmood et al. 2015
	Ooencyrtus lucidus Triapitsyn & Ganjisaffar	USA (Californie)	Triapitsyn et al. 2020
Nevroptera: Chrysopidae	*Chrysoperla carnea* Stephens	Pakistan	Cheema et al. 1973

V. Les Scelionidae : des candidats de choix contre les punaises invasives ?

1. Taxonomie

La Super-Famille des Platygastroidea regroupe 4461 espèces décrites en deux principales familles : les Scelionidae et les Platygastridae (Austin et al. 2005). Les Scelionidae sont la plus grande des deux familles avec 3308 espèces réparties en 147 genres dont les genres *Telenomus*, *Trissolcus* et *Gryon*, souvent étudiés à des fins de lutte biologique (Fig. 20) (Austin et al. 2005). Il persiste une confusion taxonomique entre les familles Scelionidae et Platygastridae, utilisées indifféremment dans la nomenclature des espèces mais pourtant taxonomiquement distinctes. Il est ainsi courant que la famille Platygastridae soit attribuée à des espèces du genre *Trissolcus* ou *Telenomus*, des Scelionidae d'après les classifications les plus récentes (Talamas et al. 2017 ; Ogburn & Walgenbach 2020 ; Queiroz et al. 2019).

Les Scelioninae constituent la plus grande des Sous-Familles de Scelionidae avec 16 tribus (Masner 1993), et de nouvelles espèces sont encore régulièrement décrites (Veenakumari et al. 2018 ; Lomeli-Flores et al. 2019 ; Triapitsyn et al. 2020). L'identification de certaines espèces susceptibles d'être utilisées en biocontrôle reste toutefois complexe, et fait l'objet de mises à jour récentes à la lumière des outils génétiques, comme pour le genre *Trissolcus* (Talamas et al. 2017).

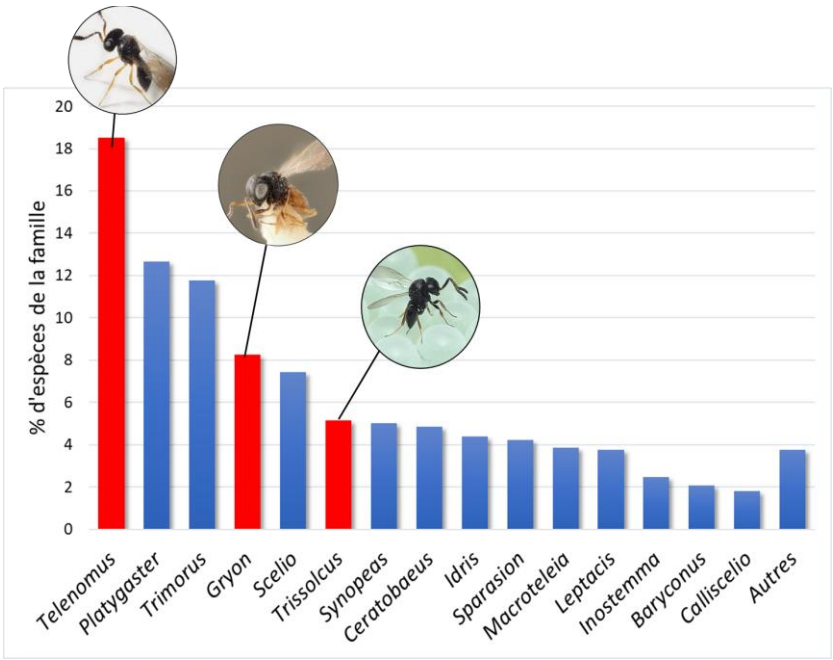


Figure 20 : Principaux genres de la famille des Scelionidae rangés par ordre d'importance (pourcentage d'espèces) sur un total de 3308 espèces. Les genres représentés en rouge sont les principaux utilisés en lutte biologique.

2. Biologie

2.1. Physiologie et diversité d'hôtes

Tous les scelionidés connus à ce jour sont des endoparasitoïdes oophages solitaires. Beaucoup d'entre eux se développent dans des environnements chauds : ils présentent un optimum de température proche de 30°C et ne se développent pas ou mal en dessous de 15 °C (Orr et al. 1985 ; Torres et al. 2002 ; Asante et al. 2000 ; Canto-Silva et al. 2005 ; Dasilao & Arakawa 2005). Les scelionidés ont généralement une fécondité variable, y compris au sein d'un même genre : *Trissolcus cristatus* Johnson élevé sur *Podisus maculiventris* Say pond par exemple 32 œufs en moyenne au cours de sa vie tandis que *T. basalis* élevé sur *N. viridula* pond en moyenne 292 œufs (Awan et al. 1990 ; Orr & Boethel 1990). Le genre *Gryon* présente quant à lui une fécondité inférieure à 100 descendants / femelle pour la plupart des espèces connues (Dasilao & Arakawa 2004 ; Romeis et al. 2000 ; Canto-Silva et al. 2006b), avec quelques exceptions comme *Gryon pennsylvanicum* pouvant produire 144 œufs / femelle (Peverieri et al. 2012).

Les scelionidés s'attaquent à des œufs d'insectes ou d'araignées d'ordres divers, mais leur degré de spécificité envers leur(s) hôte(s) est généralement élevé, la plupart étant associés à un seul genre ou une seule famille (Taylor 1975 ; Masner 1991 ; Austin et al. 2005). Le genre *Scelio* est par exemple spécialiste des œufs d'orthoptères (Dangerfield et al. 2001), tandis que le genre *Idris* est connu pour s'attaquer aux œufs d'araignées (Austin 1981, Iqbal and Austin 2000 ; Margaria et al. 2016). Il existe toutefois certaines espèces qui s'attaquent à un large nombre d'hôtes au sein d'une même famille, comme *T. basalis* ou *T. euchisti*, des parasitoïdes de *Nezara viridula* pouvant parasiter un grand nombre de punaises pentatomides (Follet et al. 2000).

Parmi les hôtes connus des scelionidés, beaucoup sont des espèces considérées comme nuisibles en agriculture (*N. viridula*, *H. halys*, *Locusta migratoria* L (Orth., Acrididae)), en foresterie (*L. dispar*, *Leptoglossus occidentalis* Heidemann (Hem., Coreidae)), mais également en santé animale et humaine (punaises hématophages du genre *Triatoma* ou *Rhodnius* (Hem., Reduviidae) et les taons (Dipt., Tabanidae)).

2.2. Mode de reproduction

Les scelionidés sont des espèces haplodiploïdes : les femelles diploïdes sont issues d'oocytes fécondés tandis que les mâles sont haploïdes et proviennent d'oocytes non fécondés (parthénogenèse arrhénotoque). Après accouplement, les femelles peuvent stocker le sperme mâle dans leur spermathèque et « décider » de féconder ou non les œufs qu'elles pondent, et donc le sexe de leur descendance. Chez les parasitoïdes, la disponibilité des œufs et leur mode de maturation varie en fonction des espèces, et permet de les classer en deux catégories principales (Jervis et al. 2008) :

- Chez les espèces strictement pro-ovigénique (type 1, Fig. 21), les oocytes maturent au cours du développement larvaire. La femelle dispose d'un stock fini d'œufs immédiatement après son émergence.
- Chez les espèces **s**trictement synovipogénique (type 4, Fig. 21) les oocytes ne sont pas matures à l'émergence. Ils continuent à se développer dans les premiers jours de la vie de la femelle. Au cours de sa vie, la femelle produit de nouveaux oocytes continuellement. Ceux qui ne sont pas utilisés sont réabsorbés pour récupérer les ressources.

Entre ces deux catégories extrêmes, il existe un continuum de modes de maturation des œufs (Types 2 et 3, Fig.21) (Jervis et al. 2001 ; 2008). On retrouve parmi les espèces pro-ovigéniques de nombreuses espèces de parasitoïdes oophages dont les Scelionidae, tandis que les espèces synovipogéniques regroupent plutôt des parasitoïdes larvaires tels que les Braconidae.

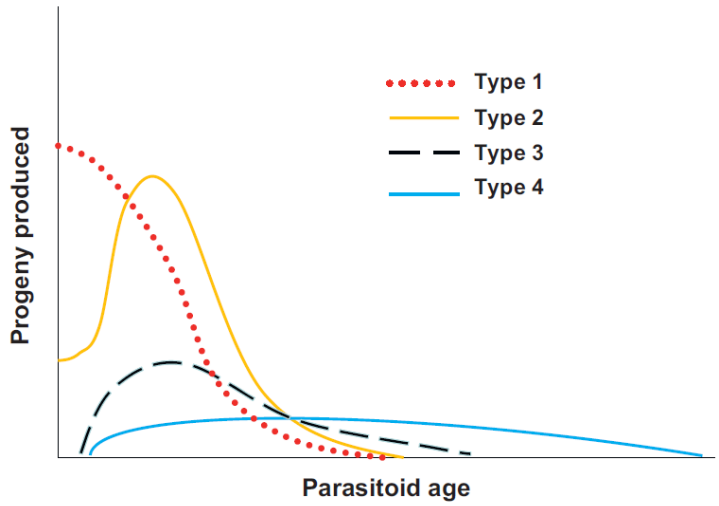


Figure 21 : Représentation graphique des quatre catégories de courbes de fécondité chez les parasitoïdes (d'après Jervis et al. (2008))

2.3. Alimentation

Comme la plupart des parasitoïdes oophages, les scelionidés ont besoin de se nourrir au stade adulte car les réserves accumulées pendant leur développement larvaire sont limitées, et généralement allouées à la reproduction. L'alimentation au stade adulte permet d'augmenter la longévité, l'activité de recherche d'hôte et de reproduction (Jervis et al. 2008). Le scelionide *Gryon pennsylvanicum* Ashmead est par exemple incapable de vivre plus de 4 jours sans nourriture tandis que sa longévité moyenne est de 35 jours s'il s'alimente (Peverieri et al. 2012). Certains parasitoïdes comme *Trichogramma platneri* Nagarkatti et *T. pretiosum* peuvent également acquérir des ressources complémentaires en se nourrissant de leur hôte (Mills & Kuhlmann 2004) mais ce n'est pas le cas des scelionidés dont les principales sources de nourriture sont le nectar et le miellat produit par les plantes, riches en sucres (Austin et al. 2005).

3. La relation hôte parasitoïde : des barrières à franchir

Pour un organisme de la taille d'un scelionide (< 1 mm), même une parcelle agricole en monoculture peut s'avérer être un environnement complexe dans lequel détecter, atteindre et parasiter un hôte nécessite des aptitudes particulières. Au cours de l'évolution, les parasitoïdes oophages ont ainsi développé une grande variété de capacités de perception en fonction de l'habitat de leur hôte (sol, bois, feuillage, milieu aquatique, etc.), basées sur des stimuli olfactifs, tactiles ou visuels (Vinson 1976, 1984).

3.1. Les étapes pré-ovipositionnelles

Chez les parasitoïdes oophages, la détection d'un hôte adéquat pour le développement de leur progéniture (*i.e.* bonne espèce et bon stade) repose sur l'utilisation de signaux (généralement chimiques) présents dans l'environnement à des concentrations variables (Fig. 22).

Certains sont émis à longue distance par les plantes (Composés Organiques Volatiles, ou COVs) suite à l'attaque d'insectes herbivores (Volatiles de Plantes Induits par l'Herbivore, ou VPIHs) ou à leur oviposition (Volatiles de Plantes Induits par l'Oviposition, ou VPIOs), et indiquent donc la présence de ces derniers. Ces COVs sont présents en abondance dans l'environnement et donc faciles à détecter par un parasitoïde qui peut les utiliser pour atteindre un hôte potentiel. Par exemple, *Trissolcus basalis* (Colazza et al. 2004 ; Frati et al. 2017 ; Salerno et al. 2019) et *Telenomus podisi* (Blassioli Moraes et al. 2005 ; Michereff et al. 2011) sont attirés par les légumineuses infestées par *N. viridula* ou *E. heros*. En revanche, ces signaux peuvent être induits par une multitude d'espèces herbivores et ne sont donc pas spécifiques. Une fois l'habitat de l'hôte atteint, les parasitoïdes oophages utilisent donc des signaux plus spécifiques de leur hôte (donc plus fiables), mais plus difficilement détectables (généralement à courte distance). Ces signaux appelés kairomones (Wajnberg & Ris 2007) peuvent être des phéromones (Boyle et al. 2020) ou des composés de contact (non-volatiles). Par exemple, les traces chimiques laissées par le passage de femelles *H. halys* stimulent l'activité de recherche de *T. japonicus* (Arif et al. 2021), tandis que *T. basalis* et *T. brochymenae* sont stimulés par les traces laissées par *N. viridula* et *M. histrionica* (Colazza et al. 1999 ; Conti et al. 2004).

L'utilisation de ces signaux à longue et courte distance pour trouver un hôte est ce que Vet et al. (1990) appellent un « détour infochimique ». Ce détour repose sur un « compromis de détection-identification » entre l'utilisation de signaux faciles à détecter mais peu spécifiques (les COVs) et des signaux spécifiques de l'hôte mais difficilement détectables (Vet & Dicke 1992 ; Martinez 2017).

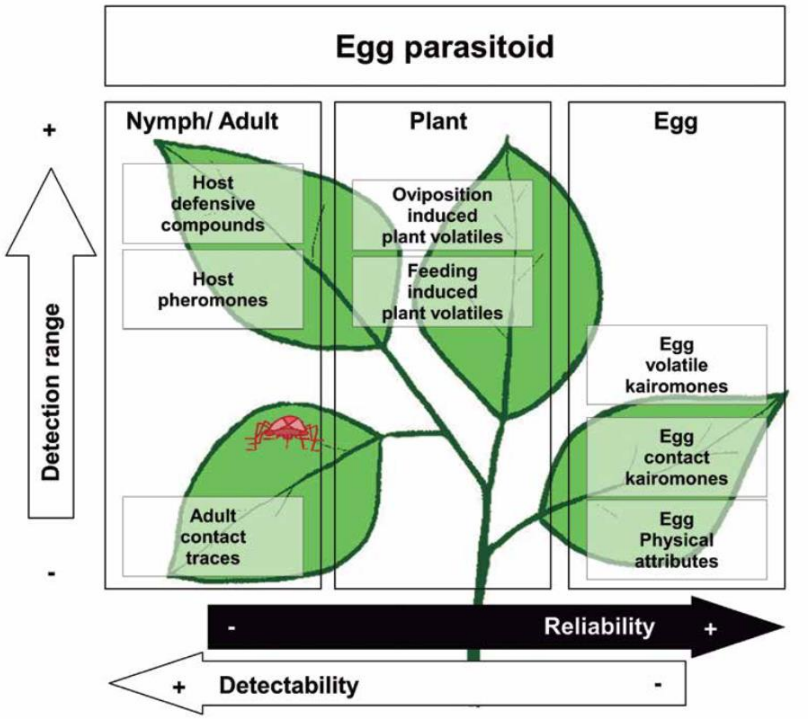


Figure 22 : Représentation schématique du compromis entre « détection » et « identification » de l'hôte par les parasitoïdes oophages (d'après Martinez, 2017).

3.2. Comportement de ponte des scelionidés

Le parasitisme se décompose en quatre phases (Fig. 23).

- 1) **Antennation (ou "drumming")** : le parasitoïde entre en contact avec son hôte et en palpe la surface avec ses antennes. C'est au cours de cette étape que le parasitoïde va évaluer la qualité de l'hôte à travers différents critères de sélection comme l'âge de l'œuf, sa taille, l'espèce ou bien la présence d'un parasitoïde déjà en développement à l'intérieur (Vinson, 1998 ; Rocha et al. 2006a ; Hougardy & Hogg 2021). Cette étape est généralement de courte durée (environ une minute), mais varie en fonction de l'espèce (Romeis et al. 2000 ; Wiedemann et al. 2003 ; Rocha et al. 2006b ; Hougardy & Hogg 2021)
- 2) **Oviposition :** si l'hôte est accepté, la femelle se place alors en position de ponte. Chez les Scelionidae, l'ovipositeur au repos est maintenu à l'intérieur du metasoma dans un tube de tissus souples (Austin 1983, Austin et al. 2005). Une fois l'ovipositeur sorti, le parasitoïde perce le chorion de l'œuf. Un seul œuf est généralement pondu, mais des cas de superparasitisme peuvent exister (Hougardy & Hogg 2021). L'oviposition est la phase la plus longue, et peut durer une à trois minutes en fonction des espèces (Romeis et al. 2000 ; Wiedemann et al. 2003 ; Rocha et al. 2006b ; Hougardy & Hogg 2021)
- 3) **Marquage chimique** : une fois l'oviposition terminée, la femelle retire son ovipositeur et commence un marquage chimique en frottant son abdomen à la surface de l'hôte pour y répandre des phéromones (Wiedemann et al. 2003). Ces phéromones permettent à la femelle de reconnaître les œufs qu'elle a déjà parasités, et de prévenir d'éventuelles compétitrices que la place est déjà occupée. Cette étape de marquage dure entre 10 et 20 secondes (Romeis et al.

2000 ; Wiedemann et al. 2003 ; Rocha et al. 2006b) mais peut cependant être absente chez certaines espèces du genre *Ceratobaeus* (parasitoïde d'araignées, Austin 1984).

- 4) **Alimentation/repos** : le parasitoïde peut marquer une période de repos, de nourrissage ou bien chercher directement un nouvel hôte à parasiter.

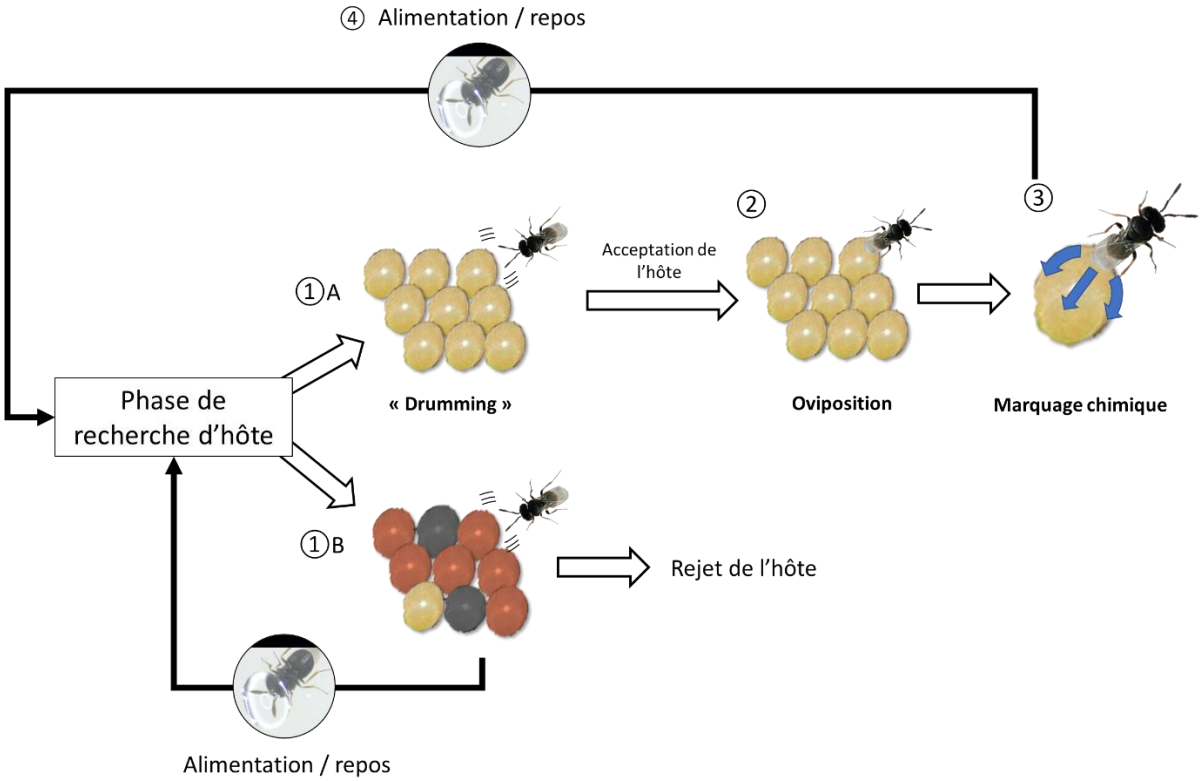


Figure 23 : séquence comportementale de parasitisme chez les Scelionidae

4. Les scelionidés en lutte biologique

Jusqu'en 1981, seules 20 espèces de scelionides ont été introduites comme auxiliaires (Luck 1981). Un des succès les plus importants est le contrôle de *Oxydia trychiata* Guenée (Lep., Geometridae) par *Telenomus alsophilae* Viereck en Colombie (Bustillo & Drooz 1977 ; Orr 1988). Depuis 1985 seulement 3 espèces ont fait l'objet d'introductions intentionnelles (Driesche & Hoddle 2016) : *Trissolcus basalis* entre 1987 et 1989, pour réguler *Nezara viridula* aux Etats-Unis et particulièrement à Hawaii ; *Telenomus remus* entre 1988 et 1989, contre *Spodoptera frugiperda* aux Etats-Unis et au Venezuela à la fin des années 1990 et *Telenomus lucullus* entre 1989 et 1996 contre *Eudocima fullonia*. Comme nous l'avons vu dans la **partie III**, des scelionidés natifs ont également été évalués pour contrôler *M. histrionica* aux Etats-Unis, sans grand succès et *T. basalis* a également été utilisé en lutte biologique inoculative contre *N. viridula* et *E. heros* au Brésil (Correa-Ferreira & Moscardi, 1996). Cette pauvreté en termes d'introductions est à mettre en miroir avec les autres familles de parasitoïdes. En effet, entre 1985 et 2015, les différents programmes mondiaux de LBC contre des insectes nuisibles ont

mené au lâcher de 154 espèces de parasitoïdes (Driesche & Hoddle 2016) représentant 19 familles dont cinq majoritaires : les Braconidae (27 espèces), les Encyrtidae (26 espèces), les Aphelinidae (22 espèces) les Eulophidae (21 espèces) et les Ichneumonidae (12 espèces) (Fig. 24).

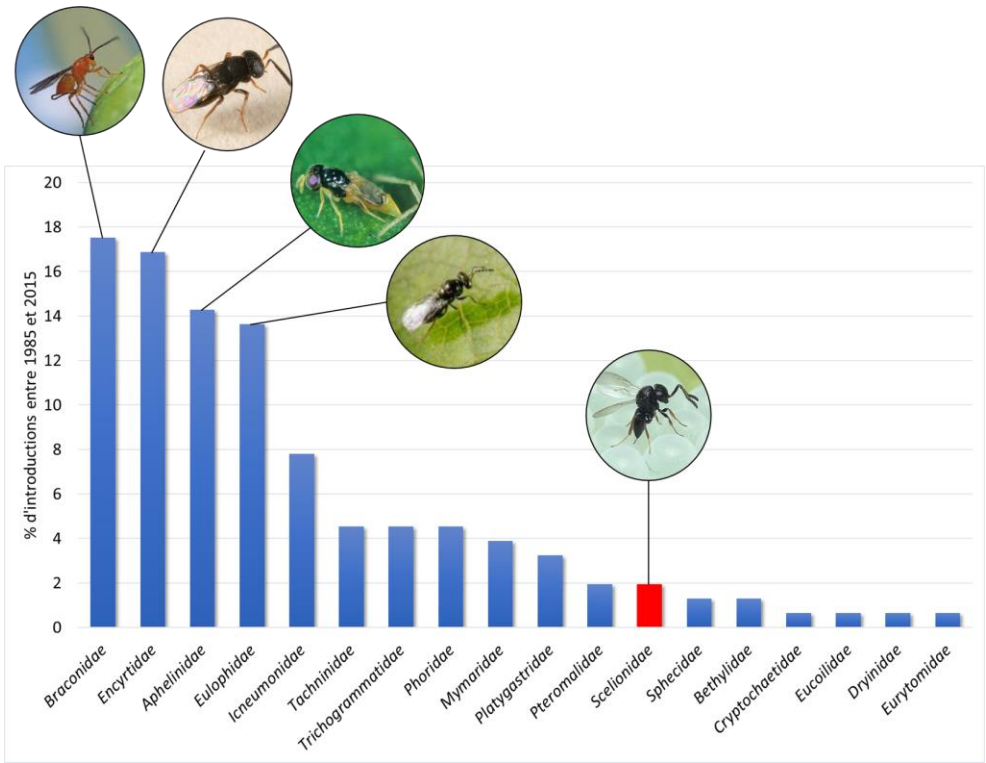


Figure 24 : Familles d'hyménoptères parasitoïdes utilisées en lutte biologique classique entre 1985 et 2010 avec en rouge, les Scelionidae (d'après Driesche & Hoddle 2016)

5. Le genre *Gryon*

5.1. Taxonomie et Biologie

Le genre *Gryon* Haliday appartient à la tribu des Gryonini et est le 4e genre le plus important des Scelionidae avec 273 espèces (330 espèces d'après Perioto et al. 2019). La taxonomie du genre *Gryon* est mal décrite et peu étudiée (Polaszek & Kolberg 2008), de même que sa biologie avec à peine une quinzaine d'espèces étudiées en laboratoire ou dans leur milieu naturel. Ces parasitoïdes sont de très petite taille (< 1 mm), et attaquent généralement les œufs de punaises appartenant aux Coreidae, Pentatomidae, Scutelleridae, Lygaeidae, Reduviidae et Phymatidae (Peter & Rajmohana 2014 ; Ramani et al. 2019). La plupart des espèces attaquées par *Gryon* sp. sont d'ailleurs des punaises nuisibles pour l'agriculture et la foresterie humaine (Ramani et al. 2019) comme *Spartocera dentiventris* Berg (Hem., Coreidae) sur le tabac, *Clavigralla* sp. (Hem., Coreidae) sur le pois d'Angole ou *Leptoglossus occidentalis* Heidemann (Hem., Coreidae) sur les conifères.

5.2. Le genre Gryon en lutte biologique : des tentatives

Contrairement aux genres *Trissolcus* et *Telenomus* qui ont été évoqués régulièrement au cours de cette revue bibliographique, les parasitoïdes du genre *Gryon* ont été très peu étudiés à des fins de lutte biologique. Délaissés au cours du 20e siècle à quelques exceptions près (Sankaran & Nagaraja 1975 ; Taylor 1975 ; Morrill & Amazan 1990), ils sont davantage étudiés depuis le début du 21e siècle. Plusieurs programmes de recherche ont ainsi vu le jour pour réguler des punaises présentant des enjeux économiques significatifs, la plupart du temps en impliquant un « *Gryon* » natif (Fig. 25). C'est le cas de *G. japonicum*, *G. philippinense*, *G. gallardoi*, *G. fulviventris* et *G. clavigrallae*. *Gryon pennsylvanicum*, originaire d'Amérique du Nord, est le seul à avoir fait l'objet d'un programme de LBC dans un autre pays (Italie).

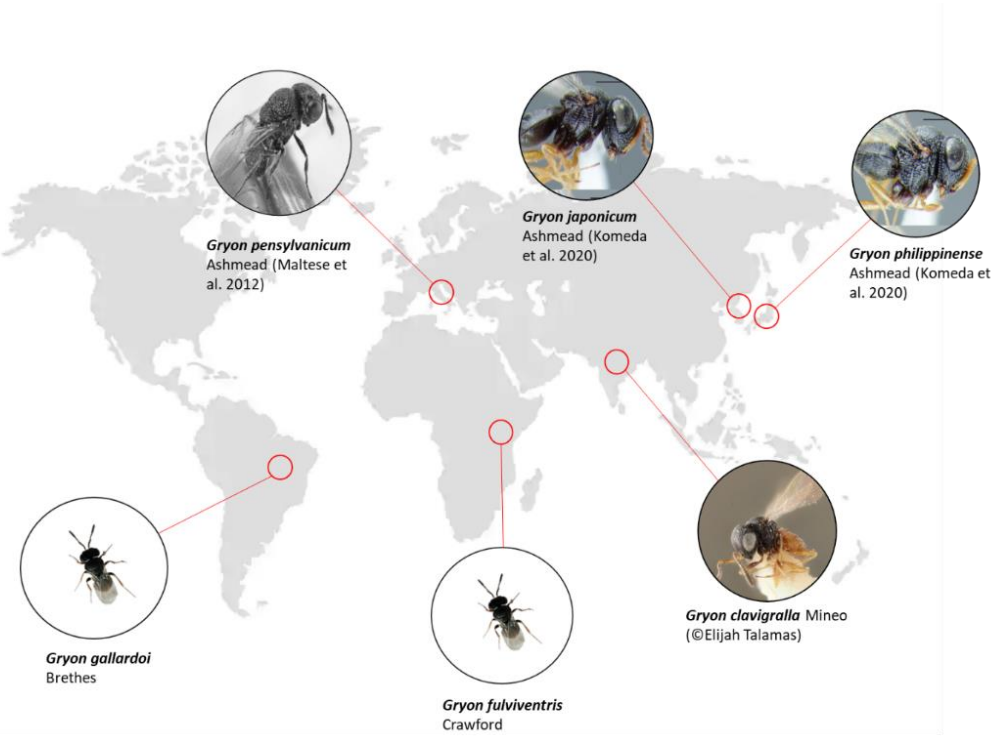


Figure 25 : Espèces du genre *Gryon* étudiées dans le cadre d'un programme de lutte biologique contre différentes punaises d'importance économique à travers le monde.

Toutefois, la plupart de ces projets de recherche n'a fait l'objet que d'études préliminaires sur la biologie du parasitoïde (Shanower et al. 1996 ; Asante et al. 2000 ; Romeis et al. 2000 ; Dasilao & Arakawa 2004, 2005). Seules trois espèces ont fait l'objet d'études approfondies : *G. pennsylvanicum*, *G. japonicum* et *G. gallardoi*.

Au début des années 2000 au Brésil, *Gryon gallardoi* Brethes a fait l'objet d'un projet de thèse visant à l'évaluer comme auxiliaire contre *Spartocera dentiventris* (Berg) (Hemiptera : Coreidae), un ravageur du tabac. La physiologie, le comportement de recherche d'hôte en ou encore la dispersion du parasitoïde ont été abordés (Wiedemann et al. 2003 ; Canto-Silva et al. 2005, 2006a, b) et se sont

poursuivies jusqu'en 2008 (Rocha et al. 2006a, 2008). Cela n'a cependant pas été suffisant pour statuer sur l'efficacité du parasitoïde et le projet semble abandonné depuis.

Le projet de Lutte biologique classique contre *Leptoglossus occidentalis* Heidemann (Hemiptera : Coreidae) en Italie a débuté en 2010 par la mise en quarantaine d'une souche canadienne de *Gryon pennsylvanicum* (Roversi et al. 2011). Sa biologie, son efficacité et sa spécificité envers *L. occidentalis* ont été évalué en laboratoire (Peverieri et al. 2012 ; Peverieri et al. 2013 ; Paoli et al. 2013, 2014 ; Roversi et al. 2014 ; Peverieri et al. 2015). Les tests de spécificité ont conclu à une apparente spécificité de *G. pennsylvanicum*, mais les auteurs ont suggéré des tests complémentaires sur des espèces d'autres régions du globe. En Italie, le programme de LBC sur *L. occidentalis* semble cependant inactif depuis 2015 et n'a pour le moment abouti à aucun lâcher.

Gryon japonicum, natif de Corée du Sud et du Japon, a fait l'objet de plusieurs études depuis 1990 mais à des fins de lutte biologique par conservation sur *Riptortus pedestris* et *Riptortus clavatus*. Plusieurs aspects ont été explorés comme sa spécificité (Noda 1990), sa distribution spatiale et temporelle (Mainali et al. 2012b), son rôle en complément de cultures pièges ou de pièges phéromonaux (Youn & Jung 2008 ; Lim & Mainali 2013) ainsi que des études en laboratoire pour décrire sa physiologie (Paik et al. 2009 ; Mainali et al. 2012a). Ainsi, *G. japonicum* semble prometteur pour réguler *R. pedestris* et *R. clavatus* et peut être recruté via des pièges à phéromones pour augmenter sa densité localement.

Finalement, il subsiste une méconnaissance importante de la biologie du genre *Gryon* en conditions de laboratoire et *in natura*. Contrairement aux genres *Trissolcus* ou *Telenomus*, l'évaluation d'une espèce de *Gryon* est donc dans le cadre d'un programme de lutte biologique est donc difficile à remettre en perspective.

5.3. Un candidat contre bagrada : *Gryon gonikopalense*

En Mai 2014, des œufs sentinelles de bagrada sont exposés dans la région le district Toba Tek Singh, dans les plaines du Punjab au Pakistan (Mahmood et al. 2015). A cette localité, bagrada se nourrit sur *Brassica juncea* et *B. napus.* Ces œufs sentinelles ont permis la capture d'un Encyrtidae natif : *Ooencyrtus* sp (décrit comme une nouvelle espèce *Ooencyrtus mirus* par Triapitsyn & Power en 2020 (Power 2020) ; et deux scelionides natifs : *Trissolcus hyalinipennis*, et *Gryon* sp. identifié comme *Gryon gonikopalense*, qui fait l'objet de la présente thèse.

Les adultes *G. gonikopalense* mesurent environ 0.8 mm de long. La tête, le thorax et l'abdomen sont entièrement noir (Fig. 26). Les dix premiers segments des antennes (antennomères) sont noirs, le scape est jaune foncé. Les antennes des femelles sont constituées de 12 antennomères, caractéristiques des Scelioninae (Talamas et al. 2017) et sont distinguables des mâles par l'élargissement des 5 premiers antennomères en une massue (Fig. 26). Les pattes sont bicolores : le fémur est noir (le haut du fémur peut être jaune foncé) tandis que le tibia et le tarse sont jaune foncé.

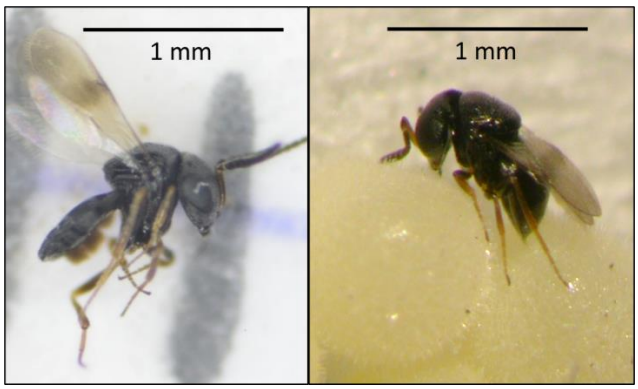


Figure 26 : Adultes *Gryon gonikopalense* mâle (à gauche) et femelle (à droite) (Photos Martel G. et Roche M.)

6. Pourquoi un Scelionidae serait-il un bon candidat contre une punaise invasive ?

Les Scelionidae remplissent de nombreux critères qui caractérisent un "bon" auxiliaire. C'est ce que souligne Orr dans son article de 1988 où il dresse la liste des atouts des scelionidés comme auxiliaires en LBC. Le premier est leur condition de parasitoïde puisque ce groupe représente 80% des succès en biocontrôle. Ils sont également plus efficaces que les prédateurs contre les hémiptères (Hokkanen & Sailer 1985) dont le stade œuf est le plus vulnérable (Abram et al. 2020).

Les scelionidés ont un développement rapide par rapport à leur hôte (Orr et al. 1985 ; Canto-Silva et al. 2005 ; Arakawa & Namura 2002), une fécondité importante couplée à une sex-ratio généralement biaisée en faveur des femelles (Peverieri et al. 2012 ; Forouzan et al. 2015 ; Yang et al. 2018). Le taux d'accroissement de leur population est donc supérieur à celui de leur hôte et compris entre 0,15 et 0,4 (Orr 1988). De plus, ce sont des espèces pro-ovigéniques, et les femelles sont donc capables de parasiter leur hôte dès leur émergence. Elles ont ainsi un impact sans délai sur leur hôte. D'autre part, du fait d'une coévolution souvent étroite avec ce dernier, la période d'activité des scelionidés à l'échelle d'une journée ou des saisons est généralement synchronisée avec leur hôte (Orr 1988). De cette coévolution résulte également une compatibilité écologique qui facilite l'établissement des scelionidés dans l'aire invasive de leur hôte (Orr 1988).

Comme nous l'avons également vu, le degré de spécificité des Scelionidae est élevé. Seul *T. basalis* introduit à Hawaii contre *N. viridula* a fait l'objet d'effets non intentionnels en ayant réduit de 50% la population de la punaise endémique *Coleotichus blackburniae* White (Follet et al. 2000 ; Abram et al. 2020). La spécificité d'hôte des scélionidés peut également être liée à une forte spécialisation dans la recherche de leur hôte comme *Scelio* sp. qui creuse le sol pour parasiter les œufs d'orthoptères (Dangerfield et al. 2001). Or, cette spécialisation est un des principaux critères de sélection d'un auxiliaire en LBC.

Enfin, la production en laboratoire est réalisable et peut être optimisée sur plusieurs aspects. Le stockage des œufs de l'hôte au froid sur de longues périodes et leur utilisation programmée est possible que ce soit dans le cadre expérimental ou dans une optique de production de masse (Peverieri et al. 2015 ; McIntosh et al. 2019 ; Wong et al. 2021).

Synthèse des objectifs et questions de recherche

Au cours de cette étude bibliographique, nous avons mis en évidence trois points importants :

- La lutte biologique contre les pentatomidés doit s'inscrire dans un ensemble de méthodes de gestion dont la mise en application est encore limitée aux Etats-Unis.
- La régulation des populations de bagrada implique des connaissances solides sur sa biologie, notamment son comportement d'oviposition. Ce dernier devrait être particulièrement important à considérer dès lors que l'on souhaite faire intervenir des parasitoïdes oophages.
- L'utilisation des parasitoïdes scelionides en LBC requiert d'approfondir nos connaissances sur leur biologie et leur comportement. La difficulté à identifier les espèces que l'on souhaite utiliser illustre, entre autres, les connaissances lacunaires dont nous disposons.

Mon travail de thèse s'inscrit dans un processus visant à déterminer un ou plusieurs auxiliaires pour réguler bagrada aux Etats-Unis par LBC (Fig. 27). L'optimisation de la mise en élevage et l'évaluation du parasitoïde *G. gonikopalense* comme agent de biocontrôle ont donc été le cœur de mon activité de recherche qui s'est organisée autour des objectifs et questions de recherche suivants :

- Quel est le cycle de développement de *G. gonikopalense* ? Quelles sont ses capacités de reproduction ? Ces capacités sont-elles proches des autres espèces de *Gryon* et de Scelionidae ? La physiologie de *G. gonikopalense* lui permet-elle de s'acclimater et de s'établir en Californie ?
- *Gryon gonikopalense* est-il capable de surmonter la barrière physique du sol et parasiter les œufs enterrés de bagrada ? Ce comportement dépend-il de composés chimiques émis par bagrada ? Ce comportement est-il un atout significatif contre la punaise, ou bien ne pond-elle que marginalement ses œufs dans le sol ? *Gryon gonikopalense* est-il capable de parasiter les œufs de bagrada sur sa plante hôte, ou bien est-il restreint au sol ?
- L'élevage de *G. gonikopalense* est-il optimisable par le stockage des œufs de bagrada ? Les conditions de stockage permettent-elles d'assurer un parasitisme efficace de la part de *G. gonikopalense* ? Le stockage des œufs a-t-il des conséquences sur le développement des parasitoïdes et leur survie ?
- *Gryon gonikopalense* est-il spécifique de bagrada ? L'évaluation précoce de la spécificité de *G. gonikopalense* peut-elle permettre d'anticiper l'arrivée de bagrada en Europe ?

In fine, mon travail de thèse devra permettre d'apporter des arguments en faveur ou en défaveur de *G. gonikopalense* comme agent de lutte biologique contre bagrada. Ces arguments devront pouvoir être utilisés dans le cadre de la rédaction d'une pétition pour une introduction dans les cultures attaquées par la punaise.

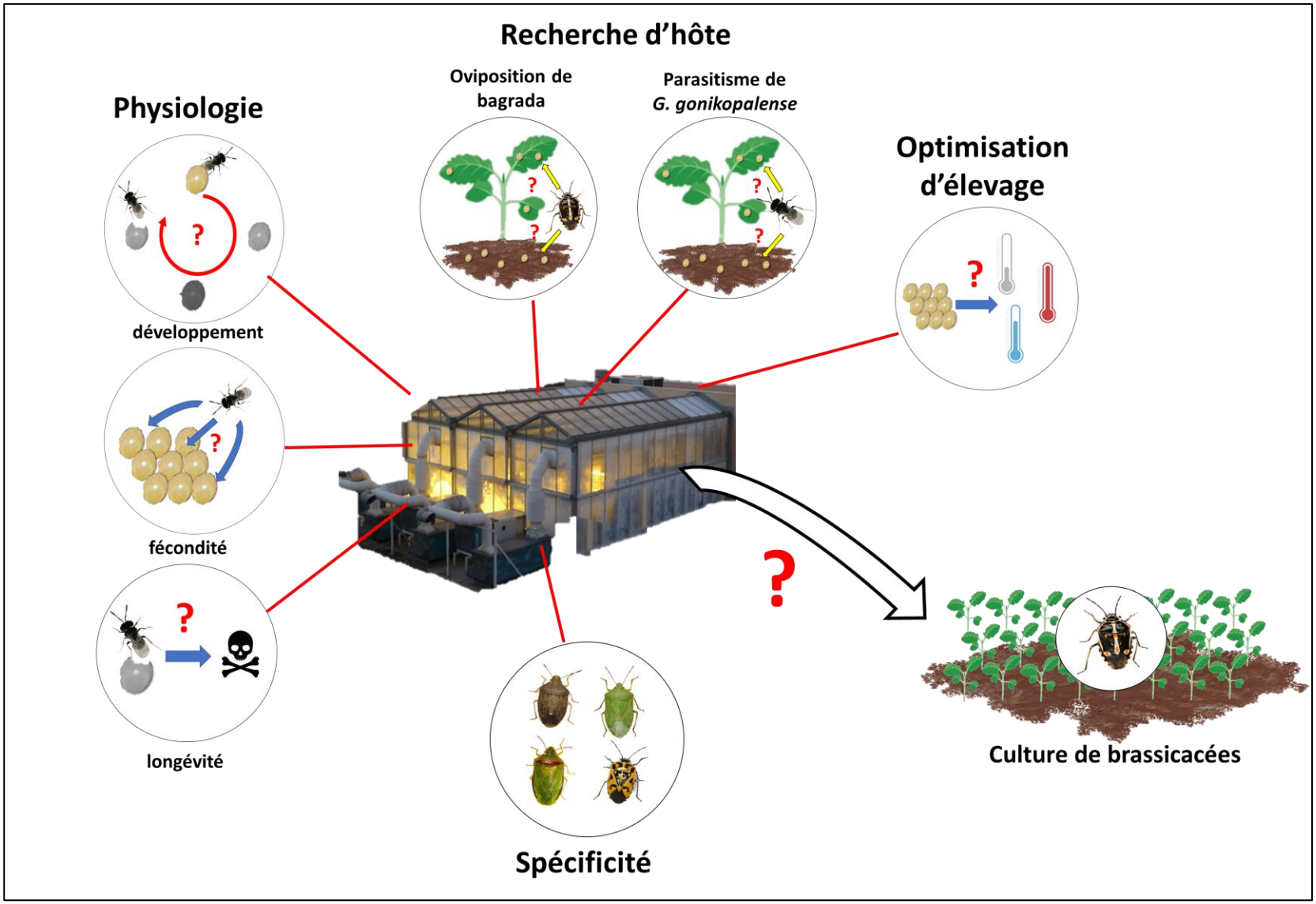


Figure 27 : Synthèse des objectifs de recherche portés par la thèse et mis en œuvre dans la quarantaine EBCL

Préambule technique.

Maintien d’un système tri-trophique continu en milieu confiné

L'activité de quarantaine de l'EBCL est soumise à un agrément (Arrêté préfectoral n°R76-2019-163 du 15 juillet 2019) conformément à la directive Européenne n°2000/29/CE. Les organismes vivants qui y sont manipulés sont soumis à un permis d'importation, de détention et de reproduction à des fins scientifiques, et un confinement strict. La punaise bagrada et son parasitoïde *G. gonikopalense* font donc également l'objet d'un agrément (Arrêté n° R76-2019-162 du 15 Juillet 2019). Ces documents sont délivrés par l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) et le Service régional de l'alimentation (SRAL) à Montpellier.

Dans ce projet de recherche, le contexte de quarantaine a donc été une composante principale de mon projet de recherche en conditionnant la mise en place et le maintien du système tri-trophique constitué par la punaise, ses plantes-hôtes et son parasitoïde. Les principales limites ont été 1) l'espace disponible pour la mise en place du système d'élevage et expérimental et 2) un compromis constant entre la productivité des élevages et la gestion des risques liés à leur détention. La présence en quarantaine impose le port d'une combinaison intégrale, de surbottes et d'une charlotte ou capuche pour couvrir les cheveux (Fig. 33C).

Par ailleurs, une part importante de mon activité de thèse a été dédiée au maintien de ce système tri-trophique dont dépendent les expérimentations. Un travail d'optimisation a donc été réalisé en amont pour rendre ce système le plus efficace et productif possible. Les différents essais et améliorations proposées ont porté sur trois points principaux : 1) la production des plantes hôtes de bagrada, 2) la production et la collecte des œufs de bagrada et 3) la gestion des risques et des déchets.

1. Choix des plantes hôtes

Bagrada est une espèce polyphage mais nous avons vu en introduction qu'elle se nourrissait principalement de brassicacées. De nombreuses espèces ont donc été testées entre 2017 et 2018 (Tableau 5, Fig. 28A, C) pour déterminer les plus pratiques et rentables à produire pour nourrir les colonies de bagrada en continu et maximiser son activité de reproduction. Les principaux critères de sélection étaient la surface foliaire, la présence de boutons floraux particulièrement appréciés par bagrada et l'appétence générale de la plante. Le chou cabus (*Brassica oleracea var. capitata* L.) et le radis (*Raphanus sativus* L.) ont par exemple été abandonnés car les plants obtenus étaient peu attaqués par bagrada, offraient une surface foliaire peu importante, et ne développaient pas de boutons floraux. A l'inverse, l'alysse maritime (*Lobularia maritima* L.) développait de nombreuses fleurs et était très attractive pour bagrada, mais offrait peu de surface foliaire et devait donc être remplacée très fréquemment.

Mes efforts de production se sont finalement concentrés sur deux plantes très attractives pour bagrada qui se développaient bien en conditions de quarantaine : le brocoli (*Brassica oleracea var. cymosa* Duschene), et la moutarde blanche (*Sinapis alba* L.). La première offrait une importante surface foliaire à la punaise tandis que la deuxième offrait des boutons floraux et des siliques. D'autre part, ces deux espèces sont les principales cibles de bagrada dans son aire native (moutarde en Inde et Pakistan) et invasive (brocoli aux USA), et étaient donc pertinentes pour conduire les élevages et les

expérimentations (Fig. 28B). La fausse roquette (*Diplotaxis erucoides* L.) s'est également avérée très attractive pour bagrada et était collectée régulièrement pour approvisionner les élevages (Fig. 28D).

Tableau 5 : Liste des espèces cultivées dans la quarantaine de l'EBCL en 2018 pour nourrir *Bagrada hilaris*

Espèce	**Nom vernaculaire**
Brassica oleracea var. capitata L.	Chou cabus
Brassica oleracea var. cymosa Duschene	Brocoli
Sinapis alba L.	Moutarde blanche
Raphanus sativus L.	Radis
Brassica napus L.	Colza
Eruca sativa Mill	Roquette
Lobularia maritima L.	Alysse maritime
Diplotaxis erucoides L.	Fausse roquette

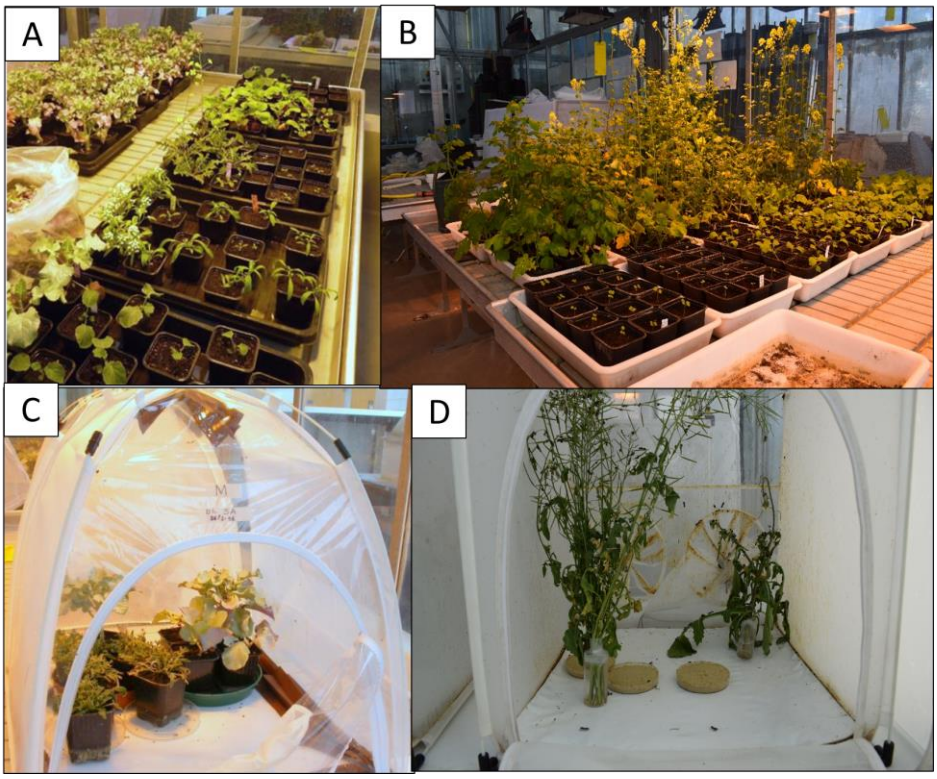


Figure 28 : (A) : Culture de brassicacées pour l'élevage *de Bagrada hilaris* en 2018 en serre de quarantaine ; (B) : production de moutarde blanche et de brocoli en 2020 ; (C) : cages d'élevage de *B. hilaris* contenant diverses espèces de brassicacées en 2018 ; (D) : cages d'élevage de *B. hilaris* contenant de la fausse roquette en 2020.

2. Collecte des œufs de *Bagrada hilaris*

2.1. Méthode initiale

Comme nous l'avons vu en introduction, bagrada dépose ses œufs individuellement sur tout type de support. En l'absence d'un support de ponte de prédilection, cela conduit à une importante dispersion des œufs dans les cages d'élevage, et donc un frein à leur collecte rapide et précise. Les œufs de bagrada sont également plus fragiles que les œufs d'autres espèces de pentatomidés comme *H. halys,* et les décrocher un à un de leur support de ponte est une opération délicate et chronophage.

Une première solution pour concentrer les pontes de bagrada a été de suspendre des serviettes en papier marron foncé au sommet des cages où les punaises se regroupent souvent. Les femelles y déposaient leurs œufs et ces derniers étaient collectés tous les deux jours (Fig. 29A). Pour éviter toute manipulation, les œufs étaient découpés individuellement avec leur support papier, puis collés avec une colle neutre (UHU stic ®, Germany) les uns à côté des autres sur du carton dans le but de constituer des ooplaques artificielles (Fig. 29B). Ils étaient ensuite utilisés au cours d'expérimentations ou pour permettre la reproduction des parasitoïdes (Fig. 29C, D).

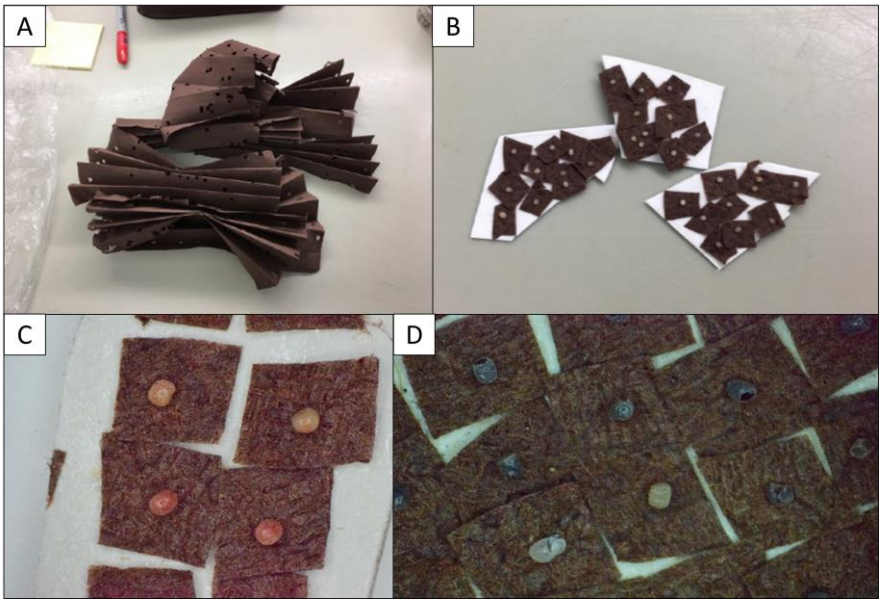


Figure 29 : (A) : collecte des papiers de ponte de *Bagrada hilaris* ; (B) : agencement et collage des œufs récoltés sur du carton ; (C) : œufs de *B. hilaris* non parasités âgés de deux jours (jaune) et quatre jours (rouge) ; (D) : œufs de *B. hilaris* parasités par *Gryon gonikopalense* et devenus noirs. Photos Martel G.

Cette méthode présentait cependant plusieurs inconvénients majeurs : 1) le temps de manipulation de la découpe au collage des œufs représente entre 20 et 30 secondes par œuf, ce qui pouvait représenter 25% du temps de travail hebdomadaire ; 2) la collecte des papiers de ponte et la découpe des œufs en dehors des cages d'élevage représentait un risque d'échappement de la punaise et 3) les œufs pouvaient se décrocher du papier de ponte.

2.2. Optimisation de la collecte des œufs

En se basant sur la biologie connue de bagrada et sur les méthodes d'élevage utilisées aux Etats-Unis (Taylor et al. 2014, 2015), l'utilisation d'arènes de ponte (boite de Pétri, diamètre= 9cm hauteur 1cm) remplies de sable (DIALL®, sable jaune, granulométrie = 200µm, Espagne) pour obtenir les œufs semblait la meilleure solution. Plusieurs substrats ont été testés au préalable en fonction de différents paramètres : prix / couleur / origine / présence de composés chimiques (colorants par exemple) / granulométrie.

Les arènes de ponte étaient réparties dans les cages (Fig. 30A). Leur nombre a été ajusté pour à la fois 1) maximiser la quantité d'œufs qui y sont pondus, 2) minimiser la quantité de sable à tamiser et 3) optimiser l'espace dans la cage (partagé avec les plantes) et ne pas compliquer leur nettoyage trois fois par semaine. La collecte des œufs s'effectuait ensuite par tamisage du sable (Figure 30B, C).



Figure 30 : (A) : Arènes de ponte remplies de sable dans une cage d'élevage de *Bagrada hilaris* ; (B) collecte des arènes de ponte avant tamisage du sable ; (C) récolte des œufs pondus dans les arènes par tamisage du sable ; (D) œufs de *B. hilaris* récoltés et couverts de particules de sable. Photos Martel G.

Ce système présente l'avantage de concentrer plus facilement les œufs et de les collecter simultanément par tamisage de l'ensemble des arènes de sable (Tamis Hogentogler®, maille = 425µm). Cette opération est donc indépendante du nombre d'œufs obtenus. La création d'ooplaques artificielles est également simplifiée par cette méthode : les œufs n'ont plus à être collés un à un, mais peuvent être saupoudrés sur une surface collante (Fig. 32B) comme cela est fait avec les œufs d'*Ephestia kuehniella* Zeler (Lep., Pyralidae) pour reproduire *Trichogramma* sp (Hym., Trichogrammatidae) (Hansen & Jensen 2002). Grâce à ce travail d'optimisation, la quantité utilisable d'œufs de bagrada collectés a pu être largement augmentée, de telle sorte qu'à l'automne 2020, la production de deux cages d'élevage était en moyenne de 900 œufs par jour, contre à peine 200 sur la même période en 2017 (Fig. 30D, Fig. 31).

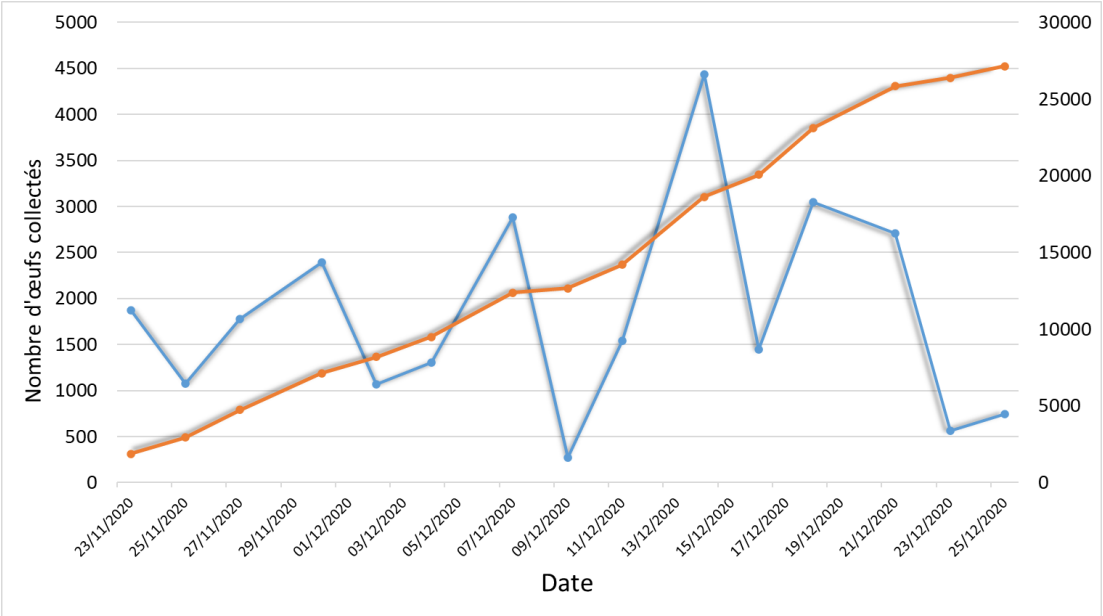


Figure 31 : Nombre d'œufs de *Bagrada hilaris* obtenus après collecte (en bleu) et en cumulé (en orange) entre le 23/11/2020 et le 25/12/2020 avec deux cages d'élevage.

3. Elevage de *G. gonikopalense*

La reproduction du parasitoïde en quarantaine n'a pas particulièrement fait l'objet d'améliorations au cours de cette thèse puisque le système mis initialement en place était déjà suffisant. Les ooplaques sont fournies aux parasitoïdes une fois par semaine, mais cette cadence peut être augmentée en fonction des besoins. Les individus adultes sont regroupés par sessions d'expositions dans des boîtes en plastique. Ils sont nourris avec du miel dilué à 50 % déposé sur les parois de la boîte ou bien sur les mailles en nylon qui permettent également l'aération de la boîte (Fig. 32A). Les expositions d'œufs de bagrada sont effectuées avec les parasitoïdes les plus jeunes, mais les individus sont conservés pendant un mois comme « population de secours ». Le système d'ooplaques est également utilisé au cours des expérimentations, et la méthode utilisée permet d'en constituer rapidement un grand nombre (Fig. 32C). La manipulation des parasitoïdes s'effectue le plus souvent dans des « chambres claires », pour éviter tout risque d'échappement des individus (Fig. 32D), bien que ce soient des insectes dociles et faciles à manipuler (ils ne volent pas).

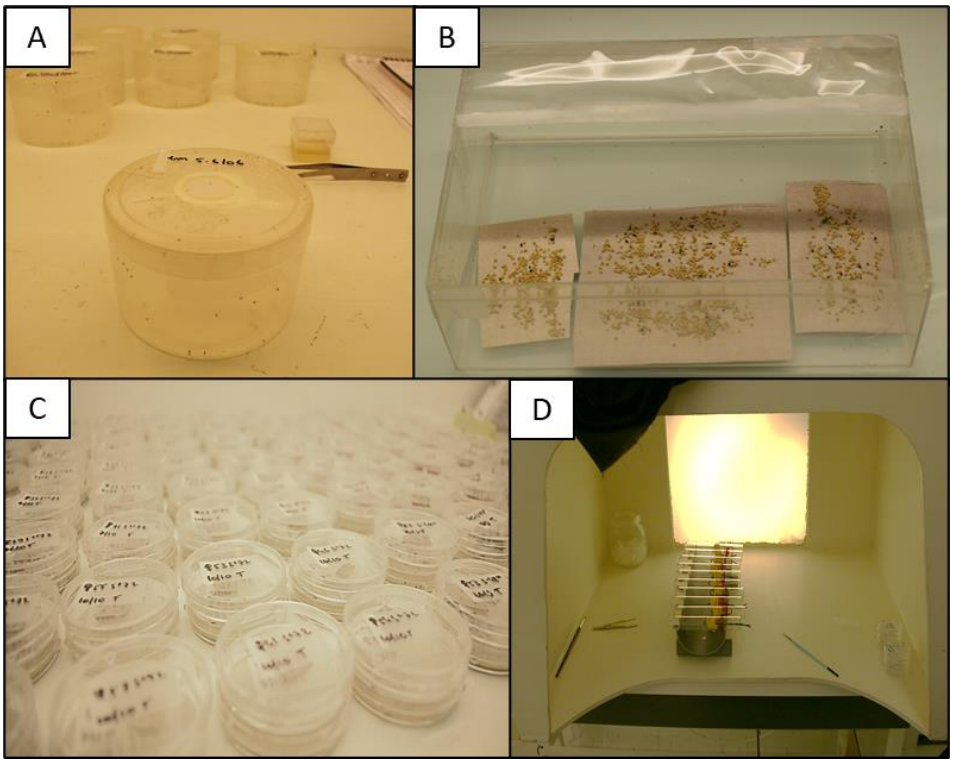


Figure 32 : (A) Nouvelle boîte d'émergence de *Gryon gonikopalense* (au premier plan) et boîtes contenant des individus plus âgés (arrière-plan) ; (B) Boîte d'exposition contenant des ooplaques s'œufs de *Bagrada hilaris* en train de se faire parasiter par *G. gonikopalense* ; (C) Un grand nombre de boîtes d'expérimentations conduites en parallèle des élevages ; (D) Expérimentations en chambre claire. Photos : Martel G.

4. Traitement des déchets et aménagement de l'espace de travail

L'élevage de bagrada en continu est la source d'une importante quantité de déchets (essentiellement organiques) qu'il est impératif de pouvoir traiter chaque semaine efficacement dans un environnement de quarantaine. Les élevages de *G. gonikopalense* et les expérimentations menées au sein de la quarantaine sont également des sources de déchets à considérer. Afin d'éviter tout risque d'échappement de la punaise, la totalité du matériel ayant été en contact avec elle doit être éliminé ou stérilisé avant réutilisation. Dans un souci d'économie de matériel et de tri des déchets, les éléments organiques (végétaux et terreau) sont séparés du matériel réutilisable (pots en plastique, boîtes de Pétri). Quel que soit leur devenir, ces éléments sont stockés après utilisation dans un congélateur à -20°C pendant un minimum de 48h pour tuer tous les stades de bagrada et de *G. gonikopalense*. Après cette période, l'ensemble des déchets (organiques ou non) est autoclavé (130 °C / 3 bars pendant 30 min) pour assurer toute destruction de matériel vivant avant d'être sortis de la quarantaine. Le matériel réutilisable est quant à lui lavé pour être à nouveau mis à disposition au sein de la quarantaine (Fig. 34).

L'élevage simultané de plusieurs cages de bagrada et de plusieurs souches géographiques (Etats-Unis, Afrique du Sud, Italie et anciennement Pakistan) nécessite un espace de travail sécurisé qui minimise les risques de dispersion de la punaise. La prise en considération de ces risques a fait l'objet d'une amélioration de l'espace de travail en quarantaine durant l'hiver 2019. Deux modifications

principales ont été faites : 1) l'ajout d'une sur-cage autour des cages d'élevage de la punaise et 2) la mise en place de toiles cirées blanches sur l'ensemble des tables de la serre de quarantaine où élevage et manipulations ont lieu (Fig. 33).

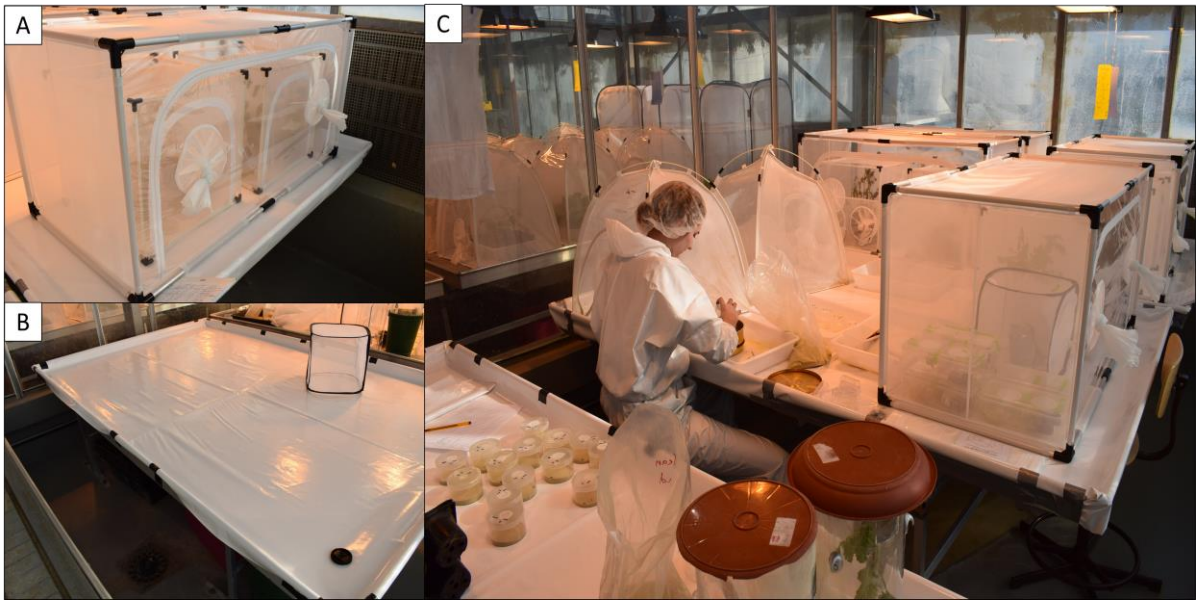


Figure 33 : (A) cages d'élevage de *Bagrada hilaris* contenues dans une sur-cage ; (B) toile cirée blanche recouvrant l'espace de travail ; (C) serre de quarantaine en période d'activité (8 cages d'élevage de bagrada). Photos Martel G.

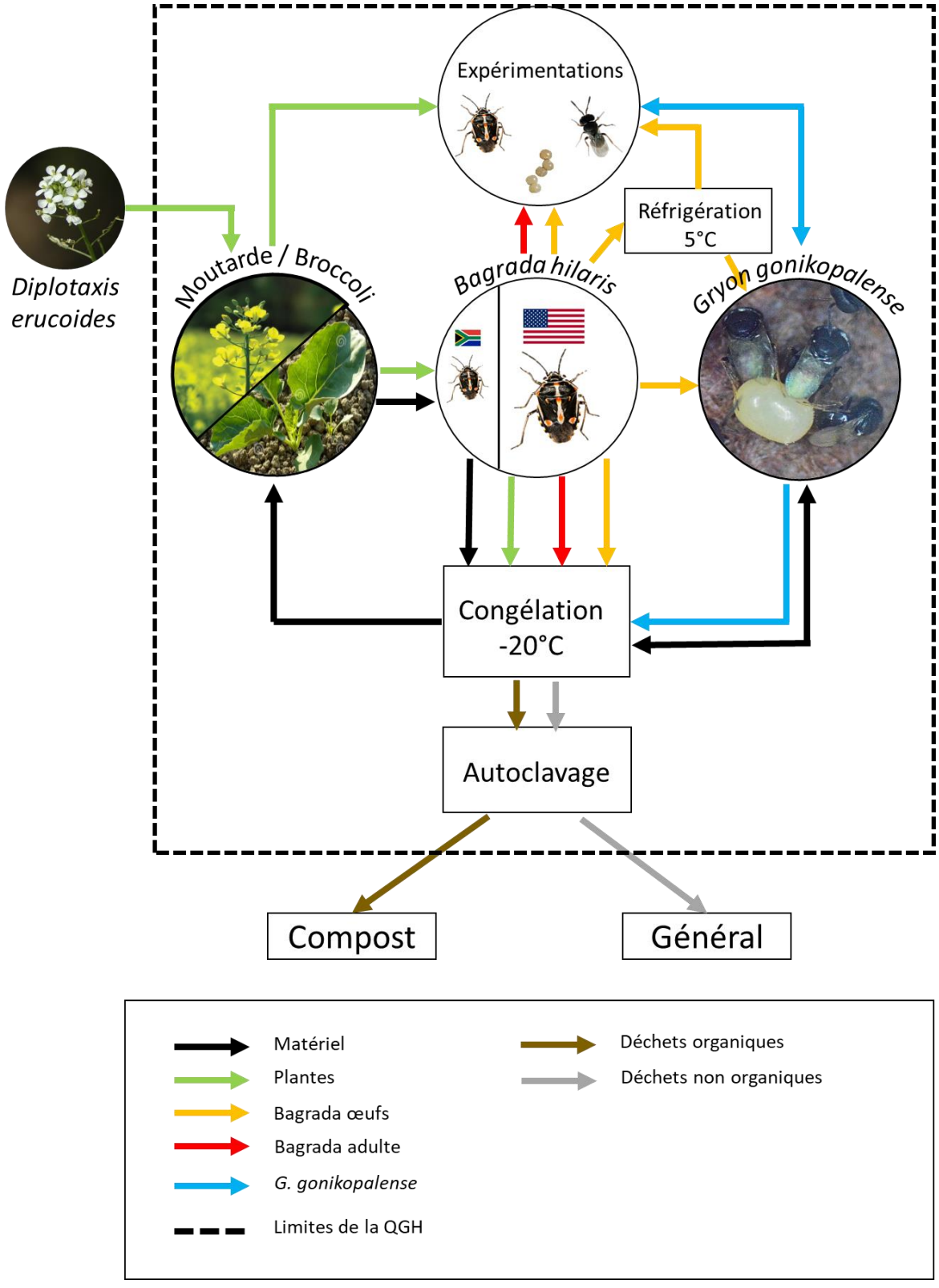


Figure 34 : Schéma récapitulatif du système de production de *Bagrada hilaris* et *Gryon gonikopalense* en milieu confiné

Chapitre 2.

Biologie générale et capacités d'acclimatation de G*ryon gonikopalense*

Article 1 : Martel, G., Augé, M., Talamas, E., Roche, M., Smith, L., & Sforza, R.F.H. (2019). First laboratory evaluation of *Gryon gonikopalense* (Hymenoptera: Scelionidae), as potential biological control agent of *Bagrada hilaris* (Hemiptera: Pentatomidae). *Biological Control* 135, 48-56 https://doi.org/10.1016/j.biocontrol.2019.04.014

Article 2: Martel, G. & Sforza, R.F.H.: Temperature effect on development, survivorship and reproduction of *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae), an egg parasitoid of *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae) in the frame of an importation biocontrol program

(En préparation pour une soumission à Insect Physiology)

Comme présenté dans le premier chapitre (section **V.5.3**), le programme de LBC contre *B. hilaris* développé par l'USDA-ARS vise à évaluer l'efficacité de plusieurs parasitoïdes scelionidés collectés au Pakistan, en particulier *G. gonikopalense* qui fait l'objet de cette thèse. Pour cela, décrire la biologie de cette espèce (jusqu'alors inconnue) est une première étape obligatoire. **L'Article 1** présente un ensemble de traits d'histoires de vie qui pourraient être liés au succès du parasitoïde une fois lâché sur le terrain. Nous proposons également dans cet article une officialisation taxonomique morphologique de l'identité du parasitoïde qui permettra de réunir les différents travaux menés dans les laboratoires de recherche sous un même nom d'espèce. D'autre part, le facteur thermique est connu pour fortement impacter la physiologie des insectes, et le succès de notre programme de LBC repose en grande partie sur la capacité d'acclimatation de *G. gonikopalense* après son introduction en Californie. Puisque *G. gonikopalense* et bagrada proviennent de la même aire native, il est attendu qu'ils partagent un optimum thermique similaire. Ainsi, nous décrivons dans **l'Article 2** comment les principaux traits d'histoire de vie de *G. gonikopalense* répondent à une gamme de températures constantes (de 15 à 40 °C) afin d'évaluer ses chances de s'établir dans le sud-ouest des Etats-Unis. L'ensemble des expérimentations présentées dans **les Articles 1 et 2** s'est déroulé en conditions contrôlées et confinées au sein de la quarantaine EBCL, telles que présenté dans le **Préambule technique**.

Le principal résultat de ce **Chapitre 2** est la capacité de *G. gonikopalense* à se développer entre 18 (46.85 ± 0.6 jours) et 37.5 °C (7.28 ± 0.09 jours) avec un taux d'émergence maximum atteint à 25°C (95.53 ± 1.96 %). Les femelles ont été capables de parasiter les œufs de bagrada à n'importe quelle température testée mais avec de fortes variations dans le nombre de descendants produits avec un maximum à 30 °C (104.47 ± 21.2 descendants/femelle). De plus, *G. gonikopalense* a été capable de parasiter des œufs de bagrada âgés de 1 à 4 jours (début d'émergence des larves de bagrada), de telle sorte que ces derniers seraient vulnérables pendant la majorité de leur développement sur le terrain. La longévité des adultes s'est avérée élevée par rapport à d'autres espèces du genre *Gryon*, avec 151.3 ± 6.8 jours obtenus à 15°C. Elle a cependant décru avec l'augmentation de la température, jusqu'à 4.28 ± 0.26 jours à 40 °C constants. Par ailleurs, nous montrons que la survie de *G. gonikopalense* est dépendante d'une source de nourriture (du miel dans notre étude) en l'absence de laquelle le parasitoïde ne peut pas vivre plus de 8 jours.

Finalement, les résultats obtenus fournissent une base de connaissances importante pour mettre en place de futures expérimentations, telles que celles qui seront présentées dans les **Chapitres 3 à 5**. Les performances de développement, de reproduction et de survie de *G. gonikopalense* suggèrent, à ce stade, que c'est un candidat prometteur pour le programme de LBC contre bagrada aux USA. La similarité confirmée de leurs optima thermiques respectifs suggère également que le parasitoïde pourrait s'établir dans les régions les plus chaudes des Etats-Unis (Californie, Arizona, Nouveau-Mexique) où bagrada prolifère particulièrement, ce qui pourrait ne pas être le cas dans les régions plus tempérées comme la côte californienne et le comté de Monterey.

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First laboratory evaluation of *Gryon gonikopalense* (Hymenoptera: Scelionidae), as potential biological control agent of *Bagrada hilaris* (Hemiptera: Pentatomidae)

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ABSTRACT

The pentatomid bug *Bagrada hilaris* is a severe invasive alien pest of Brassicaceae crops, which first appeared in California in 2008 and is expanding to several southern U.S. states and South America. To answer the demand of growers for sustainable management methods, a biological control program started in 2015. The egg parasitoid *Gryon gonikopalense* Sharma was collected in Pakistan, and after morphological identification here described, is being evaluated as a candidate for classical biological control against *B. hilaris*. Basic life history studies were performed in climatic chambers (22 ± 0.5 °C, RH 50 ± 5%, L/D 12:12). On average, *Gryon gonikopalense* developed in 25.1 ± 1.4 (mean ± sd) days and live 66.4 ± 30.7 days. Allowing females to oviposit reduced their lifespan by 52%, and food deprivation reduced it by 87%. During their life, females produced 59.2 ± 22.3 offspring on average with a maximum fecundity during the first week of their life. Mean progeny sex ratio was 46.3% female. Live host eggs from 1 to 4 days old were suitable for parasitoid oviposition, but eggs stored at −80 °C were not. Hence, the parasitoid could attack bagrada eggs for most of their development in the field but storage of bagrada eggs at very low temperature for later use in rearing or as sentinel eggs in the field is not recommended. These data provide a baseline for designing host specificity tests to help determine whether *G. gonikopalense* is suitable to use as a biocontrol agent of *Bagrada hilaris*.

1. Introduction

Bagrada hilaris (Burmeister) (Hemiptera: Pentatomidae), also known as bagrada bug (BB) or painted bug, is reported as a major pest of crucifer crops (Brassicaceae) in its native range of eastern and southern Africa and the Indian subcontinent (Ahuja et al., 2008; Kavita et al., 2014). In 2008, BB was first reported in California and since has spread to New Mexico (Bundy et al., 2012), Arizona (Palumbo and Natwick, 2010), Nevada, Utah and Texas, USA (Palumbo et al., 2016; Reed et al., 2013a,b), as well as to Mexico (Sánchez-Peña, 2014), Hawaii (Hdoa, 2014), and Chile (Faúndez et al., 2016). In California, BB attacks a wide range of cruciferous crops, including cabbage, broccoli and radish (Huang et al., 2014b; Huang et al., 2014a; Palumbo et al., 2016; Reed et al., 2013b). Yield losses caused by BB reached 25% in Arizona on crucifer crops, (Palumbo and Center, 2011) and up to 70.4% on Indian mustard crops (Ahuja et al., 2008; Joshi et al., 1989). The pest completes its life cycle in 18 days at 30 °C (Reed et al., 2017; Reed et al., 2013b) and fecundity reaches 150 eggs per female (Singh and Malik, 1993) depending on numerous factors such as host plant and population density (Madan, 2015).

To date, no efficient monitoring tools exist to manage for the long term BB populations in North America (Palumbo et al., 2016). The massive economic loss and the lack of alternative control practices, led U.S. growers to use broad-spectrum insecticides to reduce local pest populations (Palumbo et al., 2015). However, newer insecticides under evaluation in laboratory have been shown to be weakly effective on BB (Palumbo, 2011; Palumbo et al., 2015). Furthermore, Californian organic growers need an alternative and sustainable management of BB, and have funded a program on classical biological control (CBC) based on foreign exploration in the native range (Mahmood et al., 2015).

Biological control of other pentatomid pests has focussed on the use of egg parasitoids and the study of their biology (Awan et al., 1990; Forouzan et al., 2015; James and Warren, 1991; Orr and Boethel, 1990). In the Indian subcontinent, egg parasitoids from several genera

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in the family Scelionidae have been reported from BB: *Telenomus* Haliday, *Trissolcus* Ashmead and *Gryon* Haliday (Chacko and Katiyar, 1961; Narayanan et al., 1959; Rajmohana, 2006). Tachinid fly parasitoids have also been reported in Africa and India attacking *B. hilaris* adult stage (Anwar et al., 1973; Rakshpal, 1954).

Initial surveys in Pakistan, lead to the collection of three species of native BB egg parasitoids, *Trissolcus hyalinipennis* Rajmohana and Narendran (Rajmohana, 2006), *Ooencyrtus* sp. (Hymenoptera, Encyrtidae) and *Gryon* sp. (Mahmood et al., 2015). The scelionid species are mainly known to attack coreid and pentatomid bugs and have been known to provide efficient candidates for CBC programs for several decades (Colazza and Bin, 1995; Dasilao and Arakawa, 2004; Laumann et al., 2008; Nechols et al., 1989). This study focuses on the genus *Gryon*, in which several species have been studied as potential biological control candidates against coreid bugs, e.g. species of *Clavigralla* Spinola on cowpea crops in Nigeria (Asante et al., 2000), pigeon pea crops in India (Romeis et al., 2000), *Leptoglossus occidentalis* (Heidemann) on conifers in Europe (Peverieri et al., 2013; Peverieri et al., 2012), and can already represent important parasitoids in their native area (Maltese et al., 2012). However, despite a special emphasis worldwide for using Scelionidae to manage stink bug species (Forouzan et al., 2015; Nechols et al., 1989; Orr and Boethel, 1990; Peverieri et al., 2012; Romeis et al., 2000), an important lack of biological, ecological and behavioural knowledge still remains.

The aim of the present study is to clarify the species status of the *Gryon* sp. collected from BB in Pakistan, reared in the quarantine in France, and to understand its reproductive and developmental biology in the frame of a CBC for California. We studied life history traits of *Gryon* sp. on *B. hilaris* eggs under controlled conditions linked to the reproduction and the individual development as well as their longevity. Moreover, we investigated whether the age of the host and its storage condition can influence acceptance by the parasitoid.

2. Material and methods

2.1. Parasitoid identification

Comprehensive identification tools for Old World *Gryon* currently do not exist. Our determination of the Pakistani specimens as *G. gonikopalense* is based on direct comparison with the female holotype (collected in India), which was the only specimen included in its original description (Sharma, 1982). The Pakistani specimens are morphologically congruent with the holotype (Figs. A1 and A2), but they are of smaller size (1.0 mm vs. 1.3 mm). This is likely the result of emergence from eggs of different sizes, which is a known phenomenon in Scelionidae egg-parasitoids (Medal and Smith, 2015). The forewings of Pakistani *G. gonikopalense* exhibit a banding pattern (Fig. A3) that was not mentioned in the original description of *G. gonikopalense*. The holotype specimen no longer has wings, so the presence or absence of banding in the type specimen cannot be verified. The colour of appendages in *Trissolcus japonicus* (Ashmead) (Scelionidae) is known to be affected by host species (unpublished data) and we suspect that a similar phenomenon with colour, including wing colour, occurs in *Gryon*. Additionally, a current revision of Old World *Gryon* suggests that some species are widespread, and that coloration of the wings is a variable character. If *G. gonikopalense* is indeed one widespread species, the name will likely be treated as a junior synonym. We here use the name *G. gonikopalense* until the broader analysis is complete, and alert readers to the possibility of a name change.

2.2. Experimental conditions

The laboratory colonies of *Bagrada hilaris* and *Gryon gonikopalense* used in this study were maintained under official authorization in the quarantine greenhouse of the European Biological Control Laboratory (USDA-ARS-EBCL) at Montferrier-sur-Lez (France).

2.2.1. The host

Bagrada hilaris colonies were obtained in 2016 from a colony maintained at the University of California, Riverside, USA and were maintained in BugDORM® insect cages (60 × 60 × 60 cm, 680 µm opening mesh) in the quarantine greenhouse (temperature ranged from *ca.* 25 °C (night) to *ca.* 35 °C natural day light was supplemented with sodium lamps (L/D 12:12; RH: 50 ± 20%). Depending on availability, insects were fed various greenhouse-grown Brassicaceae (22 °C, RH: 50 ± 20): *Lobularia maritima* (alyssum), *Raphanus sativus* (radish), *Brassica oleracea* var. *capitata* (cabbage), *Brassica oleracea* var. *italica* (broccoli), and *Sinapis alba* (white mustard). Field collected *Diplotaxis erucoides* (false arugula) was also used.

2.2.2. The parasitoid

The colony of *G. gonikopalense* was originally obtained from BB sentinel eggs exposed and parasitized in the field in Pakistan (Mahmood et al., 2015). The parasitoid was imported into France, and then introduced in the EBCL quarantine, under the permit #16LR015 delivered by the French government of Agriculture. This breeding colony was maintained in plastic boxes (18 × 12 × 5.5 cm) made insect proof by the addition of Parafilm® at the joint between the base and the lid. The lid was aerated by a rectangular hole (15 × 6 cm) covered with a nylon mesh fabric (160 µm aperture). Boxes were maintained in a walk-in rearing chamber (22 ± 0.5 °C, RH 50 ± 5%, L/D 12:12) and insects were fed with drops of diluted Acacia honey (Lune de Miel®, France) deposited once a week on the mesh. The *G. gonikopalense* colony was routinely maintained by exposing 24 h-old fresh *B. hilaris* eggs for three to four hours to adults for oviposition twice a week.

2.2.2.1. Bagrada hilaris egg collection. Folded brown paper towels (20 × 20 cm) were hung at the top of BugDORM® cages for BB females to lay eggs on. Less than 24 h-old eggs were collected by cutting small squares of paper towels surrounding each egg. Individual eggs were then glued (UHU stic®, Germany) next to each other on a piece of cardboard (5 × 5 cm).

2.3. Development time

Of the BB eggs parasitized by *G. gonikopalense* to maintain the parasitoid colony, we daily collected emerging adult parasitoid progeny (92 males and 121 females for a total of n = 213) from the first to the last day of emergence for ten sessions of exposures. Each progeny was sexed, and the development time was calculated from the day of exposure to the emergence of an adult parasitoid. For each session of exposure, we calculated the proportion of parasitized eggs that gave an adult in order to obtain the average larval survivorship.

2.4. Parasitoid longevity

Adult longevity was measured on 112 newly emerged *G. gonikopalense* males and females individually confined in sterile Petri dishes (Falcon®, 50 × 9 mm, Corning incorporated, USA) and divided in three groups. In group 1, females (n = 17) and males (n = 16) were kept individually without mating or ovipositing. In group 2, mated females (n = 23) were daily provided with fresh BB eggs and oviposited throughout their life (see Section 2.5 for details). Males (n = 13) were each kept with one female, different from the 23 females previously described, and could mate until they died. Groups 1 and 2 were fed with honey ad libitum and supplied with water on moistened cotton. In group 3, females (n = 17) and males (n = 26) were kept individually without mating or ovipositing and were only supplied with water on moistened cotton. Individuals were kept under rearing room conditions as described above and checked every day until they died.

2.5. Parasitoid fecundity

Twenty-three pairs of newly emerged *G. gonikopalense* adults (< 24 h old) were each confined in a sterile Petri dish (Falcon®, 50 × 9 mm, Corning incorporated, USA) containing 0–2 day old BB eggs and one drop of diluted honey. Because it was very time-consuming to prepare BB eggs for presentation to the parasitoids, we minimized the number of eggs to present while aiming to provide enough to exceed the number expected to be attacked. The daily oviposition rate of other scelionids has been observed to decrease with age (Canto-Silva et al., 2006; Ehler et al., 2002; Peverieri et al., 2012), and we saw a similar pattern in pre-experimental trials with *G. gonikopalense*. Thus, the number of fresh eggs that were presented daily decreased progressively: 35 eggs on the first day, 20 from day 2 to day 6, 15 from day 7 to day 9, and 10 from day 10 until the females died. In order to determine precisely whether or not a pre-oviposition period did exist, less than 24 h-old females were kept with a virgin less than 24 h-old male for six hours before being exposed to host eggs for the first time, so that they were likely to be inseminated and time between their emergence and their first oviposition was minimized. Males were then removed from the Petri dishes to avoid disruption of female oviposition. Eggs exposed to parasitism were kept in a Petri dish under the same conditions until adult emergence. Thus, we counted for each day of oviposition the total number of parasitized eggs (indicated by a black coloration), and the number of males and females emerged. Females that produced only male progeny were excluded from analysis, on the assumption that they had failed to mate.

2.6. Parasitoid host age preference in no-choice test

One hundred and twenty newly emerged (< 24 h-old) *G. gonikopalense* virgin females were collected from the rearing colony and each was placed with a male for 48 h. Then, each female was transferred with a fine brush in a sterile Petri dish (Falcon®, 50 × 9 mm, Corning incorporated, USA) containing a 6-egg BB cluster of one of the four following age-classes: 0–1 day (n = 20), 1–2 days (n = 20), 2–3 days (n = 20), 3–4 days old (n = 20); or 0–1-day-old eggs frozen at −80 °C (n = 40). Fresh eggs were collected daily and were held in the walk-in rearing chamber for 0, 1, 2 or 3 days to produce the various age classes. For obtaining frozen eggs, clusters of 0–1 day old eggs were placed successively for 24 h at 4 °C (Electrolux Space.Plus®), 2 h at −24 °C (Ariston A Class®), and finally transferred to a −80 °C freezer (Thermo Fisher Scientific® Forma Scientific 929) outside the quarantine facility using a Quick Chill container, and kept for 2–3 days. We stepped down the temperature to limit a thermic shock between ambient temperature and −80 °C that might damage eggs. Eggs were exposed to females for one hour in the rearing room conditions for parasitism, and then eggs were kept under the same conditions until parasitoid emergence. Frozen eggs were allowed to warm up for one hour at 20 °C before exposing them to females. The eggs did not show any physical signs of dehydration, such as wrinkled chorion. For the following sections, a "parasitized egg" means a BB egg that turned black after exposure to parasitoids, even if no parasitoid progeny emerged from the egg. Non-parasitized BB eggs turn red over time, and remain yellow-brown if the bagrada larva dies during its early development, or dark brown if it dies before larva emergence. To ensure that a non-hatched egg could be considered as parasitized, all eggs were dissected after experiments, to determine whether a parasitoid larva, nymph or adult was inside.

2.7. Statistical analyses

All measured life parameters (development time, longevity, larval survivorship, number of parasitized eggs, number of progeny emerged and associated sex-ratio, post-oviposition period) were described as means ± standard deviation or means with their 95% confident interval.

Data described above were used to calculate demographic parameters of *G. gonikopalense* laboratory colony. The intrinsic rate of increase was calculated using the equation (Birch, 1948):

$$\sum lxmxe^{-rm.x} = 1$$

where x = age in days, l_x = age-specific survival rate, m_x = age-specific fecundity, r_m = intrinsic rate of natural increase. The variables l_x and m_x were measured experimentally and other demographic parameters were calculated as follows:

- Intrinsic rate of increase: $r_m = \ln(\sum lxmx)/T$
- Finite rate of increase: $\lambda = e^{rm}$
- Mean generation time: $T = (\sum x.\ lx.mx) / R_0$
- Doubling time: $T_d = \ln(2)/ r_m$
- Net reproductive rate: $R_0 = \sum lxmx$
- Gross reproductive rate: $GRR = \sum mx$

We used the Jackknife technique (Hulting et al., 1990) to estimate each parameter in order to eliminate the statistical uncertainty of calculated values (see Meyer et al., 1986). Average longevities were compared between males and females of groups 1, 2 and 3 (see part 4 of the Material and Methods) with one-factor ANOVA test followed by a Tukey post hoc test to identify differences between treatments. Correlation between female fecundity and longevity was calculated by the Pearson's coefficient. We used Kruskall-Wallis tests to compare the number of eggs parasitized by a single female and the percent emergence between the four egg age-classes and the frozen treatment as no transformation could provide normality for these data. Differences among egg classes were detected with a Nemenyi post hoc test. All statistical analyses were carried out with R software and figures were obtained with R software or Excel software. For all tests, the alpha significance threshold was set at $\alpha = 0.05$.

3. Results

3.1. Development time

Male parasitoids completed their development in 24.42 ± 1.34 days whereas females achieved it in 25.57 ± 1.33 days. Fifty percent of all male progeny emerged within two days after the first emergence day whereas females needed three days (Fig. 1). We obtained 90% of the male progeny after 25 days and 90% of the female progeny after 27 days. Ninety percent of all the progeny emerged after 27 days (Fig. 1).

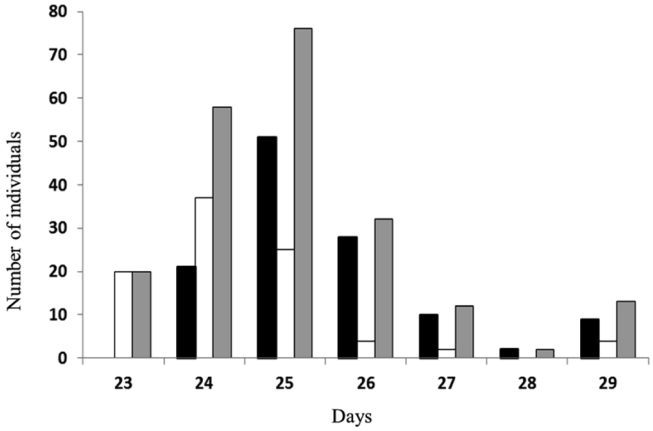


Fig. 1. Distribution of development times in *Gryon gonikopalense* males (white bars, n = 92), females (black bars, n = 121) and combined sexes (grey bars, n = 213).

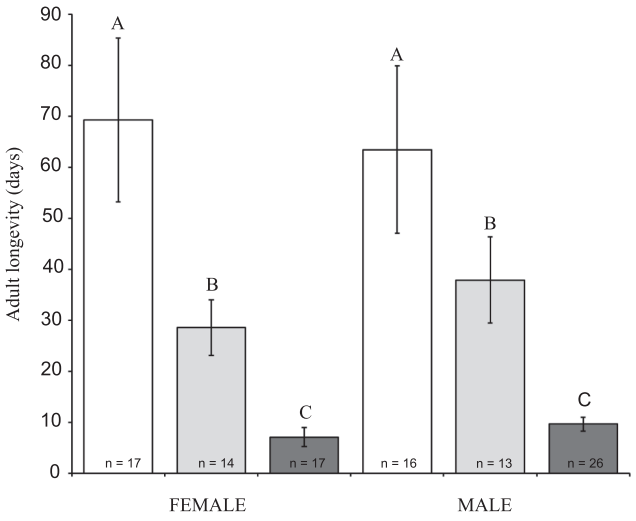


Fig. 2. Effect of reproductive activity and food access on mean longevity of male and female *Gryon gonikopalense* adults. White bars: no oviposition nor mating and food and water ad libitum; light grey bars: oviposition (females) and mating (males) and food and water ad libitum; dark grey bars: no oviposition nor mating and only water provided. Vertical bars represent 95% Confident Interval, the number at the bottom is the sample size. Results that significantly differ are represented by different letters (A, B and C) above standard deviations (ANOVA test followed by Tukey post hoc test).

3.2. Longevity

Without mating or ovipositing, adult *G. gonikopalense* fed with honey and water *ad libitum* lived 66.42 days ($CI_{95\%}$ = [55.55; 77.29] in our laboratory conditions. We observed 52% lifespan reduction when individuals had reproductive activity (31.89 days ($CI_{95\%}$ = [27.26; 36.52]) and 87% lifespan reduction when they were deprived of honey (8.60 days ($CI_{95\%}$ = [7.46; 9.74]) (ANOVA test, F = 35.03, df = 5, p-value = 2.2e−16). However, no differences were observed between males and females longevity for any treatment (Fig. 2).

3.3. Fecundity and demography

Of the 23 *G. gonikopalense* females tested, ten were excluded from analysis (three did not give any progeny and seven gave only males). Thirteen females produced male and female progeny, for a total of 770 offspring. The total offspring produced by a single female during her life ranged from 20 to 94, with a mean value of 59.23 offspring/female (Table 1). The number of BB eggs killed (including parasitized eggs that failed to emerge) by a single female during her life ranged from 35 to 139, with a mean value of 87.08 ± 28.28. All females started parasitism on the day of emergence, without an observable pre-ovipositional period.

The highest mean rate of parasitism was recorded at day 1 with 12.62 ± 9.01 eggs/female, which was then followed by a regular decrease in mean rate (Fig. 3). Fifty percent and 90% of all progeny were

Table 1
Reproduction parameters (means ± S.D.) of *Gryon gonikopalense* (Hym.: Scelionidae) reared on *Bagrada hilaris* (Hem.: Pentatomidae) eggs at 22 ± 0.5 °C, RH: 50 ± 5%, L/D 12:12 and adults fed with honey ad libitum.

Life parameters	Mean value (± S.D.)
Fecundity (offspring/female)	59.23 ± 22.29
Post-oviposition period (% of longevity)	23.33 ± 19.58
Number of males produced (male offspring/female)	32.62 ± 18.77
Number of females produced (female offspring/female)	26.62 ± 21.43
Mean progeny sex ratio (% female)	46.26 ± 22.70
Mean larval survivorship (%)	72.05 ± 14.80

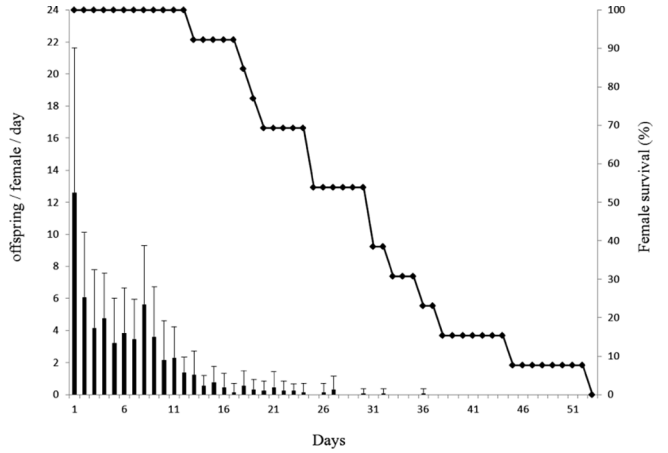


Fig. 3. Mean fecundity (black bars) of *Gryon gonikopalense* based on 13 females (black vertical lines are the standard deviations). The curve with black squares is the cohort survivorship (%) over the maximal longevity obtained during the experiment (52 days).

obtained on day 4 (359 individuals) and day 12 of emergence (692 individuals), respectively. A consistent decline in survival began at day 18, and almost 50% (53%) mortality occurred at day 24 (Fig. 3). The number of progeny produced per female was not significantly correlated to its longevity (Pearson's correlation, $r = -0.27$, $n = 13$, p-value = 0.38). The sex ratio of the total number of *G. gonikopalense* progeny obtained was 44.93% female, which is similar to the mean sex ratio obtained per female (see Table 1).

Table 2 shows the intrinsic rate of increase (r_m) of *G. gonikopalense* on BB eggs is 0.11 per day, which implies that a parasitoid colony with a stable age distribution under our laboratory conditions is able to multiply 1.11 times a day (λ). At this rate, the size of the colony doubles in 6.40 days (T_d). Females were able to produce an average of 26.4 female progeny during their lifetime and the average generation time (T) was 30.2 days.

3.4. Host age suitability

Under our laboratory conditions, *G. gonikopalense* could equally parasitize live host eggs, regardless of their age up to 3–4 days old, (Fig. 4, Table 3), but frozen eggs were much less suitable (Kruskall-Wallis test: $\chi^2 = 32.66$, df = 4, p-value < 0.0001; Fig. 4, Table 3). However, the proportion of progeny emerging from parasitized eggs did not significantly differ among the egg treatments, with emergence rate from 58.93% ($CI_{95\%}$ = [44.33; 73.52]) for 3–4 days old eggs to 87.04% ($CI_{95\%}$ = [61.59; 100]) for frozen eggs, but was lower for 2–3 days old

Table 2
Demographic parameters (means ± S.D.) of *Gryon gonikopalense* (Hym.: Scelionidae) reared on *Bagrada hilaris* (Hem.: Pentatomidae) eggs at 22 ± 0.5 °C, RH: 50 ± 5%, L/D 12:12 and adults fed with honey ad libitum. For each demographic parameter, the "True sample value" is the value calculated directly from the sample of tested females. The "Jackknife estimation" is the value obtained after applying the Jackknife method.

Parameters	True sample value	Jackknife estimation
Intrinsic rate of increase (r_m) (days-1)	0.094 ± 0.038	0.11 ± 0.0023
Finite rate of increase (λ)	1.10 ± 0.041	1.11 ± 0.0026
Mean generation time (T) (days)	27.63 ± 8.57	30.19 ± 0.14
Doubling time (T_d) (days)	6.67 ± 2.81	6.40 ± 0.14
Net reproductive rate (R_0) (female progeny/female)	26.42 ± 21.30	26.42 ± 1.78
Gross reproductive rate (GRR) (female progeny/female)	26.62 ± 21.43	26.62 ± 1.79

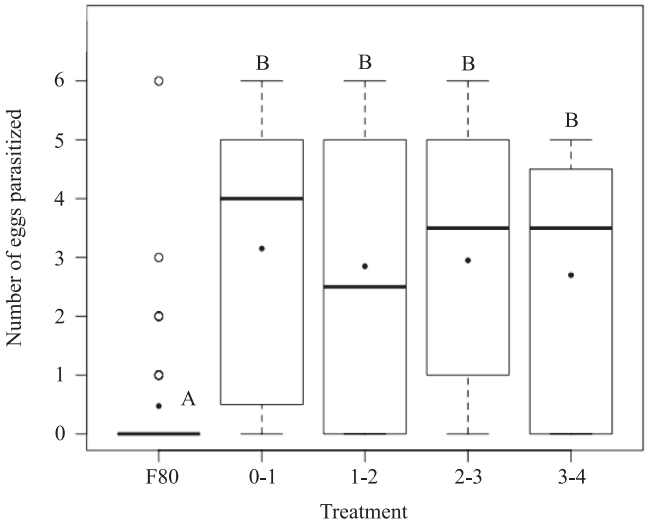


Fig. 4. Number of eggs parasitized by *Gryon gonikopalense* on live *Bagrada hilaris* eggs from 0–1 days to 3–4 days old (n = 20) or frozen 0–1-day-old eggs (F80, n = 40). Edges of each box plot represent the second and the third quartiles; the central lines in bold, the medians; the dashed lines include the first and the fourth quartiles. The black circles are the mean of each group and the white circles are outliers. Mean values associated with the same letter did not significantly differ (Kruskall-Wallis test followed by nemenyi post hoc test).

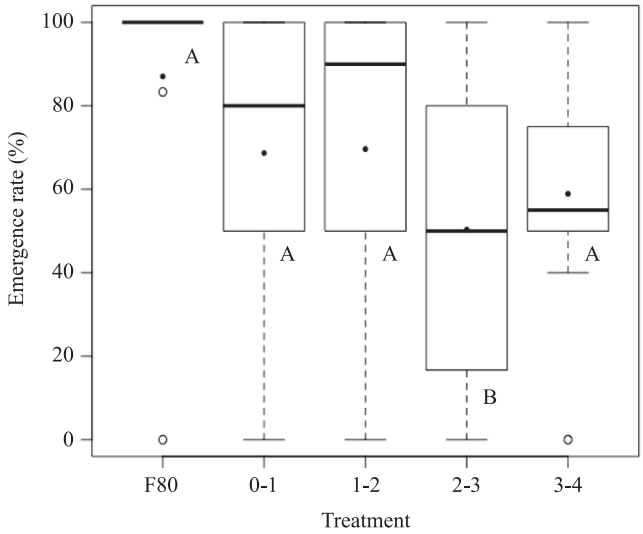


Fig. 5. Emergence rate of *Gryon gonikopalense* from parasitized eggs of *Bagrada hilaris* that were live: 0–1 days (n = 15), 1–2 days (n = 14), 2–3 days (n = 16), 3–4 days old (n = 14), or frozen 0–1-day-old eggs (F80, n = 9). Edges of each box plot represents the second and the third quartiles; the central lines in bold, the median; the dashed lines include the first and the fourth quartile. The black circles are the mean of each group and the white circles are outliers. Mean values associated with the same letter did not significantly differ (Kruskall-Wallis test followed by nemenyi post hoc test).

eggs with only 50.31% ($CI_{95\%}$ = [31.36; 69.27]) (Kruskal-Wallis test: χ^2 = 10.974, df = 4, p-value = 0.02686; Fig. 5, Table 3). Furthermore, the 17 frozen parasitized eggs gave an adult parasitoid, except one. There was no host feeding during the experiment and we know that there is no host feeding for this parasitoid during the oviposition process (data not published).

4. Discussion

We measured life history traits of *G. gonikopalense* under one set of laboratory conditions (22 °C, 12 h photoperiod). Larval development time (25.6 days) for females inside BB eggs was consistent to that obtained for other *Gryon* species, ranging from 20 to 30 days at the same temperature (Asante et al., 2000; Peverieri et al., 2012). For instance, development time at 22 °C was 25.4 days for *Gryon philippinense* (Ashmead), 30 days for *G. gallardoi* (Brethes), 24.4 days for *G. clavigrallae* (Mineo), 21.5 days for *Trissolcus plautiae* (Watanabe), and 29.6 days for *Trissolcus chloropus* (Thomson) (Canto-Silva et al., 2006; Dasilao and Arakawa, 2004; Romeis et al., 2000; Arakawa and Namura, 2002; Orr et al., 1985). Furthermore, *G. gonikopalense* adults are long-lived, and can survive over two months when fed with honey with no host available, though we have no evidence that the parasitoid can live as long in the field. A long adult longevity allows *G. gonikopalense* to survive when host availability is reduced, which occurs during the summer in California, when adult *B. hilaris* move to alternate non-crucifer host-plants (Huang et al., 2013; Palumbo et al., 2016). However, the low longevity of adults deprived of honey shows that a food source would likely be necessary for the parasitoid survivorship in the environment, similarly to what was suggested for *G. pennsylvanicum* (Peverieri et al., 2012). Thus, the potential impact of the parasitoid on *B. hilaris* populations once released in the field would likely depend on the availability of an adult food source like nectar.

In laboratory conditions, we observed that female development time was slightly longer than for males, which is consistent with other Scelionidae such as *Gryon* spp. (Dasilao and Arakawa, 2004; Romeis et al., 2000) and *Trissolcus* species (Arakawa and Namura, 2002). This protandry can be explained by at least two hypotheses: first, a shorter development time allows males to increase mating events with new emerging females; secondly, as fecundity reaches a peak during the first week after emergence, it can be advantageous for males to be ready for mating during this period (Morbey and Ydenberg, 2001). It is especially true considering that *G. gonikopalense* female fecundity rapidly decreases with time, as described by our data showing that 90% of all progeny was produced before day 12 after emergence. This measured fecundity pattern and overall number of progeny observed for *G. gonikopalense* (59.23 offspring/female) is common among *Gryon* species including *G. clavigrallae* (56.4 +/- 4.4 eggs; Romeis et al., 2000), *G. gallardoi* (67.5 ± 11.3 eggs; Canto-Silva et al., 2006), *G. pennsylvanicum* (71.8 ± 23.1 eggs; Vogt and Nechols, 1993) or *G. philippinense* (82.3 ± 14.7; Dasilao and Arakawa, 2004). Among the Scelionidae, *G. gonikopalense* has an intermediate level of fecundity. For example,

Table 3
Comparison of the number of eggs parasitized and the parasitized eggs emergence rate between five egg categories: Frozen at −80 °C (F80); 0–1 day old; 1–2 days old; 2–3 days old and 3–4 days old. Values are given as mean with associated 95% confident interval ($CI_{95\%}$) and mean values followed by the same letter did not significantly differ (Kruskall-Wallis test followed by nemenyi post hoc test).

Parameters		Host Eggs Category					Kruskall-Wallis Test		
		F80	0–1 day	1–2 days	2–3 days	3–4 days	χ^2	df	p-value
Number of eggs parasitized	Mean	0.48 (A)	3.15 (B)	2.85 (B)	2.95 (B)	2.70 (B)	32.66	4	< 0.0001
	CI 95%	[0.11; 0.84]	[2.11; 4.19]	[1.69; 4.01]	[1.97; 3.93]	[1.74; 3.66]			
Emergence rate (%)	Mean	87.04 (A)	68.67 (A)	69.64 (A)	50.31 (B)	58.93 (A)	10.97	4	0.0269
	CI 95%	[61.59; 100]	[49.76; 87.58]	[47.28; 92.02]	[31.36; 69.27]	[44.33; 73.52]			

Trissolcus basalis Wollaston, a biocontrol agent of *Nezara viridula* (Birch) (Pentatomidae), can reach an average value of 292.4 eggs per female (Forouzan et al., 2015), whereas *Telenomus podisi* (Ashmead) and *Trissolcus cristatus* (Johnson) showed a lower fecundity level than *G. gonikopalense*, with respectively 39.6 ± 4.5 and 32.2 ± 1.80 eggs (Orr and Boethel, 1990; Yeargan, 1982). On the other hand, the peak of age-specific parasitism rate at the beginning of the oviposition period of *G. gonikopalense* should provide a fast response to the host population after a hypothetical release in the field and a shortening of the generation time when hosts are abundant. Once the population of *G. gonikopalense* has reached a stable age distribution, we showed that its intrinsic rate of increase allows it to double within less than one week. Measured demographic parameters of *G. gonikopalense* indicate an overall good performance at 22 °C considering that a stable BB population is able to double in 3.7 days on rapeseed crops under field fluctuating temperatures (Naryana and Phadke, 1987), though no other study measured the BB intrinsic rate of increase on other crucifers. However, 22 °C is likely to be a low temperature for *G. gonikopalense* regarding its native area and its host's thermal optimum of 36 °C (Reed et al., 2017). This could explain why we observed a larval survivorship of only 72.05 ± 14.80%, a not so high fecundity in comparison to other *Gryon* species and a high level of variability among individual performances. Furthermore, though *G. gonikopalense* female fecundity ranged from 20 to 94 offspring, the number of BB eggs they killed was higher (from 35 to 139) because it included parasitized eggs that failed to give a viable parasitoid progeny but prevented the BB larva to develop. Thus, the fecundity value we measured could underestimate the true mortality imposed by *G. gonikopalense* on BB. In the case of inoculative releases, the latter favours massively releasing this parasitoid in the introduced range where BB is invasive. The sex ratio of *G. gonikopalense* (44.93% females) that we observed was lower than that observed in other *Gryon* spp., such as *G. pennsylvanicum* reared on *L. occidentalis* (70.42% females), *G. clavigrallae* reared on *C. scutellaris* (75% females) or *G. gallardoi* reared on S. *dentiventris* (79% females) (Canto-Silva et al., 2006; Peverieri et al., 2012; Romeis et al., 2000). It is possible that sex ratio could be impacted by an insufficient mating time for the female as only fertilized eggs can give a female progeny. However, the effect of mating time on sex ratio in Scelionidae is poorly documented, especially for the genus *Gryon*. In our study, males used to mate very fast with tested females (pers. obs.) and the latter were potentially already inseminated by other males before we isolated them from the rest of the colony. Furthermore, 4 out of the 13 females analysed gave more than 70% of females. Finally, results obtained from preliminary tests allowing males to permanently stay with a single female throughout its life (according to the method used by Peverieri et al. (2012)), lead to very low and inconsistent fecundity patterns. Several females did not manage to parasitize any host egg before at least one week and we supposed that the male could have disrupted the female oviposition. In our study, it appears from individual female performances that the sex ratio we obtained could be due to a high inter-individual variability, which is also observed for the daily and lifetime female fecundity. Increasing the sex ratio would increase the population growth rate, and it would be interesting to learn why the sex ratio was lower for *G. gonikopalense* in our study than for other *Gryon* species.

Furthermore, in the field, a parasitoid's fecundity can be limited by the host's density and its accessibility (Price, 1974). Parasitoids attacking hosts species with large egg clusters or that lay a high number of eggs are more likely to allocate energy in egg production and high fecundity (Price, 1974). Differences in fecundity between parasitoid species previously cited could be explained by differences in their host reproduction strategies. *Bagrada hilaris* dominantly lays its eggs individually so that egg clusters are rarely observed and always with a very small number of eggs (Halbert and Eger, 2010; Reed et al., 2013a; Taylor et al., 2014). Moreover, an unknown proportion of BB eggs are laid in the soil, and are likely more difficult to be found by egg parasitoid wasps (Taylor et al., 2014). The amount of energy necessary to find and parasitize these individual and buried eggs should lead to trade-offs with the fecundity (Jervis et al., 2008). However, host availability does not only concern the number of hosts in the field, but also their quality. Indeed in the field, host quality related to its age may change rapidly (Vinson, 1998). Younger eggs are therefore expected to be favoured by female parasitoids because they are more adequate for offspring development and survival, which is often reported for other *Gryon* spp., including *G. clavigrallae*, *G. nixoni* (Dodd) and *G. japonicum* (Da Rocha et al, 2006; Morrill and Almazon, 1990; Nechols, 1995; Noda, 1993; Romeis et al., 2000). The development issues due to host quality should be especially true considering the very short development time of BB (Atwal, 1959; Azim and Shafee, 1986; Kavita et al., 2014). However, we showed that *G. gonikopalense* females parasitized host eggs from less than 24 h to 4 days old without difference in parasitism rate, which is similar to *G. pennsylvanicum* (Peverieri et al., 2012) or *Telenomus podisi* (Zhou et al., 2014). The ability to utilize hosts of a wide range in age, even if older eggs lose quality, would increase fitness of females that must attack eggs that are difficult to find. We observed a lower immature survival rate of *G. gonikopalense* on older eggs (Fig. 5), but at least 50% still produced adults. Thus, the host age can affect the ability of *G. gonikopalense* larvae to complete development though the range of acceptable host is broad. Similar results were obtained with *G. pensylvannicum* (Nechols et al., 1989) and *G. gallardoi* (Da Rocha et al., 2006), and might be explained by a depletion of egg nutritive contents due to the host development over time (Vinson, 1998).

In addition, we showed that frozen host eggs were not suitable for *G. gonikopalense* female oviposition, which refused to attack most of them. Little is known about suitability of frozen host eggs for rearing Scelionidae species, especially *Gryon* spp. Among them, *G. pennsylvanicum* was able to parasitize *Leptoglossus occidentalis* eggs that were frozen at −80 °C with an average success rate from 54% to 84% (Peverieri et al., 2015) like *Trissolcus semistriatus* whom the parasitism rate remains higher than 70% on host eggs stored up to 2 months at −20 °C (Kivan and Kilic 2005). On the contrary, *Gryon gnidus* (Nixon) was unable to parasitize eggs of *Acanthomia (=Clavigralla) tomentosicollis* (Hemiptera: Coreidae) that were killed by a frozen treatment, showing that the viability of the host embryo was required to induce a successful parasitism (Egwuatu and Taylor 1977). In the case of *G. gonikopalense*, freezing of host eggs did not affect the development of parasitoid larvae, and all frozen eggs that were parasitized gave an adult progeny, though very few were parasitized. Unparasitized eggs did not show any damage induced by the parasitoid oviposition. Thus, it appears that the frozen storage of BB eggs modified the acceptance by the parasitoid (pre-ovipositional barrier) but not the survivorship of offspring inside the egg (post-ovipositional barrier). It would be interesting to determine why parasitism rate was so low despite the fact that frozen eggs appeared to be physiologically suitable for development, because understanding this might be useful for laboratory rearing. However, we currently recommend that frozen eggs should not be used for rearing *G. gonikopalense* in laboratory conditions or for field exposure as sentinel eggs, and that fresh eggs provide the best results. Despite this result, it is not excluded that a mass rearing could be foresee for *G. gonikopalense* and new experiments are currently being conducted in our laboratory to improve both host and parasitoid production.

The present study leads to the conclusion that *Gryon gonikopalense* is a promising egg parasitoid wasp for the biological control of *Bagrada hilaris*. Considering the high reproductive rate and fecundity pattern under laboratory conditions, *G. gonikopalense* is expected to have a strong parasitism pressure on BB over a short time lapse. Thus, local and rapid beneficial effects on crucifer crops could be considered. The high longevity of adult parasitoids and the wide age range of host egg suitability should also contribute to their efficiency in the field. However, our results were obtained under laboratory conditions and numerous environmental factors are expected to affect *G. gonikopalense* success in an open environment, such as food availability that may be a

limiting factor for a permanent establishment. Our results also provide a baseline for designing host specificity tests to help determine whether *G. gonikopalense* is suitable to be used as a biocontrol agent against *Bagrada hilaris*. Thus, studies must now focus on possible impact of *G. gonikopalense* on non-target species as well as interactions with other egg parasitoids species. For instance, the Scelionidae *Trissolcus hyalinipennis* that was recently found in California on BB eggs (Ganjisaffar et al., 2018) could compete with *G. gonikopalense* and limit its establishment. Finally, studies must be performed regarding the specific oviposition behaviour of *Bagrada hilaris* (Taylor et al., 2014) and the ability of *G. gonikopalense* to find and successfully parasitize burried host eggs.

Appendices

Fig. A1–A3.

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Fig. A1. *Gryon gonikopalense* (Hym.: Scelionidae), female holotype (USNMENT01109129), lateral habitus.



Fig. A2. *Gryon gonikopalense* (Hym.: Scelionidae), female collected in Pakistan (USNEMNT01109047), lateral habitus.

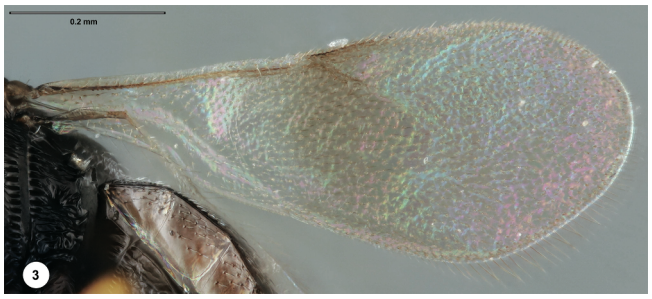


Fig. A3. *Gryon gonikopalense* (Hym.: Scelionidae), male collected in Pakistan (FSCA 00033215), fore wing, ventral view.

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Temperature effect on development, survivorship and reproduction of *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae), an egg parasitoid of *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae) in the frame of an importation biocontrol program

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Abstract:

Temperature is known to have strong effects on insect's physiology, and the success of a biocontrol program therefore relies on the biocontrol agent acclimatization capacities. Coevolved natural enemies of the target pest are expected to be better candidates as they share the same climatic range. *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae) is one of the most recent invasive pests in USA and mainly damages Brassicaceae cropping systems in California. An egg parasitoid from Pakistan, *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae), is currently evaluated as a biocontrol agent. The present study conducted in climatic chambers aimed to determine, the thermal range of *G. gonikopalense* for a possible establishment in USA. We assessed the effect of eight constant thermal regimes (from 15 to 40°C) on the parasitoid development, fecundity longevity and demographic parameters. *Gryon gonikopalense* was able to develop between 18 (46.85 ± 0.6 days) and 37.5 °C (7.28 ± 0.09) with a maximum emergence rate at 25°C (95.53 ± 1.96 %). The parasitoid could reproduce under all temperature regimes, with the highest fecundity measured at 30 °C (104.47 ± 21.2 progeny/female). In addition, the longevity of adult parasitoids reached 151.3 ± 6.8 days at 15°C and decreased with temperature to only 4.28 ± 0.26 days at 40 °C. Our results suggest that *G. gonikopalense* would be able to establish and control bagrada in the warmest regions of USA, but would not provide promising control in crucifer crops of Californian coastal regions.

Key-words: Acclimatization, life history traits, Scelionidae, biological control

1. Introduction

Importation biological control programs aim to regulate invasive species by importing selected natural enemies from their native range. The success of such programs first depends on the biocontrol agent's ability to survive and sustainably establish in a new environment (Hokkanen and Sailer, 1985). Among the many environmental barriers to overcome for the biocontrol agent, the acclimatization to the invaded range thermic conditions is described as one of the most important factors for a successful establishment (Hokkanen and Sailer 1985; Stiling, 1993; Hoelmer and Kirk, 2005). Several studies have shown that temperature has the strongest impact on egg parasitoids' biology (Kalyebi et al. 2006; Bruce et al. 2009; Dhillon and Sharma 2009; Seo et al. 2019 and many others).

Indeed, almost all biocontrol agents against weeds and insect pests are arthropods, mostly insects (Stiling and Cornelissen, 2005). Outdoor temperatures and their seasonal variations have therefore a strong influence on their development, their reproduction (Régnière et al. 2012) and even on physiological functions such as the immune system (Iltis et al. 2018). Hence, many species are known to express variations of their development cycle, their reproductive or feeding activity depending on the day or the year period (Logan et al. 2006; Régnière et al. 2012). At a larger scale, temperature is therefore expected to strongly influence population dynamics through the number of generations, the presence of an overwintering diapause or the sex ratio. Thus, the understanding of thermic requirements of a biocontrol agent before any introduction process is a decisive tool for assessing individual and populational performances regarding its main life history traits.

Since importation biocontrol mainly focuses on co-evolved enemies of a pest (Strand and Obrycki 1996), both the target pest and the biocontrol agent, originating from the same native range, share similar environmental conditions, including temperature. Co-evolved biocontrol agents are therefore expected to better match climate in invaded areas where the pest is, and adapt to this new themic environment (Smith, 2014). Among the invasive pests threatening the USA. economy, *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae) is one of the most recent (Palumbo et al. 2016, Mc Pherson 2018). First introduced in California in 2008 (Reed et al. 2013), it is now widely distributed through the New World, from Texas and Mexico to Chile (Bundy et al. 2012; Sanchez-Peña 2014; Faundez et al. 2016). It mainly

damages Brassicaceae crops, like broccoli, cabbage or mustard, (Reed et al. 2013; Palumbo et al. 2016; Sharma et al. 2020) but also non-Brassicaceae species like caper plants (Colazza et al. 2004) or tomato (Singh et al. 2011). Originated from southern Asia and East to South Africa, it completes its development under high temperatures and dry conditions, up to constant 40 °C (Reed et al. 2017). On the other hand, its lower thermic developmental threshold is relatively high (20 °C), which limits its activity during winter in the most temperate invaded areas of the world like Californian coastal regions (Reed et al. 2013, 2017). Under its thermal optimum, the pest is known to develop fast (25 days) with strong outbreaks in cole crops by the end of summer and early spring in California (Reed et al. 2013; Palumbo et al. 2016).

In order to control *B. hilaris* in the USA, an importation biological control program started in 2015 with field collections of egg parasitoids in the native range of Pakistan (Mahmood et al. 2015). Since 2016, the egg parasitoid *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae) is under laboratory evaluation as a biocontrol candidate (Martel et al. 2019; Tofangsazi et al. 2020; Martel & Sforza, 2021; Martel et al. 2021). Scelionid species are well studied biocontrol agents against stink bugs (Orr 1988; Mills 2009; McPherson 2018) and many studies have already been conducted on thermal requirements (Orr et al. 1985; James and Warren 1991; Torres et al. 1997; Arakawa and Namura, 2002; Toyama and Mishiro, 2010; Forouzan et al. 2015). These studies have shown that most of the parasitoids in the genera *Trissolcus* and *Telenomus* have high thermal requirements, with optimum values ranging from 25 to 30 °C. The genus *Gryon* meanwhile, remains poorly documented though several species worldwide seem to share similar optimum thermal values than other scelionid genera (Nechols et al. 1989; Canto-Silva et al. 2005; Dasilao and Arakawa 2005; Mainali et al. 2012).

First research studies on *G. gonikopalense* revealed "good" longevity and reproduction performances under a constant 22 °C regime (Martel et al., 2019). But since *G. gonikopalense* shares the same climatic conditions than *B. hilaris* in Pakistan, its thermal optimum is expected to be close to its host's optimum (between 30 and 36 °C) (Reed et al. 2017). Under laboratory conditions, its developmental and reproductive performances may therefore have been under-estimated (Martel et al., 2019).

The current study aimed to determine the thermal thresholds and optimum values for the development, the survivorship and the reproduction of *G. gonikopalense*, as to assess its potential for establishment in invaded areas where *B. hilaris* is spreading. New insights may help to predict biocontrol performances of *G. gonikopalense* in the field and draw preliminary hypotheses on its population dynamics over the course of the cropping period.

2. Material and methods

2.1. Breeding Conditions

The laboratory colonies of *Bagrada hilaris* and *Gryon gonikopalense* used in this study were maintained in the quarantine glasshouse of the European Biological Control Laboratory-EBCL (USDA-ARS) at Montferrier-sur-Lez (France) under the official agreement # R76-2019-162, issued by the French Ministry of Agriculture.

2.1.1. The bagrada rearing

The *Bagrada hilaris* colony is a subset of the colony maintained at the University of California, Riverside (Martel et al. 2019). The inbreed colony was routinely maintained at EBCL in BugDORM® cages (47.5 x 47.5 x 47.5 cm, 150 µm opening mesh, MegaView Science®, Taiwan) under ca. 25 °C night and 30 °C day, at an ambient photoperiod supplemented with sodium lamps (L/D 12:12), and RH: 30 ± 15 %. *Bagrada* adults and nymphs were fed weekly with a selection of 1-month-old Brassicaceae plants including *Brassica oleracea* var. *italica* Plenck (broccoli), *Sinapis alba* L. (white mustard) and *Diplotaxis erucoides* De Candolle (white wall rocket). Broccoli and mustard were grown from seeds at ca. 22°C, RH: 30 ± 10 % in a separate room of the quarantine. The white wallrocket was weekly field collected.

2.1.2. Bagrada egg collection

In the bagrada rearing cages, oviposition arenas consisted in Petri dishes (Ø = 9 cm) filled with sand (DIALL®, yellow sand, granulometry = 200 µm, Spain). From these Petri dishes, eggs were collected every two days with a fine brush after sifting sand (Hogentogler®, mesh size = 425 µm, USA). Eggs were glued next to each other (UHU stick®, Germany) on cardboard pieces (Martel and Sforza, 2021) and used either for the parasitoid rearing or subsequent experiments (see section 2).

2.1.3. The parasitoid rearing

The EBCL *Gryon gonikopalense* colony originated from Pakistan (Mahmood et al., 2015), and was maintained in insect-proof transparent plastic boxes (18 x 12 x 5.5 cm). Aeration was provided through holes in the lid (Ø = 50 mm), covered with a mesh fabric. Rearing boxes were stored in a rearing chamber (22 ± 0.5 °C, RH 50 ± 5 %, L/D 12:12) and drops of diluted acacia honey (Lune de miel®, France) were deposited on the mesh as *ad libitum* nourishment. The parasitoid colony was maintained by exposing once a week fresh bagrada eggs to female parasitoids.

2.2. Effect of constant thermal regimes on the life cycle of *G. gonikopalense*

2.2.1. Development time

We exposed 24-48 h-old bagrada eggs to randomly picked mated female parasitoids from the main laboratory colony. Once the parasitized eggs were obtained, they were separated into 8 groups. Each group was placed in an incubator (Percival Scientific, model E-30B, USA) under one of the following constant thermal regimes: 15, 18, 22, 25, 30, 35, 37.5 and 40 °C, with 50 % RH and 12:12 photoperiod. Parasitized eggs were stored at the specified temperature until all adult parasitoids emerged. For each temperature regime, from the first to the last day of adult emergences, each progeny was sexed and its total larval development time recorded (*i.e.* from parasitism date to emergence date). For each temperature, progeny obtained were then separated in two new groups corresponding to fecundity and survivorship experiments.

2.2.2. Fecundity

For each temperature regime, newly emerged females were kept with males for 24h, then each mated female was individually isolated in a clean Petri dish and placed in an incubator (Percival Scientific, model E-30B, USA) (L:D = 12:12; RH = 50 %; T °C = 18, 22, 25, 30, 35 or 37.5).

The parasitoid fecundity under 15 °C was measured with females that developed at 22 °C as no individual was able to complete its development at 15 °C. The parasitoid fecundity under 40°C was measured with females that developed at 37.5 °C as no individual was able to complete its development at 40 °C.

From its first to its last day of life, each female was daily provided with fresh bagrada eggs and diluted honey drops as nourishment. The *G. gonikopalense* fecundity follows a decreasing curve over time (Martel et al. 2019). Hence, female parasitoids were provided a decreasing number of fresh eggs as to maximize the number of females simultaneously followed given available eggs stocks: 50 eggs from day one to day four, 20 eggs from day five to day 10, 15 eggs from day 11 to day 20, and 10 eggs until the female dies.

For each day of oviposition, corresponding Petri dishes were stored at the same temperature regime until progeny emergence. We then counted the number of parasitized eggs, the number of male and female progeny emerged. All non-emerged eggs were dissected under a binocular microscope (Stemi 508, Zeiss®, Germany) in order to verify whether they contained an identifiable nymph or adult parasitoid. Eggs with unidentifiable content were considered as not parasitized. Female parasitoids that failed to parasitize any egg during their life, or produced only males were excluded from statistical analyses.

2.2.3. Adult longevity

For each thermal regime that allowed complete development and emergence of individuals (18, 22, 25, 30 35 and 37.5 °C), newly emerged male and female parasitoids were individualized Petri dishes and provided drops of diluted honey as *ad libitum* nourishment. Each individual was stored under the same climatic conditions used for development. The longevity at 15 °C was measured with male and female parasitoids that developed at 22 °C, whereas the longevity at 40 °C was measured with adults that developed at 37.5 °C.

In order to prevent honey desiccation, new drops were deposited every two days at 30, 35, 37.5 and 40 °C. At 25 °C honey was replaced every week. At 22 °C and below honey was replaced every two weeks. The parasitoid status (dead or alive) was daily checked until the last individual in the cohort has died.

2.3. Statistical analyses

Data described above were used to calculate demographic parameters of *G. gonikopalense* laboratory under each constant thermal regime. The intrinsic rate of increase was calculated using the equation (Birch 1948):

$$\sum l_x m_x e^{-r_m.x} = 1$$

Where x = age in days, l_x = age-specific survival rate, m_x = age-specific fecundity, r_m = intrinsic rate of natural increase. The variables l_x and m_x were measured experimentally and other demographic parameters were calculated as follows:

Intrinsic rate of increase: $r_m = \ln(\sum l_x m_x) / T$

Finite rate of increase: $\lambda = e^{r_m}$

Mean generation time: $T = (\sum x.l_x.m_x) / R_0$

Doubling time: $T_d = \ln(2) / r_m$

Net reproductive rate: $R_0 = \sum l_x m_x$

Gross reproductive rate: $GRR = \sum m_x$

We used the Jackknife technique (Hulting et al. 1990) to estimate each parameter in order to eliminate the statistical uncertainty of calculated values linked to the inter-individual variability (see Meyer et al. 1986). Demographic parameters were only calculated for temperatures that allowed reproduction and development. The average development time was compared between males and females and for each tested temperature with a two-way ANOVA test followed by a Tukey post hoc test to identify differences between treatments. The male and female longevity was compared for each temperature regime with a Cox proportional hazards survival models and Tukey post hoc test were performed to identify differences between each group. The average larval survivorship, females' fecundity, their oviposition and post-oviposition periods and their progeny sex ratio, were compared between each tested temperature with a one-way ANOVA followed by a Tukey post hoc test to identify differences between treatments. All values presented in tables or in the text were described as means with corresponding 95 % Confidence Interval.

All statistical analyses were carried out with R software (R Development Core Team, 2016) and figures were obtained with R software or Excel software. For all tests, the alpha significance threshold was set at $\alpha = 0.05$.

3. Results

3.1. Effect of temperature on development time of *Gryon gonikopalense*

No parasitoid developed at 15 and 40 °C (Table 1), though most of parasitized eggs had turned black after 40 days, indicating that parasitoid development at least partially occurred (Martel et al. 2019). This was further confirmed after egg dissection with presence of parasitoids that did not reach the adult stage for both temperatures. In order to test the existence of a quiescence, parasitized eggs kept at 15 °C for 60 days were warmed up one day at 22 °C then placed at 25 °C and kept at this new temperature for three weeks. No emergence was observed, and after egg dissection, most individuals did not reach the nymphal stage, and only a few reached an incomplete adult stage (data not shown). As a result, no quiescence or diapause were evidenced for this parasitoid. Between 18 and 37.5 °C, *G. gonikopalense* completed its development with successful emergence. The emergence rate varied non-linearly with increasing temperature, from 87.15 ± 8.30 % at 22°C to 77.86 % at 37.5 with a maximum of 95.53 ± 1,96 % at 25 °C (Table 1, Fig 1). The parasitoid development was influenced by both temperature (two-way ANOVA, $F_{5, 1670}$ = 19196. 4, P-value < 0.001) and gender (two-way ANOVA, $F_{1, 1670}$ = 123,9, P-value < 0.001). The development time decreased as temperature increased (Tukey test, P-value < 0.05, Table 1, Fig.2), from 46.85 ± 0.6 days at 18 °C to 7.28 ± 0.09 days at 37.5 °C. Males developed faster than females for all temperature regimes (Tukey, P-value < 0.05, Table 2), but the difference never exceeded one day (Table 1, Fig 2).

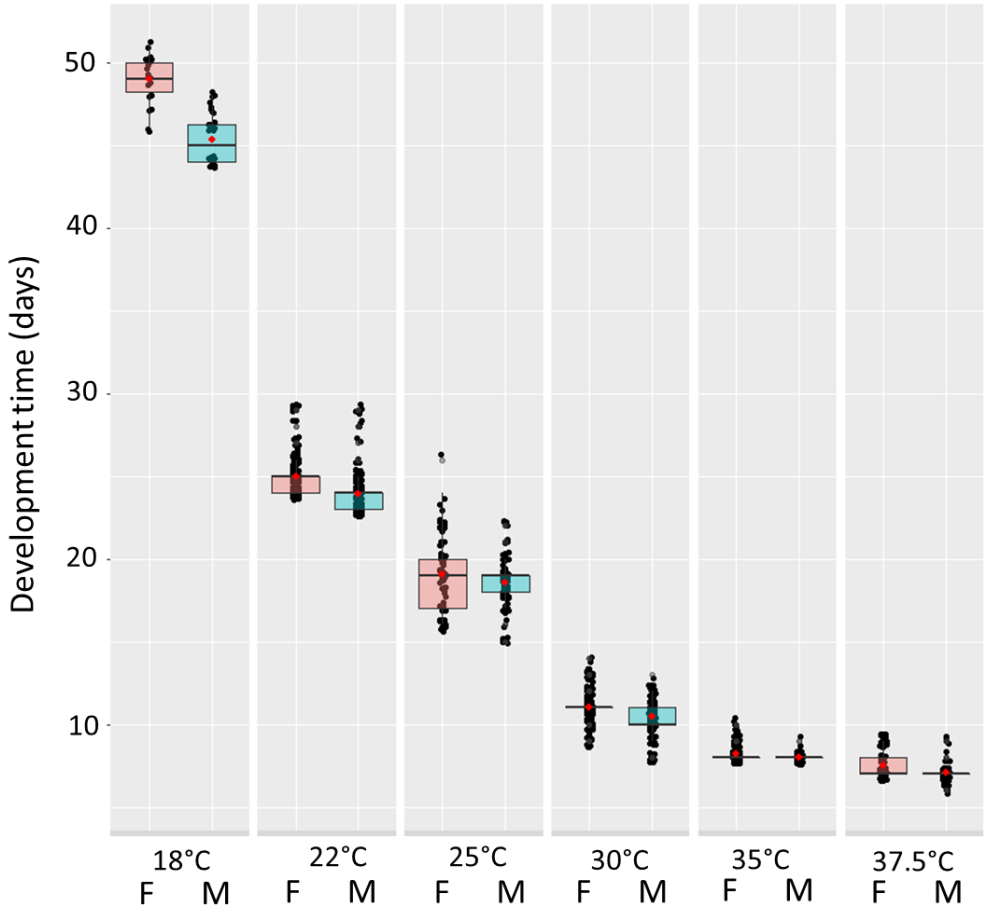


Figure 1. Duration of complete development of *Gryon gonikopalense* males ("M", blue boxplots) and females ("F", red boxplots) inside *Bagrada hilaris* eggs under eight constant temperatures from 15 °C to 40 °C. Edges of each boxplot represent 95 % data. Black dots out of boxplot whiskers are outliers. Red dots are means and horizontal black lines are medians.

Table 1. Effect of eight constant thermal regimes (from 15 °C to 40 °C) on *Gryon gonikopalense* development time, development rate and immature progeny survivorship (emergence rate). Values are given as mean ± 95 % confidence interval. For a given column, values followed by different letters statistically differ (Two-way ANOVA followed by Tukey post-hoc test; $\alpha = 0.05$). For a given variable, values followed by an uppercase letter in the female column statistically differ from value in the male column (lower case letter) (Two-way ANOVA followed by Tukey post-hoc test; $\alpha = 0.05$)

Temperature (°C)	Emergence rate (%)	Development time (days)		Development rate (days-1)	
		Male	Female	Male	Female
15	0	NA	NA	NA	NA
18	12.75 ± 4.13	45.38 ± 0.55 (a)	49 ± 0.64 (A)	0.022 ± 0.0003 (a)	0.020 ± 0.0007 (A)
22	87.15 ± 8.30	23.91 ± 0.15 (b)	24.96 ± 0.14 (B)	0.042 ±0.003 (b)	0.040 ± 0.0002 (B)
25	95.53 ± 1.96	18.57 ± 0.31 (c)	19.05 ± 0.5 (c)	0.054 ± 0.001 (c)	0.053 ± 0.001 (c)
30	91.31 ± 1.62	10.44 ± 0.2 (d)	11.04 ± 0.16 (D)	0.097 ± 0.003 (d)	0.091 ± 0.001 (D)
35	77.51 ± 2.11	8.02 ± 0.14 (e)	8.19 ± 0.05 (e)	0.12 ± 0.001 (e)	0.12 ± 0.001 (e)
37.5	77.86 ±	7.05 ± 0.08 (f)	7.49 ± 0.14 (F)	0.14 ± 0.002 (f)	0.13 ± 0.003 (F)
40	0	NA	NA	NA	NA

3.2. Effect of temperature on *Gryon gonikopalense* fecundity

Gryon gonikopalense females could parasitize bagrada eggs under all thermal regimes tested. The parasitoid's fecundity strongly varied with temperature (ANOVA, $F_{7,111} = 36.6$, P-value < 0.0001), following a non-linear curve (Table 2, Fig. 3). The lowest values were recorded at 15, 18 and 40 °C with respectively 15.2 ± 3.02, 15.07 ± 5.06 and 17.47 ± 3.2 progeny produced. From 22 to 37.5°C, *G. gonikopalense* had a better fecundity with a maximum of 104.47 ± 21.2 progeny produced at 30°C (Fig 3, Table 2).

The daily fecundity of females *G. gonikopalense* regularly decreased with time. No pre-oviposition period was recorded, and a maximum of progeny was produced on the first day of oviposition, whatever the temperature considered (Fig. 4). The oviposition period varied with temperature (ANOVA, $F_{7,111} = 8.3$, P-value < 0,0001) and was the longest at 25 °C (14 ± 3.5 days) while shortest was measured at 40 °C (2.67 ± 0.7 days). The post-oviposition period also varied with temperature (ANOVA, $F_{7,111} = 180.1$, P-value < 0,0001) and was the longest at 15 °C (44.8 ± 0.7 days)) while the shortest was recorded at 40 °C (1 ± 0.7 days) (Table 2, Fig.4).

The sex ratio of progeny did not statistically vary with temperature, expect for females exposed at 37.5 °C (ANOVA, $F_{7, 111} = 3.8$, P-value < 0.001) that only produced 38.1 % of female progeny (Table 2).

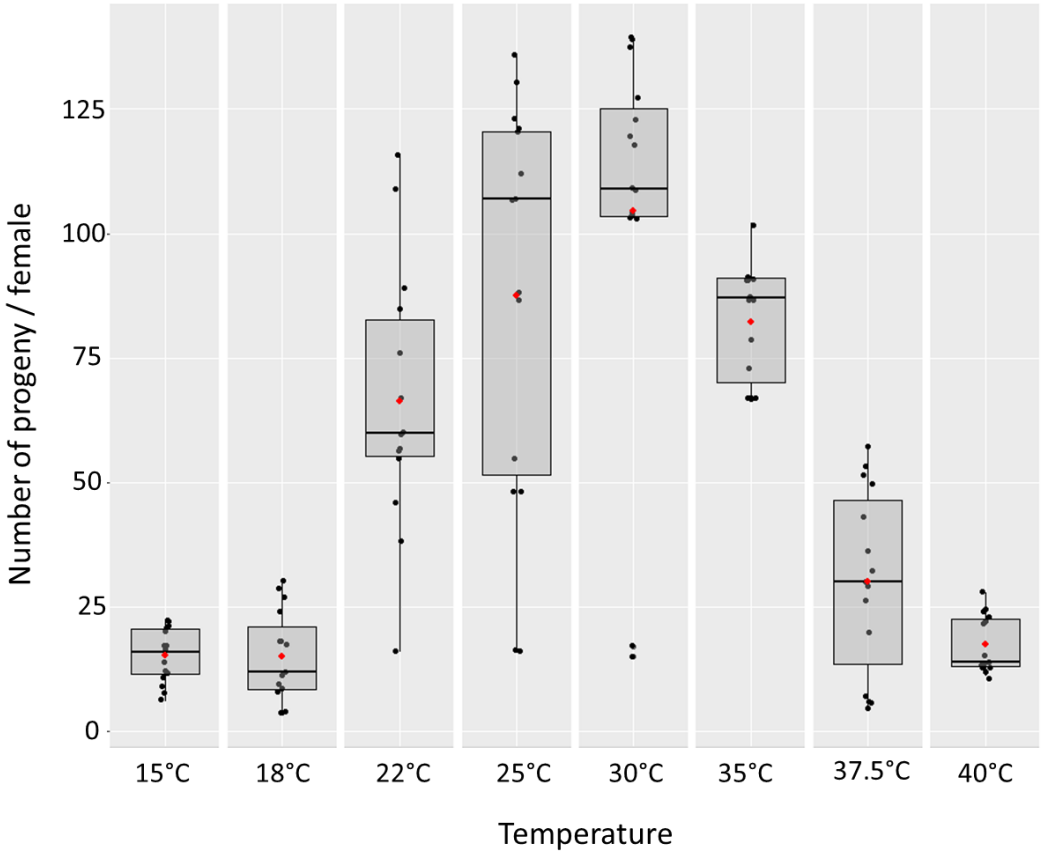


Figure 2. Number of progeny produced per female *Gryon gonikopalense* from *Bagrada hilaris* eggs under eight constant temperature from 15°C to 40°C. Edges of each boxplot represent 95% data. Black dots outside boxplots are outliers. Red dots are means and horizontal black lines are medians

Table 2. Effect of eight constant thermal regimes (from 15 °C to 40 °C) on *Gryon gonikopalense* fecundity, progeny sex ratio, oviposition and post-ovipositional periods. Values are given as mean ± 95 % confidence interval. For a given column, values followed by different letters statistically differ (One-way ANOVA followed by a Tukey post-hos test; $\alpha = 0.05$).

Temperature (°C)	Fecundity	Sex ratio (% females)	Ovipositional period (days)	Post-reproductive period (days)
15	15.2 ± 3.02 (a)	61.1 ± 10.8 (b)	5.2 ± 0.7 (a)	44.8 ± 0.7 (e)
18	15.07 ± 5.06 (a)	57.8 ± 8.1 (ab)	11.2 ± 6.3 (b)	38.6 ± 6.6(d)
22	66.43 ± 15.5 (b)	66.0 ± 15.7 (b)	12.14 ± 3.2 (b)	2.57 ± 2.0 (b)
25	87.6 ± 22.6 (bc)	61.1 ± 10.4 (b)	14 ± 3.5 (b)	21.47 ± 2.0 (c)
30	104.47 ± 21.2 (c)	62.6 ± 9.6 (b)	9.93 ± 1.5 (ab)	10.53 ± 1.8 (a)
35	82.27 ± 6.3 (bc)	49.2 ± 9.2 (ab)	7.8 ± 0.6 (ab)	9.27 ± 1.4 (a)
37.5	30.13 ± 10.2 (a)	38.1 ± 8.2 (a)	5.93 ± 1.3 (a)	3.07 ± 1.2 (b)
40	17.47 ± 3.2 (a)	64.3 ± 9.9 (b)	2.67 ± 0.7 (a)	1 ± 0.7 (b)

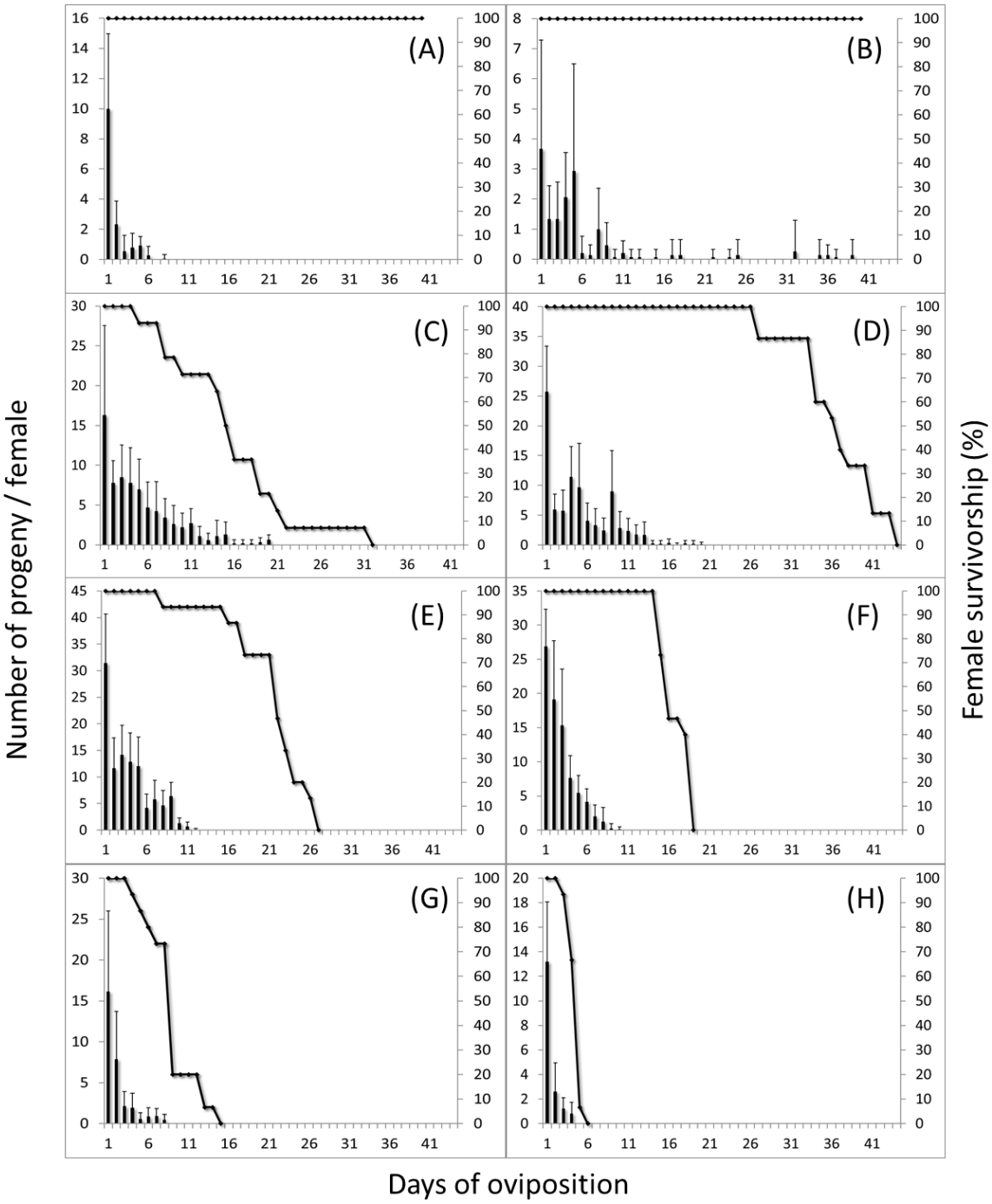


Figure 3. Daily fecundity of *Gryon gonikopalense* (black bars) under eight constant temperatures. Graphs (A), (B), (C), (D), (D), (E), (F) and (G) respectively correspond to results obtained at 15, 18, 22, 25, 30, 35, 37.5 and 40 °C. Black vertical lines are the standard deviation. The curve with black squares is the cohort survivorship (%) over the maximal longevity obtained during the experiment.

3.3. Effect of temperature on adult longevity of *G. gonikopalense*

The longevity varied with temperature, from 4.28 ± 0.26 days at 40 °C to 151.3 ± 6.8 days at 15 °C according to survival probability that decreased faster under higher temperatures (Cox proportional hazards model, Wald $\chi^2 = 880$, P-value < 0.001) (Fig. 4, 5). There was no difference between male and female longevity, except at 22 °C where females lived 107.7 ± 4.9 days on average while males lived

84.9 ± 5.3 days (Fig. 4). Both genders had the same survival probability over their lifetime at each temperature (Cox proportional hazards model, Wald $\chi^2 = 3.5$, P-value = 0.061).

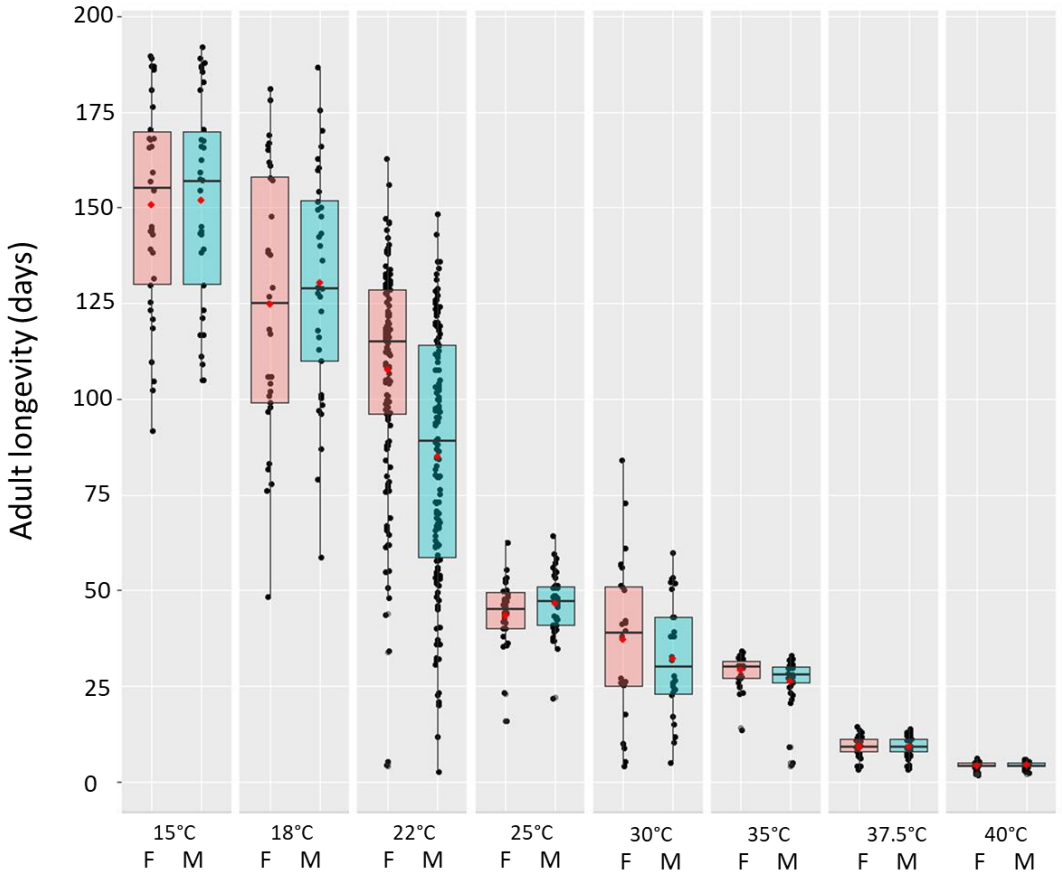


Figure 4. Longevity of adult males ("M", blue boxplots) and females ("F", red boxplots) *Gryon gonikopalense* under eight constant temperatures from 15 °C to 40 °C. Edges of each boxplot represent 95 % data. Black dots out of boxplot whiskers are outliers. Red dots are means and horizontal black lines are medians.

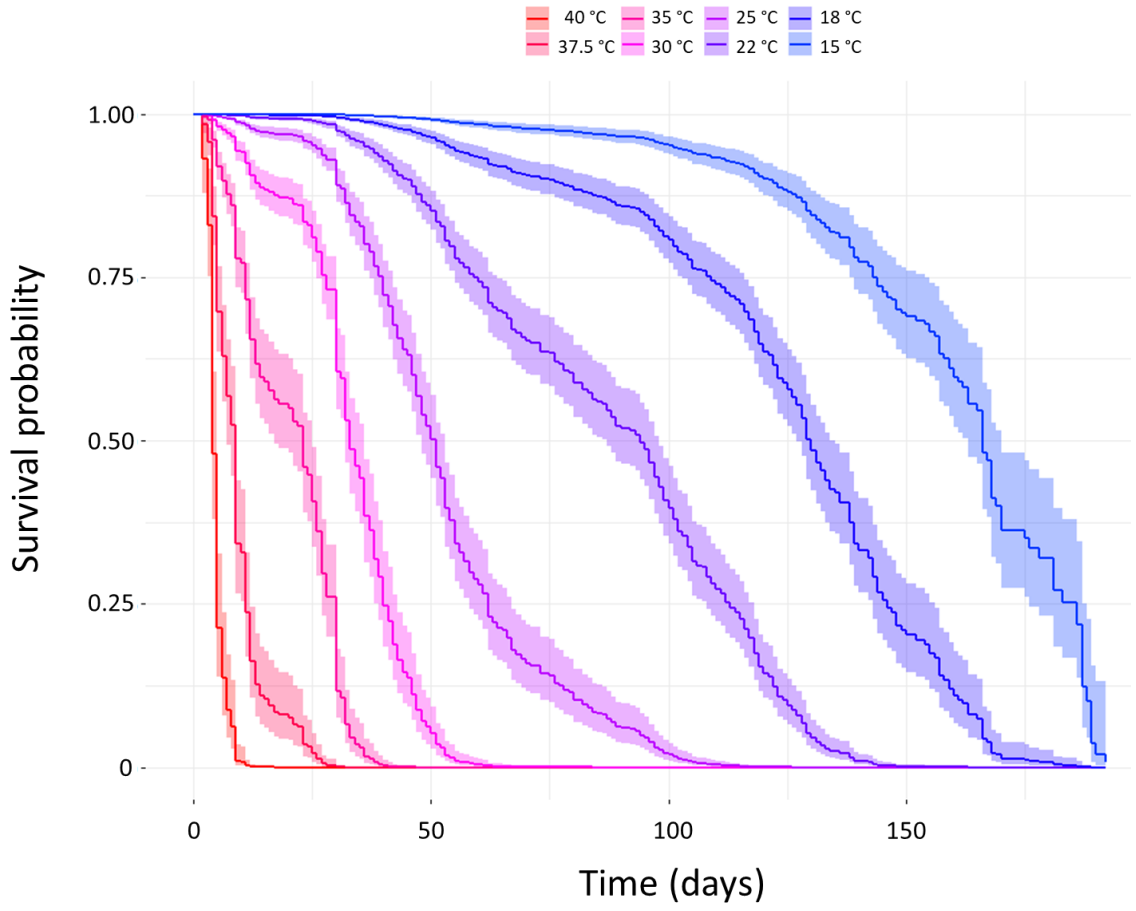


Figure 5. Survival probability of *Gryon gonikopalense* under 8 constant thermal regimes: 15, 18, 22, 25, 30, 35, 37.5 and 40 °C. Parasitoid genders are pooled for each temperature. Curves are presented with their 95% Confidence Interval.

3.4. Effect of temperature on *G. gonikopalense* demography

Development and fecundity data were used to calculate demographic parameters of a theoretical population of *G. gonikopalense* with a stable age distribution (Table 3). As no development was recorded at 15 and 40 °C, parameters were not calculated for these temperatures, and the population growth was supposed null or negative. The intrinsic rate of increase (r_m) of *G. gonikopalense* was the highest at 35 °C with 0.38, which implies that a parasitoid population is able to multiply 1.47 times a day (λ). At this rate, the size of the colony doubles in 1.81 days (T_d) with an average generation time (T) of 9.62 days. On the opposite, the population growth was near 0 at 18°C.

Table 3. Demographic parameters of *Gryon gonikopalense* reared from *Bagrada hilaris* eggs under eight constant temperatures from 15 °C to 40 °C. Each parameter was estimated with a Jackknife method. $\mathbf{r_m}$ = intrinsic rate of increase; $\boldsymbol{\lambda}$ = finite rate of increase; **T** = generation time; T_d = doubling time; $\mathbf{R_0}$ = net reproductive rate; **GRR** = gross reproductive rate

Temperature (°C)	Demographic parameter					
	$\mathbf{r_m}$	$\boldsymbol{\lambda}$	**T**	$\mathbf{T_d}$	$\mathbf{R_0}$	**GRR**
15	NA	NA	NA	NA	NA	NA
18	0.034	1.03	54.86	20.41	8.4	6.46
22	0.13	1.14	27.7	5.15	41.81	44.15
25	0.17	1.19	22.49	3.94	52.47	52.47
30	0.31	1.36	13.48	2.27	61.22	61.33
35	0.38	1.47	9.67	1.81	41	41
37.5	0.29	1.34	8.56	2.36	12.44	12.53
40	NA	NA	NA	NA	NA	NA

4. Discussion

The present study aimed to assess the influence of eight constant temperature regimes on life history traits of *Gryon gonikopalense* developing from *Bagrada hilaris* eggs. We focused on the development, the reproduction and the longevity of this biocontrol agent in order to estimate its capacity to establish after release in bagrada invasive range of California.

Under our laboratory conditions, *G. gonikopalense* successfully completed development from 22 °C to 37.5 °C, with an adult emergence rate varying from 77.5 % at 37.5 °C to 95.5 % at 25 °C. Like most of ectothermic animals (Angiletta et al. 2004), the development time of *G. gonikopalense* decreased as temperatures increased, from 24 days at 22 °C to 7 days at 37.5 °C. Males developed slightly faster than females, as commonly reported from scelionid species (Romeis et al. 2000; Arakawa and Namura 2002; Dasilao and Arakawa 2004; Martel et al. 2019). The thermal range for an optimal *G. gonikopalense* development includes high temperatures, which is consistent with other scelionid species that generally develop well up to 30°C (Yeargan, 1982; Nechols et al. 1989; Torres et al. 1997; Arakawa and Namura 2002; Canto-Silva et al. 2005; Dasilao and Arakawa, 2005; Mainali et al. 2012). When comparing the developmental optimum for *G. gonikopalense* and bagrada, it is obvious that both share similar thermal requirements, as initially hypothesized. Indeed, bagrada cannot develop well below 20 °C, while the best temperature for development rate and larval survivorship is between 34 and 36 °C (Reed et al. 2017). Our data on the developmental range of *G. gonikopalense* match with temperatures

recorded in the desert climate of Pakistan (Toba Tek Singh District, see Mahmood et al. 2015) where average lows are above 25 °C from April to September and average highs reach 40 °C in the summer (fr.climate-data.org). The present article also questions whether *G. gonikopalense* could establish in areas invaded by bagrada, and especially California where this biological control program was primarily targeted.

In the Imperial Valley (Imperial County, CA, USA), cole crops are planted from late August to October when daily average temperatures are between 24.5 and 32.5 °C (Le Strange et al. 2010; Reed et al. 2017; usclimatedata.com). During this timeframe, bagrada adults and larvae feed on seedlings (Reed et al. 2013, Palumbo et al. 2016). Mean daily temperatures are above 20 °C from late March to early November, and average highs above 20 °C the whole year (usclimatedata.com). From May to October, average temperature is continuously above 24°C (usclimatedata.com). Thus, in the desert brassica cropping systems of South California, *G. gonikopalense* could successfully develop all year round.

Regarding the parasitism pressure that *G. gonikopalense* could have on bagrada, we showed that females could well parasitize bagrada eggs from 22 to 37.5 °C. In addition, they started parasitism on their first day with a peak of progeny at this moment as already described by Martel et al. (2019) and like many scelionid species (James, 1988; Kivan and Kilic, 2005; Canto-Silva et al. 2006; Peverieri et al. 2012). Hence, there would not be a delay in the parasitism response after a hypothetical release in the field. We measured a maximum number of progeny at 30 °C (104.47 on average), whereas the thermal optimum for various scelionid species is often below 30 °C, like *Trissolcus basalis* Wollaston at 25 °C (Chabi-Olaye et al. 2001; Forouzan et al. 2015), *Trissolcus oenone* Dodd at 22.5 °C (James and Warren 1991) or *Gryon pennsylvanicum* Ashmead at 26 °C (Peverieri et al. 2012). Like several scelionid species developing under low latitude (Chabi-Olaye et al. 2001; Canto-Silva et al. 2006), demographic parameters of *G. gonikopalense* indicate an overall good performance from 25 to 37.5 °C. Considering a population of *G. gonikopalense* with a stable age distribution, its rate of increase would allow its population to double within less than four days since the temperature is 25 °C or higher. Meanwhile, a stable bagrada population was estimated to double in 3.7 days (Naryana and Phadke, 1987), so that *G. gonikopalense* could maintain parasitism pressure on its host for several most of the year, regarding

monthly temperatures previously cited. Like its development, the ability of *G. gonikopalense* to parasitize under warm conditions in the laboratory is in accordance with hot and dry climatic conditions from its native range.

Unlike fecundity, the adult longevity of *G. gonikopalense* progressively declined with increasing temperature. Although no differences were recorded between males and females, the thermal optimum for reproduction cited above corresponded to its lowest longevity: 45.1 ± 2.0 days at 25 °C and only 9.2 ± 0.6 days at 37.5 °C. However, this study focused on constant thermal regimes that are a big simplification of field reality. In California, highest average temperatures fluctuate between 23.9 and 41.2 °C in July in the Imperial Valley (usclimatedata.com). In addition, the high reproductive activity of *G. gonikopalense* under warm conditions should counterbalance a shorten lifespan regarding the impact on bagrada population. This should be especially true considering that 100 % of female's progeny was produced before their 20^{th} day, so that additional longevity would not allow any supplementary parasitism on bagrada populations at these temperatures.

While high temperature range is suitable for *G. gonikopalense*, its developmental lower thermic threshold may limit its activity during winter in Imperial county, where the coolest month (December) reaches an average high of 21 °C and an average low of 4 °C. Indeed, its development was possible at 18 °C but 87.25 % of adults could not emerge from their host, while no development was observed under constant 15 °C. Similar thermal requirements have been recorded for several scelionid species (Arakawa and Namura, 2002; Canto-Silva et al., 2005; Dasilao and Arakawa, 2005; Mainali et al., 2012) though the thermal threshold for the development of *G. gonikopalense* seems quite high compared to many other scelionids species that can develop under 15 °C (Nechols et al., 1989; James & Warren, 1991; Cividanes & Figueiredo, 1996). As no development would be possible during winter, the ability for a *G. gonikopalense* population to overwinter is a central question. We showed that *G. gonikopalense* was able to live over long periods at 18 and 15 °C (127 ± 8.1 and 151.3 ± 6.8 days respectively). Indeed, under cooler temperatures, physiological activity of ectotherms organisms is reduced, as well as energy consumption (Kern et al. 2015). As nymphal development was not possible at 15 °C, it can be hypothesized that the parasitoid is able to overwinter in the field at adult stage, though it has never been verified in its native range. Thus, *G. gonikopalense* could wait for warmer conditions in the litter as it

has been observed for *Trissolcus japonicus* Ashmead that could survive in the litter as adult up to 16 weeks in Oregon, USA (Lowenstein et al. 2019). Also, bagrada population activity decrease during winter in Imperial Valley because temperature does not allow development (Reed et al. 2013, 2017; Palumbo et al. 2016). As a consequence, the number of available hosts in the environment is reduced for *G. gonikopalense* that would undergo a long period of host deprivation. Then, another question to ask is the parasitoid's ability to attack bagrada when the stink bug activity increase in early spring. To date, there is no certitudes that it would be able to do so, but Martel and Sforza (submitted) have shown that *G. gonikopalense* is able to parasitize bagrada eggs after two months of host deprivation though it is not known whether cooler temperature may lengthen oocyte retention period in *G. gonikopalense* females.

In the Monterey County, which is the leading broccoli-producing county in California (Le Strange et al. 2010), the warmest month, September, only reaches an average high of 20.9 °C (U.S. climate data). Meanwhile, the coolest months are December and January with an average high of 14.3 and 14.6 °C and an average low of 6.6 and 6.5 °C respectively. In this region, cole crops are planted from mid-February through October, and bagrada peaks at this moment. The low or impossible development of G. gonikopalense and its low parasitism activity at 18 °C and belowwould provide a poor regulation of bagrada population. The parasitoid would have difficulties for a long-term establishment in this region and ones with similar climate. However, the maximum temperatures on the half of this period are below 22°C so that bagrada cannot develop correctly in this cool region (Reed et al. 2017). Hence, the lack of effectiveness of *G. gonikopalense* could be counterbalanced by bagrada lower population dynamic.

Conclusion

The capacity of *G. gonikopalense* to complete its life cycle under a large range of temperatures is obvious. As expected, its high thermal optimum corresponds to the one of *B. hilaris*. We can hypothesize that both species share similar population dynamics in the field. The impact of *G. gonikopalense* on bagrada population could be significant in Californian desertic brassica crops, especially during the warmer days of spring, when a high number of bagrada is available as host (Reed et al. 2013) and the parasitoid's developmental rate and reproduction activity is at its maximum. Other

southern parts of USA invaded by bagrada should also be suitable for *G. gonikopalense*'s establishment like Yuma, Arizona (Reed et al. 2013) or El Paso, Texas where temperatures may allow its development and reproduction from March to October (usclimatedata.com) as well as in Mexico. These aspects of the parasitoid's biology and other specific behaviours (Martel and Sforza 2021; Martel et al. 2021), constitute additional arguments to favour *G. gonikopalense* as a biocontrol agent. However, a high developmental threshold may restrict *G. gonikopalense* to attack bagrada in the warmest regions of southwestern USA In addition, it would not provide satisfying results in Brassicaceae crops the coastal regions of California where temperatures are too low, and it fate during winter remains uncertain. Countries with high thermal means, like Mexico where bagrada is also invasive, could consider *G. gonikopalense* as a potential candidate for biocontrol. The data provided in this article also allow us to question the possibility of using the parasitoid as biocontrol agent in new potential invasive areas for bagrada in the Mediterranean basin (Reed et al. 2017; Carajaval et al. 2019). like Pantelleria island (Sicily, Italy) (Colazza et al. 2004)

Nevertheless, temperature is important but not the only factor to be considered. Humidity parasitoid's nutrition requirement, host availability, or interspecific competition with egg parasitoids (native or introduced) may also play a role in the success of *G. gonikopalense* to sustainably establish in the USA. Also, the ability of the parasitoid to sustain on low bagrada populations during summers and winters (Reed et al. 2013) would be a key factor to consider. However, the recent discovery of *Trissolcus hyalinipennis* Rajmohana & Narendran in California from exposed bagrada sentinel eggs (Ganjisaffar et al. 2018) may open the door to a possible introduction and / or establishment of *G. gonikopalense* in the southwestern USA as both parasitoids share the same Pakistani origin (Mahmood et al. 2015).

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Website links (all visited in 2021)

https://fr.climate-data.org

https://www.usclimatedata.com/

Chapitre 3.

Le comportement de recherche d'hôte de *Gryon gonikopalense* : un atout pour la régulation de bagrada ?

Article 3 : **Martel, G.,** & Sforza, R.F.H. (2021). Catch me if you can : Novel foraging behavior of an egg parasitoid, *Gryon gonikopalense*, against the stinkbug pest, *Bagrada hilaris*. *Journal of Pest Science* https://doi.org/10.1007/s10340-020-01325-4

Article 4 : **Martel, G**., & Sforza, R.F.H (2021). How an egg parasitoid responds to an unusual stinkbug oviposition behavior: the case of *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae) and *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae). *Entomologia Generalis* (in press)

Pour détecter et trouver leurs hôtes dans un environnement complexe, les parasitoïdes oophages doivent surmonter deux principales barrières : 1) la détection chimique des œufs à parasiter dans un environnement complexe et 2) les éventuelles barrière physiques ou comportementales mises en place par l'hôte pour éviter d'être parasité. Au sein des pentatomidés, bagrada possède un comportement d'oviposition unique : ses œufs sont pondus individuellement et enterrés, contrairement aux autres espèces qui déposent leurs œufs sur leur plante hôte en ooplaques. Dans le **chapitre 2**, j'ai présenté les capacités de *G. gonikopalense* à parasiter efficacement les oeufs de bagrada. Cependant, un auxiliaire capable de réguler bagrada doit pouvoir trouver les œufs qu'elle pond dans le sol, et c'est ce que nous avons voulu déterminer dans ce **chapitre 3**.

Ainsi, nous avons testé dans **l'Article 3** la capacité de *G. gonikopalense* à parasiter des œufs naturellement enterrés par bagrada. En utilisant des œufs manuellement enterrés sous du sable ayant été en contact ou non avec la punaise, nous avons également cherché à savoir quels types de signaux chimiques pouvaient être nécessaires à leur localisation par le parasitoïde. Quoi qu'il en soit, ce comportement de recherche n'est pertinent que si la punaise pond la majorité de ces œufs dans le sol, ce qui n'a encore jamais été montré. Dans **l'Article 4**, nous avons donc décrit la distribution spatiale des œufs de bagrada, et du parasitisme de *G. gonikopalense* dans un environnement en trois dimensions, en prenant notamment en compte une influence de la plante hôte (brocoli, moutarde ou fausse roquette) et de la densité de punaises dans le système expérimental (de 1 à 15 couples).

Ces deux études constituent le résultat majeur de mon travail de thèse : d'une part, nous avons montré que bagrada pondait plus de 90% de ses œufs dans le sol, quelle que soit sa plante hôte ou le nombre de congénères (**Article 4**). Quelques œufs étaient tout de même pondus sur la plante hôte, principalement sur les jeunes feuilles ; d'autre part, les résultats obtenus dans **l'Article 3,** et les séquences filmées du parasitoïde sont sans équivoque. Ce parasitoïde est capable de détecter et parasiter les œufs enterrés de bagrada dans 71% des cas après 2h, et 100 % des cas après 72h, bien que le nombre d'œufs ainsi attaqués soit réduit en comparaison d'œufs non-enterrés. Dans le système tri-trophique mis en place dans **l'Article 4,** 87% des œufs parasités par *G. gonikopalense* étaient enterrés, bien qu'il ait été parfaitement capable d'atteindre des œufs pondus au sommet de la plante hôte.

Ainsi, ces résultats mettent en lumière que le comportement de recherche d'hôte de *G. gonikopalense* est adapté à celui d'oviposition de bagrada. Plus encore, ce comportement ne résulte pas d'un apprentissage, mais a été exprimé de manière innée par des parasitoïdes n'ayant jamais rencontré d'œufs enterrés auparavant. L'oviposition quasi-systématique de bagrada dans le sol montre quant à elle la pertinence d'utiliser *G. gonikopalense* pour réguler la punaise, ou tout du moins l'importance de considérer ce critère pour un candidat à la LBC. D'autre part, la forte diminution du parasitisme de *G. gonikopalense* en présence d'œufs enterrés manuellement suggère l'utilisation de composés chimiques de contact possiblement émis par bagrada au cours de l'oviposition. La recherche de tels composés devrait donc faire l'objet de recherches approfondies.

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ORIGINAL PAPER

Catch me if you can: novel foraging behavior of an egg parasitoid, *Gryon gonikopalense*, against the stinkbug pest, *Bagrada hilaris*

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Abstract

Host detection and parasitism by egg parasitoids involve host chemical recognition, spatial overlapping, and the ability to overcome physical barriers. Within the context of importation biological control, we examined the potential of *Gryon gonikopalense* (Hym.: Scelionidae), against the stinkbug pest, *Bagrada hilaris* (Hem.: Pentatomidae). Bagrada is invasive in the Americas where it negatively impacts cruciferous cropping systems. Within Pentatomidae, bagrada possesses a unique behavioral trait in that its eggs are oviposited buried. Our study aimed to evaluate the capacity of *G. gonikopalense* to respond to bagrada ovipositional behavior. We showed that *G. gonikopalense* exhibits a well-adapted foraging behavior observed by tracking and successfully parasitizing 2 mm-deep buried bagrada eggs, which was further supported by video recordings. Four conditions of egg detectability by parasitoids (free eggs, naturally buried eggs, and manually buried with disturbed or clean sand) and foraging durations were tested in Petri dishes. The parasitism rate of 100% was obtained after 72hours for both free and naturally buried eggs; a rate of 71% was obtained after 2 h with free eggs. There was no significant difference concerning the parasitoid success rate (< 40%) with manually buried eggs. Our data suggested that host egg detection was based on chemical cues since parasitism success was reduced by up to 20% when eggs were manually buried under a clean substrate or a substrate previously used for oviposition but disturbed. *Gryon gonikopalense* can overcome both the egg detection and physical barrier constituted by the sand to parasitize the pest despite a reduced number of progeny produced.

Keywords Egg parasitoid · Pentatomidae · Stink bug · Biological control · Foraging behavior

Key messages

- The foraging ability of the egg parasitoid *Gryon gonikopalense* toward the major crucifer pest *Bagrada hilaris* was investigated for the first time.
- The parasitoid succeeded in parasitizing buried bagrada eggs, with maximum success after 72 h.
- Success seemed to be based on chemical detection.
- With this novel and adapted foraging behavior, *G. gonikopalense* has become a promising candidate for the biocontrol of bagrada in the USA and beyond.

Introduction

The role of egg parasitoids as biocontrol agents in crop protection has been widely recognized as they are able to kill their host before it negatively impacts crops (Orr 1988; Mills 2009; Sforza 2021). In addition, egg parasitoids generally have exhibited a limited range of possible non-target hosts as their parasitism strategy results from a long-term evolution with their hosts (Strand and Obrycki 1996). Nevertheless, the success of importation biocontrol programs depends on the ability of a selected biocontrol agent to overcome several barriers that can limit or prevent its effectiveness in

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regulating a target pest, such as synchronized population dynamics, similar climatic suitability, host availability, and foraging time allocation (Hokkanen and Sailer 1985).

The first successful determinant of a newly introduced egg parasitoid is its ability to detect, find, and successfully parasitize its host (i.e., the suitable stage for its development). Since host eggs kairomones are difficult to detect in a complex environment (Vet and Dicke. 1992), egg parasitoids take an "infochemicals detour" by using easy-to-detect compounds (Vet et al. 1991). These chemicals can be produced by the host plant feeding source, that is, volatiles organic compounds (VOCs) at long distances, but usually are produced by adult host stages within a short range (Vet et al. 1991; Fatouros et al. 2008). For instance, chemical traces of the pentatomids *Nezara viridula* (L.) and *Murgantia histrionica* (Hahn) stimulate the foraging activity of their egg parasitoid *Trissolcus basalis* (Wollaston) (Colazza et al. 1999) and *Trissolcus brochymenae* (Conti et al. 2003). Similarly, cuticular hydrocarbons of *N. viridula* are also used by *T. basalis* for host recognition (Colazza et al. 2007).

The second obstacle an egg parasitoid must overcome is to access the host in order to parasitize it. In response to the selective pressure from parasitism, hosts have evolved various protective mechanisms such as avoidance strategies (Gross 1993), behavioral and morphological defenses (Greeney et al. 2012), or immune responses (Siva-Jothy et al. 2005). However, immobile life stages like eggs are more vulnerable to parasitism and several host species have developed an egg protection strategy or egg hiding behavior (Darling and Johnson 1982; Edgerly 1987; Damman and Cappuccino 1991; Taylor et al. 2014). In return, parasitoids have evolved behavioral or physical abilities to find and parasitize their host. For instance, *Scelio* sp. (Hymenoptera: Scelionidae) grasshopper egg parasitoids burrow through soil to reach host eggs and represent the only biocontrol success against locusts to date (Baker et al. 1996; Dangerfield et al. 2001).

Within less than a decade, the invasive stinkbug, *Bagrada hilaris* Burmeister (Hemiptera, Pentatomidae) (= bagrada), has become a major threat to crucifer crops in the Americas (Palumbo et al. 2016; McPherson 2018; Abram et al. 2020). It was first introduced to California in 2008, then to several U.S. states, Mexico, and Chile (Bundy et al. 2012; Sánchez-Peña 2014; Faúndez et al. 2016; Reed et al. 2013; Palumbo et al. 2016). Bagrada feeds on a broad range of Brassicaceae crops, such as broccoli, mustard, and radish (Reed et al. 2013), with yield losses reaching as high as 25% in Arizona, though they are often difficult to evaluate (Palumbo 2016).

As opposed to all known pentatomid species that lay clustered eggs aboveground on plant tissue (Mc Pherson et al. 2018), the bagrada female oviposition exhibits two main features: firstly, females lay their eggs individually, on any substrate including plant tissue (Taylor et al. 2014); secondly, females may bury single eggs belowground. To date, the latter feature is unique amongst Pentatomidae and was firstly reported by Gunn (1918), and then recently in greater detail by Taylor et al. (2014). Since then, many observations of this unique oviposition have been made with bagrada laboratory colonies (Martel and Sforza 2019). Furthermore, this behavior must be widespread within native bagrada populations since it has been observed in South Africa, Morocco, Kenya, Italy (Sforza RFH and Martel G, pers. obs.), India, Pakistan (Gunn 1918; Samuel 1949), and the U.S. (Taylor et al. 2014; Bundy et al. 2019). Therefore, it likely results from long-term evolution with natural enemies such as egg parasitoids. Egg parasitoid species that evolved abilities to detect and reach protected Bagrada eggs are therefore expected to overcome this behavioral barrier more easily than parasitoids attacking stink bugs without egg protection. Hence, specific foraging abilities should make these parasitoid species valuable candidates for an importation biological control program against *B. hilaris*. To date, two Scelionid wasp species, *Liophanurus* sp. and *Typhodytes* sp., emerging from buried bagrada eggs from the field have been described (Gunn 1918; Samuel 1949). Both wasps were able to parasitize eggs in the soil with an approximately 20% successful parasitism rate though no laboratory trials were reported (Gunn 1918; Samuel 1949). Another egg parasitoid, *Gryon gonikopalense* Sharma (Hymenoptera, Scelionidae), was recently collected in Pakistan from aboveground sentinel egg exposures (Mahmood et al. 2015). *Gryon gonikopalense* is currently under evaluation for use as a biocontrol candidate for a classical biological control program in the U.S., displaying promising biological attributes (Martel et al. 2019). The success of this egg parasitoid in the field, still unknown, relies on its ability to adapt to bagrada behavior.

If bagrada oviposition behavior has evolved as a protection against natural enemies, it should provide a fitness advantage for the pest by diminishing egg accessibility and therefore the parasitoid's success (i.e., the number of progeny they can produce). Thus, we expected that (1) the parasitism success rate of *G. gonikopalense* would be negatively impacted with buried eggs and (2) the number of parasitized eggs for a limited period of time would be lower for buried versus non-buried eggs.

On the other hand, based on the common Pakistani origin of both the Californian bagrada populations (Sforza et al. 2017) and *G. gonikopalense* (Mahmood et al. 2015; Martel et al. 2019), the current study hypothesized that this parasitoid could have evolved abilities to efficiently parasitize bagrada. Given this, we expect that (1) *Gryon gonikopalense* would be able to detect and parasitize buried bagrada eggs; (2) the parasitoid progeny would develop in buried eggs and emerge from them as easily as from non-buried eggs; and (3) the parasitoid's detection ability would be based on

recognition of specific chemicals released by its host. We therefore predict a higher parasitism rate when chemical cues are available in the environment.

The discernment of and interplay of these different factors will be examined here with particular emphasis on the ability of *G. gonikopalense* to track and parasitize buried bagrada eggs. Our study will help to assess the behavioral specificity of *G. gonikopalense* and the scope of its regulating capacity on bagrada populations in the field within the context of an importation biocontrol program.

Material and methods

Breeding conditions

The laboratory colonies of *Bagrada hilaris* and *Gryon gonikopalense* used in this study were maintained in the quarantine glasshouse of the European Biological Control Laboratory-EBCL (USDA-ARS) at Montferrier-sur-Lez (France) under the official agreement n° R76-2019–162, issued by the French Ministry of Agriculture.

Bagrada rearing

An inbred colony of *Bagrada hilaris* provided by the University of California, Riverside, has been routinely maintained since 2016 at EBCL in BugDORM® cages (47.5 × 47.5 × 47.5 cm, 150 µm opening mesh) under 26 °C (night) and *ca*. 33 °C (day), at an ambient photoperiod supplemented with sodium lamps (L/D 12:12), and RH: 30 ± 15% (Martel et al. 2019). Adults and nymphs were fed weekly with a selection of 1-month-old Brassicaceae plants including *Brassica oleracea var. italica* Plenck (broccoli), *Sinapis alba* L. (white mustard); and *Diplotaxis erucoides* De Candolle (white wallrocket). Broccoli and mustard were grown from seeds at *ca*. 22 °C, RH: 30 ± 10% in a separate room of the quarantine, and white wallrocket was field-collected weekly.

Bagrada egg collection

In rearing cages, Petri dishes (Ø = 9 cm) filled with sand (DIALL®, yellow sand, granulometry = 200 µm, Spain) were used as oviposition arenas for bagrada. In Petri dishes, eggs covered with sand particles (see Taylor et al. 2015) were collected every two days with a fine brush after sifting sand (Hogentogler®, mesh size = 425 µm, USA). Eggs were glued next to each other (UHU stic®, Germany) on cardboard pieces (Martel et al. 2019) and used either for parasitoid rearing or subsequent experiments (see Sect. 3).

The parasitoid

The EBCL *Gryon gonikopalense* colony originated from Pakistan (Mahmood et al. 2015) and was maintained in insect-proof transparent plastic boxes (18 × 12 × 5.5 cm). Aeration was provided through holes in the lid (Ø = 50 mm), covered with a mesh fabric. Rearing boxes were stored in a rearing chamber (22 ± 0.5 °C, RH 50 ± 5%, L/D 12:12) and drops of diluted acacia honey (Lune de miel®, France) were deposited on the mesh as ad libitum nourishment. The colony was maintained by regularly exposing fresh *B. hilaris* eggs to female parasitoids.

Bagrada oviposition depth

Petri dishes filled with sand were used (see 1.b above). Bagrada has been observed to deposit eggs at various locations, including against the wall of a dish (Fig. 1). After sifting sand, naturally glued eggs on a Petri dish wall were visible and used to estimate the oviposition depth of bagrada. A picture of the Petri dish wall was taken before and after sifting sand (NIKON® D3500, NIKON CORP., Japan). The two pictures were overlapped, and the distance between the sand surface and the closest part of the egg was measured from the surface. All measurements were performed with ImageJ® software. Data were used for designing parasitization experiments in Sect. 3. Under laboratory conditions, *B. hilaris* deposited eggs on average at 2.00 mm deep underneath the sand surface ($n = 116$; $CI_{95\%}$ [1.87; 2.13]).

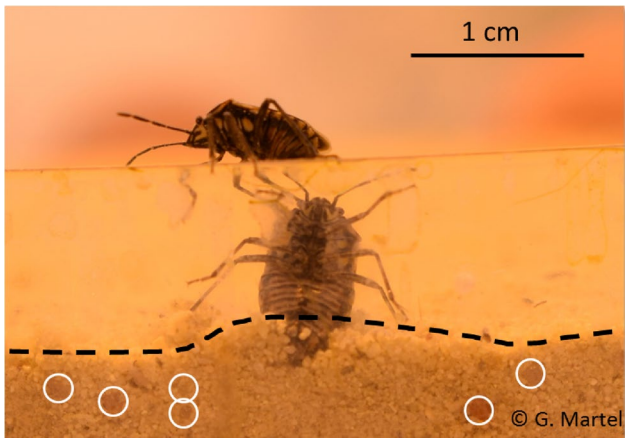


Fig. 1 Lateral view of the belowground oviposition behavior of *Bagrada hilaris* (Hemiptera: Pentatomidae) in a Petri dish filled with sand under laboratory conditions. The dotted line defines the sand surface and white circles highlight buried eggs glued on the wall of the Petri dish

Ability to parasitize free and buried eggs

In order to test the effect of buried eggs and semiochemicals released by bagrada on the parasitism success of *G. gonikopalense* (i.e., presence of at least one egg parasitized) and the number of progeny it produces, Petri dishes (Falcon®, 50 × 9 mm, Corning incorporated, USA) filled with sand were placed inside the permanent rearing cages for 24 h and removed after exposure. Four groups were formed depending on the "egg status" (Fig. 2).

- In group "Free", after sifting sand, series of 10 eggs (*n* series = 39) were constituted by manually gluing eggs onto a piece of cardboard (20 × 30 mm), which was deposited without sand into a clean Petri dish (Falcon®, 50 × 9 mm, Corning incorporated, USA).
- In group "NB" (Naturally Buried), sand was not removed from Petri dishes (n = 149) but all incompletely buried eggs were removed with a fine brush to avoid a bias in foraging behavior.
- In group "MBD" (Manually Buried with Disturbed sand), after sifting sand, series of 10 eggs (n series = 117) were constituted by manually gluing eggs on cardboard pieces positioned at the bottom of a clean Petri dish with a double-sided tape. Eggs were manually buried with sifted sand (= disturbed) originally used for 24 h by bagrada. Eggs were placed under 3 mm of sand (average measured oviposition depth of 2 mm + average size of bagrada egg of 1 mm; Azim and Shafee 1986);

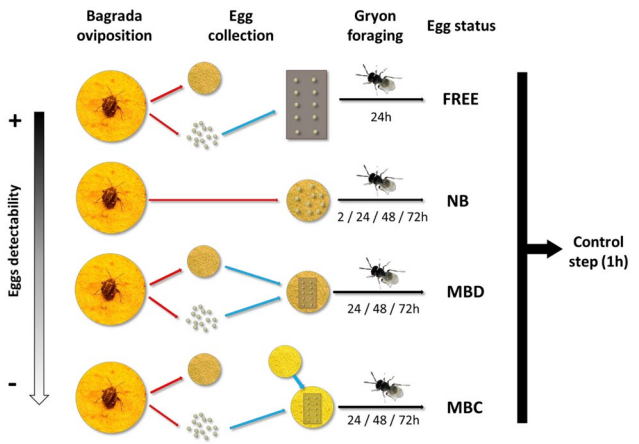


Fig. 2 Experimental design for assessing the foraging ability of *Gryon gonikopalense* (Hymenoptera, Scelionidae) on eggs of *Bagrada hilaris* (Hemiptera: Pentatomidae). Experiments were conducted with non-buried eggs (FREE), naturally buried eggs (NB), and manually buried eggs with either clean (MBC) or disturbed (MBD) sand. Eggs from MBD and MBC were grouped on a cardboard before burial. Colored arrows represent different steps: red for egg and substrate collection, blue for preparation of egg series, and black for parasitoid exposure (test followed by control)

- In group "MBC" (Manually Buried with Clean sand), after sifting sand, series of 10 eggs (*n* series = 100) were constituted by gluing eggs on cardboard pieces and manually buried under 3 mm (as described above) in new clean sand.

For groups MBD and MBC, preliminary results (not presented) showed that using a 10-egg series was sufficient since no tested parasitoid could parasitize 100% of the 10 eggs provided after 72 h foraging time. For group NB, the number of eggs deposited in the sand could not be controlled without disturbing the surface and was only determined a posteriori.

For each replicate of each group, we deposited one mated 48 h-old female *G. gonikopalense* into the corresponding Petri dish, then closed with a lid. All experiments were conducted within the parasitoid rearing chamber (see 1c). In order to assess whether *G. gonikopalense* parasitism was slowed by the presence of sand and the presence/absence of bagrada chemical cues, tested females from each of the three groups NB / MBD and MBC were randomly subdivided within three new subgroups corresponding to "foraging time": in group 1, females were allowed to search for buried eggs for 24 h; in group 2, females were allowed to search for buried eggs for 48 h, and, in group 3, females were allowed to search for buried eggs for 72 h. Free eggs were exposed to females for only 24 h. In order to assess the minimum time required for the parasitoid to successfully parasitize naturally buried eggs, an additional group with NB eggs was formed containing females allowed to search for eggs only two hours. All females were removed and singly exposed to a 10-egg series without sand for one hour in order to control their oviposition ability and avoid any type II error (false negative). After the experiment, all eggs of all groups including controls were stored under rearing chamber conditions until parasitoid emergence. For each replicate, we counted the number of parasitized eggs, the number of emerged progeny, and the development time of each emerged progeny. All non-emerged eggs were dissected under a binocular microscope (Stemi 508, Zeiss®, Germany) in order to verify whether they contained an identifiable nymph or adult parasitoid. Eggs with unidentifiable content were considered as not parasitized. Female parasitoids that failed to parasitize any egg during experimental exposures and control steps were excluded from statistical analyses.

Foraging behavior recording

The foraging behavior was recorded for *G. gonikopalense* females searching for buried bagrada eggs. Two-day-old mated females were individually deposited in a sand arena similar to what we used in the NB group (see Sect. 3). The foraging behavior was recorded with a

camera (NIKON® D3500, NIKON CORP., Japan) coupled with a binocular microscope (Stemi 508, Zeiss®, Germany) until the parasitoid located and parasitized an egg. Because belowground eggs parasitism could not be immediately established during recording, all buried eggs were collected from sifted sand and stored in the rearing chamber until any parasitoid emergence. The different steps of *G. gonikopalense* foraging behavior are presented in a supplementary video file.

Statistical analyses

The bagrada oviposition depth was described as the mean with associated 95% confidence interval. The proportion of individuals that succeeded (i.e., females that successfully parasitized at least one egg) between each variable combination of "egg status" and "foraging time" was compared with a contingency χ^2 test. A pairwise comparison to test the differences between each category was performed using a Fisher's exact test [function fisher.multcomp, package RVAideMemoire with R software (Hervé 2020)].

We compared the number of parasitized eggs between each combination of the factors "egg status" and "foraging time" with a global linear model (GLM) based on Poisson distribution, which fit the data best, followed by a Tukey post hoc test to identify differences between each treatment. Because the number of eggs laid in the group NB could not be controlled, we estimated any confounding effect of the total number of eggs on the parasitism measurements with a Spearman's rank correlation test. We measured a positive correlation between the number of parasitized eggs and the total number of eggs with each level of the factor "foraging time" except for 48 h. Hence, we corrected these data by approximating them for a total number of ten eggs. This was done in order to avoid an overestimation of the results due to a density effect and in order to compare with the data obtained in groups MBC, MBD, and FREE. We compared the development time of male and female parasitoids between buried and FREE bagrada eggs using a two-way ANOVA followed by a Tukey post hoc test to identify differences between groups. We compared the emergence rate of *G. gonikopalense* progeny between buried and non-buried bagrada eggs using a Wilcoxon test as the data were not normally distributed and no transformation was possible to reach a normal distribution. We only considered the group NB for buried eggs in order to avoid any bias linked to manual burial. All statistical analyses were carried out with R software (R Development Core Team 2016) and figures were obtained with Excel software. For all tests, the alpha significance threshold was set at $\alpha = 0.05$.

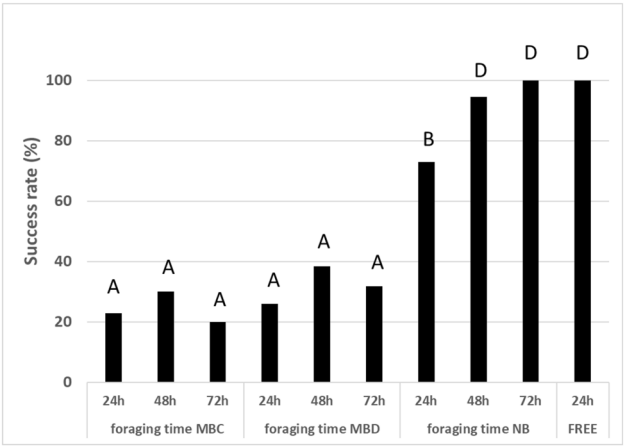


Fig. 3 Parasitism success rate of *Gryon gonikopalense* exposed to *Bagrada hilaris* non-buried eggs (FREE), naturally buried eggs (NB), and manually buried eggs with clean (MBC) or disturbed (MBD) sand. Foraging times were 24, 48, or 72 h for categories NB, MBC, MBD, and 24 h for FREE. Values associated with the same letter were not statistically different ($P > 0.05$)

Table 1 Effect of egg status, foraging time, presence of honey, and their interactions on the number of *Bagrada hilaris* eggs parasitized by *Gryon gonikopalense* under laboratory conditions. Subscripts associated with statistics indicate the degree of freedom of the corresponding test. Bold values indicate a significant effect

Factor	Test values	*P*-values
Egg status	$F_{3707} = 160.004$	< 0.001
Foraging time	$F_{3704} = 17.221$	< 0.001
Egg status × Foraging time	$F_{4699} = 3.294$	0.011

Results

Parasitism success with buried and free eggs

The *G. gonikopalense* success rate in parasitizing eggs was affected by the foraging time and the egg status ($\chi^2 = 366.22$, $df = 18$, p-value < 0.0001). The highest success rate was obtained with FREE eggs (100% success) and parasitoids searching for naturally buried eggs (NB) for 72 h reached the same value (Fig. 3). There was no significant difference concerning the parasitoid success rate with manually buried eggs (MBC and MBD) whatever the foraging time considered. Nevertheless, the maximum success rate of 38.46% obtained at 48 h with eggs manually buried under disturbed sand was significantly below that obtained with naturally buried eggs exposed for only 2 h (71.05%) ($\chi^2 = 366.22$, $df = 18$, p-value < 0.0001; data not shown).

The combined effects of foraging time and egg status affected the number of parasitized eggs (Table 1). The

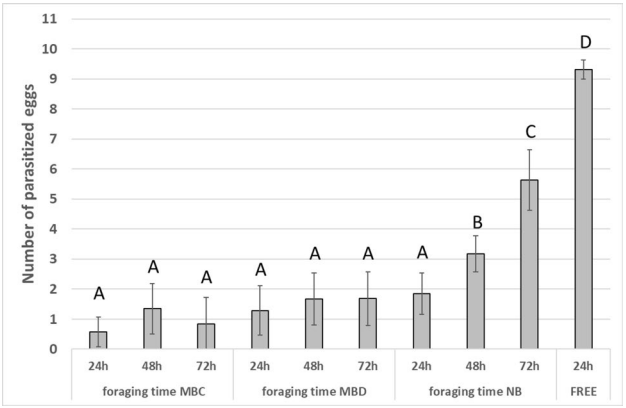


Fig. 4 Average number of *Bagrada hilaris* eggs parasitized by *Gryon gonikopalense* exposed to non-buried (FREE), naturally buried (NB), and manually buried eggs with clean (MBC) or disturbed (MBD) sand. Foraging times were 24, 48, or 72 h for categories MBC, MBD and NB, and 24 h for FREE. Each mean is given with associated 95% confidence interval (vertical bar). Means associated with the same letter are not statistically different ($P > 0.05$)

highest number of parasitized eggs was obtained when parasitoids were exposed to FREE eggs with 9.31 eggs on average ($CI_{95\%}$ [8.98; 9.63]) (Fig. 4). Parasitoids exposed to naturally buried eggs (NB) attacked on average more eggs than parasitoids exposed to manually buried eggs (MBC and MBD) when search time was equal to or greater than 48 h (Fig. 4). However, parasitoids exposed to naturally buried eggs attacked fewer eggs than those exposed to FREE eggs, even after 72 h with 5.63 eggs on average ($CI_{95\%}$ [4.62; 6.64]). The number of parasitized eggs did not differ between MBC and MBD eggs, whatever the foraging time considered (Fig. 4). There was no significant difference between the average emergence rate of *G. gonikopalense* between FREE (92.23%) and naturally buried eggs (NB) (89.46%) (Wilcoxon test, $W = 4910$, p-value = 0.65) (Fig. 5). The development time of *G. gonikopalense* was affected by both factors sex and egg status (Table 2). Males developed faster than females but adults developed slightly faster inside buried eggs, whatever the gender (Tukey test, p-value < 0.001) (Fig. 6; Table 2).

Discussion

Through the research presented here, we examined and demonstrated the ability of the egg parasitoid *Gryon gonikopalense* to find and successfully parasitize belowground eggs of the cruciferous pest *Bagrada hilaris*. We also examined supporting elements that may be implicated in the success or failure of this parasitoid, particularly host chemical recognition by *G. gonikopalense*.

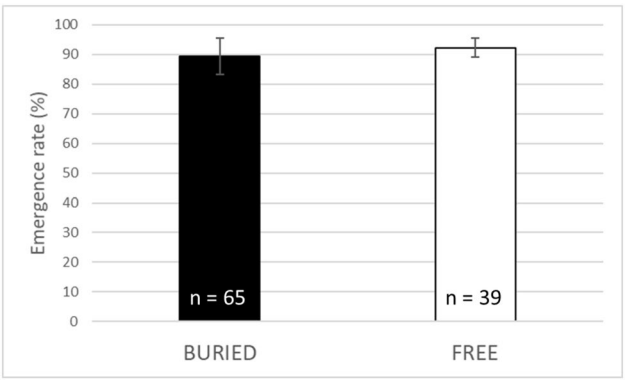


Fig. 5 Average emergence rate of *Gryon gonikopalense* developing inside buried or FREE *Bagrada hilaris* eggs. Numbers at the bottom indicate the number of replicates (n). Each mean is given with associated 95% confidence interval (vertical bar)

Table 2 Effect of *Bagrada hilaris* egg status (buried or FREE) and progeny sex and their interaction on the development time of *Gryon gonikopalense* progeny. Subscripts associated with statistics indicate the degree of freedom of the corresponding test. Bold values indicate a significant effect

Factor	Test values	P-values
Egg status	$F_{2650} = 62.358$	< 0.001
Sex	$F_{1650} = 276.336$	< 0.001
Egg status × Sex	$F_{2650} = 5.253$	0.0054

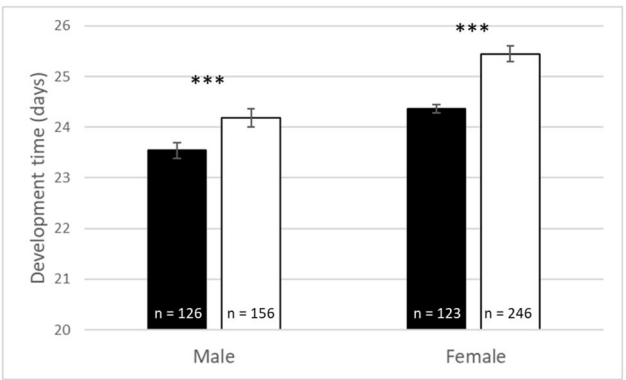


Fig. 6 Average development time of *Gryon gonikopalense* males and females inside buried (black bars) or FREE (white bars) *Bagrada hilaris* eggs. Numbers at the bottom indicate the number of replicates. Means are given with an associated 95% confidence interval (vertical bar). *** indicates a significant difference ($P < 0.05$)

For the first time, our study provided clear evidence that *G. gonikopalense* is able to locate buried bagrada host eggs from the surface and dig through the substrate to reach and parasitize them. This quantitative data were further supported by observational videos that showed the

parasitoid detecting and reaching its host through the substrate (Supplementary video). Since Samuel (1949), this is the second time that the complete foraging behavior of an egg parasitoid attacking buried bagrada eggs has been reported. However, Samuel's descriptions provided only indirect observations of parasitism behavior by counting progeny emerging from field-buried eggs, without providing informative protocols or behavioral observations. The present study therefore provides new insights into the ability of a scelionid parasitoid of *B. hilaris* to perform a well-adapted and specific response to the unique oviposition behavior of this serious pest (Taylor et al. 2014). In laboratory conditions, this foraging ability allowed *G. gonikopalense* to obtain a high parasitism success rate against naturally oviposited eggs belowground; though complete success (100%) was obtained only after 72 h of foraging, *G. gonikopalense* was able to parasitize its first eggs within only two hours with 71% success.

Moreover, our results suggested that host detection by *G. gonikopalense* was based on specific recognition of chemical cues based on the observation that the parasitoid's success rate was greatly reduced (ranging from 20 to 30%) when it was provided with MBC eggs (without any chemical trace) and MBD eggs (sand still loaded with chemicals compounds but disturbed). The specific recognition during the searching phase we recorded was relateable to a drumming behavior with antennae (Godfray 1994) when female parasitoids seemed to follow a chemical path, toward their host (See supplementary video). The use of host chemicals emitted by the host for foraging eggs has been commonly observed within scelionid species (Hokyo and Kiritani 1966; Rabb and Bradley 1970; Strand and Vinson 1983; Higuchi and Suzuki 1996; Wiedemann et al. 2003).

However, the series of experiments presented here did not allow the determination of the type of signal that could be used by *G. gonikopalense*. It was reported that under laboratory conditions mating behavior of *B. hilaris* was mediated by volatiles and short-range compounds, possibly emitted by both males and females (Guarino et al. 2008; Arif et al. 2020). These compounds could therefore be used by *G. gonikopalense* as nondirect cues (chemical detour), according to the reliability-detectability concept proposed by Vet and Dicke (1992). Surprisingly, *G. gonikopalense* was also able to find eggs buried 2 mm below clean sand, suggesting that female parasitoids were likely attracted by any compounds emitted by the bagrada egg itself. Associated compounds, such as hydrocarbons, are known to play a kairomonal role in implementing the foraging behavior of egg parasitoids (Frenoy et al. 1992; Renou et al. 1992). The ability of *G. gonikopalense* to detect bagrada eggs through a substrate with limited information would indicate a sensitive detection ability for its host. In addition, we showed that sand as a physical barrier did not represent any particular issue for offspring development or emergence, though the first was slightly shorter in buried than in free eggs.

The observed efficiency on the foraging behavior of *G. gonikopalense* was counterbalanced by notable negative consequences to its fitness that should be considered in assessing overall parasitoid efficiency in regulating *B. hilaris* populations. First, our results suggested buried host eggs were more difficult to reach than free ones: the average number of buried eggs parasitized by *G. gonikopalense* in 24 h was strongly reduced compared to non-buried ones, producing less offspring. This confirmed the direct benefit of the pest's oviposition behavior for its own fitness and the negative effect on the egg parasitoid, as hypothesized by Taylor et al. (2014). Indeed, one can presume that bagrada oviposition behavior evolved as a "refuge" against predators including egg parasitoids. As a consequence, generalist predators and egg parasitoids that did not evolve specific abilities to detect buried eggs would not have been able to reach them, as highlighted by the observation of the two other non-foraging egg parasitoids co-located in Pakistan: *Trissolcus hyalinipennis* Rajmohana & Narendran and *Ooencyrtus mirus* Triapitsyn & Power (Martel G & Sforza RFH, pers. obs.; Power; 2020). In addition, the effectiveness of bagrada oviposition behavior in the field may have been reinforced by *G. gonikopalense*'s short egg development time as first instars emerge within 3–5 days (Reed et al. 2017). Although *G. gonikopalense* successfully attacks 3- to 4-day-old eggs (Martel et al. 2019), its ability to efficiently parasitize bagrada eggs may result in a race against the clock since attacking buried eggs requires much more time than non-buried ones.

In this study, we showed that *G. gonikopalense* has adapted its behavior to bagrada oviposition, and is, to date, the only investigated parasitoid from Pakistan (Mahmood et al. 2015) known to attack buried eggs with such efficiency. Since no stinkbugs besides *B. hilaris* are known to lay belowground eggs, parasitoids able to track them are likely to be highly specialized for this host. Though it is not possible to predict whether *G. gonikopalense* is an isolated case or whether other species share the same ability, the discovery of this specialized behavior is a promising step toward the use of this egg parasitoid as a biocontrol agent against the cruciferous pest. We suggest this ability may have resulted from a long-term evolution as female parasitoids innately (without any prior learning) exhibited the ability to search for bagrada eggs in the sand under laboratory conditions. However, the spatial distribution of bagrada eggs in cropping systems is currently unknown, although it is hypothesized they oviposit mainly in soil (Taylor et al. 2014; Bundy et al. 2019). Bagrada egg distribution in its short-range environment (plant and soil surrounding) must be examined in detail to assess the relevance of *G. gonikopalense* behavioral response to its efficacy as a future biocontrol candidate. Other biotic factors such as intra- and inter-specific competition should also be investigated, as these can influence how parasitoids exploit their host (Goubault et al. 2005; Le Lann et al. 2008; Cusumano et al. 2012).

Although several egg parasitoid species have been described to attack *B. hilaris* in its native and invasive area (Mahmood et al. 2015; Ganjisaffar et al. 2018; Felipe-Victoriano et al. 2019; Lomeli-Flores et al. 2019), these have not shown any aptitude to date for attacking buried eggs. Therefore, *G. gonikopalense* would be expected to be both more effective and complementary to the other parasitoids in North and South Americas, where the pest is rapidly spreading.

Author contributions

GM and RFHS involved in conceptualization; GM involved in methodology and formally analysed and investigated the study; GM participated in writing of the original draft preparation; GM and RFHS participated in writing of the review and editing; RFHS involved in funding acquisition, resources and supervision. Both authors read and approved the manuscript.

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Complaince with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Availability of data and material complete.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Supplementary Information Supplementary file 1 (WMV 10841 kb)

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How an egg parasitoid responds to an unusual stinkbug oviposition behavior: the case of *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae) and *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae)

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With 5 figures, 1 table and 1 supplement

Abstract: The phytophagous stinkbug pest *Bagrada hilaris* (Hemiptera: Pentatomidae) (= bagrada) buries single eggs in the ground, an exception among pentatomids. To investigate potential biological control strategies to mitigate the pest's damages, an evaluation of the egg parasitoid *Gryon gonikopalense* (Hymenoptera: Scelionidae) as a biocontrol agent is ongoing. Its ability to detect and parasitize buried bagrada eggs could be a key factor in the short and long-term regulation of bagrada populations in the U.S. However, this foraging ability would be relevant only if bagrada mainly oviposits in the soil and if *G. gonikopalense* mainly parasitizes buried eggs, which is currently unknown. We assessed the oviposition pattern of bagrada in presence of three host plants (broccoli, white mustard and white rocket) and at three density levels (one, five or fifteen copulae). The prevalent oviposition site of bagrada was in the soil (92% of eggs) with no effect from the host plant neither the bagrada density. Eggs oviposited on mustard were mainly present on the top third, but no distribution pattern was observed on broccoli and white rocket. *Gryon gonikopalense* preferred parasitizing buried eggs (87% of total parasitism). It was also able to parasitize non-buried eggs whatever their location. Artificially increasing the number of non-buried eggs in the experimental design only resulted in a slight reduction in the parasitism of buried eggs (70%). As a result, this study highlights the relevance of using *G. gonikopalense* against bagrada as its specific foraging behaviour responds well to the main oviposition behaviour of its host.

Keywords: Scelionidae, pentatomid bug, parasitism, biocontrol, Brassicaceae

1 Introduction

The role of egg parasitoids as biocontrol agents in crop protection is widely recognized as they suppress their host before inducing crop damage (Orr 1988; Mills 2009; Sforza 2021). In addition, undesirable side-effects (*i.e*. attacking non-target species) are known to be intrinsically limited since egg parasitoids have usually closely evolved with their hosts (Orr 1988; Strand & Obrycki 1996; Driesche & Hoddle 2016). This is especially true for parasitoids belonging to Scelionidae that are often restricted to a single host family (Austin et al. 2005). As a result, scelionid egg parasitoids were selected in biocontrol programs against invasive stinkbugs (Hemiptera, Pentatomidae) that are negatively impacting various vegetables and fruits of economic importance worldwide and in the U. S. in particular (McPherson 2018). In recent time, these programs have selected *Trissolcus basalis* Wollaston against *Nezara viridula* L. (Wilson 1960; Rao et al. 1971; Correa-Ferreira and Moscardi, 1996; Abram et al. 2020), *Gryon pennsylvanicum* Ashmead against *Leptoglossus occidentalis* Heidemann (Roversi et al. 2011; Peverieri et al. 2012) and more recently *Trissolcus japonicus* Ashmead against *Halyomorpha halys* Stål (Conti et al. 2020; Haye et al. 2020). The use of biological control strategies could contribute to more ecological and sustainable management of these pests by reducing the use of chemical control.

In less than a decade, *Bagrada hilaris* Burmeister (Hemiptera, Pentatomidae) (= bagrada) has become a major threat to crucifer crops in the Americas (McPherson 2018; Conti et al. 2020) inducing yield losses as high as 25 % in

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Arizona (Palumbo 2016). Originated from Asia and Africa, bagrada was first discovered in California in 2008, and rapidly spread to other U.S. states including Arizona, and New Mexico (Bundy et al. 2012; Reed et al. 2013; Palumbo et al. 2016). The pest rapidly reached Mexico in 2014 and Chile in 2016 (Sánchez-Peña 2014; Faùndez et al. 2016). Bagrada is a gregarious and highly polyphagous pest that mainly feeds on a broad range of Brassicaceae crops (Reed et al. 2013; Huang et al. 2014; Palumbo et al. 2016) but also on caper (Colazza et al. 2004), tomato (Singh et al. 2011) and other Fabaceae species (Reed et al. 2013).

As a rule, pentatomid bugs deposit their barrel-shaped eggs in irregular round or hexagonal clusters or longitudinal rows on above ground tissue of their host plants (Southwood 1956; Hinton 1981; McPherson 2018). As an exception within the Pentatomidae family, bagrada exhibits an unusual oviposition behavior by laying single eggs on the aerial portion of the plant (non-buried) as well as below the soil surface (buried) (Taylor et al. 2014). This unusual oviposition behaviour was first reported by Gunn (1918) and recently detailed by Taylor et al. (2014). Such behaviour has been documented in bagrada laboratory colonies (Martel & Sforza 2020; Bundy et al. 2019) and in the field, including South Africa and Italy (Martel G. & Sforza R.F.H., pers. obs.).

This specialized oviposition behavior most likely evolved from a long-term association with native natural enemies like egg parasitoids, as predation or parasitism pressure can lead to very specific protective behaviors (Darling & Johnson 1982; Edgerly 1987; Damman & Cappuccino 1991; Gross 1993). Hiding eggs buried may have played an important role in the successful bagrada invasion in North and South America. Indeed, bagrada oviposition plays a protective role, as a refuge, decreasing egg accessibility for egg parasitoids (Martel & Sforza 2020; Power 2020; Tofangsazi et al. 2020). In the U.S. and Mexico, several native egg parasitoids were found to parasitize bagrada sentinel eggs (i.e. eggs glued on cardboards and exposed in the field) (Ganjisaffar et al. 2018; Felipe-Victoriano et al. 2019; Lomeli-Flores et al. 2019). However, the apparent lack of enemies with specific foraging aptitudes to locate buried bagrada eggs in invaded areas could imply a lack of top-down control, as predicted by the enemy release hypothesis (Roy et al. 2011).

The egg parasitoid *Gryon gonikopalense* Sharma (Hymenoptera, Scelionidae), collected from bagrada eggs in Pakistan (Mahmood et al. 2015) is currently under evaluation as a promising biocontrol candidate for an importation biological control program in the U.S. (Martel et al. 2019; Tofangsazi et al. 2020). Beyond its reproductive capacities and its high longevity, its major advantage is its ability to detect and parasitize bagrada buried eggs (Martel & Sforza 2020; Tofangsazi et al. 2020). This innate and well-adapted foraging behavior must have resulted from a long-term evolution with *B. hilaris*, and could be a key factor in the regulation of *B. hilaris* populations in invaded countries, especially in the U.S. As a consequence, we aim at considering both pest and parasitoid oviposition behavior as a main driving factor in the discovery of efficient, biologically-based management methods.

To date, a central question remains as whether eggs in nature are mainly buried or on the host plant (= non-buried eggs) and whether oviposition behavior depends on the host plant or the pest density. Indeed, morphological features of the host plant can influence the oviposition of phytophagous insects (Robbins & Daugherty 1969; Martinez 2017). The density of pest can also affect the spatial distribution of its eggs (Zitsanza et al. 2006; Bawin et al. 2014).

As a prerequisite to observations in the field, this laboratory study aims to 1) describe the oviposition pattern of bagrada at the scale of its close environment (plant and soil): If buried oviposition is a protective behavior against natural enemies acquired through evolution, we expect that bagrada will oviposit more buried eggs than on the host plant. However, morphological features of the host plant can influence the oviposition of a phytophagous insect (Robbins & Daugherty 1969; Martinez 2017), just as the pest density can affect the spatial distribution of its eggs (Zitsanza et al. 2006; Bawin et al. 2014). We therefore test whether 2) the host plant or the pest density may influence bagrada oviposition behavior.

Since attacking buried eggs limits *G. gonikopalense* fitness by reducing the number of parasitized eggs (Martel & Sforza 2021), we question whether the parasitoid would attack more buried eggs or those present on the host plant. In addition, the parasitoid foraging behavior could be influenced by host plant morphology (Sütterlin & van Lenteren 1997; Lovinger et al. 2000). We therefore attempted to describe 3) *G. gonikopalense* parasitism behavior under controlled conditions and 4) whether this foraging behavior is influenced by the host plant species.

2 Material and methods

2.1 Insects rearing

Laboratory colonies of bagrada and *G. gonikopalense* were maintained in the quarantine greenhouse of the European Biological Control Laboratory-EBCL (USDA-ARS) at Montferrier-sur-Lez (France) under the official agreement # R76-2019-162, issued by the French Ministry of Agriculture.

The bagrada colony is a subset of the colony maintained at the University of California, Riverside (Martel et al. 2019). The inbreed colony was routinely maintained at EBCL in BugDORM® cages (47.5 × 47.5 × 47.5 cm, 150µm opening mesh, MegaView Science®, Taiwan) under 25°C (night) and *ca*. 30°C (day), at an ambient photoperiod supplemented with sodium lamps (L:D 12:12), and 30 ± 15% RH. Bagrada adults and nymphs were fed weekly with a selection of 1-month-old Brassicaceae plants including *Brassica oleracea* var. *italica* Plenck (broccoli), *Sinapis alba* L. (white mustard) and

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Diplotaxis erucoides De Candolle (white rocket). Broccoli and mustard were grown from seeds in a separate room of the quarantine (22°C, 30 ± 10% RH), and the white rocket was field collected weekly in the area surrounding the laboratory. Petri dishes (diameter = 9cm) filled with sand (DIALL®, yellow sand, granulometry = 200µm, Spain) were used as oviposition arenas for bagrada and were installed within the rearing cages. In Petri dishes, eggs covered with sand particles (see Taylor et al. 2015) were collected every two days with a fine brush after sifting sand with a sieve (Hogentogler®, mesh size = 425µm, USA). Eggs were glued on cardboard pieces (Martel et al. 2019) and used either for the parasitoid rearing or subsequent experiments (see section 3).

The EBCL *G. gonikopalense* colony originated from Pakistan (Mahmood et al. 2015) and was maintained in insect-proof transparent plastic boxes (18 × 12 × 5.5 cm). Aeration was provided through holes in the lid (Ø = 50 mm), covered with a mesh fabric. Rearing boxes were stored in a climatic chamber (22 ± 0.5°C, 50 ± 5 %RH, L:D 12:12) and drops of diluted acacia honey (Lune de miel®, France) were weekly deposited on the mesh as *ad libitum* nourishment. The parasitoid colony was maintained by exposing once a week fresh *B. hilaris* eggs to female parasitoids.

2.2 Experimental tri-trophic system and general method

The experimental system (see Fig. SM1, Supplementary material) used for all following experiments was a custom-built cage that included three trophic levels: the host plant, the pest and the egg parasitoid. The cage consisted of two parts: an aerated Plexiglas cylinder equipped with a hermetic lateral door and closed at one edge by a metallic mesh and a wooden base with a central square hole. The cylinder was 43.2 cm high and 20.0 cm in diameter to accommodate the plant. Both the lid and the wall of the cylinder contained circular holes (Ø = 3 cm) covered with a nylon mesh fabric (160 µm mesh) to allow air circulation. The door was closed tight through the use of a toggle clamp and a door gasket located between the door and the cylinder wall. One potted plant (9 × 9 × 10 cm) was inserted into the wooden base, so that the upper side of the pot was precisely aligned with the bottom surface of the base (see Fig. SM1, Supplementary material). The container was previously covered with a plastic film to prevent water flowing from the soil into the sand (see Fig. SM1, Supplementary material). Moist sand is considered unsuitable for *B. hilaris* oviposition (Bundy et al. 2019) and makes egg collection difficult. Thus, a 2-cm sand layer was added above the container and entirely covered the wooden base surface. Finally, the Plexiglas cylinder covered the plant and was tightly fixed to the wooden base by 3 rubber bands (see Fig. SM1, Supplementary material). Once insects were added to the system, the whole study design was placed in a BugDORM® insect cage (60 × 60 × 120 cm, 160 ìm opening mesh), which guaranteed pest and parasitoid-proof conditions.

Three host plants were selected: broccoli (*Brassica oleracea* var. *cymosa* Duchesne), white mustard (*Sinapis alba* L.) and white rocket (*Diplotaxis erucoides* L.). Broccoli was chosen because it is the most cultivated brassicaceous crop in the bagrada's invasive range of California (U.S. Department of Agriculture-National Agricultural Statistics Service [USDA-NASS] 2017). Mustard is a major Brassicaceae cultivated in the native range of Pakistan and India (Sharma et al. 2020) and one of the most susceptible hosts of *B. hilaris* (Huang et al. 2014). White rocket (*Diplotaxis erucoides*) is a wild winter Brassicaceae that mainly grows in the Mediterranean basin and is also present in California coastal regions (www.gbif.org).

The three chosen host plants are morphologically different. Broccoli is short in size (on average 11.78 cm ($CI_{95\%}$ [10.84; 12.73]) with large simple smooth leaves with no trichomes, whereas white mustard is taller (on average 34.88 cm $CI_{95\%}$ [34.12; 35.45] with compound leaves and stem harboring evident trichomes. White rocket has a mustard-like growth (on average 39.02 cm high ($CI_{95\%}$ [38.21; 39.78]) but possesses, unlike mustard, hairless stems and leaves, with the bottom leaves shaped as a rosette. Numerous seedpods are located along the stem.

Six-week old broccoli plants with four well developed true leaves and five-week old white mustards with about 10 true leaves were used for the following experiments. Field-collected white rockets were used; only plants presenting bottom leaves and seedpods were chosen for experiments. Plants ages and sizes were chosen to 1) ensure an adequate food supply and as a trade-off between the cylinder size and plant size, and 2) enable the plant to resist 72 h (see below) under the experiment conditions without watering.

Bagrada hilaris mating couples and 1-3 d old mated *G. gonikopalense* females were used. All adults were never before exposed to the tritrophic system. The age of mated *G. gonikopalense* females was chosen because it coincided with highest fecundity (Martel et al. 2019).

2.3 Experiment 1: the pest oviposition choice

Preliminary experiments using only one bagrada copula (= mating pair) showed that oviposition occurred in the system in only 40% of the cases with broccoli and 60% with mustard, and with only one to ten eggs per replicate (data not shown). For this reason, the oviposition distribution was assessed using five copulae in experiment 1 as well as *G. gonikopalense* parasitism distribution in experiments 2. These copulae were randomly collected from the laboratory colony and ensured the presence of non-virgin females for the experiment.

2.3.1 Effect of host plant on bagrada oviposition distribution

In this experiment, five bagrada copulae were added to the tritrophic system containing one broccoli, mustard or white rocket plant, as described in subsection 2.2. Individuals were

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maintained in the system for 72 h then removed from the cylinder. Replications with broccoli (n = 21), white mustard (n = 21) and white rocket (n = 31) were always conducted concomitantly in order to avoid any confounding factors linked to temperature, humidity or light variation. For each replication all eggs oviposited in the sand after 72 h, on the plant or on the cylinder wall were collected. For replications with mustard, the plant height was quantified and eggs deposited on each leaf were counted separately in order to assess the spatial distribution all over the entire aerial portion of the plant.

2.3.2 Effect of bagrada density on its oviposition distribution

Here, we also compared bagrada egg distribution with three densities (D): one copula (D1, n = 36); five copulae (D5, n = 42) and 15 copulae (D15, n = 30). Mating couples of *B. hilaris* were added to the system containing one potted broccoli or one potted mustard plant. The bagrada egg distribution in the system was compared between each density level and host plant. No replication with white rocket could be performed due to a lack of sufficient bagrada individuals in the rearing cages at the time of experiment.

2.4 Experiment 2: parasitoid oviposition choice

2.4.1 Parasitism behavior with natural bagrada oviposition

In this experiment, we assessed whether *G. gonikopalense* would parasitize a higher number of eggs oviposited below or non-buried (i.e. on the host plant or the cylinder wall) in association with the three chosen host plants including broccoli (n = 21), mustard (n = 21) or white rocket (n = 23) or. In order to maximize parasitism response and minimize escape, five female parasitoids were used for each replication. *Gryon gonikopalense* females were added to the system together with five bagrada copulae. In order to avoid bagrada egg hatching before the end of the experiment, parasitoids were allowed to search for eggs for only 72 h. After 72 h, all insects were removed from each replicate. The sand was then sifted and all eggs removed, and if present, eggs were also collected from the plant and cylinder wall. After collection, eggs were stored in a rearing chamber (22 ± 0.5°C, 50 ± 5 %RH, L:D 12:12) until parasitoid emergence. For each egg location, we counted the number of adult parasitoids that emerged from EGGS. Unhatched eggs were dissected under a stereomicroscope (Stemi 508, Zeiss®, Germany) to determine the parasitization state. For replications with mustard, eggs deposited on leaves were stored separately for each leaf in order to assess the egg and associated parasitism spatial distribution over the plant.

2.4.2 Parasitism behavior with artificially increased bagrada oviposition

The number of non-buried eggs obtained in the experimental system was very low in all replications. Hence, we could not clearly determine whether *G. gonikopalense* exhibited a preference for buried or non-buried eggs. In order to explore the possibility of choice, we therefore repeated experiment 2. by supplementing the host plant with twenty 2-d old eggs, distributed following the oviposition distribution of *B. hilaris* we described for mustard plants (see Table SM1, Supplementary material) or randomly placed on leaves (broccoli). All conditions were similar between the initial experiment and its repetition. Prior to start, eggs were therefore manually glued (UHU stic®, Germany) on leaves of host plants with a fine brush as follows: five eggs per leaf on broccoli; 1-5 eggs per leaf on mustard, mainly on the leaves in the upper third of the plant, randomly varying their distribution among replicates. Parasitized egg count, unhatched eggs and egg storage were treated as described in section 2.4.1.

2.5 Statistical analyses

In order to determine the host plant effect on *B. hilaris* oviposition, we compared the total number of eggs laid in the tritrophic system, the number of eggs laid in the sand and the number of non-buried eggs between the three host plant species with a Kruskal-Wallis test as data distribution could not be normalized with any transformations. Differences between groups were determined with a Nemenyi post-hoc test.

The effect of pest density, the host plant and their interaction on the oviposition distribution of *B. hilaris* were analyzed with a Global Linear Model with a quasi-Poisson link function, followed by a Tukey post-hoc test.

The egg and parasitism distribution over the plant height (mustard only) were described as percentages over the total number of eggs / parasitized eggs obtained on the plant with pooled replications. In experiment 2, the total number of parasitized eggs, the number of buried and non-buried parasitized eggs were compared between the three tested host plants (mustard, broccoli or white rocket) with a Kruskal-Wallis test as the data could not be normalized. In the repetition of experiment 2, the effect of host plant (mustard or broccoli), the experimental system (natural oviposition of *B. hilaris* or supplemented oviposition on host plants) and the interaction of both on *G. gonikopalense* parasitism distribution were analyzed with a Global Linear Model with a quasi-Poisson link function followed by a Tukey post-hoc test. All statistical analyses were carried out with Rstudio software (R Development Core Team, 2016) and figures were obtained with Excel software. For all tests, the alpha significance threshold was set at $\alpha = 0.05$. All means in the result section are given as mean ± $CI_{95\%}$.

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3 Results

3.1 Effect of host plant on *B. hilaris* oviposition distribution

Host plants did not influence the total number of eggs oviposited in the tritrophic system by bagrada (Kruskal-Wallis test: $\chi^2 = 1.27$, df = 2, p-value = 0.53), with 76.27 ± 8.18 eggs obtained with five copulae over all the replicates (see detail for each plant species on Fig. 1A). The number of eggs oviposited in the sand did not differ between the host plants (Kruskal-Wallis test: $\chi^2 = 1.77$, df = 2, p-value = 0.41; Fig. 1C), so that bagrada females oviposited 71.27 ± 7.97 eggs in the sand over all the replicates. Hence, the bagrada egg distribution did not differ between broccoli, white rocket and mustard (Kruskal-Wallis test: $\chi^2 = 3.84$, df = 2, p-value = 0.15), with a mean of 92.37 ± 2.35 % eggs oviposited in the sand (Fig. 1B).

Considering the host plants, we were not able to identify any egg distribution pattern on broccoli because each leaf is at equal distance from the ground or the plant apex. On white rocket, the quasi-absence of eggs on leaves or stems did not allow us to determine any pattern. However, we could assess the height distribution of bagrada oviposition on mustard as it grows vertically rather than horizontally like broccoli, reaching on average 34.88 ± 0.57 cm (see Table SM1, Supplementary material). Under laboratory conditions, *B. hilaris* oviposited higher numbers on the top third of the plant, with 88.5% of eggs above the 6th leaf (on average 25.02 ± 1.09 cm height, Table SM1, Supplementary material). No eggs were observed on the plant stems.

3.2 Effect of density and host plant on bagrada oviposition

Egg distribution in the tritrophic system was investigated with three levels of bagrada densities (D1, D5, and D15) in relation to host plant (broccoli and mustard). For a given density, there was no host plant effect on the total number of eggs oviposited, but egg number increased with density (p-value < 0.001; Table 1) with an average of 5.25 ± 1.11 eggs at D1, 72.81 ± 11.93 eggs at D5 and 152.6 ± 66.56 eggs at D15 (Fig. 2A). There was no effect of the pest density nor the host plant on the egg distribution in the system (Table 1). On average, *B. hilaris* laid 90.77 ± 3.07 % of its eggs in the sand for all replicates except for mustard at D1 (Fig. 2B).

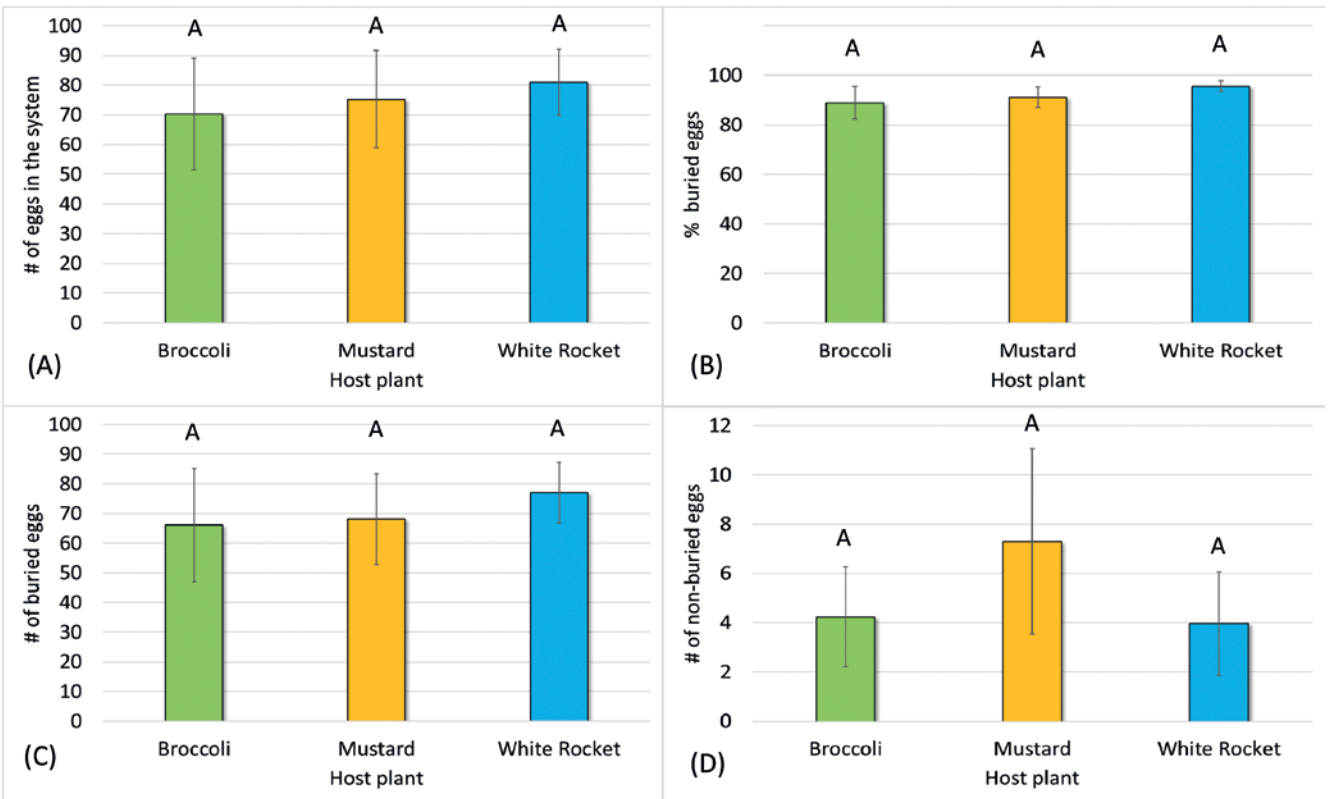


Fig. 1. Description of the oviposition distribution of five copulae of *Bagrada hilaris* in a controlled tri-trophic system involving broccoli (green bars, n = 21), mustard (yellow bars, n = 21) or white rocket (blue bars, n = 31); **(A):** total number of eggs in the system; **(B):** percentage of buried eggs; **(C):** total number of buried eggs oviposited; **(D):** total number of non-buried eggs oviposited. Vertical black bars are 95% confidence intervals and treatments with the same letter did not differ statistically (Tukey test, α = 0.05).

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Table 1. Effect of 1) the host plant, the bagrada density and their interaction and 2) the host plant, the egg supplementation and their interaction on the oviposition and the parasitism behaviour of *Bagrada hilaris* and *Gryon gonikopalense*, respectively under laboratory conditions. The term "supplementation" refers to artificially glued eggs on the host plant leaves. The significance of each factor on each variable (in bold) was tested with a Global Linear Model with a quasi-Poisson link function. Bold values indicate a significant effect of the corresponding explicative factor, with alpha significance threshold was set at α = 0.05.

	Variable and explicative factor	Test value	P-value
Bagrada hilaris	**Total number of eggs oviposited**		
	host plant	z = 1.41	0.24
	insect density	**z = 131.71**	**< 0.001**
	host plant × insect density	z = 0.34	0.71
	Total number of buried eggs		
	host plant	z = 1.31	0.26
	insect density	**z = 133.76**	**< 0.001**
	host plant × insect density	z = 0.0098	0.92
	Total number of non-buried eggs		
	host plant	z = 0.41	0.52
	insect density	**z = 10.78**	**< 0.001**
	host plant × density	**z = 7.36**	**0.001**
	Percentage of buried eggs		
	host plant	**z = 5.13**	**0.025**
	insect density	**z = 11.14**	**< 0.001**
	host plant × insect density	**z = 16.46**	**< 0.001**
Gryon gonikopalense	**Total number of parasitized eggs**		
	host plant	z = 0.17	0.68
	egg supplementation	z = 3.12	0.081
	host plant × egg supplementation	z = 0.36	0.56
	Number of buried eggs parasitized		
	host plant	z = 0.52	0.47
	egg supplementation	z = 0.17	0.68
	host plant × egg supplementation	z = 0.63	0.43
	Number of non-buried eggs parasitized		
	host plant	z = 0.59	0.44
	egg supplementation	**z = 21.16**	**< 0.001**
	host plant × egg supplementation	z = 0.035	0.85
	Percentage of parasitized eggs buried		
	host plant	z = 0.12	0.73
	egg supplementation	z = 4.39	0.04
	host plant × egg supplementation	z = 2.10	0.15

3.3 Effect of host plant on *G. gonikopalense* oviposition choice with natural oviposition of *B. hilaris*

Out of all the replications, *G. gonikopalense* females parasitized 46.03% of all available non-buried eggs (168 eggs over 365) and 30.4% of all available buried eggs (1583 over 5203). Hence, the parasitism response of *Gryon gonikopalense* was not influenced by the number of available bagrada eggs in the experimental system.

The total number of eggs parasitized by *G. gonikopalense* in the experimental system did not differ between host plants (Kruskal-Wallis test: χ^2 = 1,11, df = 2, p-value = 0.57) with on average 25.9 ± 7.86 eggs parasitized for broccoli, 26.47 ± 9.62 eggs parasitized for white rocket and 28.30 ± 5.71 eggs parasitized for mustard (Fig. 3A).

The number of buried parasitized eggs did not differ between broccoli (23.19 ± 8.09) mustard (23.00 ± 8.03) or white rocket (26.65 ± 5.71) (Kruskal-Wallis test: χ^2 = 2.02,

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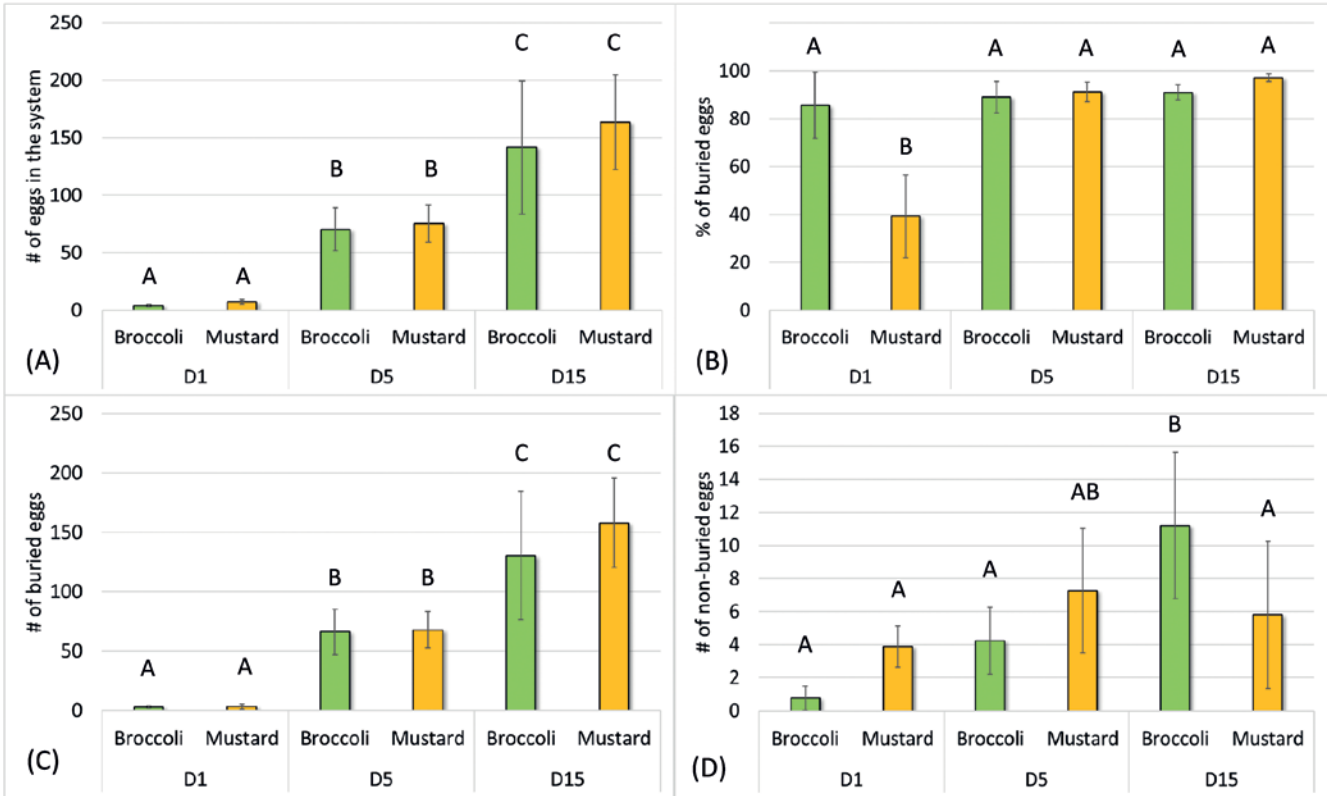


Fig. 2. Oviposition distribution of *Bagrada hilaris* in a controlled tri-trophic system involving broccoli (green bars) or mustard (yellow bars) at three bagrada densities: one copula (D1); five copulae (D5); 15 copulae (D15). **(A):** total number of eggs oviposited; **(B):** percentage of buried eggs; **(C):** total number of buried eggs oviposited; **(D):** total number of non-buried eggs oviposited. Vertical black bars are 95% confidence intervals. Categories with the same letter did not differ statistically (Tukey test, α = 0.05).

df = 2, p-value = 0.36; Fig. 3C). Likewise, no difference was observed for the number of non-buried eggs parasitized (Kruskal-Wallis test: $\chi^2 = 3.34$, df = 2, p-value = 0.19) with on average 2.71 ± 1.47 eggs parasitized on broccoli, 3.48 ± 2.42 on mustard and 1.65 ± 1.4 on white rocket.

Hence, considering pooled trials for broccoli, mustard and white rocket, we observed that 87.79 ± 5.12 % of the parasitized eggs were buried in the sand, and 12.21 ± 5.11 % were non-buried (Fig. 3B). As shown in section 3.1, there was no egg distribution pattern observed on broccoli and white rocket. However, for mustard, a greater proportion of parasitized eggs was recorded on the upper third of the plants where bagrada eggs are more numerous (see Table SM1, Supplementary material). *Gryon gonikopalense* was able to parasitize bagrada eggs located at the top of mustard plants

3.4 Effect of host plant on *G. gonikopalense* oviposition choice with supplemented oviposition of *B. hilaris*

As discussed in the Material and Methods section, the foraging preference of *G. gonikopalense* could not be properly evaluated since natural oviposition of *B. hilaris* occurs predominantly buried. With a supplementation of eggs on the host plant, non-buried eggs represented on average 20.32 ± 2.77 % of eggs in the system compared to 7.5% with natural oviposition (See 3.3).

As a result, the number of non-buried parasitized eggs increased (from 2.71 ± 1.47 to 9.43 ± 2.64; p-value = <0.001; Table 1; Fig. 4D), while the average number of buried parasitized eggs increased with supplemented eggs on broccoli from 23.19 ± 8.09 to 28.21 ± 8.62, (Tukey test, p-value = 0.013; Fig. 4C). Hence, over all replicates, 70.82 ± 7.09 % of the parasitized eggs were located in the sand, and thus 29.18 ± 7.09 % of parasitized eggs were found non-buried (Fig. 4B). On mustard replications, *G. gonikopalense* was able to reach all leaves that were supplemented with bagrada eggs, including the apical meristem. Like in presence of naturally oviposited eggs, the distribution of parasitized eggs matched with the artificial egg distribution (see Table SM1, Supplementary material).

4 Discussion

In this study, we focused on the oviposition behavior and the parasitism distribution at the host plant scale of *B. hilaris* and

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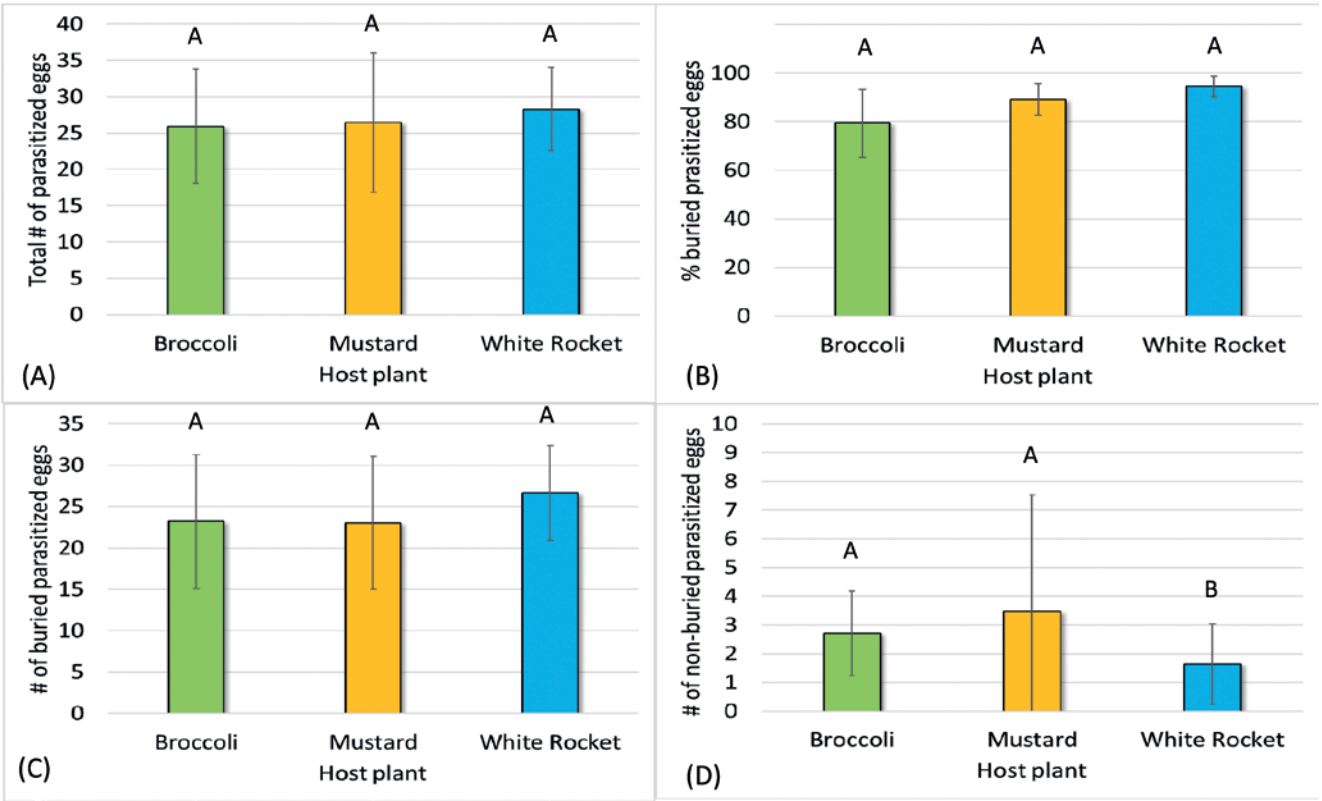


Fig. 3. Description of the parasitism distribution of five females *Gryon gonikopalense* in a controlled tri-trophic system involving broccoli (green bars, n = 21), mustard (yellow bars, n = 21) or white rocket (blue bars, n = 23); **(A):** total number of parasitized eggs; **(B):** percentage of buried parasitized eggs; **(C):** total number of buried parasitized eggs; **(D):** total number of non-buried parasitized eggs. Vertical black bars are 95% Confidence Intervals and treatments with the same letter did not differ statistically (Tukey test, α = 0.05).

G. gonikopalense, respectively. Although the unusual oviposition behavior of *B. hilaris* bagrada has been documented (Gunn 1918; Taylor et al. 2014; Bundy et al. 2019), our data describe quantitatively for the first time the pattern of this oviposition behavior. In our complex experimental system, 92.37% of bagrada eggs were deposited buried. Our result is consistent with both field observational reports (Gunn 1918; Taylor et al. 2014), and preliminary laboratory tests with 94.6% of eggs found buried in Petri dishes (Bundy et al. 2019).

We hypothesized that physical attributes of the plant could modify the oviposition choice of bagrada, as reported for *Empoasca fabae* L. (Hemiptera, Cicadellidae) (Robbins & Daugherty 1969). However, bagrada egg distribution did not differ among the plant species used despite their morphological differences. Regarding the egg distribution on the plant itself, there was no evidence for any spatial distribution pattern with broccoli and white rocket. However, eggs from mustard were mainly on the upper third of the plant (from 25 to 35 cm height) where leaves are younger. As bagrada prefers feeding on younger leaves (Reed et al. 2013), egg distribution over the plant could be attributed to individuals feeding on upper leaves, as usually observed in laboratory colonies (Martel G. & Sforza R.F.H., pers. obs.).

In our experimental system, bagrada density did not influence the oviposition pattern. From one couple (D1) to fifteen couples (D15), we only observed an increase in the total number of eggs oviposited whatever the host plant, and a slight increase of buried oviposition. However, a very low number of oviposited eggs was obtained with one couple. Because of the gregarious behavior of bagrada (Palumbo et al. 2016), it is likely that one single female was not stimulated enough for ovipositing in absence of congeners, though the percentage of buried eggs remained unchanged.

The bagrada behavior is in contrast to most herbivorous insects, including stinkbugs, that oviposit on the same plant they feed on (Martinez 2017; Kiritani et al. 1965; McPherson et al. 2018). How herbivorous insects select oviposition sites is mainly driven by cues and resources provided by their host plant that will maximize the fitness of progeny (Gripenberg et al. 2010; Desneux et al. 2012), according to the "preference performance" hypothesis (Jaenike 1978). However, polyphagous insects, like *B. hilaris* (Palumbo et al. 2016; Huang et al. 2014) undergo less pressure than specialists to find host plants and can prioritize factors (including predator avoidance) other than nutritional quality in selecting oviposition sites (Jaenike 1990; Gripenberg et al. 2010). In

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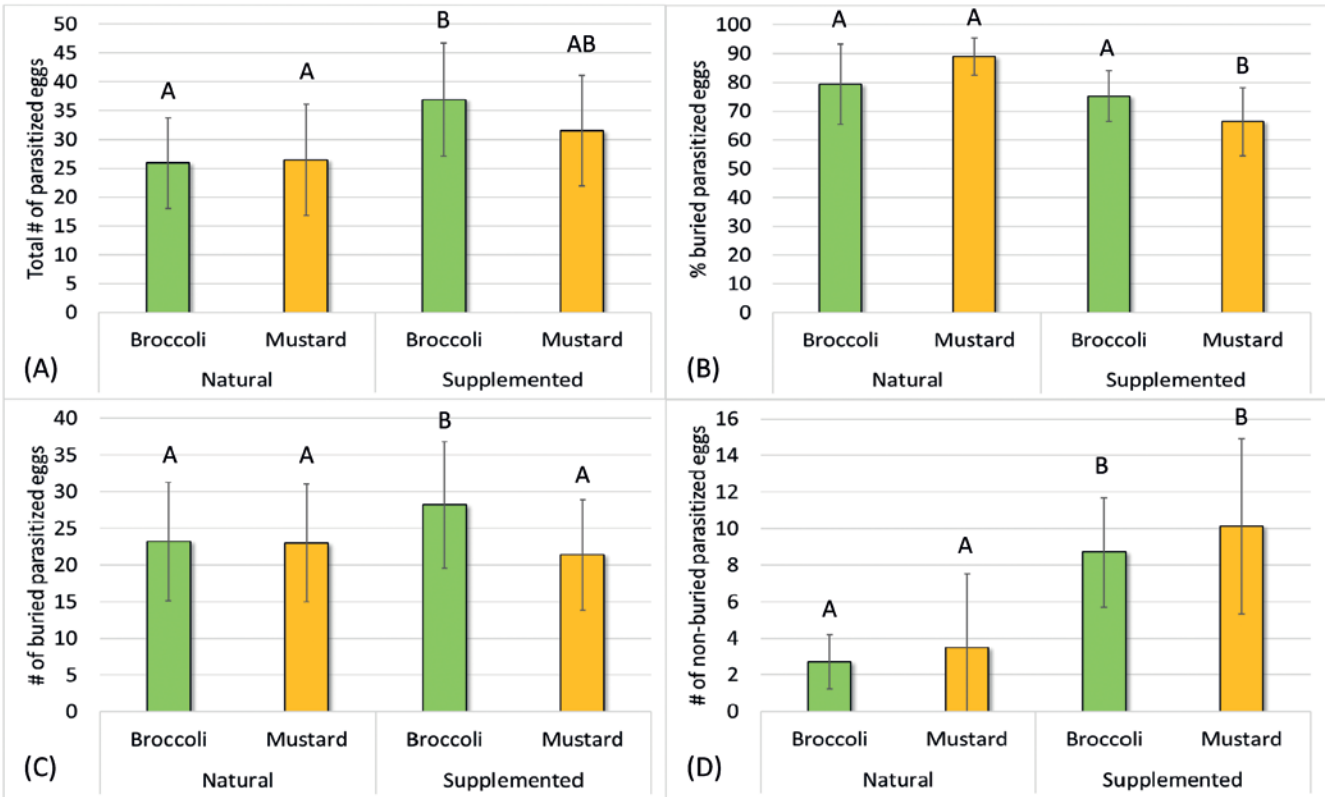


Fig. 4. Description of the parasitism distribution of *Gryon gonikopalense* in a controlled tri-trophic system involving broccoli (green bars) or mustard (yellow bars) and in presence of eggs naturally laid by B. hilaris (Natural) or supplemented with manually glued eggs on the host plant (Supplemented). **(A):** total number of parasitized eggs; **(B):** percentage of buried parasitized eggs; **(C):** total number of buried parasitized eggs; **(D):** total number of non-buried parasitized eggs. Vertical black bars are 95% confidence intervals treatments with the same letter did not differ statistically (Tukey test, α = 0.05).

the case of *B. hilaris*, our results lead to the hypothesis that the preference for buried oviposition results from evolution with generalist predators, and especially egg parasitoids. The selection of oviposition sites that are hardly accessible to egg parasitoids is a behavior shared by many herbivorous insects for parasitism and predation avoidance. For instance, *Lygus hesperus* Knight (Hemiptera, Miridae) prefers to oviposit eggs in strawberry fruits rather than in more accessible parts of the plant, so that the parasitoid *Anaphes iole* Girault (Hymenoptera, Mymaridae) cannot reach them easily (Udayagiri & Welter, 2000). In the case of bagrada, the efficiency of its oviposition strategy as a "refuge" against enemies has been recently highlighted with native egg parasitoids (Power et al. 2020; Tofangsazi et al. 2020). In addition, the innate expression of this oviposition behavior in independent populations worldwide (South Africa, Morocco, Kenya, Italy (Sforza R.F.H. & Martel G., pers. obs.), India, Pakistan (Gunn 1918; Samuel 1949), and the U.S. (Taylor et al. 2014; Bundy et al. 2019), is in favor of an old acquisition in the evolutionary lineage instead of multiple converging local adaptations. Hence, egg parasitoids that evolved specific behaviors in attacking buried bagrada eggs should constitute more valuable candidates for an importation biological control program against *B. hilaris* than parasitoids attacking unprotected pentatomid eggs.

Gryon gonikopalense is able to detect, reach and parasitize buried bagrada eggs (Martel & Sforza 2020; Tofangsazi et al. 2020). Furthermore, it is able to complete its development beneath the soil surface and emerge from buried eggs as well as from non-buried eggs (Martel & Sforza 2020). In the current study, *G. gonikopalense* females were able to parasitize both below and non-buried eggs in the tritrophic study design. More interestingly, females mostly parasitized buried eggs, without any differences between host plants. We originally hypothesized a possible host plant influence on the foraging ability of *G. gonikopalense*, due to morphological differences between broccoli (no trichomes, large and smooth leaves, bushy growth) and mustard (trichomes on stems and leaves, small leaves, vertical growth). Indeed, such physical barriers like trichomes or hairs can negatively affect parasitoids' foraging efficiency (Sütterlin & van Lenteren 1997; Romeis et al. 1998). Our results clearly indicated that the proportion of non-buried parasitized eggs on mustard was similar to that on broccoli (15.80% on average), suggesting that *G. gonikopalense* movements were not altered by the plant's physical characteristics. Instead, *G. goniko-*

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palense was able to reach bagrada eggs laid at different plant heights, up to 40 cm. This observation confirms the results of Tofangsazi et al. (2020) who showed that *G. gonikopalense* was able to attack hosts over a "neutral" 25 cm long wooden rod. An additional barrier of our laboratory study could have been the use of sifted sand that may have slightly biased the foraging ability of *G. gonikopalense*. However, fine sand is likely to be the hardest soil for the parasitoid, as bagrada eggs are much better buried and hidden. In a realistic soil, cracks or bigger particles are common, and eggs would be inappropriately buried, becoming more accessible to parasitism (and predation). As a result, *G. gonikopalense* was able to parasitize the same number of eggs in three different soil types from Californian broccoli fields (Tofangsazi et al. 2020), that all differed in particle size or clay content.

In our study, bagrada oviposited 92% of eggs buried. It was then difficult to assess whether *G. gonikopalense* showed a preference between buried and non-buried eggs. When we manually increased the number of non-buried eggs, the proportion of parasitized unburied eggs changed from 20.6% to 24.74% for broccoli and from 11.01% to 33.61% for mustard. Meanwhile, the number of buried parasitized eggs did not change between broccoli and mustard. This suggests that unprotected eggs could represent an attractive alternate host source compared to "hard-to-reach" buried eggs. Nevertheless, given that *B. hilaris* did not use the foliage as a major oviposition site, the possibility that *G. gonikopalense* would focus on non-buried eggs may be considered as an unrealistic situation and might result from an opportunistic behavior. However, it should not be ignored that other stinkbug species feeding on bagrada host plants may be hosts for *G. gonikopalense* and should therefore be evaluated as possible non-target species in the frame of an importation biocontrol program.

5 Conclusion

Our present work highlighted the protective oviposition behavior of bagrada against egg predators, especially egg parasitoids (Fig. 5). A clear understanding of both bagrada and *G. gonikopalense*'s biologies is a key factor for a realistic

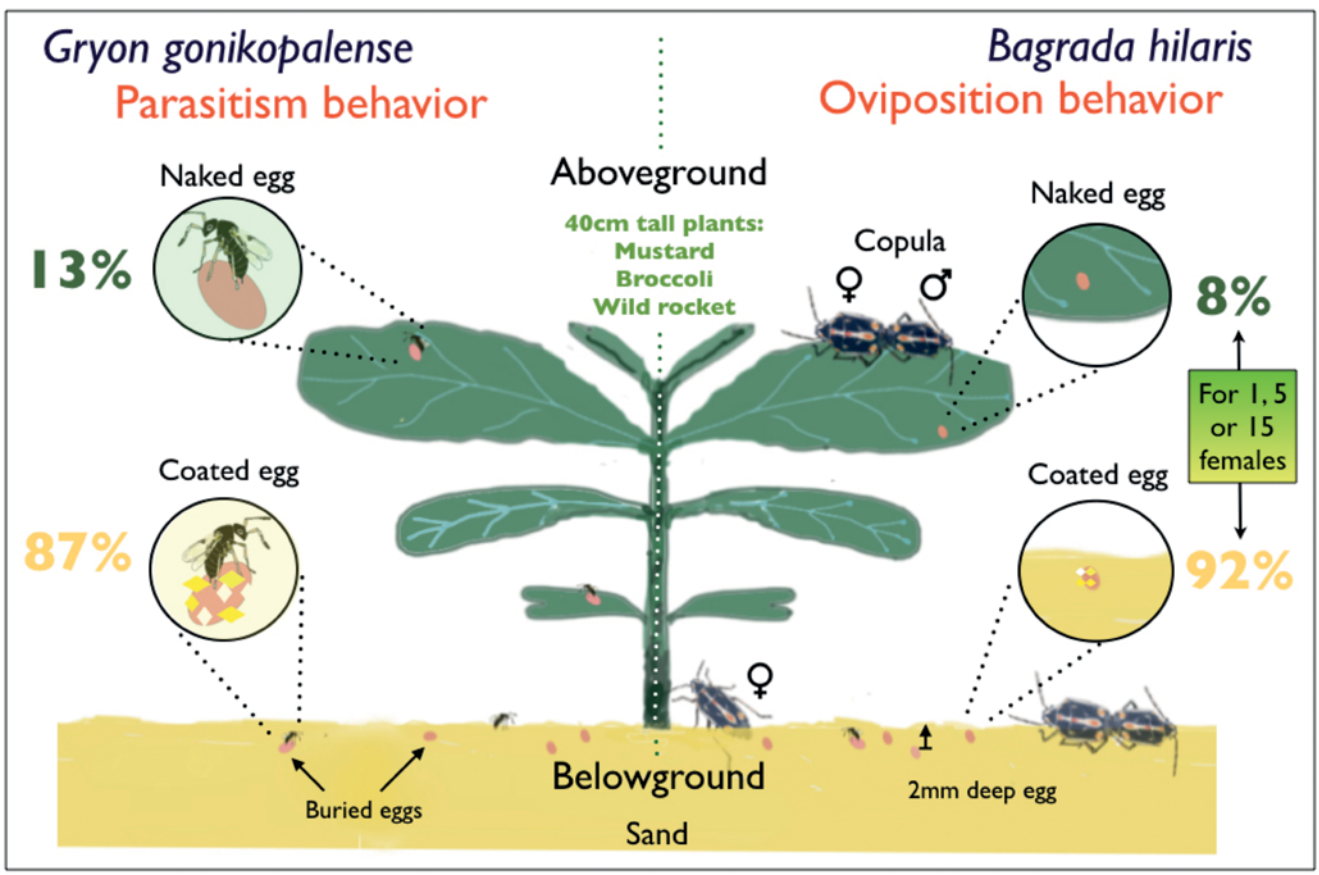


Fig. 5. Flow chart detailing the main data obtained in this study about the foraging behavior and egg parasitism of *Gryon gonikopalense* (Hymenoptera, Scelionidae) on three host plants in relation with the oviposition behavior of *Bagrada hilaris* (Hemiptera, Pentatomidae) with three different bagrada densities.

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assessment of parasitoid searching efficiency in controlling bagrada in the field. Beyond the frame of a biological control program, this should help to optimize other pest management methods like inter-cropping or push-pull strategies currently studied in various agrosystems (Sharma et al. 2020; Arif et al. 2020). For instance, the clarification of oviposition preferences of *Phthorimaea operculella* Zeller (Lepidoptera, Gelechiidae) facilitated a better local application of pesticides to control this tobacco pest (van Vuuren et al. 1998). Since bagrada oviposits mainly in the soil, one can argue that in the U.S., this pest should not be too much impacted from native stinkbug egg parasitoids attacking unprotected host eggs. On the opposite, the relevance of using *G. gonikopalense* as a biocontrol agent against bagrada is well supported by its specific foraging ability (Fig. 5).

However, the particular behavior of this promising parasitoid raises several questions since the benefits for *G. gonikopalense* to develop underneath the soil surface are still unknown. Few hypotheses can be addressed: 1) soil may limit egg predation by generalist arthropods (Morrison et al. 2016; Abram et al. 2015; Jones 2013), 2) The parasitism of buried bagrada eggs may limit intra-specific competition between *Gryon gonikopalense* females, and overall inter-specific competition with parasitoids without the same foraging abilities (Ganjisaffar et al. 2018; Felipe-Victoriano et al. 2019; Lomeli-Flores et al. 2019) and 3) buried eggs might be better protected from varying climatic conditions.

As foraging behavior of *Gryon gonikopalense* is now better understood, further investigations should now be devoted to its dispersal capacity, as they both constitute important components of the biocontrol candidates' efficiency (Wright et al. 2001; Canto-Silva et al. 2006). Finally, prior to consider *Gryon gonikopalense* for release, its host range and the possible antagonistic or synergistic interactions with other egg parasitoids need to be specified.

Authors' contributions: Conceptualization: [GM, RFHS]; Methodology: [GM, FS]; Formal analysis and investigation: [GM, FS]; Writing – original draft preparation: [GM, FS]; Writing – review and editing: [GM, RFHS]; Funding acquisition: [RFHS]; Resources: [RFHS]; Supervision: [RFHS.]. All authors read and approved the final manuscript.

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Supplementary Material (SM)

Table SM1. Average height of mustard leaves where eggs of *Bagrada hilaris* were naturally oviposited or supplemented with manually deposited eggs Leaf 1 corresponds to the first leaf after cotyledons. For each leaf, the percentage of eggs oviposited by bagrada and the percentage of eggs parasitized by *Gryon gonikopalense* is provided in both cases of "natural oviposition" and "supplemented oviposition".

Plant part	Average height (cm) ± CI 95%	Natural oviposition		Supplemented oviposition	
		% eggs oviposited	% eggs parasitized	% eggs oviposited	% eggs parasitized
Mustard Leaf 1	5.67 ± 0.46	0.74	2.63	0.35	0.98
Mustard Leaf 2	7.8 ± 0.96	1.47	0.00	0.00	0.00
Mustard Leaf 3	11.54 ± 0.99	2.94	2.63	5.23	2.94
Mustard Leaf 4	17.99 ± 1.45	0.74	0.00	6.97	9.80
Mustard Leaf 5	21.7 ± 1.39	2.94	0.00	9.41	10.78
Mustard Leaf 6	25.02 ± 1.2	12.50	10.53	10.80	7.84
Mustard Leaf 7	27.7 ± 1.02	9.56	28.95	10.80	3.92
Mustard Leaf 8	30.15 ± 1.03	13.97	13.16	9.41	11.76
Mustard Leaf 9	32.27 ± 1.34	22.79	18.42	17.77	17.65
Mustard Leaf 10	33.71 ± 0.82	19.12	2.63	10.80	19.61
Mustard Apex	34.88 ± 0.76	13.24	21.05	18.47	14.71



Fig. SM1. Photograph of the system in place in the quarantine greenhouse, containing broccoli (left tube) and mustard plant (right tube). Photo credit: Martel G.

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Chapitre 4.

Vers une optimisation de la production de *Gryon gonikopalense* en laboratoire

Article 5 : **Martel, G.,** & Sforza, R.F.H.: Evaluation of three cold storage methods of *Bagrada hilaris* (Hemiptera: Pentatomidae) and the effects of host deprivation for an optimized rearing of the biocontrol candidate *Gryon gonikopalense* (Hymenoptera: Scelionidae). *Biological Control (in press)*

Nous avons vu en introduction qu'un auxiliaire efficace mais impossible à élever en masse au laboratoire pouvait être un frein important à un programme de LBC. Or, la production d'un parasitoïde dépend de celle de son hôte. Pour que celle-ci soit assurée, le stockage des œufs de l'hôte à de basses températures est reconnue comme une méthode efficace. Cela permet en effet de pallier les variations de production et de contrôler le moment de leur utilisation pour l'élevage du parasitoïde. Cependant, des méthodes identiques peuvent conduire à des réponses très variables en fonction de l'espèce de parasitoïde considérée. Les individus se développant dans des œufs stockés au froid peuvent notamment subir des effets létaux (absence de développement) ou sub-létaux comme un allongement du temps de développement, ou bien une diminution de la longévité et de la fécondité. Une standardisation est donc difficile à mettre en place. Dans ce quatrième chapitre, nous avons testé trois températures de stockage des œufs de bagrada (-20°C, 5°C, 13°C) et six durées (d'une semaine à trois mois) pour améliorer et optimiser la production de *G. gonikopalense* en laboratoire. En parallèle, nous avons déterminé si *G. gonikopalense* pouvait subir une privation d'hôte de longue durée et être capable de produire des descendants sains lorsque bagrada était à nouveau disponible (dans le cas où l'élevage de bagrada accuserait d'importantes baisses de production).

Le principal résultat de ce chapitre est que les œufs de bagrada conservés jusqu'à 1 mois à 5°C sont parasités normalement par *G. gonikopalense,* sans effet négatif sur sa descendance. En revanche, les œufs congelés à -20°C et les œufs stockés à 13°C (sous le seuil de développement de bagrada =15°C) sont refusés par le parasitoïde dans la plupart des cas, bien que les individus s'y développent jusqu'à l'adulte, puis émergent. Par ailleurs, les œufs congelés sont parasités de la même manière quelle que soit la durée de stockage.

De ces résultats découlent trois principales avancées : 1) la quantité de parasitoïdes et le moment de leur production peuvent être contrôlés en stockant les œufs de bagrada et en les utilisant au moment opportun. Cette méthode permet de ne pas gâcher une surproduction temporaire, tout en compensant les moments de sous-production, bien que le stockage des œufs ne soit efficace que quelques semaines. 2) Deux principaux leviers permettraient de maintenir la population de *G. gonikopalense* en cas de pénurie de bagrada : le parasitoïde peut subir une privation d'hôte mais rester capable de produire des descendants permettant de maintenir un élevage, tandis que des stocks d'œufs congelés permettraient de maintenir une activité de reproduction, même minime. 3) Le faible taux de parasitisme des œufs congelés nous alerte sur la pertinence de les utiliser comme œufs sentinelles pour collecter des parasitoïdes natifs ou *G. gonikopalense* lui-même. En effet, les espèces refusant de parasiter des œufs congelés pourraient entraîner un biais d'échantillonnage.

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Evaluation of three cold storage methods of *Bagrada hilaris* (Hemiptera: Pentatomidae) and the effects of host deprivation for an optimized rearing of the biocontrol candidate *Gryon gonikopalense* (Hymenoptera: Scelionidae)

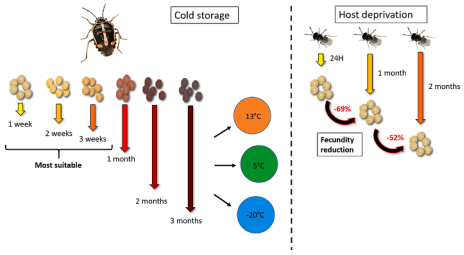
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HIGHLIGHTS

- Host stockpiling for parasitoid production is a challenge in a biocontrol program.
- *G. gonikopalense* rearing can be optimized with bagrada egg storage for biocontrol programs.
- Parasitism was seven and nine times higher with eggs stored at 5 °C than at 13 and −20 °C respectively.
- Egg storage temperature influenced the progeny's emergence rate but not longevity or body length.
- Host deprivation allowed parasitism after 60 days but fecundity was reduced by 84%.

GRAPHICAL ABSTRACT



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ABSTRACT

The cold storage of host eggs has been utilized in the production of egg parasitoids used in biological control programs. However, similar stockpiling methods can lead to various responses depending on the parasitoid species considered. Hence, standardization of storage methods is difficult. The egg parasitoid *Gryon gonikopalense* Sharma, is currently being evaluated as a biocontrol agent of *Bagrada hilaris* (Burmeister), an invasive pest on Brassicaceae crops in the Americas. In this study, we investigated cold storage methods and the effect of host deprivation on *G. gonikopalense* in order to optimize its rearing process. Laboratory tests were conducted with bagrada bug eggs stored under three temperature treatments (-20 °C, 5 °C, 13 °C) and six durations (one week to three months). In addition, we assessed the effect of host deprivation on parasitoid fecundity and progeny development. Bagrada bug eggs stored at 5 °C up to one month allowed three and nine times more parasitism than eggs stored at 13 and -20 °C respectively. Progeny emergence rate was also the best with eggs stored at 5 °C (94.1%) while eggs stored at 13 and -20 °C led to 77.7 and 89.2 % of emergence respectively. However, the different storage treatments led to similar longevities and body lengths for male and female progeny. In addition, *G. gonikopalense* did parasitize bagrada eggs after 60 days of host deprivation, but fecundity was reduced by 84%.

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The results can be used to optimize bagrada bug egg production for use in laboratory and / or field experiments and improve *G. gonikopalense* rearing and production.

1. Introduction

The on-demand production of parasitoid biocontrol agents and their hosts for research or field releases is a challenge to overcome in many biocontrol programs. Extending the parasitoid reproduction period or host suitable life stages as long as possible is therefore an important issue. For this latter purpose, various cold storage methods are generally used to preserve the living material. Depending on the life stage considered, the range of temperature used can vary from freezing (-20 °C or -80 °C for instance) (Kivan and Kilic, 2005; Egwuatu and Ajibola Taylor, 1977) to only refrigeration (between 2 and 10 °C) (Alim and Lim, 2009; Sabbatini Peverieri et al., 2015). The cold storage duration can vary from short time periods (*i.e.* several days) to much longer time intervals (*i.e.* weeks, months) (Kivan and Kilic 2005; Spínola-Filho et al. 2014; McIntosh et al. 2019; Wong et al. 2021).

The effectiveness of cold storage techniques in extending the availability period of host material has often proved successful (Orr et al., 1986; Goldson et al. 1993; Rathee and Ram 2018). However, cold storage conditions and their efficiency are not always consistent for different species, so that each case requires a specific investigation. In addition, there are important limitations to consider as cold storage may have detrimental effects on the quality of the host, its recognition by its parasitoids (Alim and Lim, 2009) and consequently, on the quantity or quality of parasitoid produced (Egwuatu and Ajibola Taylor, 1977; Kivan and Kilic, 2005; Alim and Lim, 2009; Colinet and Boivin, 2011; Sabbatini Peverieri et al., 2015; Martel et al. 2019). In addition to host acceptance by the parasitoid, lethal or sublethal effects can affect progeny developing inside previously cold-stored eggs. This can be caused by the reduction of host quality with storage temperature or duration (Lim and Mahmoud, 2009; Mainali and Lim, 2012; McIntosh et al. 2019). While lethal effects directly kill immature parasitoids, sublethal effects of storage can result in negative changes in their physiology, morphology or behaviour (Lim and Mahmoud, 2009; Mainali and Lim, 2012; McIntosh et al. 2019).

The bagrada bug, *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae) (=bagrada) is an invasive pest in the Americas that spread from California to adjacent US states, Mexico and Chile between 2008 and 2016 (Reed et al. 2013; Sánchez-Peña 2014; Palumbo et al. 2016; Faúndez et al., 2018). It mainly damages Brassicaceae cropping systems like broccoli, cabbage or mustard, (Azim and Shafee, 1986; Reed et al. 2013; Palumbo et al. 2016). The egg parasitoid *Gryon gonikopalense* (Sharma) (Hymenoptera: Scelionidae), native to Pakistan (Mahmood et al. 2015), is currently being evaluated as a biocontrol agent of bagrada (Martel et al. 2019; Martel and Sforza, 2021).

Research studies on physiology, behavior and specificity of *G. gonikopalense* are just beginning, but are promising. Also, a part of the research program remains devoted to the discovery of new biocontrol candidates in the native range of bagrada (Conti et al., 2021). An efficient storage method of bagrada eggs for parasitism by *G. gonikopalense* would be useful for field surveys using sentinel eggs to detect and collect parasitoids, and to estimate their diversity and abundance. Obviously, laboratory colony maintenance and mass production for field releases and experiments would be strongly enhanced by continuously available bagrada eggs. Martel et al. (2019) showed that deep-freezing bagrada eggs at -80 °C was not a suitable method for *G. gonikopalense* rearing, and would therefore be ineffective for production of sentinel eggs as well. Bagrada eggs were shown to be exploited by *G. gonikopalense* for up to four days at 22 °C (Martel et al. 2019), but this is far from sufficient to facilitate mass production. In this study, we compared the effectiveness of several combinations of storage temperature and duration regarding the parasitism success by *G. gonikopalense,* and the fitness of its offspring.

The main objectives of the present study were to 1) determine the effect of different combinations of storage durations and temperatures on bagrada egg viability; 2) determine the effect of these treatments on *G. gonikopalense* parasitism and 3) evaluate consequences on offspring. In addition, we assessed 4) consequences of host deprivation on *G. gonikopalense* fecundity and 5) sublethal consequences on offspring.

2. Material and Methods

2.1. Insect rearing

The bagrada colony was derived from a colony maintained at the University of California Riverside and shipped to EBCL on March 2016. This colony was first collected at Coachella Valley, CA, USA (GPS coordinates: 33°34′40.71″ N, 116°08′45.78″W). The bagrada colony used in this study was maintained in the quarantine glasshouse of the European Biological Control Laboratory-EBCL (USDA-ARS) at Montferrier-sur-Lez (France) under the official agreement # R76-2019–162, issued by the French Ministry of Agriculture. The colony was contained in BugDORM® cages (47.5 × 47.5 × 47.5 cm, 150 μm opening mesh, MegaView Science®, Taiwan) under 25 °C (night) and *ca.* 30 °C (day), at an ambient photoperiod supplemented with sodium lamps (L/D 12:12), and RH: 30 ± 15%. Bagrada adults and nymphs were fed weekly with a selection of one-month-old Brassicaceae plants including *Brassica oleracea* var. *italica* Plenck (broccoli), *Sinapis alba* L. (white mustard) and *Diplotaxis erucoides* De Candolle (white rocket). Broccoli and mustard were grown from seeds in a separate greenhouse of the quarantine (22 °C, RH: 30 ± 10%) and the white rocket was field collected twice a week in the area surrounding the laboratory.

In the rearing cages, Petri dishes (Ø = 9 cm) filled with sand (DIALL®, yellow sand, maximum diameter 200 μm, Spain) were used as oviposition arenas for bagrada (Martel and Sforza, 2021). In Petri dishes, eggs were collected every two days with a fine brush after sifting sand (Hogentogler®, mesh size = 425 μm, USA). Eggs were glued adjacent to each other (UHU stic®, Germany) on cardboard pieces (Martel et al. 2019) and used either for parasitoid rearing or subsequent experiments (see section 2).

The *G. gonikopalense* colony originated from Pakistan (Mahmood et al. 2015), and was maintained at EBCL in insect-proof transparent plastic boxes (18 × 12 × 5.5 cm), in the same quarantine greenhouse, as described above. Aeration for boxes was provided through holes in the lid (Ø = 50 mm), covered with a nylon mesh fabric (160 μm aperture). Rearing boxes were stored in a rearing chamber (22 ± 0.5 °C, RH 50 ± 5 %, L/D 12:12) and drops of diluted acacia honey (Lune de miel®, France, 50% dilution) were deposited on the mesh *ad libitum* for nourishment. The parasitoid colony was maintained by offering fresh bagrada eggs to female parasitoids once per week.

2.2. Effect of temperature and duration of bagrada eggs storage on parasitism

Bagrada eggs < 48 hold were collected from the main rearing colony and randomly assigned to three storage temperature treatments: 13 °C (incubator, Percival Scientific, model E-30B, USA), 5 °C (fridge Serie 600 FLEX Electrolux®, Sweden), and -20 °C (freezer Comfort StopFrost, Liebherr®, Germany). Relative humidity was 50% for the 13 °C and 5 °C treatments, but was not measured for the -20 °C. For each storage temperature, eggs were stored in plastic boxes and removed after 1 week (S1), 2 weeks (S2), 3 weeks (S3), 1 month (M1), 2 months (M2), 3 months (M3) (except for 13 °C) and 4 months (M4) (only for 5 °C) then exposed to *G. gonikopalense*.

For each combination of storage temperature and duration, eggs were glued on cardboards (UHU stic®, Germany) to constitute 10-egg clusters (n = 40 replications for each treatment). Each cardboard was then individually exposed to one 24 h-old mated female parasitoid for one hour in a Petri dish under rearing chamber conditions (22 ± 0.5 °C, RH 50 ± 5 %, L/D 12:12). Eggs stored at -20 °C were allowed to warm for 20 min at 22 °C before exposure to the parasitoid. After one hour, female parasitoids were removed from the Petri dish and eggs were maintained in the same laboratory conditions until all parasitized eggs emerge. Simultaneously, and for each treatment, 20-egg clusters (n = 20 replications) were warmed up at 22 °C without being exposed to parasitoid to assess the emergence rate of bagrada. For each treatment, we counted the number of parasitized eggs and emerging adult parasitoids, and the development time of each of them.

2.3. Effects of temperature and duration of bagrada eggs storage on G. gonikopalense progeny

For each treatment, the total development time (from parasitism day to adult emergence) was measured for males and females that emerged from each treatment of the previous experiment. Parasitoids were then separated individually into clean Petri dishes for each treatment and provided with diluted acacia honey drops (Lune de miel®, France, 50% dilution) in order to measure their longevity. The status of parasitoids (dead or alive) was observed daily until all individuals in the cohort had died.

Post mortem body length, from the end of the abdomen to the end of the scutellum, was measured for each individual (Fig. 1). Each parasitoid was photographed under a camera (NIKON® D3500, NIKON CORP., Japan) mounted on a binocular microscope (Stemi 508, Zeiss®, Germany, magnification × 50). body length was measured using Image J® software (Schneider et al. 2012).

2.4. Effect of host deprivation duration on parasitism capacity of Gryon gonikopalense.

Newly emerged male and female parasitoids from the laboratory colony were stored together under the rearing chamber conditions (22 ± 0.5 °C, RH 50 ± 5 %, L/D 12:12) and fed with diluted honey drops. After 24hrs, 30 and 60 days, females were randomly selected and isolated without males in individual Petri dishes (24hrs: n = 29; 30 days: n = 38; 60 days: n = 13). Each female was daily provided fresh bagrada eggs (<48hrs old) *ad libitum* and diluted acacia honey drops until its death (Martel et al. 2019). Eggs of each day of oviposition were stored in separate Petri dish under the rearing chamber conditions until progeny emergence.

The number of parasitized eggs and number of male and female progeny emerged were enumerated. All eggs that did not hatch were dissected under a binocular microscope (Stemi 508, Zeiss®, Germany) to verify whether an identifiable nymph or adult parasitoid was present. Eggs with unidentifiable content were considered not parasitized. Female parasitoids that failed to parasitize any egg during their lifetime or produced only males (*i.e.* unfertilized) were excluded from statistical analyses.

For each of the three host deprivation treatments (24 h, 30 and 60 days) total development time (from parasitism day to adult emergence) of the male and female progeny obtained were determined. Adult parasitoids obtained were separated individually into clean Petri dishes and provided drops of diluted acacia honey *ad libitum* for nourishment. Each individual was stored under the rearing chamber conditions previously described and checked daily until the death of all individuals in the cohort.

2.5. Statistical analysis

The effect of storage duration, the storage temperature and their interaction on the number of eggs parasitized by *G. gonikopalense* were analyzed with a Global Linear Model with a quasi-Poisson link function, followed by a Tukey post-hoc test. The parasitoid development time, larval survivorship and progeny size were compared between each combination of storage duration and storage temperature using a two-way ANOVA followed by a Tukey post-hoc test. The effect of storage temperature, duration and progeny sex on the longevity of progeny was tested with a Cox proportional hazards survival model with extrapolated censored data. Tukey post hoc test was performed to identify differences between each treatment.

The parasitoid fecundity was compared between each host-deprivation treatment with a one-way ANOVA followed by a Tukey post-hoc test.

The parasitoid development time was compared between each host-deprivation treatment and sex with a two-way ANOVA followed by a Tukey post-hoc test. Larval survivorship was compared between each host-deprivation treatment with a one-way ANOVA followed by a Tukey post-hoc test. The effect of host deprivation duration and progeny sex was tested with a Cox proportional hazards survival model. Tukey post hoc test was performed to identify differences between each treatment.

All tests were performed with R software (R Development Core Team, 2016). For all tests, the alpha significance threshold was set at $\alpha = 0.05$. All results are presented as a mean ± $CI_{95\%}$.

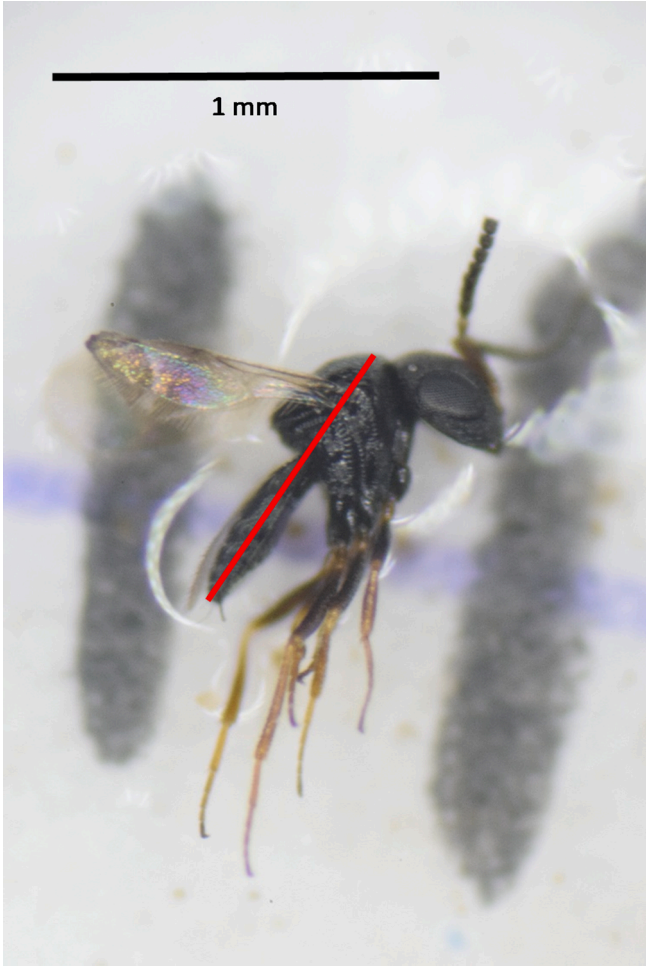


Fig. 1. Picture of a male adult *Gryon gonikopalense* (magnification × 50). The red line corresponds to the body length measured for each individual. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Effect of temperature and duration of bagrada eggs storage on parasitism

The number of bagrada eggs parasitized by *G. gonikopalense* was affected by the storage temperature and duration (Table 1). More eggs were parasitized when they were stored at 5 °C from 1 to 3 weeks (7.4 ± 0.8 parasitized eggs, pooled data) compared to the -20 °C treatment (0.8 ± 0.2 parasitized eggs, pooled data for all durations) or the 13 °C storage (2.5 ± 0.4 parasitized eggs, pooled data) (Fig. 2; Tukey test, $P < 0.05$). For a given storage temperature, the number of parasitized eggs decreased with storage duration (Fig. 2, Table 1; Tukey test < 0.05), except when eggs were placed at -20 °C. Hence, *G. gonikopalense* only parasitized 3.7 ± 2.1, 0.55 ± 0.45 and 0.25 ± 0.22 eggs when they were stored at 5 °C for one, two and three months respectively (Tukey test, $P < 0.05$; Fig. 2). Trials with eggs stored for four months did not result in any parasitism.

Between one week and one month of storage at 13 °C, there was no difference in the number of eggs parasitized by *G. gonikopalense* (Tukey test, $P > 0.05$). Meanwhile, only two eggs were parasitized after two months of storage over all trials (400 eggs tested).

The emergence of bagrada was affected by both egg storage temperature and duration (Table 1). No bagrada nymphs emerged from eggs stored at -20 °C, whatever the storage duration (Fig. 3). Bagrada could emerge from eggs stored at 5 °C up to two months, but the percentage of emergence decreased with storage duration (34.9 ± 6.4%, 28.9 ± 5.0%, 5.5 ± 3.6% and 0.33 ± 0.7 % after one (S1), two (S2), three weeks (S3) and two months (M2) respectively, (Tukey test, $P < 0.05$; Fig. 3). No bagrada nymphs emerged from eggs stored one month at 5 °C.

From eggs stored at 13 °C, the percentage of bagrada emergence was the highest after one week (50.7 ± 5.5%) but dropped to 1.1 ± 0.9% after two weeks, and only 0.2 ± 0.4 after both three weeks and two months (Tukey test, $P < 0.05$; Fig. 3). No nymphs emerged from eggs stored one month at 13 °C.

3.2. Sublethal effects of temperature and duration of bagrada eggs storage on G. gonikopalense progeny

For a given temperature, the storage duration had no effect on the percentage of emergence of *G. gonikopalense* (Table 2). Hence, 89.2 ± 8.0 % of parasitoids emerged from eggs stored at -20 °C, while 94.1 ± 4.0 % and 77.7 ± 6.1 % of them emerged from eggs stored at 5 °C and 13 °C respectively (Fig. 4). However, several combinations of storage temperature and duration only provided few individuals, according to parasitism levels presented in previous section (Fig. 2).

The development time of *G. gonikopalense* was affected by both egg storage temperature and duration (Tukey test, $P < 0.05$, Table 2). The longest development was measured when *G. gonikopalense* developed inside eggs stored at 5 °C for two months (28.7 ± 5.2 days, Fig. 5), while the shortest development was measured when *G. gonikopalense* developed inside eggs stored at 5 °C for one to three weeks (22.7 ± 0.2 days, pooled treatments, Fig. 5).

Females had better survival chances than males over time (Cox proportional hazards model, Wald $\chi^2 = 27$, df = 1, $P < 0.001$; Fig. 6). The temperature of egg storage also influenced the survival probability of adult parasitoids (Cox proportional hazard model, Wald $\chi^2 = 30$, df = 2, $P < 0.001$; Figs. 6, S1) but not the storage duration (Cox proportional hazard model, Wald $\chi^2 = 1.3$, df = 5, $P = 0.25$). Hence, parasitoids that developed inside bagrada eggs stored at -20 °C had the highest longevity: females lived on average 116.7 ± 7.1 days and males 91.5 ± 10.6 days (Fig. 6). Parasitoids that developed inside bagrada eggs stored at 5 °C lived between 100.5 ± 8.8 days and 77.6 ± 33.2 days when eggs were stored for one month (M1) and three months (M3) respectively. (Fig. 6). Parasitoids that developed inside eggs stored at 13 °C lived on average 49.4 ± 6.3 days (pooled treatments) but this represents incomplete data: nearly 80% of all parasitoids that developed inside eggs stored at 13 °C underwent a severe unintended heatwave during the course of the experiment and died, which is detailed in the discussion section.

There was no effect of storage temperature or storage duration on progeny body length, (Tukey test, $P > 0.05$; Table 2). Only parasitoids from eggs stored one month at 5 °C were smaller from other treatments (Tukey test, $P = 0.04$, Fig. 7). However, there was no difference of body length when parasitoids developed inside eggs stored at 5 °C for two or three months, compared to all other treatments (Tukey test, $P > 0.05$, Fig. 7).

3.3. Effect of host deprivation duration on the parasitism capacity of G. gonikopalense

Mated females *G. gonikopalense* were able to parasitize fresh bagrada eggs after 60 days of host deprivation. The recorded fecundity declined as the host-deprivation duration increased (ANOVA test, $F_{2,77} = 52.1$, $P < 0.001$) with only 23.66 ± 4.39 progeny produced after 30 days and 12.23 ± 6.19 progeny after 60 days (Table 3). The highest fecundity (77.4 ± 13.41 progeny) was recorded with females that were host-deprived for only 24 h.

3.4. Sublethal effects of host deprivation duration of female G. Gonikopalense on their offspring

The emergence rate of progeny did not differ among treatments, and 93.7 ± 2.1% of adult progeny emerged from bagrada eggs (ANOVA test, $F_{2,77} = 2.52$, $P = 0.087$; Table 3). There was no effect of female host deprivation on the sex ratio of their progeny (ANOVA test, $F_{2,77} = 1.23$, df = 2, $P = 0.29$). Over all treatments, females produced 69.1 ± 5.35 % female progeny (Table 3). The sex of progeny (two-way ANOVA, $F_{1, 609} = 96.2$, $P < 0,0001$) and the host deprivation duration (two-way ANOVA, $F_{2, 609} = 37.0$, $P < 0,0001$) both had an effect on their development. Males always developed faster than females, regardless of the host deprivation treatment, Tukey test, $P < 0.05$, Table 3). No difference was measured in male development time regarding the host deprivation treatment (Tukey test, $P > 0.05$, Table 3). However, females from 60-days host-deprived mother emerged on average 1.4 days before females from 30-days host-deprived mother (Tukey test, $P < 0.05$, Table 3). The progeny longevity was also affected by their mothers' host-deprivation (Cox proportional hazard model, Wald $\chi2 = 40$, df = 2, $P < 0.001$), but there was no difference between male and female parasitoids for a given treatment (Cox proportional hazard model, Wald $\chi^2 = 3.1$, df = 1, $P = 0.077$). Considering pooled males and females, progeny lived shorter (74.08 ± 5.9 days) when their mother was host-deprived for 60 days compared to 30 days and 24hrs host-deprivation treatments (respectively 88.02 ± 2.6 and 90.74 ± 6.3 days; Table 3, Fig. S2).

Table 1
Effects of *Bagrada hilaris* egg storage temperature, duration and their interaction on the number of eggs parasitized by *Gryon gonikopalense* and the emergence rate of bagrada nymphs. Values in bold indicate a significant effect of the corresponding parameter for a given variable. Effects on variable (a) were measured with a Global linear model with a quasi-Poisson link function. Effects on variable (b) were measured with a two-way ANOVA. Significance α threshold was set at 0.05.

Explicative parameters	Df	Test value	P-value
Number of eggs parasitized (a)			
temperature	2	$F_{2, 451} = 56.5$	**<0.0001**
duration	6	$F_{6, 451} = 37.1$	**<0.0001**
temperature X duration	9	$F_{9, 451} = 18.9$	**<0.0001**
Bagrada emergence rate (b)			
temperature	2	$F_{2, 342} = 10.56$	**<0.0001**
duration	6	$F_{6, 342} = 75.33$	**<0.0001**
temperature X duration	9	$F_{9,342} = 31.63$	**<0.0001**

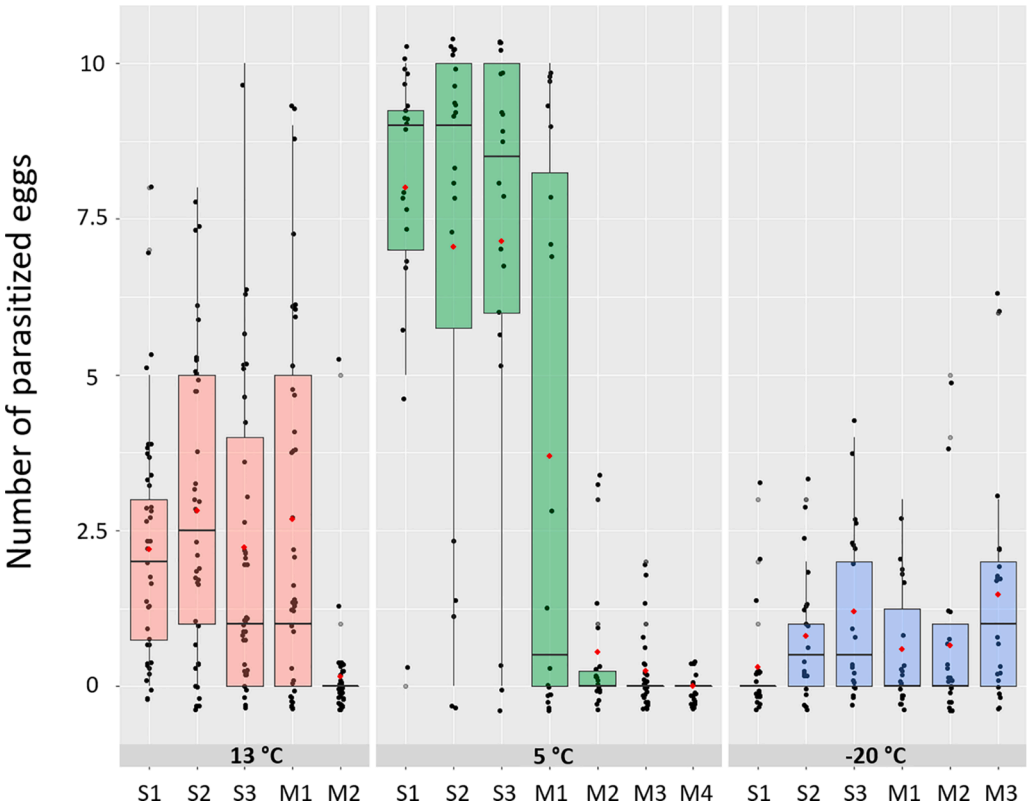


Fig. 2. Number of bagrada eggs parasitized by *Gryon gonikopalense* in one hour, depending on the egg storage temperature (13 (red), 5 (green) or −20 °C (blue)) and duration (S1 = one week; S2 = two weeks; S3 = three weeks; M1 = one month; M2 = two months; M3 = three months and M4 = four months). Black dots are observations and red dots are mean values for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

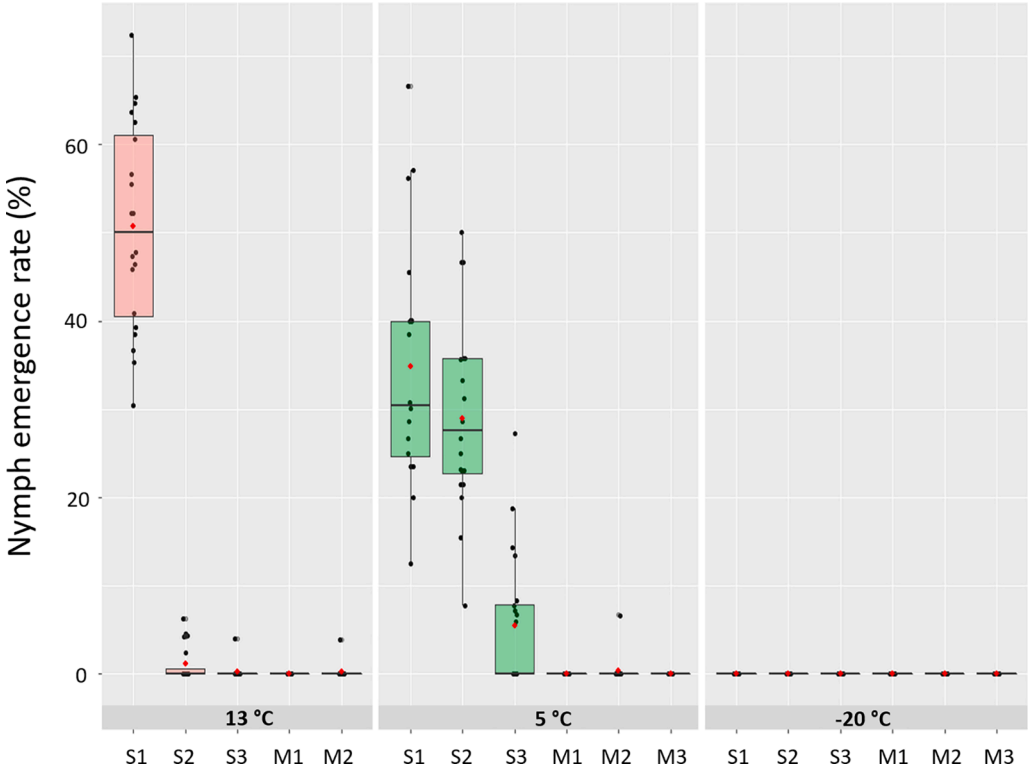


Fig. 3. Percentage of bagrada nymphal emergence from eggs stored at 13, 5 or −20 °C and for one week (S1), two weeks (S2), three weeks (S3), one month (M1), two months (M2), or three months (M3). Black dots are observations and red dots are mean values for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Effects of *Bagrada hilaris* egg storage temperature, duration and their interaction on the development time, the emergence and the body length of *Gryon gonikopalense* progeny. Values in bold indicate a significant effect of the corresponding parameter for a given variable (Two-way ANOVA, $\alpha = 0.05$).

Explicative parameters	Df	Test value	P-value
Development time			
temperature	2	$F_{2,\ 482} = 10.56$	**<0.0001**
duration	5	$F_{5,\ 482} = 75.33$	**<0.0001**
temperature X duration	8	$F_{8,\ 482} = 31.63$	**<0.0001**
Percentage of emergence			
temperature	2	$F_{1,224} = 9.75$	**0.013**
duration	5	$F_{5,224} = 1.9$	0.11
temperature X duration	9	$F_{5,224} = 0.77$	0.51
Body Length			
temperature	2	$F_{2,\ 483} = 0.0029$	0.32
duration	6	$F_{6,\ 483} = 0.0031$	0.29
temperature X duration	8	$F_{8,\ 483} = 0.0063$	**0.012**

4. Discussion

Our study developed guidelines for an efficient laboratory standard procedure for a long-term storage of bagrada eggs with the purpose of improving rearing of its parasitoid *G. gonikopalense*. We focused on bagrada eggs stored under various temperature treatments (-20 °C, 5 °C, 13 °C) and durations (one week to three months). We also assessed the ability of female parasitoids to produce offspring after a long period of host deprivation as a complementary tool of maintaining a colony in the laboratory.

Bagrada eggs stored at 5 °C for up to one month were the best for successful reproduction of *G. gonikopalense*. Refrigerating bagrada eggs at 5 °C allowed parasitism rate ranging from 71 to 80% between one and three weeks. We expected sublethal effects on parasitoid progeny, since exposure of host eggs to low temperatures can reduce host quality for subsequently developing immature parasitoids (Vinson 2010; Skillman and Lee, 2017; McIntosh et al. 2019, Wong et al. 2021). Instead, we observed a high survival of immature parasitoids (96%) and no apparent detrimental effects on the offspring development time, size or longevity compared to other storage temperatures. However, bagrada eggs stored beyond three weeks at 5 °C were mostly rejected by the parasitoid females. As such, *G. gonikopalense* was a more selective parasitoid compared for instance to *Trissolcus japonicus* Ashmead (Hym.: Scelionidae), whose parasitism emergence was only reduced by 9.61% with 2-month refrigerated at 8 °C *Halyomorpha halys* Stal (Hem.: Pentatomidae) eggs (Wong et al. 2021).

Eggs stored at -20 °C provided the lowest parasitism rate for *G. gonikopalense* (8%), but no negative impacts on development time, emergence rate, size and longevity of progeny were detected comparing with the 5 °C treatment. In addition, the average number of eggs parasitized did not vary with the duration of storage, suggesting that bagrada eggs could be stored longer than three months, unlike eggs refrigerated at 5 °C. Our data confirm Martel et al. (2019) on the unsuitability for *G. gonikopalense* to use frozen bagrada eggs at -80 °C, though the storage at -20 °C in the current study led to more parasitism. Little is known about the suitability of frozen host eggs for rearing scelionid species. As the freezing temperature storage tested here did not alter the progeny emergence and development of *G. gonikopalense* (post-ovipositional barrier), it can be hypothesized that the egg acceptance by the parasitoid (pre-ovipositional barrier) was reduced through a possible alteration of chemical or morphological cues (Zachariassen and Hammel 1976; McIntosh et al. 2019). For instance, *Gryon gnidus* Nixon was unable to parasitize eggs of *Acanthomia* (=*Clavigralla*) *tomentosicollis* (Hem.: Coreidae) that were stored at -4°C, showing the need of a viable host embryo to induce successful parasitism (Egwuatu and Ajibola Taylor, 1977). To a lesser extent, the parasitism rate of *T. japonicus* reared from

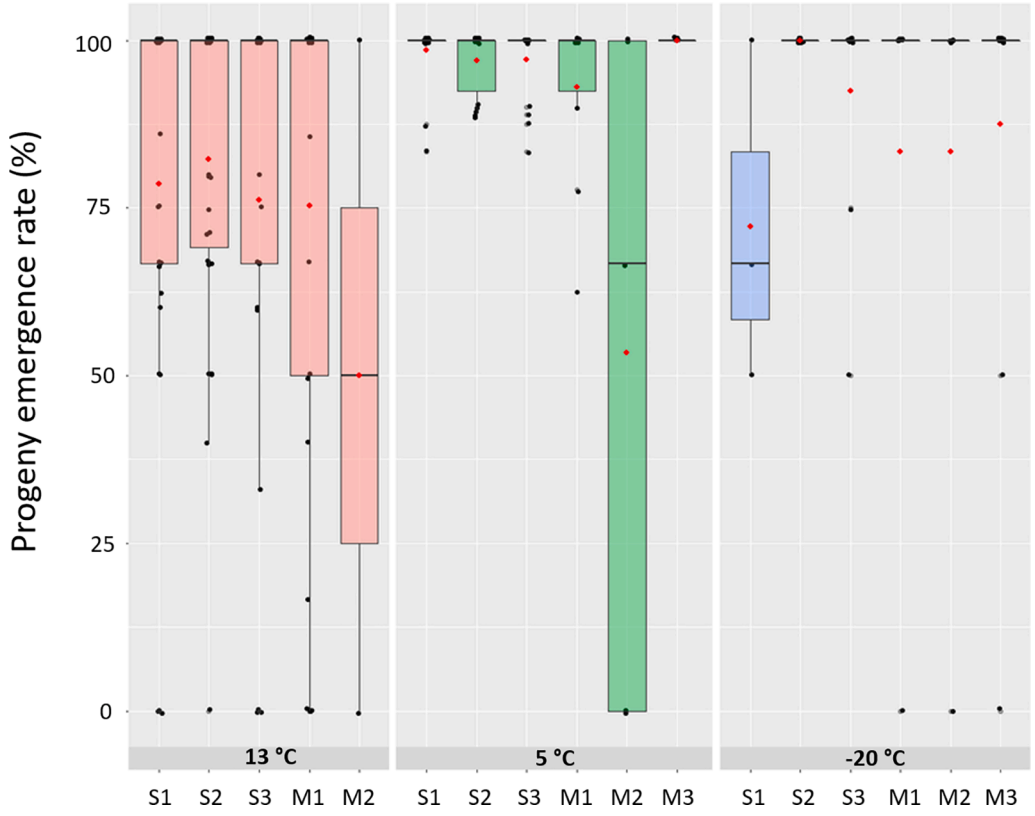


Fig. 4. Percentage of *Gryon gonikopalense* emergence from *Bagrada hilaris* eggs stored at 13, 5 or -20 °C for one week (S1), two weeks (S2), three weeks (S3), one month (M1), two months (M2), three months (M3). Black dots are observations and red dots are mean values for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

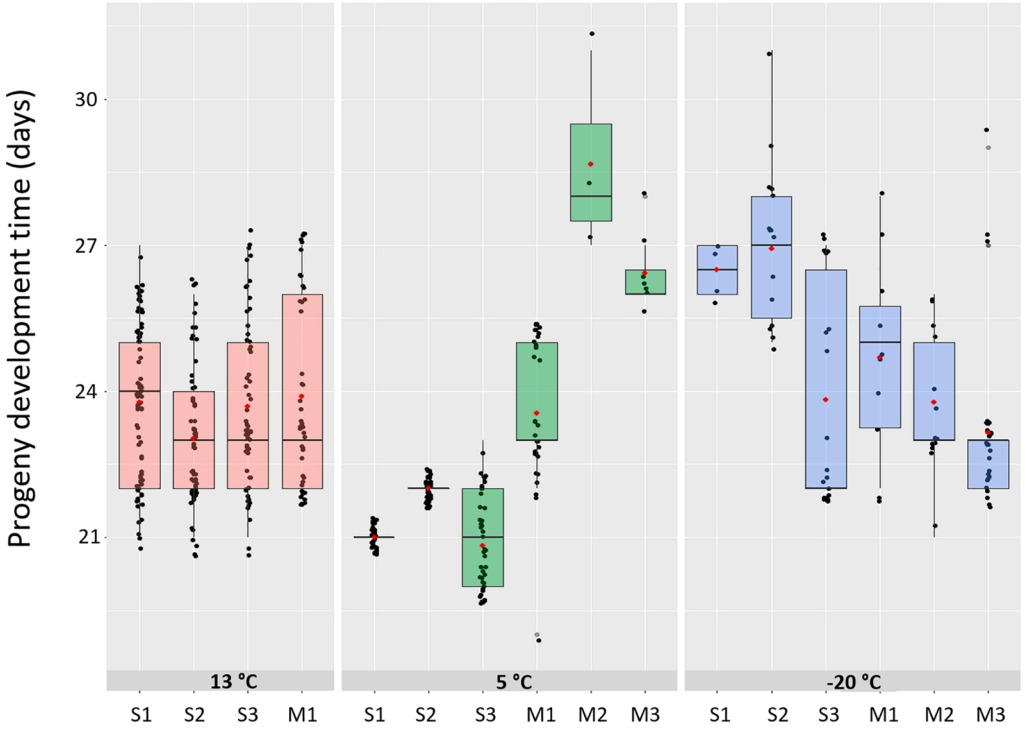


Fig. 5. Development time of *Gryon gonikopalense* inside *Bagrada hilaris* eggs stored at 13, 5 or −20 °C and for one week (S1), two weeks (S2), three weeks (S3), one month (M1), two months (M2), three months (M3). Black dots are observations and red dots are mean values for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

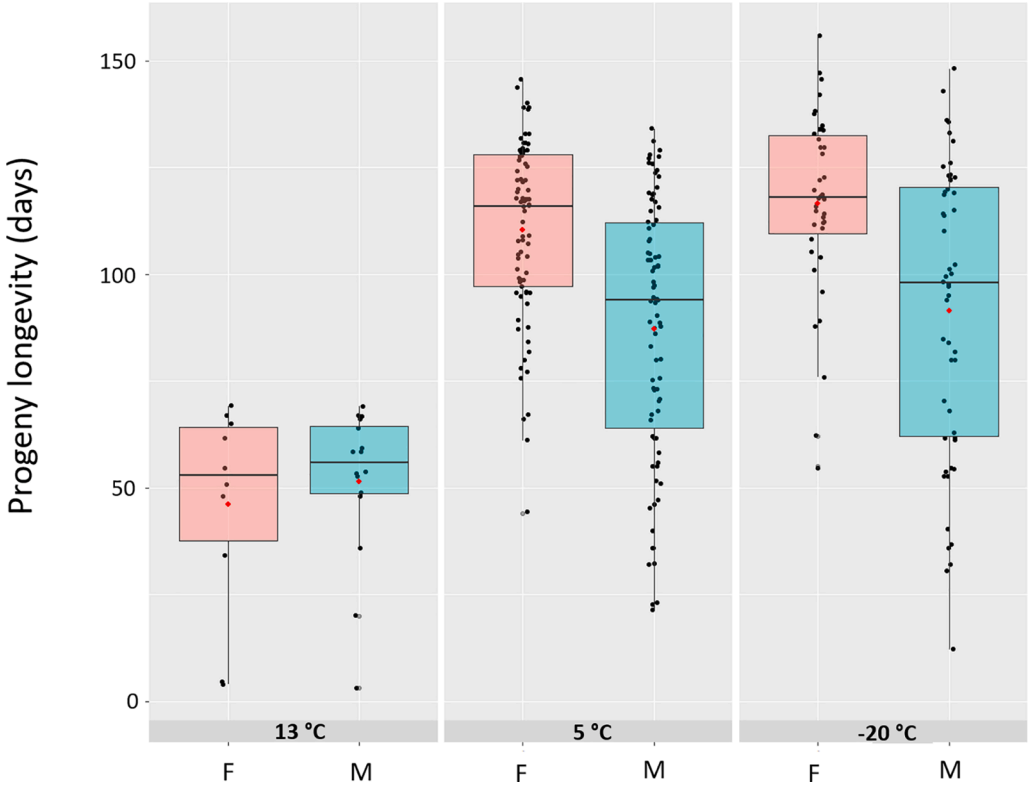


Fig. 6. Longevity of females (F, red boxplots) and males (M, blue boxplots) *Gryon gonikopalense* developed from *Bagrada hilaris* eggs stored at 13, 5 or −20 °C. Storage durations (from one week (S1) to three months (M3) are pooled for each storage temperature. Black dots are observations and red dots are mean values for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

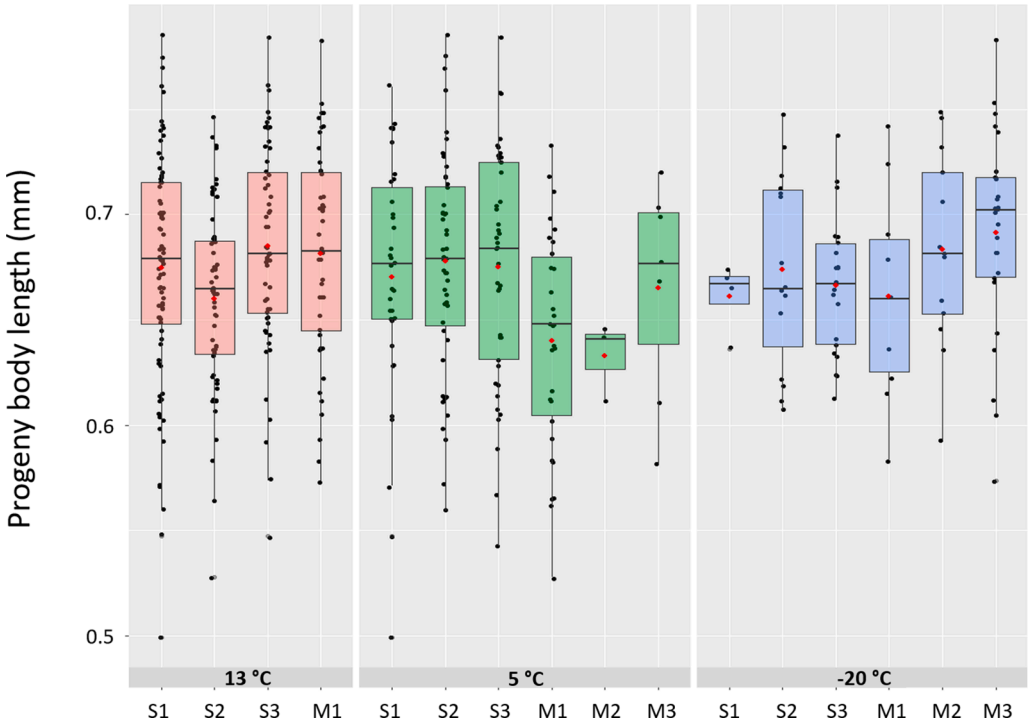


Fig. 7. Body length of *Gryon gonikopalense* adults that developed inside *Bagrada hilaris* eggs stored at 13, 5 or -20 °C and for one week (S1), two weeks (S2), three weeks (S3), one month (M1), two months (M2), or three months (M3). Black dots are observations and red dots are mean values for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Effect of host deprivation (eggs of *Bagrada hilaris*) duration (24hrs ; 30 or 60 days) on *Gryon gonikopalense* fecundity, progeny sex ratio, development time, longevity and immature progeny survivorship (% of emerged progeny). Values are given as mean with the corresponding 95% confidence interval. Within a given row, values followed by different lower-case letters differ significantly (One-way ANOVA). Values of male and female development time (Two-way ANOVA) and longevity (Cox proportional hazard model) followed by different uppercase letters differed significantly (tests were followed by Tukey post-hoc test; $\alpha = 0.05$).

Measured trait	Host deprivation duration					
	# replicates	24 h	# replicates	30 days	# replicates	60 days
Female fecundity (progeny / female)	29	77.4 (a)[63.9 ; 90.8]	38	23.7 (b)[19.2 ; 28.1]	19	12.2 (c)[6.1 ; 18.4]
Progeny Sex ratio (% females)	29	63.5 (a)[54.8 ; 72.2]	38	76.2 (a)[70.8 ; 81.6]	19	72.4 (a)[59.3 ; 85.5]
Proportion of emerged progeny (%)	29	91.5 (a)[87.3 ; 95.6]	38	96.1 (a)[93.9 ; 98.4]	19	91.7 (a)[85.5 ; 97.9]
Male progeny development time (days)	134	23.0 (Aa) [22.7 ; 23.3]	60	23.5 (Aa) [23.2 ; 23.7]	40	22.8 (Aa) [22.2 ; 23.7]
Female progeny development time (days)	112	24.2 (Bab) [23.9 ; 24.5]	178	24.8 (Ba) [24.7 ; 24.9]	134	23.4 (Bb) [23.2 ; 23.6]
Male progeny longevity (days)	62	92.8 (Aa) [84.3 ; 101.2]	40	84.8 (Aa) [79.0 ; 90.7]	26	76.6 (Aa) [63.8 ; 89.5]
Female progeny longevity (days)	62	83.3 (Aab) [74.0 ; 92.5]	78	93.8 (Aa) [91.3 ; 96.3]	78	73.2 (Ab) [67.3 ; 79.1]

H. halys frozen at -80 °C was reduced by 58% (Wong et al. 2021). Nevertheless, the effects of host freezing storage vary among Scelionidae species, and some can parasitize frozen eggs well, despite a reduction in the rate of parasitism (Sabbatini Peverieri et al., 2015; Kivan and Kilic 2005; McIntosh et al. 2019).

Finally, egg storage at 13 °C allowed a better host viability than 5 and -20 °C, as this temperature is slightly below the bagrada developmental threshold (Reed et al. 2017). With nearly 50%, the bagrada emergence rate was the highest after one-week storage, then declined after two weeks, to reach 1% after two months. At 13 °C, the rate of parasitized eggs by *G. gonikopalense* was intermediate (25–30%). As opposed to storage at 5 °C and -20 °C, that completely stopped the development of bagrada, eggs stored at 13 °C may only have slowed their development. We can hypothesize that in these conditions, egg resources continued to decline and so did the egg quality and acceptability for *G. gonikopalense*, (Barrett and Schmidt 1991; Vinson 2010; Skillman and Lee, 2017). Nevertheless, no detrimental effects were observed on parasitoid development time, emergence and body length compared to 5 and -20 °C treatments. Only the progeny longevity was apparently lower, but our experiment was interrupted by a heatwave during summer 2019. Nearly 80% of developing parasitoids were killed. As a result, the average longevity (50 days) was based on only 20% of individuals. Considering the missing data, we observed no difference in longevity between storage durations at 13 °C. Also, we may expect that adding the longevity of missing individuals would have led to the same values than the freezing and refrigeration treatments, respectively 102 and 99 days on average.

Concomitantly to egg storage tests, we observed that females *G. gonikopakense* were able to parasitize bagrada eggs after up to 60 days of host deprivation. However, the fecundity was reduced by 69% after one month of host deprivation and by 84% after two months. Our data are consistent with other parasitoid families, including Scelionidae (Navasero & Elzen, 1992; Terkanian, 1993; Vogt & Nechols, 1993; Hougardy and Mills, 2006; Bruce et al. 2009), but to our knowledge, the effect of host-deprivation over >10–15 days in scelionid species was never explored. In the present study, we used a larger time scale and the parasitism ability of a scelionid species after a 60-day host deprivation is surprising. The observed fecundity decline may be caused by a decreasing number of viable eggs and an egg resorption as observed for the scelionid *Telenomus isis* (Polaszek) (Bruce et al. 2009). On the other

hand, a loss of unused oocytes quality may have occurred, as it is generally observed in both proovigenic and synovipogenic parasitoid species (Vogt & Nechols, 1993; Jervis et al. 2008). Despite this fact, no direct negative impacts were observed on the progeny sex ratio, development time, and emergence. However, the progeny's longevity was reduced by 17% after 60 days of host deprivation. Overall, our results indicate that *G. gonikopalense* could be used for reproduction after several weeks of absence of bagrada eggs, which would be helpful in maintaining the parasitoid colony with no measured negative fitness consequences. However, further experiments on possible sublethal effects on progeny fecundity or behavioral traits should be considered (Wong et al. 2021).

By this study, we aimed to support sustainable egg production of laboratory bagrada colonies and to facilitate the rearing of *G. gonikopalense*. Indeed, a low bagrada egg production may occur during routine laboratory production. At their highest rate, bagrada colonies maintained at EBCL can produce nearly 30,000 eggs per month (Martel G. & Sforza RFH, unpublished data). However, in the summertime, the collected egg number can drop below 500 per week. This has severe consequences both on maintenance of *G. gonikopalense* colonies and ongoing experiments requiring bagrada eggs. Regarding the production aspects, our results show that the refrigeration technique for bagrada eggs storage is efficient, and freezing storage can be used as a complement. From the parasitoid side, host deprivation is well supported by females and could be employed as a last resort in case of a low bagrada egg production.

Our study also brings new insights in field collection of bagrada parasitoids with sentinel eggs. In fact, freezing eggs at -20 °C killed bagrada nymphs, avoiding the risk of accidental introduction in the field. Frozen eggs could then be used as sentinel eggs in outdoor exposure. However, the unsuitability of frozen eggs for *G. gonikopalense* (8% parasitism) could imply a sampling bias for this species during field surveys. Other egg parasitoid species may also avoid these unsuitable sentinel eggs as observed for instance with old sentinel eggs (Jones et al. 2014). Hence, the diversity of bagrada egg parasitoids may be underestimated in the field though good biocontrol candidates may exist among them. Alternatively, eggs stored at 5 or 13 °C were more suitable for parasitism up to one month but showed some bagrada emergences. The use of sentinel eggs stored at these temperatures is therefore a risky option in areas where bagrada is absent. Hence, a compromise has to be found between suitable refrigerated eggs for parasitism with the remaining risk of bagrada emergence, and frozen eggs that guarantee zero bagrada emergence but lead to a low acceptance by *G. gonikopalense*, and possibly other egg parasitoid species. Finally, freezing -refrigeration methods could potentially be expanded to nontarget native stinkbug eggs for estimating levels of nontarget attack by *G. gonikopalense* in the field. This could be especially useful for univoltine stinkbug species that produce low numbers of egg masses simultaneously. If the storage effects we observed with bagrada eggs are similar when applied to other stinkbugs, this may help in understanding the impact of *G. gonikopalense* on such nontarget species in the frame of a biocontrol program.

Taken as a whole, our data helped understanding how to best store bagrada eggs in the laboratory for 1) optimizing their use for concomitant experiments and *G. gonikopalense* rearing and 2) anticipating host population variations and consequences on quality and quantity of the parasitoid colony. This study should also provide information for 3) a better accurate use of bagrada sentinel eggs regarding parasitoid sampling and 4) setting up a mass rearing for release of *G. gonikopalense*.

CRediT authorship contribution statement

Guillaume Martel: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - original draft. **René F.H. Sforza:** Writing - original draft, Funding acquisition, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2021.104759.

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Supplementary data

Figure S1. Survival probability of *Gryon gonikopalense* after it developed in eggs of *Bagrada hilaris* stored at 13, 5 or -20°C. Storage durations (from one week (S1) to three months (M3)) and progeny sexes are pooled for each storage temperature. Curves are presented with their 95% Confidence Interval.

Figure S2. Survival probability of *Gryon gonikopalense* progeny from 24 h, 30 days or 60 days host deprived mother. Progeny sexes are pooled for each host deprivation category. Curves are presented with their 95% Confidence Interval.

Figure S1

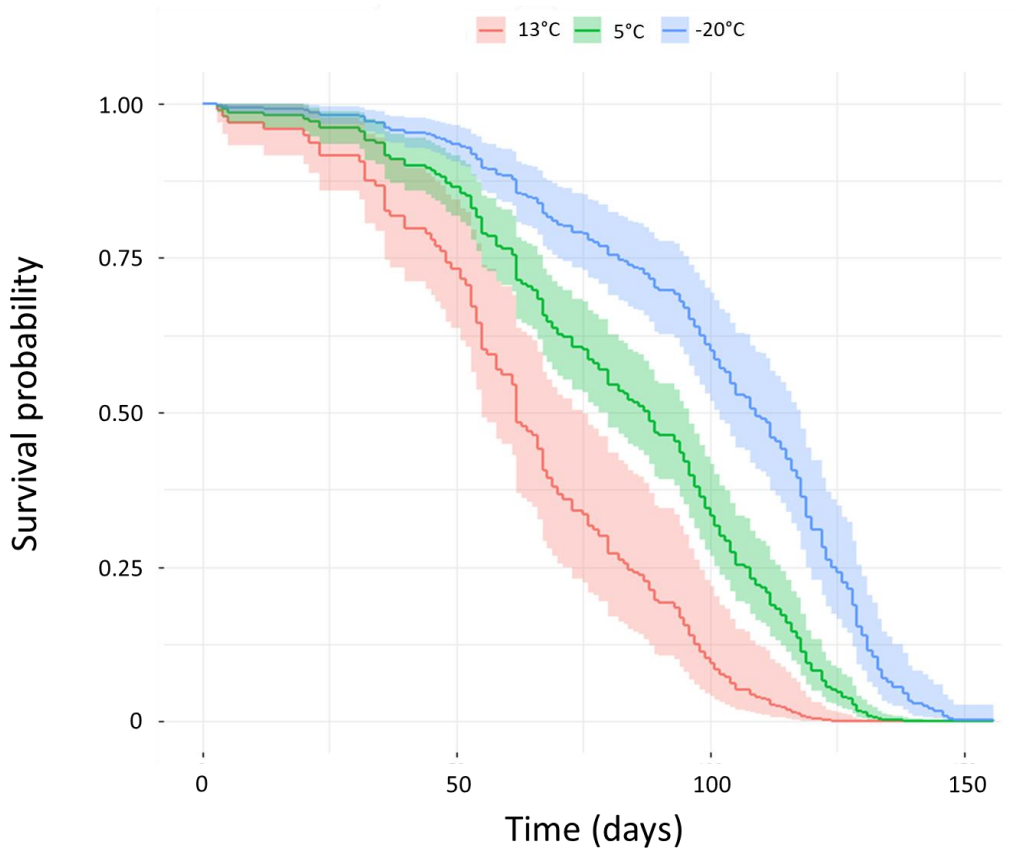
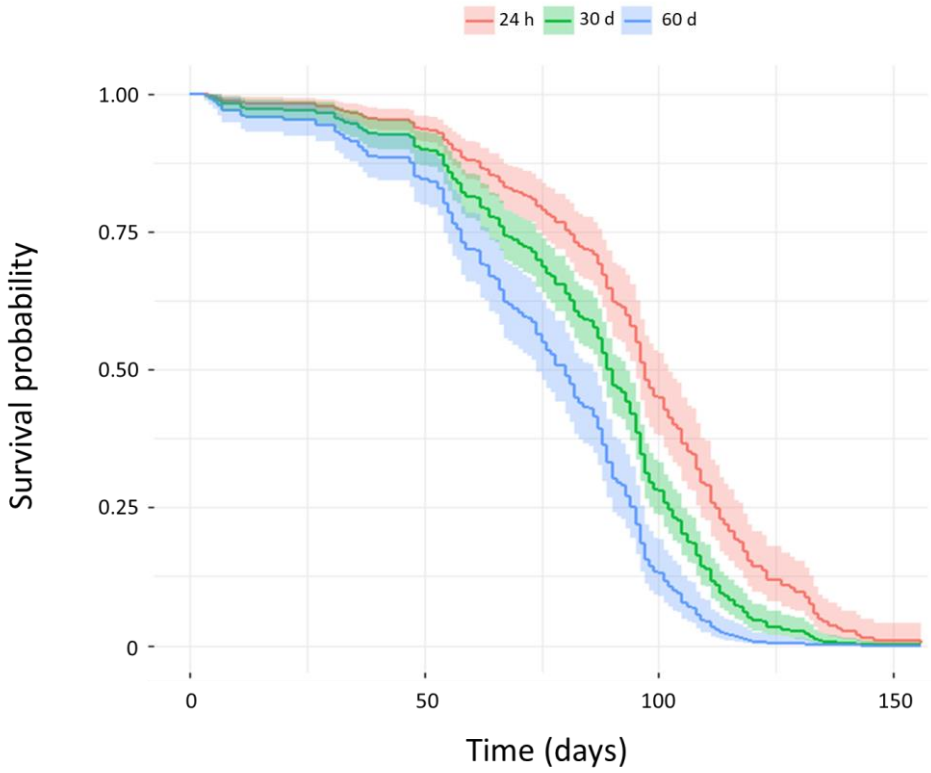


Figure S2


24 h
30 d
60 d
1.00
0.75
0.50
0.25
0
Survival probability
0
50
100
150
Time (days)

Chapitre 5.

Une ouverture sur l'Europe à la lumière de la spécificité de *Gryon gonikopalense*

Article 6 : **Martel, G.,** & Sforza, R.F.H.: Anticipating the arrival of a new stinkbug pest in continental Europe: what can we learn from preliminary host specificity tests for biocontrol?

(Non soumis)

Au cours des précédents chapitres de cette thèse, nous avons pu constater que *G. gonikopalense* rassemblait un grand nombre de qualités faisant de lui un candidat prometteur pour contrôler bagrada aux Etats-Unis. Sa physiologie est comparable à celle à d'autres scelionidés utilisés en LBC, et son comportement de recherche d'hôte spécialisé semble être son principal atout. De plus, *G. gonikopalense* peut être assez simplement produit en grandes quantités en laboratoire. Le dernier point à vérifier est donc sa spécificité vis-à-vis de bagrada. La validation de ce critère est en effet cruciale pour pouvoir le lâcher dans l'environnement sans craindre qu'il s'attaque à d'autres espèces non-cibles. Des tests de spécificité sont actuellement en cours en Californie, et évaluent depuis 2017 la gamme d'hôte physiologique de *G. gonikopalense* sur des pentatomidés Nord-Américaines et Californiennes. Dans ce dernier chapitre avant la discussion générale, je présente des tests similaires menés en serre de quarantaine sur des pentatomidés d'origine euro-méditerranéenne. Ces tests ont deux objectifs : 1) complémenter la gamme d'hôtes décrite en Californie et déterminer des points communs ou de divergence entre les espèces parasitées ; 2) adopter une démarche d'anticipation en évaluant les risques d'introduction de *G. gonikopalense* dans le sud de l'Europe continentale où bagrada pourrait se répandre dans les années à venir depuis les populations déjà installées du Maroc, de Malte ou de Pantelleria (Sicile).

Les résultats obtenus montrent que quatre des 10 espèces de pentatomidés testées sont dans la gamme d'hôte de *G. gonikopalense*, avec des taux de parasitisme mesurés entre 21.8 et 61.3 % : *Carpocoris mediterraneus*, *Dolycoris baccarum*, *Graphosoma italicum* et *Ancyrosoma leucogrammes*. Cependant, le taux de parasitisme a été plus faible en conditions de choix (en présence de bagrada) qu'en non-choix (absence de bagrada) avec un maximum de 26 %. Etonnamment, trois espèces du genre *Eurydema*, que nous pressentions comme des hôtes possibles de *G. gonikopalense* (même tribu que bagrada et affinité pour les brassicacées) n'ont pas du tout été parasitées. Aucun parasitisme n'a également été observé sur les deux espèces invasives cosmopolites que sont *Nezara viridula* et *Halyomorpha halys*, ni sur le Rhopalidae *Liorhyssus hyalinus*.

Mis en commun, les tests Californiens et Européens montrent que *G. gonikopalense* n'est pas spécifique de bagrada en conditions de laboratoire. Certaines espèces parasitées comme *A. leucogrammes* et *C. mediterraneus* sont restreintes au Sud de l'Europe et au bassin méditerranéen et pourraient empêcher le lâcher de *G. gonikopalense* dans les populations invasives de bagrada de cette région comme celle de Pantelleria. D'autre part, certaines espèces comme *Graphosoma semipunctatum* ont été testées trop peu de fois et il serait imprudent de s'en tenir aux résultats négatifs obtenus. De nouvelles expérimentations seront réalisées en 2021 pour confirmer ces tests préliminaires et éventuellement inclure de nouvelles espèces. Par ailleurs, les conditions de choix ou de non-choix en laboratoire ne sont pas simplement transposables à la réalité du terrain, et nous pouvons nous demander si *G. gonikopalense* pourrait réellement rencontrer ces espèces non-cibles dans son environnement. Enfin, la spécialisation comportementale du parasitoïde que j'ai présentée au **Chapitre 3** pourrait contrebalancer un manque apparent de spécificité envers bagrada.

Anticipating the arrival of a new stinkbug pest in continental Europe: what can we learn from preliminary host specificity tests for biocontrol?

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Abstract:

The egg parasitoid *Gryon gonikopalense* is a promising candidate for biocontrol of the painted bug *Bagrada hilaris* in the Southwestern USA. Specificity tests are running in California to evaluate the physiological host range of *G. gonikopalense* and assess the risks of its introduction. In the present study, we aim to evaluate the parasitoid's host range in the European range of bagrada: the Mediterranean basin. These preliminary tests should complement those performed in California and provide risk information of a release of *G. gonikopalense* in southern continental Europe where bagrada could spread in the future years.

The results showed that four species over the 11 tested were in the physiological host range of *G. gonikopalense*, with parasitism rate ranging from 21.8 % to 61.3 %: *Carpocoris mediterraneus*, *Dolycoris baccarum*, *Graphosoma italicum* and *Ancyrosoma leucogrammes*. However, parasitism rates were lower in choice than in no-choice conditions with a maximum of 26 %. Surprisingly, the three *Eurydema* sp. that belong to Strachinii tribe and feed on brassicaceae like bagrada, were not parasitized. No parasitism was observed on the cosmopolitan invasive pentatomid *Nezara viridula* and *Halyomorpha halys*, nor on the Rhopalidae *Liorhyssus hyalinus*. The adult parasitoid size was correlated with the host egg size. Parasitoids from non-target species were bigger than ones developing on bagrada and took more time to complete their development. The likely consequences of releasing *G. gonikopalense* in Mediterranean basin are discussed regarding the parasitized species *A. leucogrammes* and *C. mediterraneus* which are restricted to this region. The parasitism of non-target species is also discussed regarding the specialized foraging behavior of *G. gonikopalense* demonstrated against buried bagrada eggs. Further experiment should be conducted to confirm results, and eventually include new species to test.

1. **Introduction**

The painted bug, *Bagrada hilaris* Burmeister (Hemiptera, Pentatomidae) (= bagrada) is a worldwide invasive pest on Brassicaceae cropping systems (McPherson 2018; Conti et al. 2021). The native range of this stinkbug is from Asia to a vast part of eastern and southern Africa. It was first discovered in North America in 2008 in Californian broccoli and cabbage fields where yield losses were up to 25% (Palumbo et al. 2016). Since then, bagrada rapidly spread to other U.S. states including Arizona, Texas and New Mexico (Bundy et al. 2012; Reed et al. 2013; Palumbo et al. 2016). The pest reached Mexico in 2014 and Chile in 2016 (Sánchez-Peña 2014; Faùndez et al. 2016). Bagrada is a gregarious and highly polyphagous pest that mainly feeds and impacts crucifer species (Reed et al. 2013; Huang et al. 2014; Palumbo et al. 2016) but also tomato (Singh et al. 2011) and Fabaceae species (Reed et al. 2013; McPherson 2018).

To date, like most invasive stinkbug species, bagrada is mainly controlled in North America by large-spectrum pesticides (Joseph et al. 2016; Sosa-Gomez et al. 2019). Various Integrated Pest Management (IPM) strategies are also explored (Sharma et al. 2020; Arif et al. 2020). Among them, importation biological control in USA may constitute a serious alternative to pesticide management, especially in organic cropping systems where farmers have currently no efficient method to control bagrada. As a result, the egg parasitoid *Gryon gonikopalense* Sharma (Hymenoptera, Scelionidae) originated from Pakistan (Mahmood et al. 2015; Martel et al. 2019) is under evaluation in the quarantine facility of the European Biological Control Laboratory (Montpellier, France). Initial studies revealed promising physiological and behavioral traits of this parasitoid (Martel et al. 2019; Martel and Sforza 2021; Martel et al. 2021), especially regarding the below-ground oviposition of bagrada (Gunn 1918; Taylor et al. 2014).

Since the efficiency of this egg parasitoid is promising, host specificity test with native north American pentatomids have to be simultaneously conducted in order to assess a possible negative impact on non-target species in the invaded range. Such tests are currently running in California where the importation of a biocontrol agent against bagrada is considered and should bring new insights about the feasibility of the biocontrol program with *G. gonikopalense*. But what about non-USA invaded territories where bagrada is present and susceptible to spread and negatively impact crops? Indeed, the pest is absent from the continental Europe, but present on islands of Malta and Pantelleria (Sicily, Italy) where it feeds on caper plants (Capparaceae) (Colazza et al. 2004; Arif et al. 2020). Recent scenarios modeling the bagrada's invaded range expansion regarding climate change and human routes predicted its possible introduction in several Mediterranean type climate countries (Carjaval et al. 2018). In the Mediterranean basin, the latter authors especially listed southern Spain, Portugal and inland Italy. Anticipating the arrival of bagrada, a proactive strategy for management and control, including biocontrol, can be set up in light of actions taken in New Zealand as to anticipate the future arrival of the Brown marmorated stink bug (*Halyomorpha halys* Stal (Hemiptera: Pentatomidae)). Researchers have assessed the safety of releasing the egg parasitoid *Trissolucs japonicus* Ashmead (Hym: Scelionidae), a promising biocontrol agent against *H. halys* (Charles et al. 2019; Conti et al. 2021). For a biocontrol agent to be released in the environment of either North America or Europe, a permit to release is mandatory by regulatory bodies. As a prerequisite, host specificity tests are considered a key step in the process of building up a permit request for evaluating the safety of the selected biocontrol candidate (Fauvergue et al. 2020). These tests aim to measure the parasitism activity and direct or indirect impacts that the candidate may induce to a selection of host species in choice and no-choice tests. Recent studies have evaluated scelionid species against stinkbugs by selecting a range of a dozen non-target hosts (Power et al, 2020; Haye et al, 2020).

Hence, the purpose of the present study was to conduct host specificity tests with *G. gonikopalense* against several European native and invasive pentatomids, with a special emphasis on Mediterranean species. We targeted several tribes of the family Pentatomidae, including the Strachinii tribe to which belongs bagrada. These investigations should provide useful information on the host range of this egg parasitoid and on its safety in the frame of a biocontrol program. As most of non-target species tested in our study are absent from the USA, we also expect our results to complement host specificity tests conducted in California with North American pentatomids, in order to have a wider overview of the physiological host range of *G. gonikopalense*.

2. MATERIAL AND METHODS

2.1. Insects rearing

2.1.1. Bagrada and *G. gonikopalense* colonies

The laboratory colonies of *Bagrada hilaris* and *Gryon gonikopalense* used in this study were maintained in the quarantine glasshouse of the European Biological Control Laboratory-EBCL (USDA-ARS) at Montferrier-sur-Lez (France) under the official agreement n° R76-2019-162, issued by the French Ministry of Agriculture.

The *Bagrada hilaris* colony is derived from a colony maintained at the University of California Riverside and shipped to EBCL on March 2016. This colony was first collected at Coachella Valley, CA, USA (GPS coordinates: 33°34'40.71" N, 116°08'45.78"W). It has been routinely maintained since 2016 at EBCL in BugDORM® cages (47.5 x 47.5 x 47.5 cm, 150µm opening mesh) under 26°C (night) and *ca*. 33°C (day), at an ambient photoperiod supplemented with sodium lamps (L/D 12:12), and RH: 30 ± 15% (Martel et al. 2019a). Adults and nymphs were fed weekly with a selection of one-month-old Brassicaceae plants including *Brassica oleracea var. italica* Plenck (broccoli), *Sinapis alba* L. (white mustard) and *Diplotaxis erucoides* De Candolle (white wallrocket). Broccoli and mustard were grown from seeds at *ca*.

22°C, RH: 30 ± 10% in a separate room of the quarantine glasshouse, and white wallrocket was weekly field-collected.

In rearing cages, Petri dishes (Ø = 9cm) filled with sand (DIALL®, yellow sand, granulometry = 200µm, Spain) were used as oviposition arenas for bagrada. In Petri dishes, eggs covered with sand particles (Taylor et al. 2015) were collected every two days with a fine brush after sifting sand (Hogentogler®, mesh size = 425µm, USA). Eggs were glued next to each other (UHU stic®, Germany) on cardboard pieces (Martel and Sforza 2021) and used either for parasitoid rearing or subsequent experiments.

The EBCL *Gryon gonikopalense* colony originated from Pakistan (Mahmood et al. 2015), and was maintained in insect-proof transparent plastic boxes (18 x 12 x 5.5 cm). Aeration was provided through holes in the lid (Ø = 50 mm), covered with a mesh fabric. Rearing boxes were stored in a rearing chamber (22 ± 0.5°C, RH 50 ± 5 %, L/D 12:12) and drops of diluted acacia honey (Lune de miel®, France) were deposited once a week on the mesh as *ad libitum* nourishment. The colony was maintained by regularly exposing fresh *B. hilaris* eggs to female parasitoids.

2.1.2. Non-target species

Non-target (NT) stinkbugs tested as alternative hosts of *G. gonikopalense* included ten Pentatomidae species and one Rhopalidae species. All specimens were collected as adults in the field at Monferrier-sur-Lez (France, 43°41’3.14” N; 3°52’32.13” E) excepted *Halyomorpha halys* provided by INRA Sophia Antipolis, *Eurydema oleracea* collected in Saint-Privat (France, 44°37’45.7” N; 4°24’28.8” E) and *Ancyrosoma leucogrammes* collected in Les Matelles (France, 43°44’01.1” N; 3°49’19.9” E). Each species was field collected by hand from its host plant or directly with its host plant (Table 1)

All NT species were separately reared in the EBCL quarantine greenhouse (25 ± 1°C, RH 50 ± 5 %, L/D 12:12) in cages with nylon mesh (Vermandel® 30 x 30 x 30 cm, mesh =

500µm, Netherlands). Nymphal and adult stages were fed with field collected host plant or eventually with broccoli and mustard grown in the quarantine facility (Table 1). Fresh plants were collected and provided to stinkbug species every two days.

Table 1. List of the 10 stinkbug (Pentatomidae) species and one plantbug (Rhopalidae) used as non-target hosts for *Gryon gonikopalense*. The diet of each species includes the host plant on which it was collected from and/or the plant species used for rearing. The selection criteria indicate why each stinkbug was selected.

Alternate Host Species	Tribe	Collecting period	Selection criteria	Diet
Eurydema ventralis	Strachinii	March-May	Host plant overlap, close relatedness	Various cultivated Brassicaceae including cabbage and broccoli
Eurydema ornata		March-May	Host plant overlap, close relatedness	
Eurydema oleracea		April-June	Host plant overlap, close relatedness	
Carpocoris mediterraneus	Carpocorini	March-August	Native Mediterranean species, close relatedness	Various wild Asteraceae like *Carduus* sp., *Cynara* sp. and *Apiaceae* (*Foeniculum vulgare*, *Daucus carota*)
Dolycoris baccarum		March-August	Native European species, close relatedness	
Graphosoma italicum	Graphosomatini	May-August	Native European species, close relatedness	Apiaceae (*Foeniculum vulgare*, *Daucus carota*)
Graphosoma semipunctatum		June-August	Native Mediterranean species, close relatedness	
Ancyrosoma leucogrammes		May-August	Native Mediterranean species, close relatedness	
Nezara viridula	Pentatomini	April	Pest in Europe, close relatedness	Fabaceae (*Phaseolus* sp. Fresh Green bean)
Halyomorpha halys	Capeini	Laboratory colony	Pest in Europe, close relatedness	Fabaceae (*Phaseolus* sp.); grape (*Vitis vinifera*), apple, tomato
Liorhyssus hyalinus	Rhopalidae, Rhopalini	April	Phylogenetic outgroup	Asteraceae

Eggs of each species were daily collected and assigned to no-choice or choice tests. Egg masses were used as laid. Eggs laid on plant tissue were not detached from it.

2.2. Experimental procedure:

To maximize fecundity (Martel et al. 2019), all females *G. gonikopalense* used for tests were newly emerged individuals that mated for 24h with newly emerged males. Bagrada eggs used as control in no-choice or choice tests were maximum 48h old and eggs of NT species less than 24h old.

Sequential no-choice tests

For no-choice tests, each NT egg mass was deposited in one individual clean Petri dish (Falcon® 50 x 9 mm, Corning incorporated, USA) (number of replicates per species varying from 4 to 53). For each replicate, one female *G. gonikopalense* was placed in the Petri dish and left for 24h in the rearing chamber conditions (22 ± 0.5°C, RH 50 ± 5 %, L/D 12:12). After this period, NT egg masses were stored in the Petri dish in the same conditions until emergence of parasitoids. Tested females were transferred in a new clean Petri dish and provided with 15-20 fresh bagrada eggs as a control, in order to ensure that they were able to parasitize eggs. After 24h, females were removed and control eggs were stored in the rearing chamber until emergence of parasitoids.

Choice tests

From the 11 NT species that passed the no-choice tests, only three were used to perform choice tests: *Dolycoris baccarum* (n replicates = 30), *Carpocoris mediterraneus* (n = 27) and *Graphosoma italicum* (n = 19). Although *Ancyrosoma leucogrammes* was parasitized by *G. gonikopalense* during choice tests, no more eggs were available from this species to perform choice tests. Each NT egg mass was deposited in individual clean Petri dishes (Falcon® 50 x 9 mm, Corning incorporated, USA) in presence of bagrada eggs assembled as an artificial egg

mass as described in section 2.b. The size of bagrada egg masses was 15 eggs, and was arbitrary determined to be equivalent to NT egg masses and ensure eggs *ad libitum*. For each replicate, one female *G. gonikopalense* was placed in a Petri dish and left for 24h in the rearing chamber conditions (22 ± 0.5°C, RH 50 ± 5 %, L/D 12:12). After this period, the female was removed from the Petri dish. Bagrada and NT eggs were separated in two different Petri dishes and stored under the same conditions until parasitoid emergence.

For each no-choice and choice trials and for both NT and bagrada, we counted the number of parasitized eggs, the number of emerged parasitoids, the development time of male and female parasitoids, and the size of emerged parasitoids. For NT species tested in choice trials, we also measured the length and width of all eggs obtained (n = 40 for each species).

All non-emerged eggs were dissected under a binocular microscope (Stemi 508, Zeiss®, Germany) in order to verify whether they contained an identifiable nymph or adult parasitoid. Eggs with unidentifiable content were considered as not parasitized. Female parasitoids that failed to parasitize any NT or control eggs, were excluded from statistical analyses.

Only NT species that were parasitized by at least one female *G. gonikopalense* in no-choice tests were used for choice tests.

2.3. Statistical analysis

In no-choice tests, the egg mass size was compared between NT species with a 1-way ANOVA followed by a Tukey post hoc test in order to test de differences between groups. The percentage of egg masses parasitized was compared between each NT species with a contingency χ^2 followed by a pairwise multiple comparison [function fisher.multcomp, package RVAideMemoire with R software (Hervé 2020)]. The percentage of eggs parasitized in egg masses, the percentage of emerged parasitoids, the percentage of emerged host nymphs, the parasitoid sex ratio developing in NT eggs and in bagrada eggs were compared between NT

species with a Kruskal-Wallis test followed by a Nemenyi post hoc test in order to assess the differences between categories.

In choice test, the egg mass size was compared between NT species with a one-way ANOVA followed by a Tukey post hoc test in order to test differences between groups. As bagrada oviposits single eggs, egg masses were manually constituted and were therefore not included in this statistical analysis. The percentage of egg masses parasitized was compared between each NT species with a contingency χ^2 followed by a pairwise multiple comparison. Mean percentage of eggs parasitized in egg masses, percentage of emerged parasitoid and percentage of emerged host nymph, were compared between NT species with a Kruskal-Wallis test followed by a Nemenyi post hoc test in order to assess the differences between categories. For each NT species, the same variables were compared between bagrada and the NT species with a paired-Wilcoxon test in order to assess the choice of *G. gonikopalense* between both hosts.

Development time and body size of male and female parasitoid progeny that emerged from bagrada and NT species eggs were compared using a two-way ANOVA followed by a Tukey post hoc test in order to identify differences among categories. The correlation between parasitoid size and host eggs length was tested with a Pearson correlation test.

All statistical tests were performed with Rstudio software, with a significant threshold set at $\alpha = 0.05$.

3. RESULTS

3.1. No-choice tests

The egg mass size differed between NT species (ANOVA $F_{10,276} = 50.14$, df = 10, P-value < 0.001), varying from 5.5 ± 0.6 eggs for *A. leucogrammes* to 24.8 ± 1.8 eggs for *H. halys* (Table 2). Over all tested NT species, only four were parasitized in no-choice conditions: *C. mediterraneus*, *D. baccarum*, *G. italicum* and *A. leucogrammes*. The parasitism rate of *G. gonikopalense* varied among these species (Pearson's chi-square test: $\chi^2 = 104.5$, df = 10, P-value < 0.001) from 21.8% for *G. italicum* egg masses parasitized to 61.3% for *D. baccarum* (Table 2). Considering the egg mass itself, *G. gonikopalense* parasitized a greatest proportion of eggs when host was *A. leucogrammes* (37.8 ± 22.3 eggs) (Kruskal-Wallis test, $\chi^2 = 108.06$, df = 10, P-value < 0.001). The emergence rate of *G. gonikopalense* progeny also varied among host species (Kruskal-Wallis test, $\chi^2 = 79.668$, df = 3, P-value = 0.02) with a greater emergence rate on *A. leucogrammes* (93.7 ± 14.8%) (Table 2). *Gryon gonikopalense* progeny sex ratio did not differ significantly between NT species (Kruskal-Wallis test, $\chi^2 = 6.01$, df = 3, P-value = 0.11).

Table 2. No-choice tests: Parasitism success of *Gryon gonikopalense* exposed to eggs of 11 non-target species in absence of its host *Bagrada hilaris*. Values are given as mean ± 95% confidence interval. For a given column (variable), values followed by the same letter did not statistically differ (Kruskal-Wallis test followed by a Nemenyi post hoc test (lowercase letters) or a one-way ANOVA followed by a Tukey post hoc test uppercase letters), $\alpha = 0.05$).

Host Species	Mean egg mass size	# Females tested	Total % of egg masses parasitized (#)	Mean % of eggs parasitized in egg mass	Mean % emerged parasitoids	Mean % of emerged host nymphs	Parasitoid sex ratio (NT) (% female)
Eurydema ventralis	11.7 ± 0.2 (A)	47	0 (a)	0 (a)	NA	83.3 ± 6.6 (ab)	NA
Eurydema ornata	11.7 ± 0.3 (A)	53	0 (a)	0 (a)	NA	90.8 ± 4.3 (a)	NA
Eurydema oleracea	11.7 ± 0.2 (A)	18	0 (a)	0 (a)	NA	94.3 ± 4.2 (a)	NA
Carpocoris mediterraneus	13.1 ± 1.2 (AB)	35	42.8 (bc)	18.1 ± 7.6 (bc)	69.2 ± 13.4 (b)	56.1 ± 21.8 (c)	37.5 ± 16.2 (a)
Dolycoris baccarum	15.9 ± 2.1 (B)	31	61.3 (b)	27.2 ± 9.5 (c)	82.9 ± 11.0 (ab)	33.3 ± 13.9 (d)	58.3 ± 11.1 (a)
Graphosoma italicum	13.1 ± 2.0 (AB)	32	21.8 (c)	7.8 ± 5.6 (b)	74.3 ± 20.7 (ab)	62.5 ± 13.5 (bc)	36.1 ± 21.2 (a)
Graphosoma semipunctatum	13.7 ± 0.8 (AB)	4	0 (a)	0 (a)	NA	89.1 ± 20.4 (ab)	NA
Ancyrosoma leucogrammes	5.5 ± 0.6 (D)	18	44.4 (bc)	37.8 ± 22.3 (c)	93.7 ± 14.8 (a)	52.9 ± 22.8 (cd)	35.7 ± 29.3 (a)
Nezara viridula	17 ± 4.6 (BC)	4	0 (a)	0 (a)	NA	77.8 ± 24.9 (abc)	NA
Halyomorpha halys	24.8 ± 1.8 (C)	29	0 (a)	0 (a)	NA	78.7 ± 7.7 (abc)	NA
Liorhyssus hyalinus	20.4 ± 1.4 (BC)	16	0 (a)	0 (a)	NA	93.3 ± 3.7 (a)	NA

3.2. Choice tests

Like for no-choice replicates, egg masses size varied between the three NT species tested (ANOVA, $F_{2,73} = 10.8$, P-value < 0.001) with *D. baccarum* laying the biggest egg masses (17.8 ± 2.4 eggs) (Table 3). The total percentage of egg masses parasitized (Pearson's chi-squared test, $\chi^2 = 0.11$, df = 2, P-value = 0.95), the proportion of eggs parasitized in egg masses (Kruskal-Wallis test, $\chi^2 = 0.015$, df = 2, P-value = 0.99), the progeny emergence rate (Kruskal-Wallis test, $\chi^2 = 1.56$, df = 2, P-value = 0.45), and the emergence rate of the host (Kruskal-Wallis test, $\chi^2 = 5.6$, df = 2, P-value = 0.06) did not differ between NT species (Table 3).

Considering paired-choices between each NT species and bagrada, *G. gonikopalense* behaved similarly for each of the three NT species: bagrada egg masses were more often parasitized than NT species in presence of *C. mediterraneus* (Pearson's chi squared test, $\chi^2 = 24.5$, df =1, P-value < 0.001), *D. baccarum* (Pearson's chi squared test, $\chi^2 = 19.4$, df =1, P-value < 0.001) and *G. italicum* (Pearson's chi squared test, $\chi^2 = 10.6$, df =1, P-value = 0.0011). The mean percentage of eggs parasitized was also higher in bagrada than *C. mediterraneus* (paired Wilcoxon test, v =36, P-value < 0.001), *D. baccarum* (paired Wilcoxon test, v =47, P-value = 0.0011) and *G. italicum* egg masses (paired Wilcoxon test, v =0, P-value < 0.001). The mean emergence rate of *G. gonikopalense* developing on bagrada or NT species did not differ (Table 3). The mean host emergence rate was higher in *C. mediterraneus* (paired Wilcoxon test, v =257, P-value = 0.002) and *G. italicum* (paired Wilcoxon test, v =128, P-value = 0.002) versus bagrada, but did not differ between bagrada and *D. baccarum* (paired Wilcoxon test, v =147, P-value = 0.51).

Table 3. Choice tests: Parasitism success of *Gryon gonikopalense* exposed to eggs of three non-target species in presence of its host *Bagrada hilaris*. Values are given as mean ± 95% confidence interval. For a given column (variable), values followed by the same letter did not statistically differ (Kruskal-Wallis test followed by a Nemenyi post hoc test (uppercase letter) or paired-Wilcoxon test (lowercase letter), α = 0.05).

[1]Only replicates where eggs of NT species or *B. hilaris* were parasitized

[2]Compared with a Pearson's chi-squared test (α = 0.05) between NT species (uppercase letter) or between NT and *B. hilaris* (lowercase letter)

Host species	Mean egg mass size	# Females tested	Total % of egg masses parasitized[2]	# Females parasitizing				Mean % of eggs parasitized[1]	Mean % emerged parasitoids[1]	Mean % of emerged host nymphs[1]
				none	both	only NT	only bagrada			
Carpocoris mediterraneus	13.4 ± 1.1 (a)	27	22.2 (Aa)	1	5	1	20	14.2 ± 12.2 (Aa)	84.8 ± 18.1 (Aa)	54.0 ± 15.6 (Aa)
Bagrada hilaris	15		92.6 (b)					70.0 ± 16.6 (b)	95.4 ± 3.8 (a)	17.7 ± 12.7 (b)
Dolycoris baccarum	17.8 ± 2.4 (b)	30	23.3 (Aa)	4	6	1	19	15.8 ± 13.1 (Aa)	85.2 ± 13.8 (Aa)	30.7 ± 15.5 (Ba)
Bagrada hilaris	15		83.3 (b)					62.1 ± 16.1 (b)	89.8 ± 8.3 (a)	24.9 ± 11.9 (a)
Graphosoma italicum	12.3 ± 1.2 (a)	19	26.3 (Aa)	3	5	0	11	12.3 ± 11.8 (Aa)	80.7 ± 28.3 (Aa)	56.6 ± 18.7 (Aa)
Bagrada hilaris	15		84.2 (b)					80.0 ± 10.7 (b)	92.9 ± 4.7 (a)	5.0 ± 6.5 (b)

3.3. Effect of host egg on *G. gonikopalense* progeny development and size

The egg size differed among host species that allowed parasitism including bagrada (1-way ANOVA, $F_{4,156} = 333.5$, P-value < 0.001). The largest eggs were those of *C. mediterraneus* (1.270 ± 0.017 mm) while the smallest were those of bagrada (0.846 ± 0.023 mm) (Table 3). *G. gonikopalense* did not develop at the same rate in the different NT species (Tukey test, P-value < 0.05, Table 4). Parasitoids that developed inside bagrada eggs had the shortest development time (23.4 ± 0.1 days, male and female pooled) and parasitoids that developed inside *Carpocoris mediterraneus* eggs had the longest development time (27.3 ± 0.8 days, male and female pooled) (Fig 1). Males developed faster than females whatever the host species considered (Tukey test, P-value < 0.05, Table 4, Fig. 1).

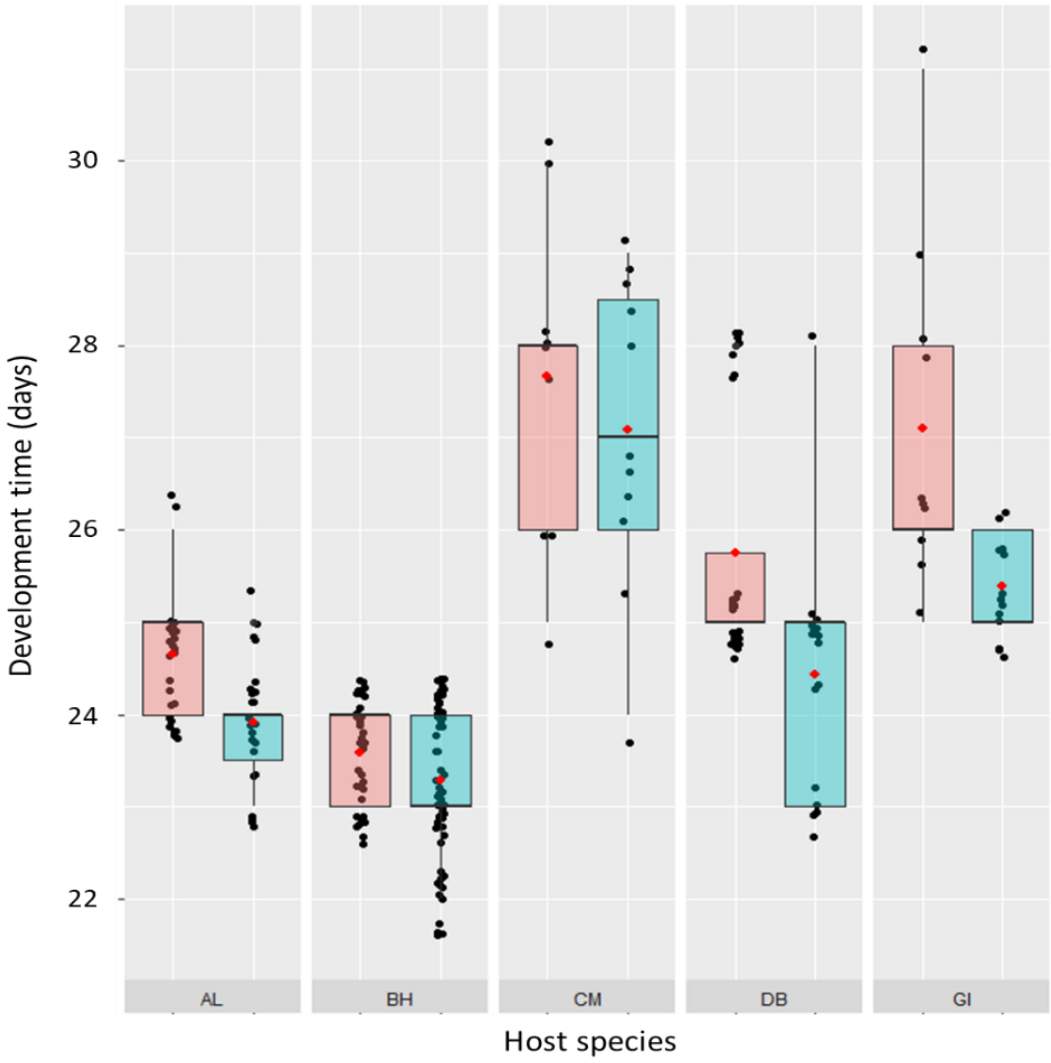


Figure 1. Development duration of male (blue boxplots) and female (red boxplots) *Gryon gonikopalense* progeny developing inside eggs of five host species: *Ancyrosoma leucogrammes* (AL); *Bagrada hilaris* (BH); *Carpocoris mediterraneus* (CM); *Dolycoris baccarum* (DB) and *Graphosoma italicum* (GI).

The size of *G. gonikopalense* progeny differed between host species (Tukey test, P-value < 0.05). However, there was no difference between males and females for a given species excepted when host was *G. italicum* (Table 4, Fig. 2). Parasitoids that developed inside bagrada eggs were the smallest (0.670 ± 0.006 mm, n = 255) and parasitoid that developed inside *Carpocoris mediterraneus* eggs were the largest (0.947 ± 0.01 mm, n = 111) (Fig 2). The

parasitism size was positively correlated with the host length (Pearson correlation, $R^2 = 0.91$, P-value = 0.03).

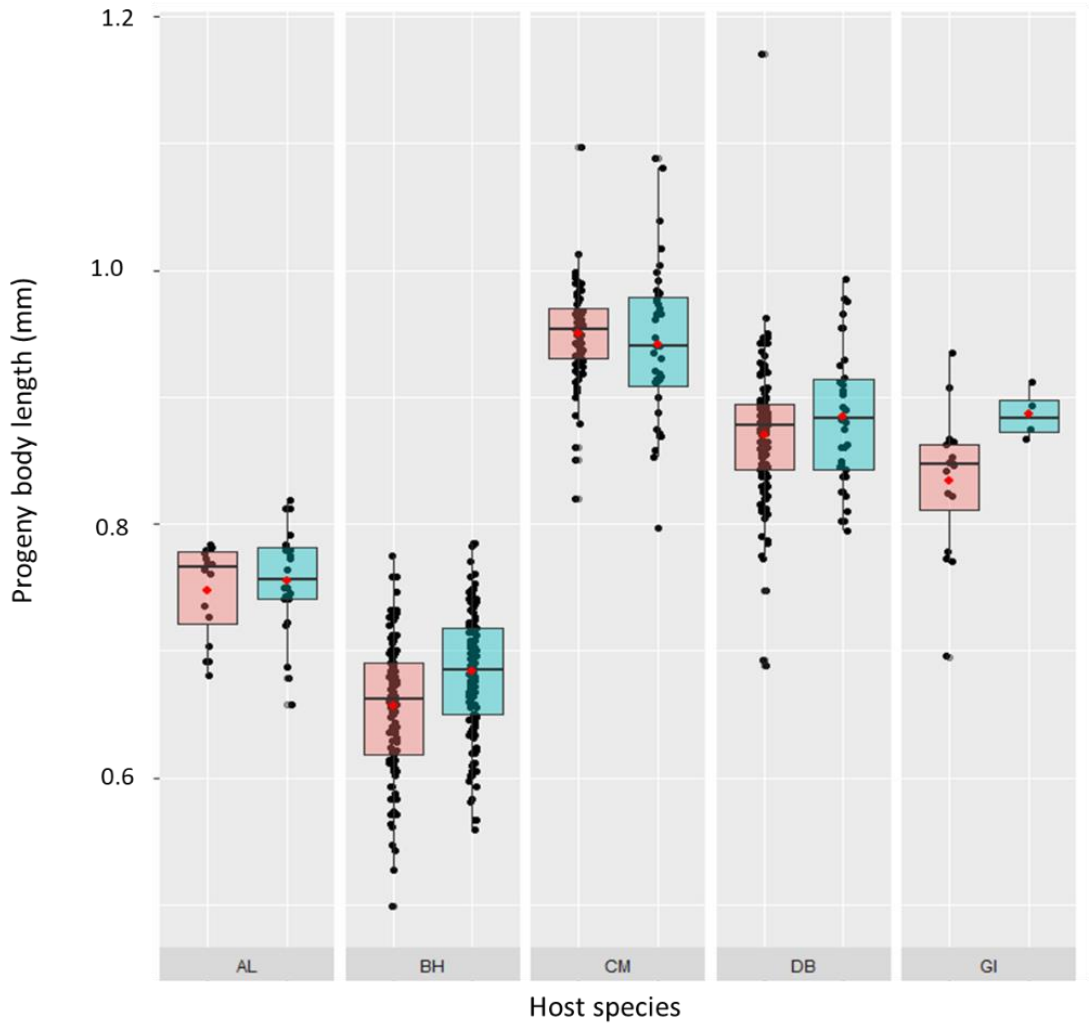


Figure 2. Body length of male (blue boxplots) and female (red boxplots) *Gryon gonikopalense* progeny developing inside eggs of five Pentatomidae host species: *Ancyrosoma leucogrammes* (AL); *Bagrada hilaris* (BH); *Carpocoris mediterraneus* (CM); *Dolycoris baccarum* (DB) and *Graphosoma italicum* (GI).

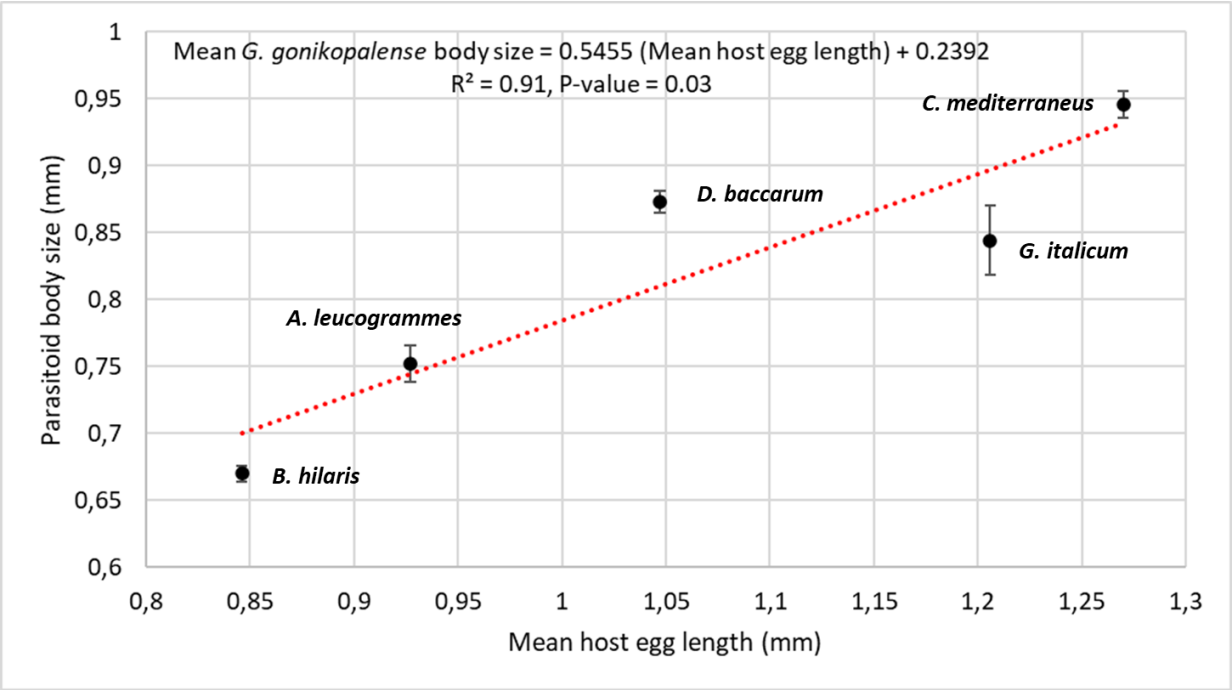


Figure 3. Graphical representation of the correlation between the mean host egg length and the body size of *Gryon gonikopalense* progeny. Each black dot represents the average host egg length (with 95% confidence interval) of a non-target species (*Bagrada hilaris, Ancyrosoma leucogrammes, Dolycoris baccarum, Graphosoma italicum* and *Carpocoris mediteraneus*). The dashed red dot line is the linear regression (with corresponding equation given at the top of the figure).

Table 4. Effect of the host species, the sex of individuals and their interaction on the development time and the body size of *Gryon gonikopalense.* Values in bold indicate a significant effect of the corresponding of the corresponding parameter for a given variable (Two-way ANOVA, $\alpha = 0.05$).

Explicative parameter	Df	Test value	P-value
Development time			
host species	4	$F_{4, 224} = 92,5$	**<0,0001**
sex	1	$F_{1, 224} = 30,9$	**<0,0001**
host species X sex	4	$F_{4, 224} = 3,3$	**0,011**
Body size			
host species	4	$F_{4, 591} = 762,6$	**<0,0001**
sex	1	$F_{1, 591} = 14,1$	**0,0001**
host species X sex	4	$F_{4, 591} = 2,8$	**0,025**

4. Discussion

The purpose of the present study was to test the host specificity of the egg parasitoid *G. gonikopalense* among 10 European and Mediterranean non-target pentatomid species. The test list comprised two major cosmopolitan stinkbug pests from Europe and USA: *Nezara viridula* and *Halyomorpha halys*, in order to examine a possible parasitism pressure of *G. gonikopalense* on these species. Finally, we tested the possibility for the parasitoid to attack an outgroup non-pentatomid bug, *Liorhyssus hyalinus*.

The main result from choice and no-choice tests is that *G. gonikopalense* was not restricted to bagrada eggs, and could attack species outside the Strachiini tribe, *i.e*. the Carpocorini and Graphosomatini tribes. Out of the eleven species exposed to *G. gonikopalense*, four were parasitized, at least during no-choice tests: *Dolycoris baccarum*, *Carpocoris mediterraneus*, *Graphosoma italicum* and *Ancyrosoma leucogrammes*. Two of them, *C. mediterraneus* and *A. leucogrammes* are near exclusive to the Mediterranean basin (http://www.gbif.org), while others are present in all western continental Europe. However, the parasitoid showed a clear preference for bagrada eggs, with higher parasitism rates against this species in both no-choice and choice tests. In no-choice conditions, *D. baccarum* was the most suitable NT species for *G. gonikopalense* with 61.3% of egg masses parasitized. On the opposite, the parasitoid only parasitized 23.3% of *D. baccarum* egg masses during choice tests and the NT host was parasitized alone in only one replicate over 30.

We did not record each oviposition steps of *G. gonikopalense* on NT species eggs. However, we noticed that the parasitoid oviposited on *G. italicum* most of the time, which was not consistent with the parasitism rate we measured (only 21.8% of parasitized egg masses). The inclusion of behavioral observations should be considered in further tests as it allows to follow the fate of each parasitized egg in relation with the mother behavior. For instance, some NT species can constitute evolutionary traps (emergence of host larva despite parasitoid

oviposition) (Abram et al. 2014; Haye et al.2015), or die due to parasitism but fail to produce parasitoid offspring (parasitoid-induced host egg abortion, Abram et al. 2016).

Gryon gonikopalense developing on NT species with larger eggs resulted in larger parasitoids (from 1.11 to 1.41 times larger with *A. leucogrammes* and *C. mediterraneus* eggs respectively). The positive correlation between host size and parasitoid size is well described for many egg parasitoid species (Bai et al. 1992; Grenier et al. 2001; Durocher-Granger et al. 2011), including Scelionidae like *Gryon gallardoi* Brèthes (da Rocha et al. 2007) In addition, the body size is positively correlated to reproductive traits, including initial egg load, potential and realized fecundity and life span (Sagarra et al. 2001; Jervis et al. 2003; Arakawa et al. 2004; Jervis et al. 2008). Indeed, larger individuals have access to more resources during their immature stage and have more resources to allocate to reproduction at adult stage (Jervis et al. 2008). For instance, *Trissolcus mitsukurii* (Hym: Scelionidae), a native egg parasitoid of *Nezara viridula* in Japan, was larger when developing on larger host like *Halyomorpha halys* or *Plautia crossota* Stal (Arakawa et al. 2004); fecundity and longevity were also increased. Hence, we may hypothesize that *G. gonikopalense* developing on larger hosts like *C. mediterraneus*, *D. baccarum* or *G. italicum* would have better fecundity and longevity, which will require further experiments. In addition, one should investigate whether larger parasitoids are better competitors than smaller ones, or bigger parasitoids are more prone to attack stink bugs laying bigger eggs, inducing a possible host shifting overtime.

We expected *Eurydema* spp to be parasitized by *G. gonikopalense* for three reasons: 1) All species belong to the Strachinii tribe and are therefore phylogenetically related to bagrada; 2) like bagrada, *Eurydema* spp develop and feed on brassica host plants, 3) eggs of *Eurydema* spp are morphologically similar to those of the American pentatomid *Murgantia histrionica* (Pentatomidae: Strachinii) that feeds on brassicas and was parasitized by *G. gonikopalense* in

no-choice tests (Pickett et al. 2021). But surprisingly in the latter report, *Eurydema ventralis*, *E. ornata* and *E. oleracea* were not parasitized by *G.gonikopalense*.

One explanation may be the difference in egg morphology between *Eurydema* spp and bagrada. The firsts had a thicker chorion and were more resistant to pressure and stings perforation (authors, pers. obs). By contrast, bagrada eggs were more fragile and easier to burst, though we did not quantify these traits. This difference of hardness could be explained by egg vulnerability level in the field: Usually, pentatomids lay eggs on their host plant without particular protection (McPherson 2018). As eggs are fixed on their substrate, they are exposed to predators and parasitoids, so that a thick chorion is an advantage against parasitoid oviposition. Alternatively, most bagrada eggs are oviposited under the soil surface, and egg surface is protected by glued soil particles that limit access to parasitoids (Taylor et al. 2014; Taylor et al. 2015; Tofangsazi et al. 2020; Martel et al. 2021). Hence, we may argue that a lower investment in egg hardness is necessary. As *G. gonikopalense* lives in sympatry with bagrada (Mahmood et al. 2015), it may have developed abilities towards its host and be able to parasitize buried bagrada eggs (Martel & Sforza 2021). Stinkbug species that invested in robust eggs may therefore be less suitable for *G. gonikopalense*.

However, this should not be the only explanation since parasitized NT species also lay robust eggs compared to bagrada. As none of tested non-target species live in sympatry with *G. gonikopalense*, the lack of chemical recognition of unknown host eggs by the parasitoid may have induced less parasitism on NT species. For instance, *Trissolcus simoni* and *Trissolcus brochymenae* can positively respond to chemical compounds of their natural hosts *Eurydema ventralis* and *Murgantia histriconica* respectively (Conti et al. 2004). Although both parasitoids can partially respond to chemicals emitted by a non-native host (*Nezara viridula*), the parasitism was residual and led to only few adult emergences (Conti et al. 2004). Hence, a better understanding of mechanisms driving host preference or specificity of *G. gonikopalense* could

improve its further use in biological control and predict its efficiency regarding NT species. In the current study, *G. gonikopalense* revealed a high flexibility in parasitizing eggs of different size, from the smallest ones (bagrada and *A. leucogrammes*) to the biggest (*C. mediterraneus* and *G. italicum*).

Our data on to stinkbug pests are contrasting. Thus, *Nezara viridula* was not attacked by *G. gonikopalense*, but more replicates are definitely needed as parasitism was observed in trials conducted in USA (Pickett et al. 2021). On the contrary, we can clearly state that *Halyomorpha halys* is an unsuitable host for *G. gonikopalense* since no parasitism was observed while the stinkbug emergence rate was high (78.7 ± 7.7%). Hence, *G. gonikopalense* would not help in attacking this pest if released, but may eventually attack *N. viridula*.

To conclude, the egg parasitoid *Gryon gonikopalense* was not exclusive to bagrada and was able to attack stink bugs from Carpocorini and Graphosomatini tribes in addition to Strachinii (bagrada). Species tested in USA did not include any Graphosomatini, or Capeini species, while our study did not include Asopinae or Nezarini species (Pickett et al. 2021). Our results are complementary to those conducted in USA, with a total of 20 species tested, belonging to 8 tribes. However, *G. gonikopalense* attacked less European than North American NT species (Pickett et al. 2021). In addition, species successfully parasitized in no-choice conditions were much less parasitized in presence of bagrada eggs (choice tests), indicating a preference for its natural host. Importantly, parasitized NT species included stinkbugs distributed in the Mediterranean basin like *A. leucogrammes* and *C. mediterraneus*. Also, more replicates would be necessary concerning *G. semipunctatus* which was not parasitized in our study but could be, since it shares the same genus than *G. italicum*. Similarly, choice tests should be performed with the critical species, *A. leucogrammes.* In case of parasitism by *G. gonikopalense*, it would prevent its introduction and release in southern Europe. Furthermore, studies on the dispersal ability of the parasitoid would complement host specificity tests. *Gryon*

gonikopalense is not known to fly, though wings are functional. The parasitoid was only observed walking, even during host foraging behaviour (Tofangsazi et al. 2020; Martel et al. 2021). When having the choice between bagrada eggs laid in the soil or on the plant, *G. gonikopalense* was mainly recorded parasitizing eggs in the soil (Martel et al. 2021). As a consequence, it can be hypothesized that it would mainly encounter bagrada eggs rather NT species laying eggs on upper parts of the foliage.

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Chapitre 6.

Discussion générale

L'objectif principal de mon travail de thèse a été de construire une base de connaissances nouvelles sur la biologie du scelionide *G. gonikopalense* afin de l'utiliser pour un programme de LBC contre bagrada aux Etats-Unis. Au-delà de cet aspect appliqué, mes recherches avaient également vocation à développer des connaissances sur les scelionides et en particulier le genre *Gryon.* Celles-ci demeurent en effet lacunaires tant du point de vue de la taxonomie, que de la physiologie et du comportement. Je me suis donc employé à structurer cette thèse autour d'expérimentations permettant de répondre à ces deux objectifs, en décrivant :

- Les similitudes rattachant *G. gonikopalense* à ses congénères (mêmes genre ou famille)
- Ce qui fait de ce parasitoïde un candidat « pas comme les autres » pouvant être valorisé dans un programme de LBC contre bagrada.

L'adéquation des performances physiologiques et comportementales de *G. gonikopalense* avec les critères de sélection d'un auxiliaire de LBC fera l'objet d'un **premier volet** de discussion. Dans un **second volet**, je proposerai une réflexion plus globale sur la faisabilité d'un programme de LBC aux USA avec *G. gonikopalense* et les implications d'une introduction. Dans un **troisième et dernier volet** je proposerai des perspectives de recherches qui permettraient, en s'appuyant sur les bases apportées par cette thèse, de consolider nos connaissances sur *G. gonikopalense* et d'aboutir le cas échéant à une pétition autorisant son lâcher dans l'aire invasive de bagrada. J'élargirai également la problématique de bagrada à son aire invasive européenne, particulièrement à Pantelleria en Italie, discuterai de la possibilité d'utiliser la LBC dans ce milieu insulaire.

1. La biologie de *G. gonikopalense* : quel bilan ?

1.1. Des traits physiologiques prometteurs mais insuffisants

Dans le **chapitre 2** nous avons vu que la physiologie de *G. gonikopalense* était proche des autres espèces du genre *Gryon* et plus largement des genre *Trissolcus* ou *Telenomus*, tant du point de vue du développement que de la fécondité. *Gryon gonikopalense* se développe rapidement (entre 11et 18 jours à l'optimum thermique) et chaque femelle peut produire plus d'une centaine d'individus dont près de 60% de femelles en moyenne. A titre de comparaison, *T. japonicus*, meilleur auxiliaire connu contre la punaise diabolique (*H. halys*) et introduit en 2020 à Zurich (Suisse), achève son développement en 12 jours à 25°C et chaque femelle peut produire près de 80 descendants dont plus de 70 % de femelles (Yang et al. 2018 ; Peverieri et al. 2020). *Gryon gonikopalense* est également une espèce longévive, capable de survivre plus deux mois jours dans la plupart des conditions thermiques et jusqu'à près de 150 jours en dessous de 20°C (**Article 2**). Là encore, les performances observées sont proches de *T. japonicus* (Peverieri et al. 2020).

Comme présentés dans la partie 2 de l'introduction, les critères de sélection d'un parasitoïde en LBC privilégient les espèces maximisant fécondité, sex-ratio et longévité, tout en minimisant le temps de développement. De ce point de vue, *G. gonikopalense* apparaît comme un candidat prometteur et

remplit complètement ces critères, de telle sorte que son taux d'accroissement maximum (0.38) (**Article 2**) est près de deux fois supérieur à celui de bagrada (entre 0.18 et 0.20) (Naryana & Phadcke 1987)

Nous pouvons toutefois questionner l'efficacité de *G. gonikopalense* à contrôler bagrada en considérant uniquement ces caractéristiques physiologiques. En effet, les performances mesurées ici sont inférieures à celles du principal auxiliaire utilisé en LBC contre les punaises pentatomidés : *Trissolcus basalis* (cf introduction). Ce parasitoïde présente une fécondité moyenne de 250 œufs / femelle, un temps de développement plus rapide que *G. gonikopalense* (seulement 10 jours à 27°C) et peut se développer à des températures inférieures à 18°C contrairement à *G. gonikopalense*. Malgré ces caractéristiques "optimales", *T. basalis* n'est pas parvenu à réguler efficacement *N. viridula* à Hawaii par exemple, et est encore régulièrement relâché en Amérique du Nord contre cette punaise (Abram et al 2020).

D'autre part, j'ai considéré que le principal facteur environnemental impliqué dans l'établissement de *G. gonikopalense* était la température du milieu, avec l'objectif d'une première description de l'optimum thermique du parasitoïde et de ses limites. Mais l'utilisation de températures constantes en incubateur est bien entendu une simplification exagérée de la réalité écologique (Colinet et al., 2015 ; Estay et al., 2014 ; Fischer et al., 2011). En milieu naturel, les organismes sont exposés à des températures qui fluctuent dans le temps (ex : variations nycthémérales) et dans l'espace (ex : mosaïque de conditions microclimatiques) (Colinet et al., 2015 ; Potter et al., 2013). Les conséquences de ces fluctuations sur les performances individuelles de l'espèce considérée dépendent donc à la fois de la température moyenne autour de laquelle la variabilité thermique s'opère et la sensibilité de l'espèce à ces variations thermiques en elles-mêmes (Bozinovic et al., 2011 ; Colinet et al., 2015 ; Estay et al., 2014). Des comparaisons de performance entre régimes constants ou fluctuants (intégrant la manière dont les fluctuations peuvent interagir avec différentes températures moyennes pour affecter la physiologie d'une espèce) pourraient donc conduire à des conclusions différentes quant à la réponse physiologique de *G. gonikopalense*. En outre, le facteur thermique n'influe jamais seul sur les organismes, et d'autres facteurs devraient être pris en compte pour évaluer les performances du parasitoïde dans son environnement comme l'humidité, la photopériode ou même la force du vent qui peuvent influencer la dispersion du parasitoïde (Canto-Silva et al. 2006a). Les résultats obtenus en laboratoire devront donc être confirmés en conditions de terrain, ce qui restera impossible tant que *Gryon gonikopalense* sera étudié hors de son aire native (*i.e*. Pakistan).

1.2. La recherche d'hôte, l'atout maître de *G. gonikopalense*

1.2.1. Une réponse comportementale adaptée à l'oviposition de bagrada

Dans le **Chapitre 3**, nous avons montré que l'élément clé de la relation hôte-parasitoïde entre bagrada et *G. gonikopalense* est le comportement de ponte de la punaise qui utilise le sol comme une barrière physique à d'éventuels agresseurs. L'**Article 4** met en évidence que l'oviposition de bagrada dans le sol est un comportement quasi-systématique (90% des œufs), qui ne dépend ni de la densité de

punaises, ni de la plante hôte sur laquelle elles se nourrissent. Cela confirme donc les observations « à vue » faites sur le terrain, notamment au Nouveau-Mexique (Taylor et al. 2014). Plus important encore, cela implique que les critères de sélection d'un auxiliaire doivent inclure la capacité à parasiter les œufs de bagrada dans le sol.

Le résultat majeur de mon travail de thèse a été la mise en évidence d'un tel comportement chez *G. gonikopalense* qui s'est montré capable de détecter et parasiter les œufs enterrés de bagrada (**Article 3 et vidéos associées)**. Ce comportement original de "foraging" s'est déclenché spontanément en conditions contrôlées. Il entraîne cependant une diminution du taux de parasitisme (par rapport aux œufs non-enterrés) puisque le temps de recherche d'œufs est augmenté. *Gryon gonikopalense* est capable de parasiter aussi bien des œufs couverts de particules de sable que des œufs nus, et trouve les œufs enterrés dans 100% des cas après 72 h (A**rticles 3 et 4**). L'originalité de *G. gonikopalense*, et son potentiel comme auxiliaire s'observe également par son comportement de recherche d'hôte. Comme je l'ai proposé dans le **Chapitre 3**, l'origine de ce caractère inné devrait être génétique, du moins en partie, et résulter d'une co-évolution avec bagrada. L'originalité de *G. gonikopalense* repose sur ce comportement de recherche d'hôte dont les caractéristiques ne sont pas partagées par les deux autres parasitoïdes collectés sur bagrada au Pakistan en 2014, *i.e*. *Trissolcus hyalinipennis* et *Ooencyrtus mirus* (Mahmood et al, 2015).

En effet, dans nos conditions de laboratoire, *T. hyalinipennis* échoue à parasiter les œufs de bagrada couverts de particules de sable (granulométrie = 425 µm), bien qu'ils ne soient pas enterrés (données non publiées et non présentées dans cette thèse). Dans un sol moins fin et homogène (40 % de particules > 1 mm, qui génèrent plus d'espace entre les particules de sol recouvrant les œufs), *T. hyalinipennis* n'attaque que les œufs en surface à un taux < 60% tandis que les œufs enterrés ne sont pas parasités (Tofangsazi et al. 2020). De son côté, *O. mirus* est inapte à parasiter les œufs enterrés de bagrada (Power 2020).

Dépourvus de ce comportement spécifique observé chez *G. gonikopalense*, *T. hyalinipennis* et *O. mirus* s'avèrent être également plus généralistes (Pickett et al. 2021 ; Power et al. 2020). En effet, *O. mirus* est capable de parasiter les familles de punaises Rhopalidae et Coreidae et même *Helicoverpa zea* Boddie (Noctuidae), un lépidoptère attaquant le coton et la tomate notamment. *Trissolcus hyalinipennis* peut quant à lui parasiter les mêmes espèces que *G. gonikopalense*, mais à des taux plus élevés : *Euschistus conspersus* est par exemple parasité dans 0.9 % des cas par *G. gonikopalense* et dans 36 à 57 % des cas avec *T. hyalinipennis* (Pickett et al. 2021). Ainsi, la spécialisation comportementale de *G. gonikopalense* pourrait permettre de contrebalancer un éventuel manque de spécificité obtenu en laboratoire, duquel on peut en outre discuter (ce que nous verrons ultérieurement).

1.2.2. Du laboratoire au terrain : recul méthodologique

Comme pour la description de la physiologie de *G. gonikopalense*, il est important de garder à l'esprit les conditions contrôlées dans lesquelles ce comportement prometteur a été décrit. On peut

notamment questionner dans quelle mesure l'utilisation d'un sable fin comme substrat pour bagrada est représentatif de la réalité du terrain, à la fois du point de vue de son oviposition dans le sol et du parasitisme de *G. gonikopalense.* En effet, différents types de sols (plus ou moins argileux, plus ou moins sec, granulométrie plus ou moins fine, etc.) influencent l'oviposition de bagrada (Bundy et al. 2019 ; Tofangsazi et al. 2020). En Californie cependant, le sol des cultures de brassicacées est composé de 60-70 % de sable avec 60 % de particules inférieures à 1 mm dans certaines localités, et aucune particule supérieure à 2mm (Tofangsazi et al. 2020). Le sable utilisé pour cette thèse était quant à lui homogène (granulométrie = 425 µm) permettant de standardiser les expérimentations à la fois en boîte de Pétri (A**rticle 3**) et dans un environnement tri-trophique en trois dimensions (**Article 4**). Bien entendu, je ne peux pas exclure que son utilisation ait facilité l'oviposition de bagrada dans notre système expérimental qui aurait pu varier avec un autre sol. Cependant, certaines populations de bagrada vivent réellement dans des environnements sablonneux comme dans les fermes de rooibos en Afrique du Sud, les cultures de câpriers au Maroc (Sforza R, comm. pers.) ou sur l'île de Pantelleria en Italie où j'ai pu constater que la granulométrie est très proche de celle que nous avons utilisée.

Contrairement à bagrada, le sable fin et sec utilisé dans cette thèse a probablement été une contrainte plus importante pour le parasitisme de *G. gonikopalense* puisque les œufs étaient parfaitement enterrés et protégés. Sur le terrain, des anfractuosités ou des particules plus grossières sont communes et les œufs auraient été plus accessibles au parasitoïde. *Gryon gonikopalense* est d'ailleurs capable de parasiter bagrada de manière équivalente dans trois sols différents issus de champs de brocoli en Californie qui diffèrent par leur granulométrie ou leur taux d'argile (Tofangsazi et al. 2020).

1.2.3. La recherche d'hôte : un processus complexe

Au-delà de la simple relation entre bagrada et *G. gonikopalense,* nos conditions de laboratoire ne permettent pas de prendre en compte la complexité du processus de recherche d'hôte du parasitoïde et du « détour infochimique » qu'il doit effectuer pour trouver les œufs de bagrada (cf introduction). Ainsi, leur détection dans un environnement naturel devrait reposer dans un premier temps sur l'utilisation de signaux chimiques peu spécifiques émis par la plante-hôte tels que le brassicadiene, un diterpène émis par les brassicacées en réaction à une attaque d'un insecte phytophage et attractif pour bagrada (Guarino et al. 2018 ; Arriola et al. 2020). Dans un second temps, *G. gonikopalense* devrait utiliser des signaux spécifiques émis par bagrada (adultes ou œufs) (**Article 3**) comme cela a été montré chez *T. basalis* utilisant le n-nonadécane émis par *N. viridula* ou *T. japonicus* utilisant le n-tridecane émis par *H. halys* (Colazza et al. 2007 ; Zhong et al. 2017). Dans notre étude, ces composés n'ont pas été identifiés chimiquement mais pourraient par exemple être le (E)-2-octenyl acétate, une phéromone sexuelle émise par les adultes bagrada (Guarino et al. 2008 ; Arif et al. 2020).

La recherche d'hôte devrait également dépendre des capacités de dispersion de *G. gonikopalense*. Contrairement à *T. hyalinipennis* (observations personnelles) ou *O. mirus* (Power 2020), *G. gonikopalense* n'a jamais été vu en train de voler, ni dans nos boîtes d'élevage, ni dans le système

tri-trophique mis en place (**Article 4**). Cette absence de vol a été par ailleurs confirmée par Tofangsazi et al. (2020). Le vol est pourtant actif chez d'autres espèces de *Gryon* (Romeis et al. 2000 ; Dasilao & Arakawa 2005 ; Canto-Silva et al. 2006a) et plus largement chez les Scelionidae (Farrow et al. 1981 ; Vogt & Nechols 1991 ; Talamas et al. 2015). De plus, les ailes de *G. gonikopalense* sont musculairement fonctionnelles, puisque j'ai pu observer à plusieurs reprises des femelles en phase d'oviposition battre frénétiquement des ailes à intervalles plus ou moins régulier. Cette apparente absence de vol, d'un point de vue comportemental, et non morphologique, pourrait donc être une adaptation liée à son comportement de recherche d'hôte au sol et souterrain. Le déplacement de *G. gonikopalense* par la marche pourrait en outre être un facteur important dans son mode de dissémination sur le terrain, et donc dans son efficacité à contrôler bagrada au champ. En effet, la capacité de dispersion joue un rôle dans la stabilisation des dynamiques populationnelles des insectes (Stein et al., 1994 ; Canto-Silva et al. 2006a), notamment dans le cas de lâchers en LB inondative (Saavedra *et al*., 1997 ; McDougall & Mills, 1997), mais aussi pour l'établissement durable des insectes introduits (Hopper & Roush, 1993). Toutefois, une importante dispersion chez un parasitoïde n'est pas forcément corrélée à son efficacité comme auxiliaire : une forte dispersion peut en effet nuire à l'efficacité du contrôle, comme cela a été montré pour *Telenomus podisi* lâché au Brésil contre *Euschistus heros*, et dont l'importante dispersion a nécessité environ 5000 individus / ha à chaque lâcher (Correa-Ferreira 2002 ; Conti et al. 2021). Une faible dispersion peut également être synonyme de succès, en augmentant alors la densité du maillage des lâchers, comme pour *Neodryinus typhlocybae* Ashmead (Hym., Dryinidae) sur *Metcalfa pruinosa* Say (Hem., Flatidae) (Malausa et al 2006)

1.2.4. <u>Implications pour la dynamique hôte-parasitoïde et sur le contrôle de bagrada :</u>

Le fait que bagrada ponde quasi-systématiquement ses œufs dans le sol implique une barrière physique qui joue un rôle de refuge pour la punaise en limitant l'accès aux ennemis naturels, dont *G. gonikopalense*. Or, l'existence d'un refuge pour un ravageur est un des éléments favorisant théoriquement un point d'équilibre dans la dynamique populationnelle hôte-parasitoïde (Fernandez-arhex & Corley 2003), bien que cela soit discuté dans la communauté scientifique (Murdoch & Briggs 1996). La protection des œufs de bagrada pourrait impliquer un parasitisme densité-dépendant (Murdoch & Briggs 1996) puisqu'à faible densité ils seraient potentiellement plus difficiles à détecter. Le relâchement de la pression parasitaire de *G. gonikopalense* permettrait à la population de bagrada de se maintenir à faible effectif puis d'augmenter à nouveau. D'un autre côté, on peut supposer que la spécialisation comportementale de *G. gonikopalense* lui permettrait de contrôler plus efficacement bagrada par rapport à des parasitoïdes comme *O. mirus*. A faible densité d'hôte, *G. gonikopalense* serait ainsi capable de se maintenir dans l'environnement et d'accroître à nouveau sa population une fois que l'effectif de bagrada est devenu plus important.

1.3. Production d'auxiliaire en laboratoire :

J'ai présenté au **Chapitre 1** les problématiques et les enjeux liés à la production d'un auxiliaire en laboratoire pour un programme de LBC. Dans la partie « **Préambule technique** » et le **Chapitre 4**, j'ai montré qu'une optimisation de la production de bagrada et *G. gonikopalense* en laboratoire était possible. Premièrement, la prise en compte de l'oviposition particulière de la punaise a mené à développer une méthode adaptée pour concentrer ses pontes dans les cages d'élevage, et faciliter leur collecte régulière. Cela a permis de multiplier par trois le nombre d'œufs utilisables quotidiennement (900 œufs/ jour aujourd'hui en moyenne avec deux cages d'élevage). Dans la quarantaine EBCL, le nombre de colonies de bagrada pourrait être augmenté pour atteindre facilement une production de plus de 100 000 œufs / mois, mais nécessiterait des installations dédiées. Deuxièmement, les œufs de bagrada peuvent être stockés au froid (idéalement à 5°C) tout en étant correctement parasités par *G. gonikopalense* même après 3 semaines. Au-delà, les oeufs de bagrada étaient majoritairement refusés par le parasitoïde. Sur ce point, *G. gonikopakense* s'est montré relativement sélectif, en comparaison de *Trissolcus japonicus* par exemple, qui est capable de parasiter des œufs de *H. halys* stockés à 8°C pendant 2 mois, avec seulement 9.6% de réduction du taux d'émergence (Wong et al. 2021). Toutefois, aucun effet létal ou sub-létal n'a été observé sur la descendance de *G. gonikopalense*. En revanche, les faibles taux de parasitismes observés sur des œufs congelés à –80 °C et -20 °C A**rticles 1 et 5)** nous ont amené éliminer l'utilisation de la congélation comme méthode de stockage des œufs pour l'élevage de *G. gonikopalense*. Etant donné que les œufs parasités se développaient normalement, il est probable que la congélation entraîne un refus de *G. gonikopalense* (barrière pré-ovipositionnelle) plutôt qu'un effet sur sa descendance (barrière post-ovipositionnelle). Une explication possible est la perte de signaux chimiques, ou une dénaturation de la structure du chorion de l'œuf nécessaires à la reconnaissance de l'œuf par le parasitoïde (Zachariassen & Hammel 1976 ; McIntosh et al. 2019). Ces œufs congelés pourraient toutefois être utilisés en dernier recours puisque le taux de parasitisme a été le même après 1 semaine ou 3 mois de stockage. Ces résultats questionnent également la pertinence d'utiliser des œufs sentinelles congelés pour échantillonner les populations de parasitoïdes dans l'aire native et invasive de bagrada. Si la congélation des œufs entraîne un important refus de la part de certaines espèces de parasitoïdes, y compris *G. gonikopalense*, cela pourrait entraîner un biais d'échantillonnage et sous-estimer la diversité de parasitoïdes dans l'environnement comme cela est le cas avec des œufs trop âgés (Jones et al. 2014).

Enfin, la question du transport et du stockage des parasitoïdes devra également se poser si *G. gonikopalense* peut être lâché aux Etats-Unis. La plupart des macroorganismes ne peuvent pas être stockés sur de longues durées, ne résistent pas bien au transport et aux fluctuations de température associées (Fauvergue et al. 2020). J'ai toutefois effectué au cours de ma thèse des envois répétés de G. gonikopalense pour nos collaborateurs de l'USDA en Californie laboratoire ISPH. Les parasitoïdes ont été envoyés sous forme d'ooplaques d'œufs parasités de bagrada, et le taux d'émergence était de plus de 80% à la réception. Les durées de développement obtenues en fonction de différents régimes

thermiques (A**rticle 2**) permettent en outre de ralentir ou d'accélérer le développement de *G. gonikopalense* en fonction des besoins, sans que les individus subissent d'effets négatifs. Cela pourrait également servir à synchroniser l'émergence de plusieurs ooplaques parasitées sur le terrain pour maximiser la concentration de *G. gonikopalense*.

1.4. Effets non-intentionnels : spécificité physiologique de *G. gonikopalense*

Dans un programme de LBC, deux critères sont évalués pour valider un agent de lutte : son impact et sa spécificité. Dans le cas de *G. gonikopalense*, l'impact est vérifié, reste alors à valider sa spécificité d'hôte. Comme il a été mentionné précédemment, *G. gonikopalense* présente l'avantage d'être plus spécialiste de bagrada que *T. hyalinipennis* et *O. mirus,* du point de vue de son comportement. Cependant, nous avons montré dans le **Chapitre 5** que *G. gonikopalense* n'était pas tout à fait spécifique de bagrada. Bien que les tests de spécificité présentés dans ce chapitre aient été menés sur des espèces absentes du continent nord-américain, les résultats obtenus complémentent la liste des espèces non-cibles testées en Californie (Pickett et al. 2021). De plus, ils s'inscrivent dans une démarche d'anticipation vis-à-vis de l'arrivée possible de bagrada sur le continent européen, inspirée des travaux menés sur *T. japonicus* en Nouvelle-Zélande (Charles et al. 2019 ; Saunders et al. 2021). Ensemble, les tests européens et californiens, ont montrés qu'au moins 11 espèces de pentatomidés pouvaient être parasités par *G. gonikopalense* sur 20 espèces testées. En Californie, sept espèces ont été parasitées en conditions de non-choix, dont une espèce d'intérêt *Podisus maculiventris,* tandis qu'en Europe, quatre espèces de pentatomidés ont été parasitées (Tableau 6) Ces résultats peuvent être discutés sur deux aspects.

Tableau 6 : Liste des espèces non-cibles Californiennes et Européennes utilisées pour tester la spécificité de *Gryon gonikopalense* pour *Bagrada hilaris*

Origine	Espèce	Tribu	Parasitisme
Californie	*Podisus maculiventris*	Asopinae	Oui
	Holcostethus sp.	Carpocorini	Oui
	Chlorochroa ligata	Nezarini	Non
	Chlorochroa uhleri		Non
	Banasa sordida	Pentatomini	Oui
	Euschistus conspersus		Oui
	Euschistus servus		Non
	Thyanta pallidovirens		Oui
	Murgantia histrionica	Strachinii	Oui
Europe / Californie	*Nezara viridula*	Pentatomini	Oui
Europe	*Halyomorpha halys*	Capeini	Non
	Carpocoris mediterraneus	Carpocorini	Oui
	Dolycoris baccarum		Oui
	Ancyrosoma leucogrammes	Graphosomatini	Oui
	Graphosoma italicum		Oui
	Graphosoma semipunctatum		Non
	Eurydema oleracea	Strachinii	Non
	Eurydema ornata		Non
	Eurydema ventralis		Non
	Liorhyssus hyalinus	Rhopalidae	Non

1.4.1. Quel crédit apporter aux tests de choix ou de non-choix ?

La méthodologie des tests de choix et de non-choix, souffrent par essence d'un manque de transposabilité à la réalité du terrain. En effet, les tests de non-choix menés sans tests de choix ont tendance à surestimer la gamme d'hôte potentielle d'un parasitoïde car ce dernier est « forcé » d'attaquer l'espèce non-cible dans des conditions contrôlées (Van Driesche & Murray 2004). Les tests de choix seuls peuvent quant à eux produire des faux négatifs qui montreraient effectivement une préférence pour l'hôte principal, mais passeraient à côté du risque de parasitisme de l'espèce non-cible en absence de l'hôte principal (Van Driesche & Murray 2004 ; Murray et al. 2010). Pour cette raison, les résultats préliminaires obtenus en Californie (non-choix seul) restent discutables dans leur représentation de la réalité. Les tests menés sur les espèces européennes (choix et non-choix) seraient plus robustes de ce point de vue.

Nous pouvons en outre nous questionner sur le risque réel de rencontres entre *G. gonikopalense* et les espèces non-cibles en Californie (et en Europe). Comme je l'ai évoqué dans la partie 1.2, on peut supposer que la spécialisation comportementale de *G. gonikopalense* pourrait en réalité permettre de contrebalancer son manque de spécificité observé en laboratoire (facteur négatif pour envisager son lâcher en Californie). De plus, la plupart des espèces non-cibles se développent sur des plantes-hôtes différentes de bagrada, qu'elles soient sauvages comme la carotte ou le fenouil (*G. italicum* et *A.*

leucogrammes), cultivées comme les haricots (*E. conspersus)*, ou des arbres comme le noyer ou le Cyprès de Lambert (*T. pallidovirens* et *B. sordida*).

Bien que *G. gonikopalense* soit capable de se déplacer en hauteur sur les plantes consommées par des hôtes potentiels (**Article 4**), un déplacement se limitant majoritairement au sol par la marche pourrait entraîner un évitement de certaines espèces comme *B. sordida* ou *T. pallidovirens* qui se développent dans les arbres. En outre, si la marche implique une dispersion moins efficace que le vol, le risque réel pour *G. gonikopalense* de se retrouver dans des cultures très différentes des brassicacées où prolifèrent d'autres pentatomidés est questionnable. La rencontre d'hôtes alternatifs pourrait toutefois être envisagée en cas de dispersion non-intentionnelle (par le vent comme *Scelio fulgidus* Crawford par exemple (Farrow 1981)) ou dans le cas d'une rotation de culture sur la même parcelle, avec des ombellifères ou des légumineuses remplaçant les brassicacées sans destruction du précédent cultural.

1.4.2. Sélectionner les espèces à tester : quelles limites ?

Un deuxième point de discussion est la pertinence des critères de sélection des espèces non-cibles à tester, et la limite qui a été fixée dans cette thèse (similaire à ce qui a été fait pour *T. japonicus* par exemple (Lara et al. 2019)). Les tests de spécificités reposent généralement sur la méthode de la phylogénie centrifuge (Wapshere 1974 ; Abram et al. 2021). Son principe est de proposer à l'auxiliaire candidat des hôtes phylogénétiquement de plus en plus éloignés de l'espèce cible à contrôler afin d'en estimer l'amplitude taxonomique la plus large. Pour *G. gonikopalense*, le plus bas niveau taxonomique testé a été la tribu des Strachinii, comportant *M. histrionica* (Californie), *Eurydema ornata*, *E. oleracea* et *E. ventralis* (**Article 6**), tandis que le plus haut niveau taxonomique testé a été la famille avec une espèce de Rhopalidae (*Lyorhissus hyalinus*). L'hypothèse que la gamme d'hôte est déterminée par la phylogénie est en effet supportée par plusieurs études chez les insectes herbivores ou entomophages (Desneux et al., 2012, Haye et al., 2015 ; Hinz et al. 2020, Paynter et al., 2020).

Cependant, d'autres facteurs comme la niche écologique de l'hôte (sa plante hôte notamment) peuvent être plus importants que la phylogénie (Kulmann et al. 2006). D'autres facteurs comme l'intérêt patrimonial, ou à l'inverse le statut de ravageur d'une espèce (comme *H. halys* ou *N. viridula*) peuvent conduire à la tester. Hormis des espèces prédatrices comme *P. maculiventris,* il existe peu d'espèces de pentatomidés d'intérêt pour l'humain et les espèces peu communes, rares ou endémiques sont généralement peu connues, difficile à collecter ou à élever et donc à tester. Contrairement aux végétaux, ou à des insectes plus "populaires" comme les lépidoptères ou les coléoptères, le statut « d'espèce menacée ou patrimoniale » n'existe pas chez les punaises. D'ailleurs ne les appelle-t-on pas « stink bugs » en anglais (insectes qui puent) ? A titre d'exemple, la liste des insectes protégés sur le territoire Français n'inclue aucune espèce de l'ordre des hémiptères (www.legifrance.gouv.fr) alors même que des espèces extrêmement localisées ou rares mériteraient peut-être cette reconnaissance, y compris au sein des pentatomides comme *Eurydema sea* Péricart & De la Rosa (Sannier 2019)

Les résultats obtenus (Californie et Europe) n'ont pas permis de mettre en évidence la prévalence d'un critère en particulier. Pour s'assurer que *G. gonikopalense* est bien restreint aux

pentatomidés, d'autres familles généralement parasitées par le genre *Gryon* devraient être testées comme les Coreidae (*Leptoglossus* sp. attaqué par *G. pennsylvanicum* par exemple (Peverieri et al. 2012)). Au sein des pentatomidés, la prévalence du critère phylogénétique et écologique n'est pas évidente puisque *E. ventralis*, *E. oleracea* et *E. ornata*, trois espèces de Strachinii (proximité phylogénique), consommant des brassicacées (proximité écologique) n'ont pas été parasitées par *G. gonikopalense*. A l'inverse, des espèces d'autres tribus, consommant des plantes de familles différentes ont été parasitées (*G. italicum* sur fenouil par exemple). Même des critères liés à la morphologie des œufs ou des ooplaques (taille, forme) ne semblent pas non plus permettre de dresser des tendances sur les espèces parasitées par *G. gonikopalense* : des espèces de grande taille (*C. mediterraneus*) ont été parasitées aussi bien que des espèces de petite taille (*A. leucogrammes*), et des espèces pondant de larges ooplaques (*H. halys*) n'ont pas été parasitées par rapport à des espèces pondant des ooplaques plus petites (*A. leucogrammes*). Au vu de ces données préliminaires, il me semble qu'aucune conclusion ne peut encore être avancée sur le degré de spécificité d'hôtes de *G. gonikopalense*. En outre, il me paraitrait judicieux d'approfondir ce volet avec davantage d'espèces européennes à la lumière du questionnement ci-dessus.

2. Mise en place de la LBC en Californie : un projet réaliste ?

2.1. Modèle agricole vs lutte biologique classique

Dans les grandes cultures, la mise en place de LBC ou LB inondative est rare car les coûts sont beaucoup plus importants que les traitements phytosanitaires (Fauvergue et al. 2020). En France par exemple, le seul macroorganisme utilisé sur plus de 100 000 ha est le trichogramme *Trichogramma brassicae* Bezdenko contre la pyrale du maïs, dont la production (entre 30 et 50 €/ha) est compatible avec les contraintes économiques de certains systèmes de production (Fauvergue et al. 2020). Pour des surfaces plus faibles, et surtout confinées comme des serres, les producteurs peuvent au contraire arriver à combiner des macroorganismes pour lutter contre la plupart des ravageurs (Fauvergue et al. 2020).

En Californie, la surface moyenne d'une parcelle de brocoli est d'environ 520 ha (Takele 2001) et la plupart des cultures sont traitées avec des produits phytosanitaires divers correspondant au grand nombre de ravageurs de brassicacées qui existent (cf Introduction, **IV. 4.2.1**). Pour la période 2019-20, les cultures biologiques de brocoli en Californie représentaient 10 % de la surface totale de brocoli produite (Ross 2020). Les plus grandes d'entre elles, comme dans le Comté de Monterey ou l'Imperial Valley sont toutefois basées sur un modèle de production intensif en monoculture et sur de grandes surfaces (Figure 35A, Kremen et al. 2012). Dans un tel contexte agronomique, la faisabilité d'un programme de LBC contre bagrada n'est donc pas une évidence. Cependant, sur les 44 comtés de Californie produisant le brocoli en agriculture biologique, 31 cultivent moins de 10 ha de brocoli comme les Comtés de Sacramento ou San diego (Ross 2020). Dans ces parcelles de petites tailles (Fig. 35B, C), le lâcher d'auxiliaires comme *G. gonikopalense* serait beaucoup plus envisageable, de même que le suivi de la dynamique populationnelle du parasitoïde, mais également la combinaison de méthodes de gestion comme les cultures pièges ou l'utilisation de phéromones.



Figure 35: (A) Parcelle de brocoli en agriculture biologique de grande taille dans la Salinas Valley (Californie, USA) ; (B-C) parcelles de brocoli et de chou de petites tailles dans une ferme bio-urbaine de Sacramento (Californie, USA) attaquée par *Bagrada hilaris* en 2016. Photo A tirée de Kremen et al. 2012 ; Photos B et C par Sforza R.F.H.

2.2. Etablissement de *G. gonikopalense* en Californie : quelles perspectives ?

2.2.1. Acclimatation du parasitoïde

Le climat de la zone géographique où l'on souhaite mettre en place un programme de LBC peut être un facteur d'échec ou de succès (Hokkanen & Sailer 1985). Puisqu'ils sont ectothermes, les parasitoïdes ont plus de facilités à s'établir dans un écosystème thermiquement stable, à la fois à l'échelle d'une journée, mais aussi au cours de l'année. C'est pourquoi la plus grosse proportion de succès en LBC est enregistrée dans les régions à climat méditerranéen comme la côte ouest des USA et le sud-ouest de l'Australie, ou à climat tropical (température constante toute l'année) comme en Amérique du sud et en Asie du sud-est (Hokkanen & Sailer 1985).

Au regard de l'optimum thermique de *G. gonikopalense* et de ses capacités de développement et de reproduction (A**rticle 2**), il est tout à fait envisageable qu'il s'établisse dans les régions les plus chaudes de l'aire invasive aux USA (Los Angeles et Imperial Valley en Californie ; Arizona ; Texas) qui présentent des températures moyennes hivernales au-dessus de 12 °C. En revanche, *G. gonikopalense* devrait avoir des difficultés dans les zones plus tempérées de la côte ouest, notamment dans le comté de Monterey en Californie où les températures entre novembre et mars ne permettent pas son développement (**Chapitre 2**). En Outre, l'hypothèse de l'établissement de *G. gonikopalense* en Californie est soutenue par la très récente introduction et l'établissement accidentel de *T. hyalinipennis* près de Los Angeles (Ganjisaffar et al. 2018). Pour rappel, *T. hyalinipennis* et *G. gonikopalense* sont originaires des mêmes localités au Pakistan (Mahmood et al. 2015).

2.2.2. Dynamiques populationnelles

Concernant la dynamique populationnelle (spatiale et temporelle) de *G. gonikopalense* en Californie, de très nombreuses inconnues subsistent. Aucune donnée n'existe à ce jour et des captures régulières du parasitoïde dans son aire native (Pakistan) à l'aide d'œufs sentinelles sur une année complète seraient nécessaires. Les optima thermiques de bagrada et *G. gonikopalense* suggèrent des périodes d'activité similaires (**Article 2**). Comme son hôte, le parasitoïde serait principalement actif en journée (températures les plus élevées), et des pics démographiques devraient avoir lieu entre mars et octobre (températures supérieures à son seuil de développement). La collecte du parasitoïde au mois de mai au Pakistan va dans ce sens (Mahmood et al. 2015). En prenant en compte les résultats présentés dans le **Chapitre 2**, nous pouvons toutefois proposer une estimation du nombre de générations effectuées par *G. gonikopalense* dans l'Imperial Valley en Californie, (Figure 36). En considérant que le développement est impossible entre décembre et février, le parasitoïde pourrait effectuer environ 17 générations, avec un pic démographique entre juin et août où les températures moyennes journalières correspondent à son optimum thermique la plupart du temps.

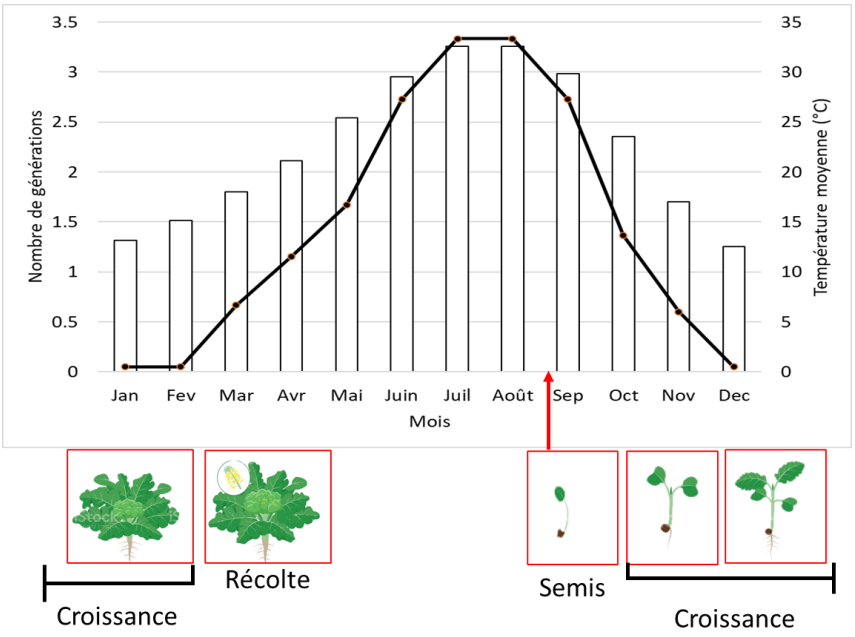


Figure 36 : Dynamique temporelle théoriques de *Gryon gonikopalense* dans les cultures de brocoli de l'Imperial Valley (Californie, USA). Les barres verticales représentent la température moyenne mensuelle pour l'année 2020 (usclimatedata.com). La courbe noire en trait plein représente le nombre moyen de générations de *G. gonikopalense* par mois (calculé d'après les données de **l'Article 2**)

Cependant, le nombre de générations possibles dépend de nombreux facteurs, notamment la dynamique populationnelle de bagrada. Or, comme nous l'avons vu en introduction, cette dernière présente deux principaux creux en été (période post-récolte du brocoli et disparition de la plante hôte) et en hiver (températures trop basses, hivernation) On peut donc en réalité s'attendre à ce que *G. gonikopalense* ait la même dynamique populationnelle aux mêmes périodes. Plusieurs scénarii selon la saison peuvent être alors envisagés quant au maintien du parasitoïde dans l'environnement :

En hiver, aucun développement n'est possible pour *G. gonikopalense* et sa reproduction devrait également être faible voire nulle (**Chapitre 2**) sur bagrada ou de potentiels hôtes alternatifs. Deux scénarii sont alors possibles pour les populations du parasitoïde :

- Elles ne survivent pas à une hivernation et meurent.
- Elles hivernent et peuvent attaquer bagrada quand les températures remontent. L'hivernation au stade adulte a été décrite chez plusieurs espèces de scelionides du genre *Telenomus* et *Trissolcus*, qui survivent généralement dans la litière ou les anfractuosités du sol (Torgersen et al. 1981 ; Orr 1988 ; Lowenstein et al. 2019). Elle devrait donc être envisageable pour *G. gonikopalense*, notamment en considérant un mode de vie essentiellement au sol.

En été, la ressource limitante pour *G. gonikopalense* est son hôte qui se déplace des zones de cultures récoltées vers des plantes hôtes alternatives. La question est donc de savoir si les populations de *G. gonikopalense* vont suivre leur hôte et cinq scénarii sont envisageables :

- Elles suivent bagrada et se maintiennent sur cet hôte principal.
- Elles suivent bagrada et se maintiennent à la fois sur cet hôte principal et sur des hôtes alternatifs.
- Elles ne suivent pas bagrada et estivent au stade adulte.
- Elles ne suivent pas bagrada et meurent au cours de l'été.
- Elles ne suivent pas bagrada mais se maintiennent sur des hôtes alternatifs.

Comme nous l'avons vu dans les sections précédentes de cette discussion, des connaissances supplémentaires sur les capacités de dispersion du parasitoïde sont nécessaires pour estimer la vraisemblance de chaque scénario. De même, plusieurs pentatomidés californiens seraient susceptibles de servir d'hôte au parasitoïde, mais il est difficile à ce stade de savoir s'ils seraient véritablement exploités en conditions naturelles. Il est également envisageable que même dans le cas de la mort du parasitoïde au cours de l'hiver (ou de l'été), il y aurait la possibilité de réaliser deux lâchers au début de chaque pic de population de bagrada en suivant le cycle de la culture (Fig. 36) : un premier au moment des semis de septembre, et un deuxième juste avant le printemps. Le programme de LBC deviendrait alors un programme de LB inoculative.

2.2.3. Sources de nourriture : un prérequis pour *G. gonikopalense*

Nous avons vu au **Chapitre 2** (**Article 1**) que *G. gonikopalense* ne pouvait pas vivre plus de 8 jours sans nourriture en moyenne, et qu'un apport en eau seul n'était pas suffisant. Il est donc nécessaire qu'une source de nourriture soit disponible à proximité des cultures où le parasitoïde serait lâché. La piqûre de bagrada entraîne généralement l'apparition de gouttes de sève sur sa plante hôte qui pourraient être consommées par *G. gonikopalense*, mais ce sont principalement les plantes à nectar qui pourraient servir à l'alimentation du parasitoïde comme cela a été montré chez *T. basalis* ou *Telenomus laeviceps*

se nourrissant sur le sarrasin (*Fagopyrum esculentum* Moench) ou le bleuet (*Centaurea cyanus* L.) (Rahat et al. 2005 ; Foti et al. 2017 ; Barloggio et al. 2019) ou encore la coriandre et l'aneth qui représentent une source de nectar permettant d'allonger considérablement la durée de vie de *T. japonicus* (McIntosh et al. 2020).

2.2.4. « Des » parasitoïdes contre bagrada : quelle place pour *G. gonikopalense* ?

L'introduction de *G. gonikopalense* en Californie devrait prendre en compte la présence d'autres parasitoïdes de bagrada identifiés après leur capture à l'aide d'œufs sentinelles (Ganjisaffar et al. 2018 ; Felipe-Victoriano et al. 2019 ; Lomeli-Flores et al. 2019 ; Ganjisaffar & Perring 2020 ; Ganjisaffar et al. 2020). Ces parasitoïdes n'ont cependant jamais été confrontés à bagrada auparavant et ont évolué avec des punaises pondant leurs œufs sur leur plante hôte. Le fait qu'ils parasitent des œufs sentinelles non enterrés peut relever d'une forme d'opportunisme (c'est notamment le cas d'*Idris elba*, un parasitoïde d'araignées (Lomeli-Flores et al. 2019)) et on peut supposer que ces parasitoïdes ne seraient pas des compétiteurs sérieux pour *G. gonikopalense*.

L'introduction récente de *T. hyalinipennis* en Californie (Ganjisaffar et al. 2018) soulève quant à elle la question d'une possible compétition avec *G. gonikopalense*. En conditions de laboratoire et sur des œufs non-enterrés, un évitement réciproque des œufs déjà parasités par l'autre parasitoïde a été observé chez *G. gonikopalense* et *T. hyalinipennis* (Hougardy & Hogg 2021). La coexistence de deux parasitoïdes qui exploitent le même hôte est généralement dû à deux stratégies qui se contrebalancent mutuellement (Cusumano et al. 2012 ; Cingolani et al. 2013, Mohammadpour et al. 2014, Peri et al. 2014) : l'un des parasitoïdes est un meilleur compétiteur extrinsèque (meilleures capacités dispersion, de recherche d'hôte), généralement un spécialiste comme semble l'être *G. gonikopalense.* L'autre est un meilleur compétiteur intrinsèque (temps de développement plus court, fécondité plus importante), plus généraliste comme semble l'être *T. hyalinipennis.*

Cependant, du fait que *G. gonikopalense* soit le seul parasitoïde connu pour parasiter efficacement les œufs enterrés de bagrada, il est possible qu'il n'ait pas de compétiteurs en Californie. En conditions naturelles, *G. gonikopalense* et *T. hyalinipennis* pourraient alors cohabiter dans deux niches écologiques différentes et ne pas entrer en compétition. Nous pouvons même imaginer que *G. gonikopalense* serait le principal parasitoïde des œufs de bagrada dans le sol, tandis que *T. hyalinipennis* ou d'autres espèces natives pourraient attaquer la portion d'œufs pondue sur les plantes ou des supports inertes ne conférant pas de protection particulière.

Perspectives de recherche

Au cours de cette thèse, j'ai abordé un grand nombre d'aspects de la biologie de *G. gonikopalense* et de sa relation avec bagrada, mais également des aspects plus techniques de sa production en laboratoire. Mon travail a donc ouvert de nombreuses portes, et je présenterai dans cette dernière section les multiples perspectives de recherches qui en découlent.

1. L'identité de *G. gonikopalense*

La phylogénie du genre *Gryon* est très complexe et mal connue. La détermination des espèces passe par l'identification de caractères morphologiques qui divergent parfois avec les outils moléculaires (barcoding). Il est donc difficile de comparer un nouveau spécimen découvert à une base de données établie ou à des spécimens de référence parfois en mauvais état, ce qui est le cas de l'holotype de *G. gonikopalense* (Sharma 1982). Ce dernier fait également partie d'un complexe d'espèces, le complexe myrmecophilum nommé d'après *Gryon myrmecophilum* Ashmead. La délimitation des espèces au sein de ce complexe pose des problèmes pour l'identification claire de *G. gonikopalense*, qui pourrait être une espèce cryptique, ou une sous-espèce. Son identité n'est donc pas définitive. Le nom *Gryon gonikopalense* utilisé au cours de cette thèse et dans les articles publiés est la première occurrence dans la littérature scientifique depuis sa description. Ce nom a également été utilisé par Tofangsazi et al. 2020 (deuxième occurrence), mais commence à être publié sous le nom *Gryon* sp. near *gonikopalense* (Hougardy & Hogg 2021). C'est par souci d'homogénéité entre les différentes publications présentées dans cette thèse que nous avons choisi de conserver l'appellation initiale. Cependant, *G. gonikopalense* deviendra dans un futur proche un synonyme du nouveau nom qui sera donné. Une nouvelle clé de détermination des espèces paléarctiques du genre *Gryon* est en cours d'édition, et devrait permettre de grandement simplifier leur identification comme cela a été fait récemment pour le genre *Trissolcus* (Talamas et al. 2017).

2. Inclure la LBC dans une gestion intégrée :

Comme nous l'avons vu en introduction, l'emploi de la LBC est difficilement envisageable comme unique méthode de contrôle des punaises invasives, et son succès repose notamment sur la mise en pratique d'autres moyens de gestions intégrés des cultures. Le bilan de nombreuses pistes de recherches sont évoquées dans **l'Article 7 (Annexe 1).** En ce qui concerne la gestion intégrée de bagrada, la plupart des pistes ont été présentées en introduction, comme les pratiques culturales visant à limiter l'installation de la punaise au sein des cultures. Mais ces méthodes ne permettent pas à elles seules de diminuer sensiblement les populations de bagrada. Aux USA, le mode de production des brassicacées est encore basé sur un modèle intensif (Kremen et al. 2012) et ces pratiques ne sont que peu considérées au bénéfice de l'utilisation de produits phytosanitaires. Pour augmenter l'efficacité de la LBC, deux principaux leviers pourraient être utilisés : 1) favoriser le maintien du parasitoïde introduit

et des parasitoïdes natifs dans les cultures et 2) concentrer le ravageur pour faciliter son exploitation par les parasitoïdes.

D'une manière générale, pour se nourrir, les parasitoïdes sont attirés par des espèces végétales nectarifères. Le maintien de ces espèces à proximité des cultures permet d'augmenter le recrutement des parasitoïdes et prolonger leur maintien dans l'écosystème ce qui augmente leur effet « top-down ». C'est par exemple le cas de *Trichopoda pennipes* F., dont le parasitisme de *N. viridula* est plus important en présence d'asclépiades (Tillman & Carpenter 2014) et de sarrasin (Tillman 2017 ; Conti et al. 2021). Cette approche serait en adéquation avec l'exigence de *G. gonikopalense* d'avoir une source de nourriture présente pour se maintenir, et pourrait permettre un meilleur impact du parasitoïde sur bagrada (**Article 1)**.

Simultanément, la synthèse et utilisation de phéromones attractives pourrait être envisagée pour concentrer les populations de bagrada, et faciliter leur détection par *G. gonikopalense*. Le (E)-2-octenyl acétate (**Discussion**, **1.2.3**), une phéromone impliquée dans l'accouplement mais également l'agrégation des larves, est une piste d'étude (Guarino et al. 2008 ; Arif et al. 2020). Toutefois, l'utilisation au champ de ces composés a montré des effets limités en termes de portée et de durabilité, et des études supplémentaires sont nécessaires (Arif et al. 2020). Ces conclusions vont par ailleurs dans le sens de l'effet limité des phéromones sexuelles ou d'agrégation montré pour le contrôle de *N. viridula* et *H. halys* (Millar et al. 2002 ; Morrison et al. 2018). La piste des cultures pièges pourrait alors être une autre manière de détourner bagrada de sa plante hôte, bien qu'aucune plante n'ait été montrée réellement attractive aux Etats-Unis pour le moment. L'alyssum (*Lobularia maritima*) pousse naturellement en bordure de cultures et est attractive pour de nombreux insectes bénéfiques comme les syrphes (prédateurs de pucerons) (Brennan, 2013). Elle semble cependant peu attractive pour bagrada face à une culture de brassicacées. (Huang et al. 2014). A l'inverse, l'utilisation de brassicacées dans les cultures d'autres familles de plantes pourrait s'avérer efficace. De récentes expérimentations menées en laboratoire et sur l'île de Pantelleria (Sicile, Italie) ont montré que les brassicacées *Brassica oleracea* var. botrytis (chou-fleur) et *Eruca sativa* (roquette) pourraient être envisagées pour attirer bagrada hors des cultures de câprier, une des principales ressources agronomiques de l'île (Arif et al. 2020).

Gryon gonikopalense pourrait également être utilisé dans une combinaison de plusieurs auxiliaires. En effet, la présence de plusieurs espèces de parasitoïdes (ou prédateurs) complémentaires peut engendrer des effets synergiques ou additifs en comparaison d'un seul auxiliaire (Driesche & Hoddle 2016). Dans une méta-analyse, Stiling & Cornelissen (2005) montrent notamment que la combinaison de plusieurs auxiliaires diminue l'abondance du ravageur de 27% par rapport à un auxiliaire seul. Comme je l'ai exposé antérieurement, l'impact de *G. gonikopalense* pourrait être complémenté par d'autres parasitoïdes n'attaquant pas bagrada dans le sol. Et bien que *G. gonikopalense* soit à ce jour le candidat le plus prometteur, la recherche de nouveaux candidats dans l'aire native de bagrada continue, notamment au Kenya et en Afrique du Sud. Ces nouveaux parasitoïdes pourraient également présenter des adaptations à bagrada, et pourquoi pas, être de meilleurs candidats. En parallèle

des parasitoïdes, de nouvelles opportunités ont également émergé du côté des champignons entomopathogènes tels que *Zoophthora radicans* (Torres Acosta et al. 2016), *Beauveria bassiana*, *Metarhizium anisopliae*, et *Isaria fumosorosea* (Barrera-Lopez et al. 2020). L'utilisation de ces champignons pour contrôler bagrada dans les cultures pourrait être une piste complémentaire au projet de LBC développé dans cette thèse.

Une dernière méthode pourrait être considérée dans l'ensemble des stratégies de gestion de bagrada : la technique de l'insecte stérile (TIS) (Oliva et al., 2020). Bien qu'elle encore soit inutilisée pour la régulation des populations de pentatomidés invasives, un intérêt croissant émerge pour cette méthode qui pourrait permettre un contrôle (au moins partiel) de certaines espèces sans les risques liés à l'introduction d'un parasitoïde. Bien qu'embryonnaires, les recherches d'application de la TIS sur *H. halys* montrent par exemple que la stérilité des mâles se transmet à la génération F1, ce qui entraîne des taux d'émergence des œufs à la F2 très faibles (Nguyen et al. 2020). Chez *N. viridula*, les mâles irradiés sont jusqu'à 99% stériles, sans effet apparent sur leur longévité, et la TIS pourrait être efficace (Horrocks et al. 2020). Mais cette technique appliquée aux pentatomidés serait trop coûteuse d'après Michael D. Toews (McPherson 2018), qui estime l'élevage de ces punaises trop coûteux (chaque individu revenant à plusieurs dollars de production). Le lâcher de plusieurs milliers d'individus mâles par hectare serait alors inenvisageable. De plus, la TIS n'empêche pas les dégâts occasionnés par les individus mâles sur les cultures. Enfin, les punaises se reproduisent généralement plusieurs fois, avec différents partenaires, ce qui multiplie les chances d'accouplement avec un individu non-stérile (McPherson 2018). Chez bagrada, j'ai pu observer ce qui semble être un comportement de "mate guarding" de la part des mâles : ces derniers restent sur le dos de la femelle pendant l'oviposition et se ré-accouplent ensuite, de telle sorte qu'une femelle s'accouple plusieurs fois avec le même mâle. La TIS pourrait être investigué au regard de ce comportement qui pourrait limiter le nombre de partenaires différents pour une femelle et donc rendre un mâle stérile efficace, dans le cas où les effets de la stérilisation se transmettent de génération en génération comme pour *H. halys*. J'ai également pu observer de nombreuses autres interactions de compétition entre plusieurs mâles tentant de prendre la place d'un autre mâle gardant « sa » femelle. L'effet de la stérilisation sur les performances de compétiteurs des mâles pourraient alors être à prendre en considération, comme cela a été montré chez *H. halys* (Suckling et al. 2019)

3. Approfondir la biologie de *G. gonikopalense* et ses interactions avec son environnement

L'étude de *G. gonikopalense* en quarantaine a permis de décrire de nombreux aspects de sa biologie et de ses interactions possibles avec bagrada, mais la transposabilité de ces résultats au terrain reste limitée et de nombreuses inconnues subsistent. Il est désormais nécessaire de complexifier les expérimentations pour intégrer plus de facteurs biotiques et/ou abiotiques et obtenir une réponse plus "réaliste" du parasitoïde. Les futurs recherches devraient ainsi se pencher plus finement sur les mécanismes de détection de bagrada par *G. gonikopalense*, notamment du point de vue des signaux chimiques impliqués et leur utilisation par le parasitoïde. J'ai également montré que le comportement

de *G. gonikopalense* était inné et obtenu avec des individus naïfs, mais la question se pose de savoir si la détection de bagrada relève uniquement de capacités innées. Depuis une vingtaine d'années, des études ont montré qu'il existe des capacités d'apprentissage chez les parasitoïdes (Boer et Dicke 2006 ; Dukas 2008 ; Giunti et al. 2015), qui reposent notamment sur des informations perçues par l'individu lors des premières expériences de sélection des hôtes.

J'ai également évoqué l'importance des capacités de dispersion du parasitoïde dans son efficacité à contrôler bagrada. Même si j'ai constaté l'absence total de vol chez *G. gonikopalense* et que cette observation est partagée par les collaborateurs de l'ISPH qui étudient également ce parasitoïde, elle peut être liée aux conditions de quarantaine. De plus, elle n'exclue pas d'autres moyens de dispersion passives (vent, phorésie, etc.). Un point important à élucider est notamment la capacité de *G. gonikopalense* à suivre les populations de bagrada qui, après récolte, se déplacent sur des plantes adventives en bordure de champ.

Enfin, il me semble important d'étudier les mécanismes de la reproduction chez *G. gonikopalense* qui sont pour le moment inconnus. J'ai pu constater une légère protandrie au sein de la colonie de quarantaine, avec des femelles se faisant féconder par les mâles très rapidement après leur émergence. Cependant, de nombreuses pistes de recherche peuvent être explorées : combien de fois les individus s'accouplent-ils au cours de leur vie ? Existe-t-il une discrimination des mâles / femelles déjà accouplés par l'autre sexe ? Si la dispersion de *G. gonikopalense* est faible, cela implique-t-il un taux de rencontre faible entre individus et un risque de consanguinité au sein de la population ?

4. Une ouverture sur l'Europe : bagrada et *G. gonikopalense* en méditerranée ?

Comme je l'ai présenté en introduction, plusieurs populations de bagrada associées au câprier sont présentes en méditerranée sur les îles de Pantelleria (Italie) et Malte, ainsi que sur la côte atlantique du Maroc. Bien qu'elles n'aient pas encore colonisé le continent en plus de 40 ans, le réchauffement global associé aux flux humains et de marchandises pourrait étendre l'aire de répartition de bagrada à partir de ces populations. Les principales régions à risque sur le continent Européen sont le sud de l'Espagne, de l'Italie (producteurs de câpres) et du Portugal (Carjaval et al. 2018). En France, la majeure partie des productions de brassicacées est effectuées dans la moitié nord du pays (agriculture.gouv.fr) dont le climat ne permettrait pas l'installation de bagrada.

Mais à l'heure actuelle, la punaise a déjà le statut de ravageur dans la culture de la câpre, notamment à Pantelleria dont c'est une des principales ressources. A l'image de la Nouvelle-Zélande qui a mené des tests de spécificité avec *T. japonicus* pour anticiper l'arrivée de *H. halys* (Charles et al. 2019), j'ai voulu élargir la problématique américaine de bagrada au bassin méditerranéen et envisager l'introduction de *G. gonikopalense* dans cette région.

Dans un premier temps, une mission de terrain sur l'île menée en juillet 2020 m'a permis de vérifier que la population locale de bagrada pondait naturellement ses œufs dans le sol, comme les individus nord-américains. Comme je l'ai mentionné dans la section **1.2.2** de cette discussion, le sol de

l'île est une terre sablonneuse issue de la roche volcanique et possède une granulométrie très fine, proche des protocoles expérimentaux utilisés pendant cette thèse. Le comportement de recherche d'hôte de *G. gonikopalense* serait donc un atout efficace pour réguler la punaise.

D'autre part, l'île de Pantelleria est de très petite taille (83km²) dont 66,4 km ^{2} sont occupés par le parc national et donc non cultivés. La plupart des parcelles de câpriers sont de petite taille (< 0,1 ha) (Fig. 37), et donc propices à des actions de biocontrôle, au suivi de la dynamique populationnelle de parasitoïdes et à la combinaison de méthodes de gestion comme les cultures pièges ou l'utilisation de phéromones et la LBC (Arif et al. 2020).

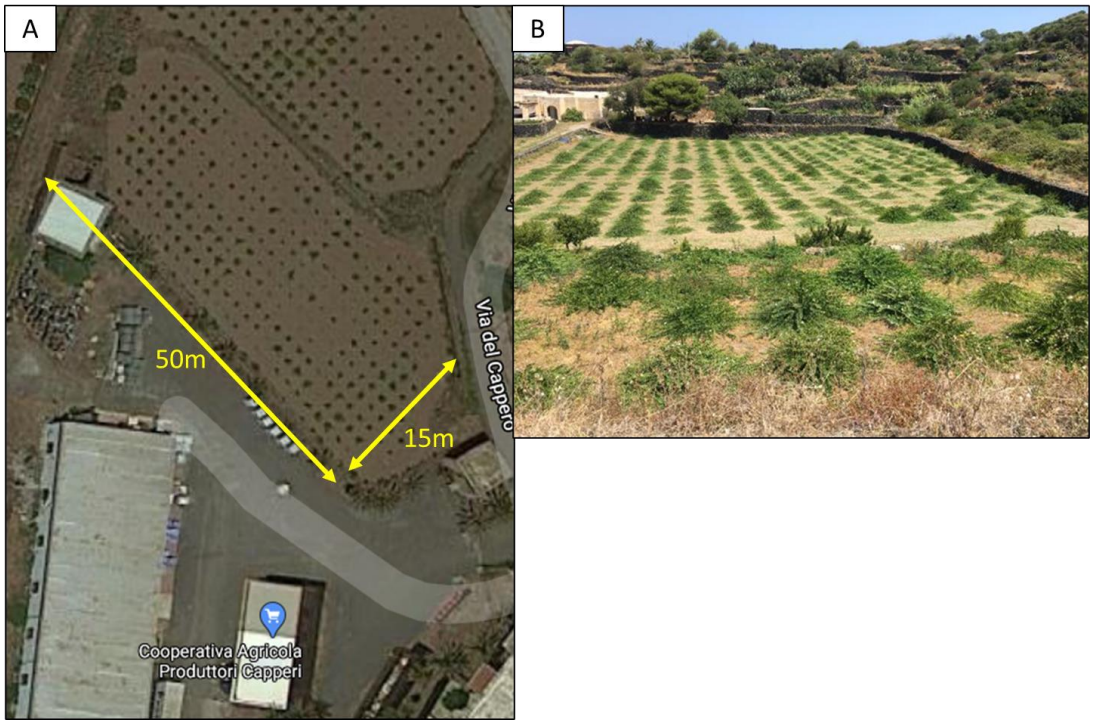


Figure 37 : (A) Vue aérienne des dimensions d'une parcelle de câpriers de la Coopérative Agricole des Producteurs de Câpres de Pantelleria (Sicile, Italie) ; (B) parcelle de câpriers en vue réelle à Pantelleria (Photo : Sforza R.F.H.)

Cependant, les tests de spécificité menés en laboratoire ont montré que *G. gonikopalense* pouvait attaquer certaines espèces européennes, en particulier une localisée dans le bassin méditerranéen : *Ancyrosoma leucogrammes* (**Article 6**), qui est peu commune et n'est pas un ravageur. Nous avons également vu que les tests de spécificité ne permettaient pas de délimiter la gamme d'hôte du parasitoïde. D'autres espèces peu communes méditerranéennes devraient être testées, notamment au sein de la tribu des Strachinii comme *Eurydema herbacea* Herrich-Schäffer, mais également des espèces nuisibles comme *Palomena prasina* L. Puisque *Graphosoma italicum* a été parasitée, des tests supplémentaires sont également à réaliser sur l'espèce *Graphosoma semipunctatum*, qui n'a pas été attaquée par *G. gonikopalense* en conditions de non-choix, mais avec un faible nombre de réplications (A**rticle 6**).

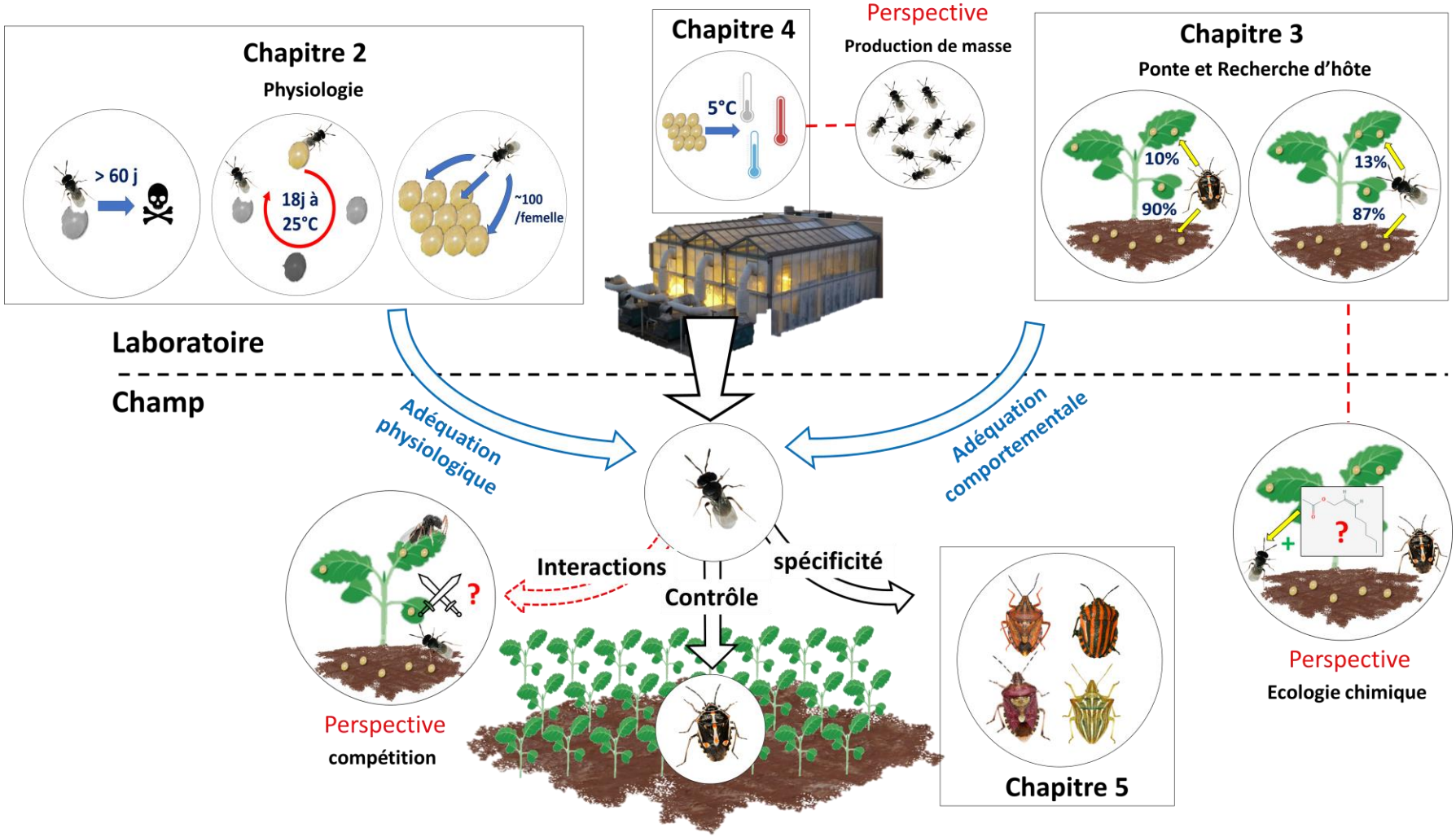


Figure 38 : synthèse des résultats et perspectives de la thèse

Conclusion générale

Le programme de LBC conduit par l'USDA-ARS contre la punaise bagrada a bien évolué depuis la découverte de trois parasitoïdes candidats au Pakistan en 2015. Au cours de cette thèse, j'ai exploré plusieurs aspects physiologiques et comportementaux de *G. gonikopalense* ayant pour but de nourrir l'objectif que nous avions à l'esprit lorsque ce projet de thèse a été mis en place en 2018 : la rédaction d'une pétition pour le lâcher du parasitoïde en Californie. Alors que les traits d'histoire de vie liés à son développement, sa reproduction et sa longévité font de lui un scelionide proche de certaines espèces déjà étudiées en LBC, j'ai pu mettre en évidence la singularité de son comportement de recherche d'hôte qui pourrait être son principal atout. Son origine commune avec bagrada devrait également lui permettre de s'acclimater en Californie, bien que la transposition au champ des résultats obtenus en laboratoire soit à considérer avec une certaine réserve. Les données obtenues constituent toutefois une base solide de connaissances de *G. gonikopalense* et permettent déjà de discuter son rôle d'auxiliaire contre bagrada aux USA et ailleurs. Des recherches supplémentaires sont nécessaires, notamment concernant les tests de spécificité qui seront poursuivis dès l'été 2021. Néanmoins, je souhaite de tout cœur que *G. gonikopalense*, à qui j'ai consacré tant d'heures d'observations, puisse un jour jouer un rôle dans la régulation de la punaise bagrada, elle-même surprenante à bien des égards.

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Annexes.

Article 7 : Conti, E., Avila, G., Barratt, B., Cingolani, F., Colazza, S., Guarino, S., Hoelmer, K., Laumann, R. A., Maistrello, L., **Martel, G.,** Peri, E., Rodriguez-Saona, C., Rondoni, G., Rostás, M., Roversi, P. F., Sforza, R. F. H., Tavella, L., & Wajnberg, E. (2021). Biological control of invasive stink bugs: Review of global state and future prospects. *Entomologia Experimentalis et Applicata*, *169*(1), 28-51. https://doi.org/10.1111/eea.12967

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Biological control of invasive stink bugs: review of global state and future prospects

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Abstract

Invasive stink bugs (Hemiptera: Pentatomidae) are responsible for high economic losses to agriculture on a global scale. The most important species, dating from recent to old invasions, include *Bagrada hilaris* (Burmeister), *Halyomorpha halys* (Stål), *Piezodorus guildinii* (Westwood), *Nezara viridula* (L.), and *Murgantia histrionica* (Hahn). *Bagrada hilaris*, *H. halys*, and *N. viridula* are now almost globally distributed. Biological control of these pests faces a complex set of challenges that must be addressed to maintain pest populations below the economic injury level. Several case studies of classical and conservation biological control of invasive stink bugs are reported here. The most common parasitoids in their geographical area of origin are egg parasitoids (Hymenoptera: Scelionidae, Encyrtidae, and Eupelmidae). Additionally, native parasitoids of adult stink bugs (Diptera: Tachinidae) have in some cases adapted to the novel hosts in the invaded area and native predators are known to prey on the various instars. Improving the efficacy of biocontrol agents is possible through conservation biological control techniques and exploitation of their chemical ecology. Moreover, integration of biological control with other techniques, such as behavioural manipulation of adult stink bugs and plant resistance, may be a sustainable pest control method within organic farming and integrated pest management programs. However, additional field studies are needed to verify the efficacy of these novel methods and transfer them from research to application.

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Introduction

Invasive insect herbivores are responsible for an annual loss of at least 70 billion USD globally (Bradshaw et al., 2016), with the biggest agricultural producers, USA and China, experiencing the highest costs and also representing the main potential sources of invasive pests (Paini et al., 2016). The economic loss caused by invasive insects is growing, mostly due to market globalization and climate change (Bradshaw et al., 2016). Herbivorous stink bugs (Hemiptera: Pentatomidae) serve as a good example of this, as many species are agricultural pests in their native range as well as serious invasive pests with high economic impact (Panizzi et al., 2000; McPherson, 2018). Stink bug damage is due to the feeding punctures of adults and nymphs on plant tissues, especially those of fruits and seeds and sometimes leaves and stems, resulting in large reductions in crop yield and/or quality (Panizzi et al., 2000; McPherson, 2018). Additionally, several species transmit plant pathogens (Mitchell et al., 2018).

At least two polyphagous pentatomid species, the brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hamilton et al., 2018), and the southern green stink bug, *Nezara viridula* (L.) (Esquivel et al., 2018), are globally important pests. *Halyomorpha halys* is a temperate/subtropical species native to eastern Asia that has established in North America and Europe, where it damages many crops (reviewed by Leskey & Nielsen, 2018), has recently established in Chile (Faúndez & Rider, 2017), and has regularly been intercepted at New Zealand's border in recent years (Avila & Charles, 2018; Charles et al., 2019). Unlike *H. halys*, the native geographical range of *N. viridula* (Africa, southwest Asia, or Mediterranean region) is not yet clearly defined, but this species has spread into tropical, subtropical, and warm-temperate zones of five continents and might expand further (Panizzi & Slansky, 1985; Todd, 1989; Panizzi & Lucini, 2016; Esquivel et al., 2018).

The most recent widespread invasive pentatomid species, the painted bug or Bagrada bug, *Bagrada hilaris* (Burmeister), which has African and Asian origins, now has an almost worldwide distribution and has recently become a serious concern on brassicaceous crops in the USA (Bundy et al., 2018b). To date, two additional stink bugs, *Murgantia histrionica* (Hahn) and *Piezodorus guildinii* (Westwood), are less widely distributed than *B. hilaris*, having expanded their ranges only within the American continent. Historically, *M. histrionica*, native to Central America and Mexico, could be considered the first recorded invasive stink bug, having invaded the southern part of the USA during the 19th century where it is still causing economic losses on brassicaceous crops (McPherson et al., 2018). *Piezodorus guildinii*, originally described from the Caribbean, is now a major pest of soybean and other Fabaceae in many parts of the American continent (Bundy et al., 2018a).

Stink bug expansion into new areas, especially when transported over long distances by global trade and tourism, can often be attributed to the overwintering behaviour of adults (Panizzi et al., 2000; Musolin et al., 2018). In autumn, several species aggregate in buildings and other artificial shelters to hibernate. Overwintering *H. halys* adults have been transported while hidden inside many types of material (Maistrello et al., 2018; Nixon et al., 2019). This has affected international trade due to the mandatory phytosanitary importation measures required by some countries (e.g., New Zealand and Australia). These regulations impose treatments on any type of commodities imported from countries where *H. halys* is confirmed as present (MPI, 2020).

Following the introduction of exotic species into new areas, native natural enemies sometimes adapt to the non-coevolved species. Fortuitous biological control of invasive stink bugs by native parasitoids and predators has been observed, but in most cases native parasitoids are not considered effective for biological control, especially when compared with coevolved species from the native area (McPherson, 2018). Because of a lack of effective natural enemies, combined with stink bug biology, locally favourable ecological conditions, and their capacity to cause direct damage to marketable produce, the impact of stink bugs in invaded agroecosystems is often significant, both in terms of crop loss and by disrupting established integrated pest management (IPM) practices (Panizzi et al., 2000; McPherson, 2018). It is therefore vital to develop new sustainable control methods that can be included within revised IPM strategies. In this paper, we review a century of biological control programs for invasive stink bugs and discuss the global biocontrol strategies developed against them through six case studies. These strategies include classical, augmentative, and conservation biological control, as well as an innovative pre-emptive classical biocontrol approach. Additionally, based on knowledge of stink bug and parasitoid behaviour, biology, and chemical ecology, we discuss the prospects of using semiochemical and other novel approaches to increase natural enemy efficacy and manage stink bug pests as part of sustainable, integrated control strategies.

Management of invasive stink bugs over a century of classical biological control

Recent stink bug invasions have renewed the interest in classical biological control as a potential pest control strategy. Evaluation of suitable candidates for classical

biological control of *B. hilaris* is underway in North America, with research focusing on egg parasitoids, as the tachinid parasitoids of adult stink bugs (Diptera: Tachinidae) that have been recorded in Asia are poorly known and do not seem to have a significant impact on this host (Sforza et al., 2017; Bundy et al., 2018b). Similarly, the most important natural enemies of *H. halys* appear to be coevolved egg parasitoids in the native range of Eastern Asia (Zhang et al., 2017; Buffington et al., 2018; Hamilton et al., 2018; Leskey & Nielsen, 2018). The egg parasitoid *Trissolcus japonicus* (Ashmead) [Hymenoptera: Scelionidae (syn. Platygastridae *s.l.*; Sharkey, 2007, but see Popovici et al., 2017)] is the predominant natural enemy of *H. halys* in Asia and is therefore considered a promising biocontrol agent for this invasive pest (Zhang et al., 2017; Buffington et al., 2018; Leskey & Nielsen, 2018). However, environmental risk assessment of *T. japonicus* as candidate biocontrol agent, conducted in the USA and in Europe, raised ecological concerns due to the apparent low host specificity of *T. japonicus* and consequent risk that this species might attack non-target species in the areas of introduction, including beneficial predatory stink bugs (Hedstrom et al., 2017; Botch & Delfosse, 2018; Haye et al., 2020). Legislative restrictions in Europe and the USA severely limit the introduction of exotic species by focusing on perceived risks for non-targets rather than the benefits of pest reduction (van Lenteren et al., 2006; Rondoni et al., 2021). Now that the occurrence of *T. japonicus* has been reported in northern Italy (Sabbatini Peverieri et al., 2018), in early 2020 the Italian government authorized the mass production and release of this parasitoid in Italy, where *H. halys* has caused the highest economic losses in Europe since its arrival in 2012 (Maistrello et al., 2016, 2018). This is the first officially authorized release of the parasitoid against *H. halys* in Europe. As has occurred in Italy, constraints are probably becoming less relevant in the USA because of the recent finding of adventive populations of *T. japonicus* in the invaded areas (Talamas et al., 2015).

About 1 century ago, the first attempts with biological control of stink bugs started with *N. viridula* and they are still in progress globally. Classical biological control mostly involved the egg parasitoid *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae), which was introduced and released in nearly all geographical areas invaded by *N. viridula* (Esquivel et al., 2018). Additionally, the native parasitoids of adults, *Trichopoda pennipes* (Fabricius) and *Trichopoda giacomellii* (Blanchard) (Diptera: Tachinidae), were found parasitizing this species in North and South America, respectively, and were subsequently introduced into Hawaii (USA) and Australia for classical biological control of *N. viridula* (Liljesthröm & Rabinovich, 2004; Esquivel et al., 2018). *Trichopoda pennipes*, which attacks adults and occasionally nymphs, was then accidentally introduced in Italy (Colazza et al., 1996) from where it spread to several countries in Europe and the Mediterranean (Tschorsnig, 2017). Despite the numerous classical biocontrol programs against *N. viridula*, this species is still an economically important pest worldwide except in South America (Panizzi & Lucini, 2016).

In contrast with other invasive stink bugs, no biocontrol efforts have been made against *M. histrionica* in the USA. However, several native natural enemies – the most common ones are the parasitoids *Trissolcus brochymenae* (Ashmead), *Trissolcus euschisti* (Ashmead) (Hymenoptera: Scelionidae), and *Ooencyrtus johnsoni* (Howard) (Hymenoptera: Encyrtidae) – have been recorded since the end of the 19th century but generally with low reported efficacy (McPherson et al., 2018).

Overall, naturally occurring and classical biological control of stink bugs, although useful in significantly reducing the exotic pest populations, are often inadequate in maintaining the pest below the economic injury level (McPherson, 2018). Therefore, additional approaches are being evaluated and developed regularly to improve the efficacy of parasitoids as biocontrol agents and to integrate biocontrol programs with other sustainable management methods targeting adult stink bugs. In the following sections, we report relevant case studies and discuss the potential application of conservation biological control and other sustainable control methods (Figure 1).

Case study 1: Selection of candidates for classical biological control of *Bagrada hilaris*

History, invasion, and pest status

The painted or Bagrada bug, *B. hilaris*, is a worldwide pest of brassicaceous crops (Bundy et al., 2018b). Like other stink bugs, it can also be a nuisance pest for humans by sheltering in homes (Faúndez, 2018). *Bagrada hilaris* is native to Africa, the Middle East, and Asia where it sporadically damages local crops (Gunn, 1918; Ahuja et al., 2008). Since its first introduction in California (USA) in 2008, it has become invasive in southern states of the USA (Reed et al., 2013) and in Hawaii (USA), Mexico, and Chile (Faúndez et al., 2016). In the Mediterranean basin, *B. hilaris* was accidentally introduced into Italy and Malta. Pinpointing the origin of invasive populations in the Americas will enable an understanding of the invasion routes of *B. hilaris* and ultimately help to find the parasitoid best suited to control this pest. A preliminary phylogeographical study using the DNA barcode region COI identified Pakistan as a source for the invasive *B. hilaris* in North America (Sforza et al., 2017).

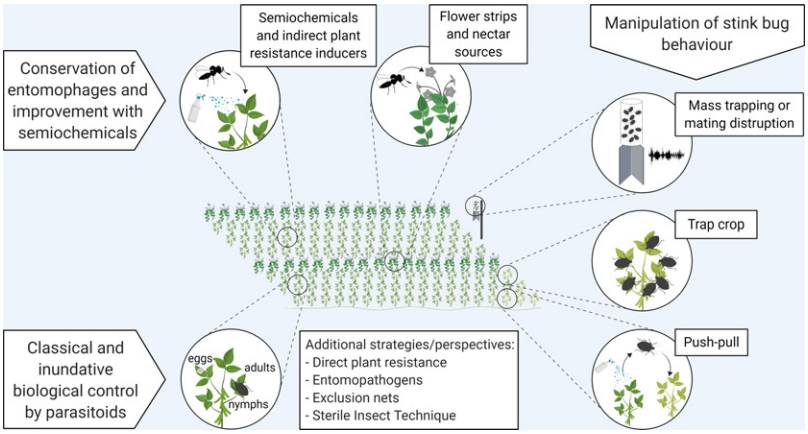


Figure 1 Possible strategies for successful stink bug management based on the integration of biological control (classical, inundative, or conservation) with manipulation of the natural enemy and/or herbivore behaviour, or with additional strategies (figure created with BioRender.com). [Colour figure can be viewed at wileyonlinelibrary.com]

This pest is multivoltine with gregarious behaviour (Reed et al., 2013; Bundy et al., 2018b). Unlike most of the 4 722 species of Pentatomidae (Rider, 2016) that glue eggs together in clusters onto plant material, *B. hilaris* deposits many of its eggs singly into the soil (Gunn, 1918; Taylor et al., 2014), which may affect egg parasitism (see below). Due to the economic impact of *B. hilaris* injury on crops in southwestern USA, its control currently relies on pesticide applications (Palumbo et al., 2016). Classical biological control could provide sustainable and long-term control, especially if implemented as part of an IPM program. To date, no classical biocontrol program is in place in any of the countries in the invaded range. However, the egg parasitoids *Gryon myrmecophilum* (Ashmead), *Telenomus podisi* (Ashmead), and *Tr. basalis* (all Hymenoptera: Scelionidae) were found parasitizing *B. hilaris* in Mexico (Felipe-Victoriano et al., 2019), suggesting a possible role for them in conservation biological control.

Exploration for natural enemies in the native range

Surveys for natural enemies began in countries where *B. hilaris* is native, including South Africa and India. As for other invasive stink bugs (see below), predators and resident North American parasitoids have been reported (Bundy et al., 2018b). However, we present only data on egg parasitoids from the native geographical range of the pest. Gunn (1918) first recorded the presence of an egg parasitoid emerging from artificially buried eggs of *B. hilaris* in cabbage and cauliflower fields. Subsequently, egg parasitoids in the scelionid genera *Telenomus*, *Typhodytes* (Mani, 1942; Samuel, 1942), and *Trissolcus* (Chacko & Katiyar, 1961) were collected from India. After the invasion of *B. hilaris* in the Americas, surveys were conducted in 2015 in Pakistan. Three species were collected, viz., *Trissolcus hyalinipennis* Rajmohana & Narendran (syn. *Allophanurus indicus* Subba Rao & Chacko), *Gryon gonikopalense* Sharma (Scelionidae), and an *Ooencyrtus* sp. (Encyrtidae) (Mahmood et al., 2015). The latter was the first report of an *Ooencyrtus* species on *B. hilaris* eggs. These egg parasitoid candidates are currently under evaluation in the USA and European quarantine facilities. Since 2016, new surveys for *B. hilaris* biocontrol candidates have been conducted in Kenya and South Africa within the framework of Access and Benefit Sharing agreements (Mason et al., 2018). Various collecting protocols, including sentinel eggs and Malaise trapping, are being used to study the native biodiversity of egg parasitoids of *B. hilaris* (RFH Sforza, M Kasina, P Addison, MC Bon & E Talamas, unpubl.).

Promising candidates

Among the parasitoid species collected in Pakistan in 2015, only two have been studied in the laboratory. The basic biological traits of *Tr. hyalinipennis* were described by Subba Rao & Chacko (1961). Their studies reported a very high fecundity level of 158 progeny per female and a longevity of over 1 month. Studies of *G. gonikopalense* at the USDA-ARS European Biological Control Laboratory (Montpellier, France) suggest this species is a promising biocontrol candidate (Martel et al., 2019). Both the host and parasitoid share the same thermal requirements for their development and reproduction, suggesting that their development will be synchronous in the field.

An important criterion for an effective candidate biocontrol agent is its foraging capacity for *B. hilaris* eggs buried in the soil, as was highlighted by Bundy et al. (2018b). Recent investigations under laboratory conditions have shown that *Tr. hyalinipennis* is unable to parasitize buried eggs, whereas *G. gonikopalense* readily finds and parasitizes *B. hilaris* eggs in the soil (G Martel & RFH Sforza, unpubl.). This information provides a basis for investigating whether *G. gonikopalense* effectively parasitizes *B. hilaris* eggs under natural conditions, especially considering that *B. hilaris* is able to oviposit both in the soil and on

its host plant (Taylor et al., 2014). Another criterion is the host specificity of the selected egg parasitoids, which is currently under investigation in USA quarantine facilities for *B. hilaris* and several other stink bugs. If the above-mentioned criteria are met, the release of *G. gonikopalense* in North America can be considered. In the meantime, foreign exploration in Africa will continue to search for other coevolved parasitoids.

Case study 2: Biological control of *Halyomorpha halys* in North America

History, invasion, and pest status

The brown marmorated stink bug, *H. halys*, was first detected in the USA in the mid-1990s (Leskey & Nielsen, 2018) in Allentown, PA (Hoebeke & Carter, 2003), and has since spread to more than 42 states (Leskey & Nielsen, 2018). In Canada, it was first detected in Ontario and Quebec in 2010 (Fogain & Graff, 2011) and subsequently in British Columbia (Abram et al., 2017c). This species is a polyphagous pest with a host range of more than 300 plant species (Lee et al., 2013; Bergmann et al., 2016). In North America, this includes over 170 plants from more than a dozen families, and apples, peaches, nectarines, pears, grapes, sweet corn, soybeans, and hazelnuts are among the most susceptible cultivated hosts (Leskey & Nielsen, 2018).

Natural control by native parasitoids and predators

Early biological control research in North America focused on identifying the indigenous natural enemies of *H. halys*. These studies mainly concentrated on the egg stage, using sentinel (fresh and frozen) *H. halys* egg masses to assess predation and parasitism (Dieckhoff et al., 2017; Abram et al., 2017b; Leskey & Nielsen, 2018). In general, control by existing natural enemies has been considered ineffective (Ogburn et al., 2016; Cornelius et al., 2016a; Abram et al., 2017b). In agreement with the enemy release hypothesis (Keane & Crawley, 2002), *H. halys* seems to have escaped from its natural enemies in the invaded North American areas. For example, in organic agro-ecosystems across the eastern USA, maximum levels of natural biological control were estimated at 20%, primarily caused by chewing predators (Ogburn et al., 2016) whose efficacy is dependent on the *H. halys* life stage (Morrison et al., 2016; Pote & Nielsen, 2017). In the laboratory, late-instar *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), katydids (Orthoptera: Tettigoniidae), earwigs (Dermaptera: Forficulidae), jumping spiders (Araneae: Salticidae), crickets (Orthoptera: Gryllidae), and ground beetles (Coleoptera: Scarabaeidae) were effective predators of *H. halys* eggs (Abram et al., 2015; Morrison et al., 2016). Grasshoppers (Orthoptera: Acrididae), *Coccinella septempunctata* (L.) (Coleoptera: Coccinellidae), and the spined soldier bug *Podisus maculiventris* (Say) (Pentatomidae) also preyed on eggs, whereas several hemipteran predators attacked young nymphs (Pote & Nielsen, 2017). Using surveillance cameras, Pote (2018) reported katydids feeding on *H. halys* eggs in the field. Possibly, indigenous generalist predators use pentatomid semiochemical cues to locate *H. halys* (Fraga et al., 2017).

Mortality from parasitism of *H. halys* eggs by indigenous parasitoids in North America tends to be lower than that from predation (Ogburn et al., 2016), accounting for less than 5% of parasitoid emergence from eggs in 87% of all surveys (Abram et al., 2017b). However, levels of parasitism and predation seem to vary depending on habitat (Cornelius et al., 2016a; Tillman et al., 2020), with parasitism greater than predation in woody ornamental nurseries (Jones et al., 2017), whereas the opposite is true for organic agroecosystems (Ogburn et al., 2016). Three parasitoid families are commonly found attacking *H. halys* eggs in the USA and Canada: Scelionidae (*Telenomus*, *Trissolcus*, and *Gryon* spp.), Eupelmidae (*Anastatus* spp.), and Encyrtidae (*Ooencyrtus* spp.) (Abram et al., 2017b; Leskey & Nielsen, 2018). According to Abram et al. (2017b), *Anastatus* and *Trissolcus* tend to dominate ornamental, semi-natural/urban, and forest habitats, whereas *Te. podisi* dominates field/vegetable crops and orchard habitats (see also Herlihy et al., 2016, and Tillman et al., 2020). One explanation for the low parasitism rates observed of *H. halys* eggs is that even when eggs are attacked, the native parasitoids frequently fail to develop to emergence, although they may still kill the host (Cornelius et al., 2016b). Abram et al. (2014) showed that *H. halys* eggs are attacked by *Te. podisi* at rates similar to indigenous *P. maculiventris* eggs. However, successful development occurred only in the latter species. They argued that *H. halys* acts as an evolutionary trap for this parasitoid, which could result in an increase in population levels of indigenous pentatomids. It is also worth noting that, although convenient, studies using sentinel eggs may underestimate the levels of parasitism (Jones et al., 2014; but see Herlihy et al., 2016, and Dieckhoff et al., 2017). This reduced parasitism could be due to the absence of cues used by indigenous parasitoids in host finding and recognition, i.e., host kairomones (Conti et al., 2003; Laumann et al., 2009; Tognon et al., 2016, 2017; Rondoni et al., 2017; Boyle et al., 2020) and oviposition-induced plant synomones (Colazza et al., 2004a; Conti & Colazza, 2012; Rondoni et al., 2017).

Exploration for natural enemies in the native range and biological control

To improve *H. halys* biological control, efforts were initiated in the late 2000s in the USA to introduce natural

enemies from its native range. Classical biocontrol efforts targeted egg parasitoids of the genus *Trissolcus*, including *Tr. japonicus*, the predominant egg parasitoid and most important natural enemy of *H. halys* in Asia capable of inflicting high rates of parasitism in landscapes and orchard crops (Zhang et al., 2017; Buffington et al., 2018; Leskey & Nielsen, 2018). In 2014, although still under evaluation in quarantine facilities in the USA, a population of *Tr. japonicus* was found during routine sentinel egg surveys in a wooded habitat in Beltsville, MD (Talamas et al., 2015; Buffington et al., 2018). Molecular analyses revealed that the *Tr. japonicus* specimens found were different than those maintained in quarantine, indicating that it was introduced into the USA by other means via an unknown pathway (Buffington et al., 2018). Since then, *Tr. japonicus* has been reported in at least 13 states across the USA (Milnes et al., 2016; Hedstrom et al., 2017; Morrison et al., 2018; Leskey & Nielsen, 2018; Jarrett et al., 2019) and two provinces in Canada (Abram et al., 2019; Gariepy & Talamas, 2019). They comprise three distinct haplotype populations, indicating that there have been several independent introductions whose source regions have not yet been clearly identified (MC Bon, pers. comm.). Although *Tr. japonicus* has been reported mainly from unmanaged landscape habitats, it has also been found in peach orchards (Kaser et al., 2018).

Laboratory testing of *Tr. japonicus* against North American pentatomids has shown a marked preference for *H. halys* but not complete host specificity (Hedstrom et al., 2017; Botch & Delfosse, 2018; Lara et al., 2019). Potential alternate hosts may be less physiologically suitable for development than *H. halys* if they are accepted for oviposition, producing smaller and less fit progeny (Botch & Delfosse, 2018). The first field study of a *Tr. japonicus* population in North America appears to confirm these laboratory studies, at least for several native pest stink bugs in the Pacific Northwest (Milnes & Beers, 2019). A degree of specificity is also conferred by chemicals associated with *H. halys* that are used by *Tr. japonicus* in host finding and recognition. In choice tests, *Tr. japonicus* remained longer on surfaces with *H. halys* contact kairomones (Hedstrom et al., 2017). Furthermore, they responded more strongly to *H. halys* kairomone traces deposited on leaves of apple, maple, and soybean than to traces of the predatory *P. maculiventris* (Boyle et al., 2020), a non-target species that is an important predator in agricultural habitats of several pests, including *H. halys* (Pote & Nielsen, 2017). Parasitoid females detected and responded in a similar manner to kairomone traces on leaves of host plants, increasing their search time in a Y-tube olfactometer, and female *Tr. japonicus* were attracted to *n*-tridecane but repelled by (*E*)-2-decenal, two defensive compounds emitted from *H. halys* males and females (Zhong et al., 2017). In other experiments, *Tr. japonicus* females were attracted to volatiles from gravid females and mature males of *H. halys* and to volatiles induced in tomato plants by oviposition and feeding of this coevolved host, but females did not respond to similar cues associated with *P. maculiventris* (Bertoldi et al., 2019).

Although *Tr. japonicus* is also capable of parasitizing *P. maculiventris* (Hedstrom et al., 2017; Botch & Delfosse, 2018), the probability of *Tr. japonicus* locating and parasitizing *P. maculiventris* under field conditions is likely to be lower than for *H. halys* (Bertoldi et al., 2019; Boyle et al., 2020). *Trissolcus japonicus* did not appear to be attracted to the *H. halys* aggregation pheromone (Morrison et al., 2018), and it was never captured in stink bug traps baited with *H. halys* and *P. maculiventris* pheromones (Boyle, 2017). Whether populations of indigenous pentatomids in North America will be negatively impacted by *Tr. japonicus* and whether this foreign parasitoid will provide better biological control of *H. halys* remain open questions that must be answered with continued field studies.

Case study 3: Biological control of *Halyomorpha halys* in Europe

Invasion of Europe and pest status

The European invasion by *H. halys* started in Switzerland in 2004 (Haye et al., 2015b) followed by the colonization of neighbouring countries and interceptions at customs/ports/airports (Claerebout et al., 2018). Genetic analyses demonstrate a high diversity of the invading *H. halys* populations, especially in Italy (Cesari et al., 2017) and Greece (Morrison et al., 2017), indicating multiple introductions that are still ongoing from their native range in Asia and other invaded countries. Serious damage in Italy was observed on peach, pear, and hazelnut, starting from 2013 (Maistrello et al., 2017; Bosco et al., 2018). Outbreaks in northern Italy in 2019 caused more than € 356 million damage on pear, peach, and nectarine, with up to 80–100% yield losses (CSO, 2019). Damage was reported also on apple, kiwi, and other fruit crops, as well as on horticultural and row crops (peppers, tomatoes, maize, and beans). Following *H. halys* invasion, previous IPM programs were seriously disrupted and official IPM guidelines had to be revised to allow for increased numbers of treatments with broad-spectrum insecticides.

Natural control by native parasitoids and predators

Due to restrictions on the practice of classical biological control in Europe, initial studies aimed to discover native natural enemies that were able to adapt to the new invasive

species. Field surveys were performed in Switzerland, Italy, and Georgia, mainly to detect egg parasitoids and predators either using sentinel egg masses (frozen or fresh) or by collecting naturally laid egg masses. Overall, egg parasitism ranged from 3 to 39% in Switzerland (using frozen sentinel eggs), from 1 to 3% in Emilia Romagna, Italy (using fresh sentinel eggs), and from 12 to 21% in Piedmont, Italy (collecting naturally laid eggs). The generalist egg parasitoid *Anastatus bifasciatus* (Geoffroy) (Eupelmidae) was the predominant species in all surveys (Haye et al., 2015a; Costi et al., 2018; Kereselidze et al., 2018; Moraglio et al., 2020), confirming its ability to develop in both frozen and fresh eggs. Other species found included *Trissolcus cultratus* (Mayr) (Scelionidae) in Switzerland (Haye et al., 2015a), and *Trissolcus kozlovi* Rjachovsky, *Trissolcus belenus* (Walker) [syn. *Tr. semistriatus* (Nees von Esenbeck) sensu Talamas et al., 2017] (Tortorici et al., 2019), *Tr. basalis*, *Telenomus turesis* Walker (Scelionidae) and the hyperparasitoid *Acroclisoides sinicus* (Huang & Liao) (Pteromalidae) in Piedmont (Sabbatini Peverieri et al., 2019; Moraglio et al., 2020). In these surveys, a wide range of eggs were lost or showed symptoms of predation (chewing or sucking): up to 31% in Switzerland (Haye et al., 2015a), 2–5% in Emilia Romagna (Costi et al., 2018), and 0.4–9% in Piedmont (Moraglio et al., 2020).

Laboratory no-choice tests were carried out to evaluate the potential of several parasitoid species to attack eggs of European native pentatomids as well as some generalist predators. *Ooencyrtus telenomicida* (Vassiliev) (Encyrtidae) achieved 36% parasitism of *H. halys* eggs, followed by *Ooencyrtus pityocampae* (Mercet) (21%), *A. bifasciatus* (20%), and *Telenomus chloropus* Thomson (6%) (Roversi et al., 2016). In another study, seven scelionid species (six *Trissolcus* spp. and *Te. turesis*) were tested on *H. halys* eggs. All the *Trissolcus* spp. caused significantly higher egg abortion compared with unexposed eggs, but only *Tr. kozlovi* was able to produce offspring from fresh eggs (Moraglio et al., 2021), confirming what was observed in field surveys in Piedmont (Moraglio et al., 2020).

Several species of wild-collected native European generalist predators were tested for their ability to feed on eggs and nymphs of *H. halys* in no-choice tests (Bulgarini et al., 2019). Only the omnivorous *Pholidoptera littoralis* (Fieber) (Orthoptera: Tettigoniidae) was capable of preying on eggs and first and second instars. The other predators did not feed on the eggs. Young *H. halys* nymphs were preyed on by *Nagusta goedelii* (Kolenati), *Rhynocoris iracundus* (Poda) (both Hemiptera: Reduviidae), and two *Himacerus* species (Hemiptera: Nabidae), and *R. iracundus* also fed on the adults (Bulgarini et al., 2019). In a laboratory study, the arboreal ant *Crematogaster scutellaris* (Olivier) (Hymenoptera: Formicidae) did not prey on eggs but proved to be effective at preying on all nymphal instars (Castracani et al., 2017). In addition, the predatory potential of bats (Chiroptera) was assessed by screening for the presence of *H. halys* DNA (Piemontese et al., 2019) in guano samples from nine Italian bat species collected in natural and agricultural environments. Two genera of bats, *Myotis* and *Nyctalus* (both Vespertilionidae), showed evidence of feeding on *H. halys* (Piemontese et al., 2019). In a similar approach, analysis of the gut contents of field-collected arthropods identified *H. halys* DNA in predatory insects and arachnids belonging to Dermaptera, Orthoptera, Hemiptera, Opiliones, and Araneae (G Bulgarini, L Piemontese, M Cesari, R Guidetti & L Maistrello, unpubl.).

Control by exotic parasitoids and possible impact on native non-target stink bugs

Recently, field surveys in Switzerland and Italy discovered the presence of populations of *Tr. japonicus* and *Trissolcus mitsukurii* (Ashmead), which are considered the most effective egg parasitoids in China and Japan, respectively (Arakawa & Namura, 2002; Zhang et al., 2017), although the higher prevalence of *Tr. mitsukurii* over *Tr. japonicus* in Japan is debated (KA Hoelmer, unpubl.). *Trissolcus japonicus* was found first in Canton Ticino, Switzerland, in 2017 and in Piedmont and Lombardy, Italy, in 2018 (Sabbatini Peverieri et al., 2018; Stahl et al., 2019b; Moraglio et al., 2020). A genetic analysis using the barcode mitochondrial DNA determined the closest match of the 'Ticino populations' with Japanese populations, but the pathways of entry into Switzerland remain unknown (Stahl et al., 2019b). *Trissolcus mitsukurii* was first recorded in northeastern Italy starting from 2016 (Scaccini et al., 2020). After the discovery of populations of *Tr. japonicus* and *Tr. mitsukurii* in Italy, the situation has been changing, as highlighted by the increased parasitism rate in Piedmont where, together with *A. bifasciatus*, *Tr. japonicus* was detected in 2018 (Moraglio et al., 2020). Given the availability of their host, the distribution of these exotic egg parasitoids is expected to expand, even in the absence of human intervention.

The prospective host range of *Tr. japonicus* in Europe was evaluated in no-choice tests, followed by large-arena choice tests (Haye et al., 2020). The developmental suitability of European non-target host species for *Tr. japonicus* was demonstrated in no-choice tests by offspring emergence from 11 out of 13 non-target species tested (85%). In large-arena choice tests, the degree of non-target parasitism was substantially reduced (three out of four suitable species were parasitized), whereas parasitism of *Palomena prasina* L. (Pentatomidae) eggs was comparable to that of *H. halys* (Haye et al., 2020). Field data from the

invaded areas in Switzerland and Italy can contribute to a risk-benefit evaluation of releasing or re-locating *Tr. japonicus* populations into other parts of Europe.

Augmentative releases and efficacy of native parasitoids

Given that *A. bifasciatus* is the most prevalent native egg parasitoid of *H. halys* in field surveys (Haye et al., 2015a; Costi et al., 2018; Moraglio et al., 2020) and that it is capable of developing in viable *H. halys* eggs (Roversi et al., 2016; Abram et al., 2017b), this species was selected as a candidate for augmentative releases. *Anastatus bifasciatus* can exploit cues associated with the non-coevolved host during its searching, as parasitoid females responded positively both to adult *H. halys* male volatiles and to *H. halys*-induced plant volatiles (Rondoni et al., 2017). Trials were performed in four apple orchards in Switzerland for 3 years and in an organic pear orchard in northern Italy for 1 year, releasing an equivalent of 11 000–26 000 *A. bifasciatus* females per ha (Stahl et al., 2019a). Parasitism averaged 6% (range: 2–16%) on frozen *H. halys* sentinel egg masses and 8% on frozen egg masses of lepidopteran non-target species. At the Italian site, parasitism on naturally laid eggs was considerably higher (49%) than on frozen sentinel eggs (16%). This suggests that although large quantities of frozen eggs are easier to obtain for experiments, data based upon their use in the field are likely to underestimate the actual impact of parasitism.

In 2019, further trials were performed in Emilia Romagna, Italy, where 10 000 *A. bifasciatus* females per ha were released over 5 weeks from the beginning of egg laying by *H. halys* (June–early July) in a small wooded area adjacent to a pear orchard. Parasitism averaged 9% on naturally laid eggs and 2.5% on frozen sentinel egg masses (Maistrello et al., 2020). However, these field trials underestimated the overall impact of *A. bifasciatus*, as they did not account for host eggs killed due to host feeding. According to previous laboratory trials, host feeding by *A. bifasciatus* may double the estimated host mortality (Stahl et al., 2019a).

Case study 4: Pre-emptive biological control of *Halyomorpha halys* in New Zealand – a world first

Risk of invasion, potential impact, and pre-emptive biocontrol approach

In New Zealand, *H. halys* presents a major risk not only to the sustainability and economics of food production but also to conservation due to its biology and very broad host range (Duthie, 2012; Lee et al., 2013; Bergmann et al., 2016). A recent assessment of the potential economic impact of *H. halys* in New Zealand found that if it established, it could cost the horticulture and arable industries 4.2 billion NZD in 2038 (New Zealand Institute of Economic Research, 2017). In addition, some New Zealand native plant species may be at risk of attack by *H. halys* as well (Duthie, 2012).

Although *H. halys* is not yet known to be present in New Zealand, there is a high risk of entry and establishment, with more than 212 live interceptions of *H. halys* adults at New Zealand's border during the latest high-risk season (i.e., September 2018–April 2019), totalling more than 1 500 adults. Invasive pests can sometimes be eradicated on arrival, but if eradication attempts fail or are deemed technically or economically unfeasible, then there will be a need to undertake area-wide pest management. If this is the case, classical biological control is expected to become a key strategy for reducing *H. halys* populations throughout New Zealand. The severity and imminent nature of *H. halys* incursion meant that there was an urgency for developing a pre-emptive classical biocontrol approach before the arrival of *H. halys*. Pre-emptive biological control is a novel approach that has the potential to enhance preparedness for a possible invasion of pest species and accelerate the response to invasive pests. With this approach, natural enemies can be selected, risk assessment studies carried out, and their potential release pre-approved by regulators for timely release after arrival of the pest. This would significantly reduce pest densities and rates of spread, ultimately reducing the economic or environmental damage associated with the pest (Hoddle et al., 2018). In preparation for a potential arrival/establishment of *H. halys* in New Zealand, a pre-emptive classical biocontrol program for this pest using the egg parasitoid *Tr. japonicus*, the most promising biocontrol agent of *H. halys*, was initiated in December 2015. This program aimed to determine the biosafety of *Tr. japonicus* to potential non-target species in New Zealand and to pre-approve its release to be ready for the anticipated arrival of *H. halys*.

Risk assessment for biological control of *Halyomorpha halys*

The potential host range of *Tr. japonicus* in New Zealand was investigated in quarantine using imported parasitized *H. halys* eggs from USDA-ARS (Newark, DE, USA) between November 2015 and December 2016. Egg masses of seven species of New Zealand Pentatomidae, including one sub-species, were individually exposed to naive mated female *Tr. japonicus* in no-choice laboratory experiments. All tested species were non-endemic but naturalised species to New Zealand. The only Pentatomidae species endemic to New Zealand, *Hypsithocus hudsonae* Bergroth, was not available at the time and could not be tested (Charles et al., 2019). Results from laboratory host-specificity testing demonstrated that the predatory Pentatomidae *Cermatulus nasalis* (Westwood) *nasalis*, *C. nasalis hudsoni*,

and *Oechalia schellenbergii* (Guérin), and the phytophagous *Monteithiella humeralis* (Walker), *Dictyotus caenosus* (Westwood), *Glaucias amyoti* (Dallas), and *Cuspicona simplex* Walker were all physiological hosts for *Tr. japonicus*, although not all were equally susceptible to parasitism (Charles et al., 2019). No development or emergence of *Tr. japonicus* from eggs of *N. viridula* was observed (Charles et al., 2019).

In addition, a CLIMEX bioclimatic model was developed to estimate the potential global distribution of *Tr. japonicus* with particular reference to New Zealand and to investigate possible overlaps with the current distribution of potential non-target species (Avila & Charles, 2018). In the native range of *Tr. japonicus*, the model predicts its presence or potential expansion coinciding with the native range of *H. halys* (confirmed by specimen collection records that show it occurs throughout the entire native range of *H. halys*) into most humid-subtropical and humid-continental areas (Avila & Charles, 2018). Globally, the model projected that many temperate, Mediterranean, and subtropical areas could be suitable for the establishment of *Tr. japonicus*. Laboratory studies also demonstrated that *Tr. japonicus* and *Tr. cultratus* could survive periods of winter temperatures as cold or colder as tolerated by their host, *H. halys* (Nystrom et al., 2017). In New Zealand, the north appears moderately to highly suitable for *Tr. japonicus*, whereas southern regions are mostly marginal. The risk posed by *Tr. japonicus* to non-target species in New Zealand is predicted to vary between different pentatomids (Avila & Charles, 2018). CLIMEX projections of the potential distribution of *Tr. japonicus* provide useful guidance for selecting release sites if importation/release of this parasitoid needs to be carried out in New Zealand or worldwide.

Approval to release *Trissolcus japonicus* in New Zealand

The importation, development, and release of new organisms into New Zealand are under strict regulation and must be approved by the Environmental Protection Authority (EPA), which implements the Hazardous Substances and New Organisms (HSNO) Act 1996 (Ehlers et al., 2019). An applicant seeking approval to release a candidate biocontrol agent must submit an application that includes a risk/benefit analysis providing evidence to support the proposal for release and demonstrating that it meets the minimum standards of the HSNO Act. The application submitted to the EPA must provide information about potential adverse effects and the expected economic, social, and cultural benefits of the introduction. The EPA then conducts a full evaluation and review of the benefits and risks associated with the proposed biocontrol agent (Barratt & Ehlers, 2017; Ehlers et al., 2019). If the perceived benefits outweigh the risks, then EPA may grant approval for release, subject (or not) to further conditions.

In March 2018, an application was submitted to the EPA by a representative group from the horticultural industries (BMSB Council) to seek pre-approval to import and release *Tr. japonicus* into New Zealand in the event of a *H. halys* incursion. The application included host range testing, bioclimatic modelling, and substantial additional information on economic and social benefits. As a result of this application, in August 2018, the EPA granted approval for a conditional release of *Tr. japonicus* in the event that a *H. halys* incursion is detected in New Zealand, and it is subject to the development of an appropriate release plan (EPA, 2018). This is the first approval granted to release a biocontrol agent into New Zealand prior to the arrival of its target pest and the first example worldwide to approve the future release of *Tr. japonicus* before the arrival of *H. halys*. The successful implementation of this approach will provide New Zealand with the opportunity for a quicker response against a *H. halys* invasion and a greater chance of achieving an eradication or implementing an early area-wide pest management plan. This novel example of pre-emptive biological control might provide the impetus for biocontrol practitioners to adopt such an approach in the future for the early management of exotic pest incursions.

Case study 5: Biological control of stink bugs in South America

General overview of stink bugs as pests in South America

In South America, stink bugs are major pests primarily in arable crops, such as soybean, beans, and maize (Panizzi & Silva, 2012), which cover large agricultural areas. For example, the total area planted with soybean in Brazil, Paraguay, Uruguay, and Argentina is approximately 56 million ha (Trase, 2018). Since the start of soybean production and its expansion in South America, increasing problems with stink bugs have been reported. In general, these are species complexes with the dominant species varying among regions (Saluso et al., 2007; Ribeiro et al., 2009; Panizzi & Lucini, 2016; Aquino et al., 2019). The first serious pest mentioned in the literature was the cosmopolitan *N. viridula* (Panizzi & Lucini, 2016). Interestingly, its importance has changed over time. Currently, *N. viridula* is found at very low population levels in various regions of Brazil and Argentina (Panizzi & Lucini, 2016). This phenomenon could be due to adaptation to new cropping systems, expansion of soybean culture to hotter regions and broad adoption of non-tillage practices that favour native species. As a result, the dominant stink bugs in crops are currently native species such as *Euschistus*

heros (Fabricius) (Pentatomidae) in hot regions (e.g., central and north Brazil) (Aquino et al., 2019) and *P. guildinii* in cold regions (e.g., southern Brazil, Paraguay, Uruguay, and Argentina) (Panizzi & Lucini, 2016). Other than *N. viridula*, no serious problems have been observed with invasive stink bugs in South America. However, because in recent years *H. halys* and *B. hilaris* have been reported in Chile, both species represent a serious threat to South American agriculture (Faúndez et al., 2016; Faúndez & Rider, 2017).

Classical and inundative biological control

The use of parasitoids for stink bug management in Brazil started in the 1990s with inundative biological control as part of an IPM program developed in the southern region of the country (Parana state). This biocontrol program was based on the use of the exotic *Tr. basalis*, which was already established in South America for the biological control of *N. viridula*, the main soybean pest at that time, and was shown to be especially useful for small to medium cultured areas surrounded by rivers. After demonstrating effective levels of control, the program was extended to more than 18 000 ha (Corrêa-Ferreira et al., 2000; Corrêa-Ferreira, 2002). In spite of its success, the program was eventually abandoned by growers who adopted new cropping systems with non-tillage practices, transgenic cultivars, and pest management based on cheap insecticides (Panizzi, 2013).

In Brazil, current efforts focus on *E. heros* control with *Te. podisi* as its main natural enemy. Because of the high dispersal capacity of this parasitoid, release of large numbers of individuals (ca. 5 000 individuals ha^{-1}) is necessary (Corrêa-Ferreira, 2002), requiring the rearing not only of many wasps but also their host. Mass rearing systems for stink bugs, including the use of artificial diets, have improved parasitoid production (Silva et al., 2008, 2018; Mendoza et al., 2016; Hayashida et al., 2018). This rearing system, together with new technologies – such as the preservation of host eggs and parasitoids at low temperatures until field release (Silva et al., 2018), protection of parasitized eggs in cardboard capsules, drone-based deployment systems, and accurate spatio-temporal field releases – could favour biocontrol programs in the near future. The high susceptibility of egg parasitoids to insecticides (Corrêa-Ferreira et al., 2010) is also important, so the identification and use of selective products are critical for successful inundative biocontrol strategies (Stecca et al., 2017). The commercial production of *Te. podisi* in Brazil is currently under consideration for license approval, which will allow a wider availability of parasitoids to growers.

In Argentina, a biocontrol program against *N. viridula* was implemented in 1981 with the introduction of *Tr. basalis* from Australia. A few years after the release of *Tr. basalis*, *N. viridula* was reduced to low densities (Crouzel & Saini, 1983). This was the only classical biocontrol effort for stink bugs in Argentina, and no subsequent evaluations were made regarding the success of the biocontrol program in the regions of release (Molinari et al., 2008).

In Uruguay, a multi-institution project was launched in 2005 that combined public and private interests for mass rearing and release of *Te. podisi*, with the aim of increasing the levels of parasitism of *P. guildinii* and reducing the use of insecticides. Releases were initially made of parasitized eggs as a source of parasitoids, but because of high levels of natural predation, adult wasps were released in the second stage of the project. Post-release parasitism of *P. guildinii* eggs by *Te. podisi* in two regions (Dolores and Paysandú) was 67 and 69%, respectively, which was higher than natural parasitism in areas without releases, i.e., 52 and 47%, respectively (Castiglioni et al., 2007).

Conservation biological control and semiochemical-based strategies to enhance parasitoid efficacy

Because of the high diversity of natural enemies (Cingolani, 2012), especially of egg parasitoids (Scelionidae), which can reach high natural parasitism rates of 60–80% (Corrêa-Ferreira & Moscardi, 1995; Cingolani et al., 2014a,b; Paz-Neto et al., 2015; Zerbino & Panizzi, 2019), conservation biological control appears to be an effective approach for stink bug management in South America. However, due to the expansion of agricultural fields, landscape and habitat management may be necessary to enhance the ecological services provided by parasitoids (Aquino et al., 2019). Other tools that could improve conservation biological control of stink bugs include the recruitment of parasitoids using semiochemicals (Vieira et al., 2013, 2014) and the use of soybean cultivars producing volatiles attractive to parasitoids (Michereff et al., 2014, 2016). Moreover, attract-and-reward (Simpson et al., 2011) and push-pull (Khan et al., 2016) strategies could increase parasitism rates of semiochemically or naturally attracted parasitoids in the field. The potential of flowering plants to improve *Tr. basalis* attraction and parasitism rates of *N. viridula* eggs has also been demonstrated (Foti et al., 2017, 2019). An attract-and-reward strategy for *Te. podisi* and other species of South American scelionids is under investigation (RA Laumann, MF Aquino, MC Blassioli Moraes & M Borges, unpubl.). Conservation biological control of *N. viridula* and semiochemical-based strategies against stink bugs are also discussed more in detail below.

Case study 6: Conservation biological control of *Nezara viridula*

Global pest status of *Nezara viridula*

Perhaps as a result of climate warming, *N. viridula* seems to be rapidly expanding its range in both hemispheres to regions that were previously not warm enough to sustain the survival of the species. For instance, in Japan, *N. viridula* was confined to the southwestern part of the archipelago since its earliest record there in the late 19th century. However, the species has recently expanded its range into the warm-temperate zone and now reaches the central part of the archipelago (Tougou et al., 2009). Similarly, *N. viridula* is currently expanding its geographical range in Europe to the north (Salisbury et al., 2009; Marcu & Grozea, 2018). Reports of its occurrence have been documented in Slovakia (Hemala & Kment, 2017) and in The Netherlands, where an estimated 10-15% of sweet pepper greenhouses were infested in 2019 (H Hoogerbrugge, pers. comm.). Currently, *N. viridula* is considered a key pest in only a limited number of agricultural crops, mainly tomatoes and legumes (Esquivel et al., 2018). Changes in geographical distribution may profoundly affect its status as agricultural pest, particularly in the newly invaded areas. Given the need for sustainable management solutions, the possibility of combining the release of natural enemies with cost-effective approaches that involve habitat manipulation as a conservation biocontrol measure can be a valuable alternative to pesticides for controlling stink bug populations (Tillman, 2017).

Conservation biological control: habitat manipulation and cultural practices

Conservation biological control comprises a variety of methods, including habitat manipulation and cultural practices that aim at supporting natural enemy populations in the vicinity and within agricultural areas and thus reducing herbivory on crops (Gontijo, 2019). A particularly favoured measure is the planting of flower strips to provide parasitoids and predators with sugar resources, shelter, alternative prey, and a suitable microclimate (González-Chang et al., 2019). Although increasing habitat diversity *per se* may not lead to a consistent enhancement of pest control (Karp et al., 2018), it has been advocated that the 'right kind of diversity' is necessary, meaning that the traits of selected flowering plants must match the requirements of the targeted biocontrol agent (van Rijn & Wäckers, 2016). For example, corolla length and floral scent have been shown to be decisive features that confer a certain degree of specificity in the interactions between flowers and visiting insects (Tillman, 2017). In recent years, conserving and enhancing natural enemies for controlling phytophagous stink bugs by providing floral resources have been receiving increased interest (Tillman, 2017). Several studies have demonstrated that a strategic arrangement of flowering plants can attract and enhance the efficacy of these parasitoids (Tillman, 2017). In the southeastern USA, where peanuts and cotton are widely grown, stink bugs such as *N. viridula* and *Chinavia hilaris* (Say) (Pentatomidae) have become important pests in cotton. Field experiments with potted milkweed, *Asclepias curassavica* L. (Apocynaceae), placed at peanut-cotton interfaces to serve as a nectar source for natural enemies, showed that parasitism of both adult stink bug species was significantly higher in the two years of the experiment. In this case, parasitism was mainly due to the tachinid fly *Tric. pennipes* (Tillman & Carpenter, 2014). Similarly, flowering buckwheat, *Fagopyrum esculentum* Moench (Polygonaceae), was able to increase the efficacy of *Tric. pennipes* parasitizing *N. viridula* in cotton (Tillman, 2017).

Studies conducted in Sicily, Italy, have shown differing impacts of buckwheat floral nectar on the abundance of *Tr. basalis* and *O. telenomicida*, two sympatric egg parasitoids of *N. viridula* (Foti et al., 2017, 2019). In the case of *Tr. basalis*, laboratory tests to screen for suitable non-crop plants showed that buckwheat and basil flowers have a positive effect on parasitoid longevity (Rahat et al., 2005; Foti et al., 2017). Buckwheat was more attractive to *Tr. basalis* than other companion plants because of characteristic compounds in the floral scent (Foti et al., 2017). When buckwheat margins were grown alongside tomato plots, *Tr. basalis* located and parasitized more *N. viridula* egg masses during the growing season (Foti et al., 2019). However, such beneficial effects cannot be generalized and may not apply to all *N. viridula* egg parasitoids. In fact, laboratory tests showed that buckwheat scent repels female *O. telenomicida* and flowering buckwheat margins fail to increase stink bug parasitism under field conditions (Foti et al., 2019). Although a net beneficial effect can be expected due to the dominance of *Tr. basalis*, the possibility that there may be contrasting effects of floral scent within the parasitoid guild highlights the importance of selecting appropriate companion plants on a case-by-case basis. Researchers might even need to go one step further, as it can be necessary to select not only a suitable species but also the right cultivar of a given companion plant. Field studies in Florida, USA, for instance, demonstrated large differences between three sweet alyssum, *Lobularia maritima* (L.) Desv. (Brassicaceae), varieties in attracting predators of *N. viridula* when grown within tomato crops (Haseeb et al., 2018). Another obstacle for conservation biological control of *N. viridula* is the fact that parasitoids such as *Tr. basalis* tend to stay within a limited range of the flower margin, rather than moving deeper into the crop.

Future work will need to focus on the question how this obstacle can be overcome. Parasitoid distribution could be enhanced, e.g., by planting flower strips within fields and using modern GPS technology to avoid accidental spraying. Large-scale trials testing this concept are currently underway in the UK (ASSIST programme; https://www.assist.ceh.ac.uk).

Future prospects for improving stink bug control

The role of biological control in reducing invasive pest populations is widely recognized, although only a small amount (around one-tenth) of past natural enemy introductions provided satisfactory control (Cock et al., 2016). Therefore, because of the generally low economic injury levels of stink bugs (McPherson, 2018), it is not surprising that the efficacy of egg parasitoids alone is often insufficient to keep their populations under control; although it could be improved through conservation biological control (see case study 6) and applied chemical ecology methods (see below). Additionally, the prospects for integrating biological control with other sustainable control methods could improve stink bug control efficacy (Figure 1). Below, we review prospective methods for improving the efficacy of egg parasitoids and, when available, parasitoids attacking adults by using a chemical ecology approach. Also, we evaluate their possible integration with other sustainable methods targeting stink bugs.

Chemical ecology for manipulating parasitoid behaviour

In recent years, the development of semiochemical-based tactics has increased greatly, as they are considered efficient tools for manipulating insect behaviour with the aim of enhancing the biological control of crop pests (Wajnberg & Colazza, 2013). Feeding and/or oviposition by herbivorous insects induce changes in a plant's ecophysiological traits and their emission of volatile organic compounds (VOCs), either as herbivore-induced plant volatiles (HIPVs) or oviposition-induced plant volatiles (OIPVs) (Hilker & Fatouros, 2015; Pashalidou et al., 2015). Changes in VOC emission can occur locally and/or systemically and at either quantitative or qualitative levels (Dicke & van Loon, 2000; Martinez et al., 2013). Emission of HIPVs or OIPVs might alert the neighbouring plants of the same or other species (Baldwin & Schultz, 1983; Arimura et al., 2000; Karban & Maron, 2002) and/or act as an indirect plant defence by recruiting natural enemies of the herbivorous insects (Meiners & Peri, 2013; Hilker & Fatouros, 2015; Pashalidou et al., 2015). Many parasitoids and predators are known to respond to HIPVs or OIPVs during their host/prey location behaviour and can discriminate between volatiles produced by undamaged or infested plants (D'Alessandro & Turlings, 2006; Hare, 2011).

Egg parasitoids in systems involving stink bugs optimize their foraging behaviour by efficiently exploiting OIPVs (Conti & Colazza, 2012). OIPVs reliably indicate the presence of host egg masses and are produced by plants in large quantities, making them easily detectable (Fatouros et al., 2008; Conti & Colazza, 2012). For example, *Tr. basalis* (Colazza et al., 2004a,b; Frati et al., 2017; Salerno et al., 2019) and *Te. podisi* (Blassioli Moraes et al., 2005, 2009; Michereff et al., 2011) are attracted to leguminous plants infested by *N. viridula* or *E. heros*, respectively. By providing reliable information to female egg parasitoids on the presence of suitable target hosts, OIPVs increase wasp recruitment on the host-infested plants, likely increasing parasitism rates.

Feeding and oviposition by the zoophytophagous predator *P. maculiventris* also induce the emission of VOCs in *Vicia faba* L. (Fabaceae) plants that attract *Te. podisi* females (Martorana et al., 2019). Interestingly, both *Tr. basalis* (Martorana et al., 2017; Rondoni et al., 2017) and *Te. podisi* (Martorana et al., 2019) show specificity in their response to OIPVs emitted by infested plants, as neither species are attracted by plants on which the alien *H. halys* had fed and oviposited. This lack of response, probably due to the absence of a history of coevolution between the interacting species, might allow egg parasitoids to optimize their time and energy budgets by exploiting cues only from suitable (coevolved) hosts (Martorana et al., 2017, 2019).

Stink bug activity may also leave short-range cues that are exploited by egg parasitoids once they have landed on plants. For instance, *Tr. brochymenae* shows an intense egg foraging behaviour on the leaf surface, exploiting substrate-borne chemical cues emitted by brassicaceous plants as a consequence of feeding and oviposition of *M. histrionica* (Conti et al., 2010). Several egg parasitoids are also able to detect chemical traces released by stink bugs and retained by the plant epicuticular waxes (Conti et al., 2003; Colazza et al., 2009; Frati et al., 2013; Boyle et al., 2020). *Trissolcus basalis* females were shown to discriminate traces left by *N. viridula* females from those left by males, due to the absence of *n*-nonadecane, a cuticular hydrocarbon present in *N. viridula* males but absent in females (Colazza et al., 2007). In doing so, wasps can restrict their searching behaviour to an area of the plant where host eggs are more likely to be found.

Laboratory studies that demonstrated the role of HIPVs, OIPVs, and contact cues in mediating host searching behaviour of egg parasitoids have opened up new opportunities for developing strategies for herbivorous stink bug control. However, the application of semiochemical-based

techniques in the field is often difficult and provides results that are sometimes inconsistent. Several potential limitations and risks have been reported in the literature, including a potential increase in intraguild predation on natural enemies that in turn induces a reduction in the top-down control of the pests (Poelman & Kos, 2016; Peri et al., 2018). These techniques are applied in complex environments in which plants are subjected to various biotic and abiotic stresses and in which several intra- and inter-specific interactions can occur simultaneously. For example, the chewing damage from *Sitona lineatus* (L.) (Coleoptera: Curculionidae), whose adults feed on leaves and larvae feed on roots, induces a change in the OIPV profile emitted by *V. faba* plants that are concurrently infested by *N. viridula*, which in turn reduces the recruitment of the latter's egg parasitoid *Tr. basalis* (Moujahed et al., 2014). Similar disruptive effects on host egg location by parasitoids were observed in the presence of concurrent infestations of both alien (*H. halys*) and local (*N. viridula* or *P. maculiventris*) stink bugs (Martorana et al., 2017, 2019). Moreover, changes in the volatile blend emitted by plants infested by stink bugs that resulted in modification of egg parasitoid searching behaviour were also observed as a result of abiotic factors, such as water stress (Salerno et al., 2017).

To manipulate parasitoid behaviour on crops, semiochemicals can be applied directly onto the plants by spraying or by using slow-release dispensers. Their emission can be induced by applying elicitors such as plant hormones (JA, methyl jasmonate, cis-jasmone, and SA) or plants themselves could be genetically engineered to increase their emission of HIPVs (Blassioli Moraes et al., 2013; Colazza et al., 2013; Simpson et al., 2013; Peri et al., 2018). However, few examples are reported in the literature on parasitoids of stink bugs, and the results are somewhat contradictory. Positive results were obtained in attracting the tachinid parasitoid *Gymnosoma rotundatum* (L.) to persimmon orchards using methyl (*E*,*E*,*Z*)-2,4,6-decatrienoate, the aggregation pheromone of the brown-winged green stink bug *Plautia stali* Scott (Pentatomidae) (Jang & Park, 2010; Jang et al., 2011). Positive results were also obtained in soybean crops using the aggregation pheromone of *Riptortus pedestris* (= *clavatus*) (Fabricius) (Hemiptera: Alydidae), which led to an increased abundance of its egg parasitoids, *Ooencyrtus nezarae* Ishii (Encyrtidae) and *Gryon japonicum* (Ashmead) (Scelionidae) (Lim & Mainali, 2013). Similarly, application of a racemic mixture of methyl 2,6,10-trimethyltridecanoate, a component of *E. heros* pheromone, attracted scelionid egg parasitoids (Borges et al., 1998). Conversely, although an increase in parasitoid recruitment in soybean fields was observed by using slow-release dispensers impregnated with (*E*)-2-hexenal, a compound present in the metathoracic glands of *E. heros* (Laumann et al., 2007), a more in-depth study demonstrated only early parasitoid recruitment but not a sustained increase in abundance (Vieira, 2010). Moreover, the application of cis-jasmone in soybean plants had positive effects on the foraging behaviour of scelionid stink bug egg parasitoids, leading to an increase in their abundance (Blassioli Moraes et al., 2009; Vieira, 2010), but this did not lead to an improvement of parasitism rate (Vieira et al., 2013). Parasitoid recruitment using semiochemicals can be affected by the experience of the foraging females, for example when the hosts are scarce or lacking. Without the reward of suitable host eggs, scelionid parasitoid females were shown to reduce their responses to semiochemicals due to habituation (Peri et al., 2006, 2016; Abram et al., 2017a). Additionally, most parasitoids are more dependent on other cues than those from the host-plant complex, such as floral odours from nectar plants. Therefore, manipulating the habitat by creating ecological infrastructures, e.g., using companion flowering plants such as buckwheat that serve as feeding sites for recruitment of stink bug egg parasitoids, might increase parasitism efficacy (see case study 6). The integration of semiochemical-based manipulation of parasitoid behaviour and habitat manipulation, e.g., the attract-and-reward strategy (Khan et al., 2008; Simpson et al., 2011), might limit the possible negative effects of parasitoid recruitment in the absence of hosts.

Chemical ecology for manipulating stink bug behaviour

The use of semiochemicals against stink bugs might be combined with biological control to increase pest control efficacy within organic farming systems. The chemical ecology of stink bugs is characterized by a wide array of signals that drive their behaviour at both intra- and inter-specific levels. Although sex and aggregation pheromones of stink bugs are commonly used as lures for trapping (Borges et al., 1998, 2011; Leskey & Nielsen, 2018), few studies have reported the application of other semiochemicals.

Stink bugs exploit host plant volatiles to find their feeding and oviposition substrates (Martinez et al., 2013; Guarino et al., 2017a; Weber et al., 2018). The types of semiochemicals they use depend on their feeding habits and their level of polyphagy. Polyphagous species respond to blends of common plant volatiles in precise proportions, whereas monophagous and oligophagous species are more attracted by key plant-specific compounds (Guarino et al., 2017a). For example, *Eurydema pulchrum* Westwood (Pentatomidae) positively responds to volatiles from host plants belonging to different families, such as *Brassica oleracea* L., *Raphanus sativus* L. (Brassicaceae) and

Solanum lycopersicum L. (Solanaceae) (Rather et al., 2010). In contrast, *B. hilaris*, a specialist of Brassicaceae that exhibits a strong preference for certain species at the seedling stage (Huang et al., 2014; Guarino et al., 2017b), preferred *B. oleracea* and *B. napus* over *B. carinata*. This behaviour seems to be mediated by a novel diterpene hydrocarbon, still uncharacterized, emitted by *B. oleracea* and *B. napus* but missing in *B. carinata* blends (Guarino et al., 2018).

Stink bug preference for certain host plant species could be exploited for pest management using trap crops, which rely on an attractive host plant to arrest the pest and/or to concentrate it in certain zones of the field, where it can be eliminated (Hokkanen, 1991). Trap crops have shown the potential to effectively manage stink bugs in conventional and organic crop production systems (Todd & Schumann, 1988; Tillman, 2006). For example, the use of sorghum as a trap crop for *N. viridula* reduced insecticide applications in cotton, and black mustard used as a trap in sweet corn reduced pest damage by 22% (Rea et al., 2002; Tillman, 2006). Similarly, soybean and a mixture of triticale, sorghum, and sunflower were successfully used as trap crops in cotton fields for *Euschistus servus* (Say), *C. hilaris*, and *N. viridula* (Mizell et al., 2008; Tillman et al., 2015). The efficacy of the trap crop technique has also been demonstrated by combining it with pheromone-baited traps for suppressing *E. servus* attacking cotton. Such combinations of attractant stimuli increased the trap crop effectiveness in terms of numbers of attracted individuals and also consistently reduced the dispersal of *E. servus* in cotton fields (Tillman & Cottrell, 2012) and of *P. stali* in kaki persimmon orchards (Yamanaka et al., 2011). Recently, trap crops have been used for the management of invasive stink bug species. For example, *H. halys* has been controlled in pepper fields by using sunflower alone or in combination with sorghum as trap crops (Soergel et al., 2015; Mathews et al., 2017).

The use of trap crops for stink bug control may be particularly useful because there are few biologically based strategies that are available for the suppression of stink bugs in small organic farms or homeowner gardens (Mathews et al., 2017). Trap cropping might lead to (1) reduction in the use of pesticides, thus reducing the cost of pest control; (2) reduction in the selective pressure for development of pest resistance; and (3) preservation of natural enemies (Gordon et al., 2017). However, none of the cases reported have yet led to commercial implementation (Weber et al., 2018).

In order to manipulate pest behaviour more effectively, the trap crop technique can also be implemented by using a stimulus that is deterrent or repellent in nature, applied on the crop, leading to a push-pull strategy (Pickett et al., 2014). Information on the successful application of this strategy against stink bugs is scarce. However, a study carried out by Zhang et al. (2014) showed that several plant extracts obtained from clove, lemongrass, spearmint oil, and ylang-ylang plants acted as repellents toward *H. halys* and could be candidates as 'push stimuli' for this pest. The repellent stimuli could be used as extracts in dispensers or as intercropping plants to repel the pest that simultaneously could be attracted by a trap crop that serves as a 'pull stimulus'.

Intercropping with a non-host plant can directly reduce the damage to cash crops by masking the host from the pests, as some plants might have a repellent effect on herbivores (Smith & McSorley, 2000). In stink bug management, intercropping soybean with millet, cow pea, and groundnut reduced damage by *N. viridula* by 50% compared with a system containing soybean and millet only (Sastawa et al., 2004).

Future research on behavioural manipulation of stink bugs will be able to take advantage of new genomic and transcriptomic technology (Sparks et al., 2014) to identify the genes responsible for their pheromone production and behavioural responses to various odours. The objective will be, for example, to use plants or yeasts as 'factories' of stink bug attractants that can be used as 'dead end' trap crops (Møldrup et al., 2012).

Prospects for additional sustainable control methods

Additional sustainable control methods that could be considered in combination with parasitoids within organic farming systems include implementation of vibratory cues to disrupt stink bug sexual communication (Laumann et al., 2017; Čokl et al., 2019; Polajnar et al., 2019), direct plant resistance (Rondoni et al., 2018; Serteyn et al., 2020), exploitation of stink bug entomopathogens (Hajek et al., 2018), exclusion netting for fruit orchards (Candian et al., 2018), attract-and-kill methods (Morrison et al., 2019), and a novel mode of application of the sterile insect technique that combines the release of sterilized stink bugs with sterile parasitoids for pest eradication (Horrocks et al., 2020). Several of these possible methods are discussed below.

The reproductive behaviour in many stink bug species is mediated by multimodal communication, based on long-range attraction on a common substrate via chemical signals and the involvement of combinations of vibratory, chemical, and visual cues during calling and courtship behaviours (Čokl et al., 2019). Vibratory signals have been proposed as tools for mass trapping, using a bi-modal trap based on the aggregation pheromone and vibratory signal (Polajnar et al., 2019), and for mating disruption (Laumann et al., 2017). Considering that females of *Te. podisi* specifically respond to stink bug vibratory signals for

spatial orientation, such signals might also be exploited for parasitoid recruitment on infested crops (Laumann et al., 2017).

Host plant (*V. faba*) resistance to stink bugs has been investigated recently at the biological, molecular, and behavioural level (Rondoni et al., 2018; Serteyn et al., 2020). Rondoni et al. (2018) showed that plants recognize *H. halys* oviposition as a warning signal and respond by activating defences against *H. halys* nymphs, resulting in reduced size of juvenile offspring. This is possibly related to the priming of JA-dependent genes, including a cysteine proteinase inhibitor. The use of proteinase inhibitors in agriculture can be implemented through the development of transgenic plants, the use of recombinant microorganisms, or production of biopesticides (reviewed by Rodríguez-Sifuentes et al., 2020).

Conclusions

This review summarises more than a century of research on the biological control of invasive stink bugs. Invasive stink bugs are difficult to control, especially when they have already colonized entire regions and population outbreaks have occurred. A new pest often spreads rapidly after its introduction, and effective management is often subject to delays while available short-term tools are identified and a classical biological control program is initiated. Pre-emptive biological control is an innovative strategy for appropriate situations to reduce delays in research and implementation, by preparing in advance for the arrival of a pest. When a pest has already established in a new region, classical biological control can be an effective means of reducing the reservoir of populations in the wider landscape, thus reducing immigration to crops. However, classical biological control of stink bugs has limitations that have been addressed here and have been recently analysed by Abram et al. (2020). Using stage-structured matrix models, these authors showed that stink bug mortality at the adult stage would be expected to provide a greater reduction of population growth than mortality at the egg stage and that a multi-tactic approach targeting different stages would be much more effective in reducing populations below the economic injury level. Here, we show that egg parasitoids are the most common natural enemies of stink bugs globally, and for this reason they have been the most commonly studied parasitoids of stink bugs. However, parasitoids attacking adults also provide interesting perspectives, although these are only known for a few invasive stink bug species.

The combination of classical biological control followed by conservation methods and integrated with other sustainable control methods targeting the various instars should provide interesting prospects for the improvement of stink bug control efficacy (Figure 1). Conservation biological control, through a provision of suitable nectar-producing flowering plants, can attract and improve the efficacy of both parasitoids attacking adults (Tillman & Carpenter, 2014; Tillman, 2017) and egg parasitoids (Foti et al., 2017, 2019). The application of semiochemicals on crops or inducing them in plants by applying elicitors might also facilitate recruitment of parasitoid species attacking adults (Jang & Park, 2010; Jang et al., 2011) or eggs (Borges et al., 1998; Lim & Mainali, 2013), although a consequent improvement of parasitism has yet to be demonstrated with these applications (Blassioli Moraes et al., 2009; Vieira, 2010; Vieira et al., 2013). The attract-and-reward strategy, which combines behavioural manipulation and habitat manipulation, might further improve parasitoid efficacy (Khan et al., 2008; Simpson et al., 2011). Semiochemicals can be used to manipulate not only natural enemies but also stink bug behaviour by using either trap crops (Todd & Schumann, 1988; Tillman, 2006; Nielsen et al., 2016) or push-pull strategies (Pickett et al., 2014). Moreover, future tactics might exploit vibratory cues to disrupt stink bug sexual communication (Laumann et al., 2017; Čokl et al., 2019; Polajnar et al., 2019) or direct plant resistance (Rondoni et al., 2018). Ultimately, as most research has been conducted only under laboratory conditions, there is an urgent need for field validation of these data before effective implementation can become a reality.

Author Contribution Statement

All authors contributed to the writing of the paper. EC and GR conceived the idea of the article and designed the layout. EC coordinated the paper and wrote the general parts. GA and BB wrote about the pre-emptive classical biological control of *H. halys* in New Zealand. FC and RAL focused on the biocontrol of stink bugs in South America. SC and MR wrote about the conservation biocontrol of *N. viridula*. EP, SG, and EW contributed with the chemical ecology of stink bugs and egg parasitoids. RS and MG wrote about the classical biological control of *B. hilaris*. LM and LT focused on the biocontrol of *H. halys* in Europe. CRS and KH focused on the biocontrol of *H. halys* in North America.

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Bilan de l'activité de thèse

Articles parus ou en cours de parution :

Martel, G., Augé, M., Talamas, E., Roche, M., Smith, L., & Sforza, R.F.H. (2019). First laboratory evaluation of *Gryon gonikopalense* (Hymenoptera: Scelionidae), as potential biological control agent of *Bagrada hilaris* (Hemiptera: Pentatomidae). *Biological Control* 135, 48-56 https://doi.org/10.1016/j.biocontrol.2019.04.014

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Martel, G., & Sforza, R.F.H. (2021). How an egg parasitoid responds to an unusual stinkbug oviposition behavior: the case of *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae) and *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae). *Entomologia Generalis* (in press)

Martel, G., & Sforza, R.F.H. (2021). Evaluation of three cold storage methods of *Bagrada hilaris* (Hemiptera: Pentatomidae) and the effects of host deprivation for an optimized rearing of the biocontrol candidate *Gryon gonikopalense* (Hymenoptera: Scelionidae).

Article prêt pour soumission

Martel, G. & Sforza, R.F.H.: Temperature effect on development, survivorship and reproduction of *Gryon gonikopalense* Sharma (Hymenoptera: Scelionidae), an egg parasitoid of *Bagrada hilaris* (Burmeister) (Hemiptera: Pentatomidae) in the frame of an importation biocontrol program

Article en préparation

Martel, G., & Sforza, R.F.H.: Anticipating the arrival of a new stinkbug pest in continental Europe: what can we learn from preliminary host specificity tests for biocontrol?

Communications orales

Martel, G., & Sforza, R.F.H. (2019). Mélodie en sous-sol : quand le Gryon chante, Bagrada déchante. Colloque des Entomophagistes May 2019, Antibes, France

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Résumé : Dans un contexte de mondialisation et de changement climatique, de plus en plus d'insectes deviennent invasifs et affectent notamment l'agriculture à l'échelle mondiale. Au cœur de ce réseau d'invasions, les Etats-Unis subissent l'arrivée de nombreuses espèces provenant principalement d'Asie. Parmi elles, les punaises pentatomidés menacent la plupart des agrosystèmes américains et sont aujourd'hui principalement régulées par les pesticides de synthèse. *Bagrada hilaris* (bagrada), originaire d'Asie et d'Afrique, est la plus récente à avoir colonisé les Etats-Unis où elle sévit depuis 2008 dans les cultures de chou et de brocoli (Brassicaceae). Pour répondre aux besoins des agriculteurs, l'USDA-ARS a financé un programme de lutte biologique classique visant à sélectionner et introduire en Californie un ou plusieurs ennemis naturels de bagrada depuis son aire d'origine. Les travaux inclus dans cette thèse sont une partie de ce programme. Ils ont cherché à évaluer en serre de quarantaine si le parasitoïde oophage *Gryon gonikopalense* (Scelionidae), originaire du Pakistan, pourrait s'établir en Californie et contrôler efficacement bagrada. Quatre principaux objectifs ont ainsi été définis : 1) décrire la biologie générale de *G. gonikopalense* ; 2) décrire son comportement de recherche d'hôte ; 3) examiner la possibilité de le produire en masse et 4) évaluer sa spécificité vis-à-vis de bagrada avec des punaises euro-méditerranéennes. L'objectif 1 a permis de montrer que la physiologie de *G. gonikopalense* était similaire à celle de nombreux autres scelionidés : en fonction de la température, il complète son développement en 7 à 25 jours, peut vivre entre 30 et 150 jours et parasiter une centaine d'œufs au cours de sa vie. Il partage avec bagrada un optimum thermique entre 25 et 35 °C qui pourrait lui permettre de s'établir en Californie. En plus d'être un parasite efficace de bagrada, l'atout de *G. gonikopalense* réside particulièrement dans son comportement de recherche des œufs de son hôte qui sont enterrés dans le sol dans 90% des cas. Nous avons découvert que *G. gonikopalense* était capable de creuser le sol pour parasiter les œufs. En outre, nous avons montré dans un système tri-trophique que le parasitoïde attaquait principalement les pontes enterrées de bagrada, tout en étant capable d'atteindre les œufs déposés sur la plante hôte (env. 10%). Pour le 3ème objectif, nous avons montré que le stockage des œufs de bagrada à 5°C sur trois semaines permettait d'optimiser l'élevage du parasitoïde et faciliter une production de masse. Concernant l'objectif 4, toujours en cours, il en résulte que les sur 11 pentatomidés testées, au moins 4 ont été parasitées, dont deux essentiellement localisées autour du bassin méditerranéen. Ces résultats complémentent les tests menés en Californie et permettent de discuter proactivement de l'introduction de *G. gonikopalense* dans les populations méditerranéennes invasives de bagrada. Finalement, nous avons décrit au cours de cette thèse plusieurs aspects, jusqu'alors inconnus, de la biologie de *G. gonikopalense*. Sa spécialisation comportementale envers bagrada en fait notamment un candidat de premier choix pour le programme de lutte biologique Une pétition rédigée sur la base de ces connaissances remise aux autorités sanitaires des Etats-Unis restent un objectif à court terme. Ceux-ci évalueront alors les risques et les bénéfices liés à l'utilisation de *G. gonikopalense* pour contrôler bagrada en Californie.

Mots clés : pentatomidé, scelionidé, parasitoïde, lutte biologique, brassicacées

Abstract: In a context of globalization and climate change, more and more insects are becoming invasive, particularly affecting agriculture on a global scale. At the heart of this network of invasions, the United States of America is experiencing the arrival of many species, mainly from Asia. Among them, stink bugs (Pentatomidae) threaten most American agrosystems and are now mainly regulated by synthetic pesticides. *Bagrada hilaris* (bagrada), native to Asia and Africa, is the most recent species to invade the USA, where it has been reported since 2008 in Brassicaceae crops. To answer the needs of farmers, USDA-ARS funded a classical biological control program to select and introduce from its native range one or more natural enemies of bagrada into California. The research included in this PhD thesis is part of this program. It sought to assess in a quarantine greenhouse whether the oophagous parasitoid *Gryon gonikopalense* (Scelionidae), native to Pakistan, could establish in California and efficiently control bagrada. Thus, four main objectives were defined: 1) to describe the general biology of *G. gonikopalense*; 2) describe its host foraging behavior; 3) examine the possibility of its mass production and 4) assess its specificity towards bagrada using Euro-Mediterranean non-target stink bugs. Objective 1 allowed to show that the physiology of *G. gonikopalense* was similar to that of many other scelionids: depending on the temperature, it completed its development in 7 to 25 days, its lifespan ranges from 30 to 150 days and it is able to parasitize hundred eggs during its lifetime. It shares with bagrada a thermal optimum between 25 and 35 °C which could allow it to settle in California. In addition to being an effective parasitoid of bagrada, the advantage of *G. gonikopalense* particularly involves its foraging behavior for host's eggs, which are buried in the ground in 90% of cases. We found that *G. gonikopalense* was able to dig into the soil to parasitize eggs. In addition, we have shown in a tri-trophic system that the parasitoid mainly attacks the buried eggs of bagrada, while being able to reach the eggs deposited on the host plant (about 10%). For the 3rd objective, we have shown that the storage of bagrada eggs at 5 °C over three weeks made it possible to optimize the breeding of the parasitoid and facilitate mass production. Concerning the objective 4, still in progress, we showed that among the 11 pentatomids tested, at least four were suitable for the parasitoid, and two of them were mainly located around the Mediterranean basin. These results complement the tests carried out in California and open a proactive discussion on the introduction of *G. gonikopalense* in invasive Mediterranean populations of bagrada. Finally, during this thesis, we have described several aspects, hitherto unknown, of the biology of *G. gonikopalense*. Its behavioral specialization towards bagrada makes this parasitoid particularly promising for the biological control program. A petition-to-release drafted on the basis of this knowledge submitted to the health authorities of the USA remains a short-term objective. They will then assess the risks and benefits of using *G. gonikopalense* to control bagrada in California.

Key words: pentatomid, scelionid, parasitoid, biological control, brassicaceae