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# Genetic Basis of Extra-Ocular Pigmentation in Semi-Aquatic Embryos

Aidamalia Vargas Lowman

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**Aidamalia VARGAS – LOWMAN**

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Les bases génétiques de la pigmentation dans les embryons de punaise  
d'eau

Genetic Basis of Extra-Ocular Pigmentation in Semi-Aquatic Embryos

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Devant le jury composé de :

Dr. NADEAU, Nicola	DR University of Sheffield	Rapporteure
Dr. BELDADE, Patricia	DR Instituto Gulbenkian de Ciência	Rapporteure
Dr. GIBERT, Patricia	DR Université Claude Bernard Lyon 1	Examinatrice
Dr. JORON, Mathieu Examineur	DR Centre d'Ecologie Fonctionnelle & Evolutive	
MERABET, Samir	DR ENS de Lyon	Examineur
Dr. KHILA, Abderrahman	DR ENS de Lyon	Directeur de thèse



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## Abstract

Understand how the re-use of pre-existing genetic pathways control the wonderful diversity in nature is a major challenge in evolutionary biology. The diversity of colours present in Gerromorpha (semi-aquatic bugs) during the embryonic development is an excellent model to achieve this objective. In order to explore this question, I present a review on the different functions of colouration in nature and on the mechanisms known to produce colours. I also introduce the concept of genetic co-option and how pigmentation traits have been used to understand the evolutionary recruitment of pre-existing genes or pathways to produce novelty. The principal aim of this doctoral thesis was to understand the genetic basis for the diversification of the extra-ocular pigmentation in Gerromorpha embryos. Most of the semi-aquatic bugs develop a variability of yellow or red colours patterns in legs and antennae during the embryonic stage. Since the red colour in appendages was similar to the one present in the eyes, we hypothesized that the extra-ocular colours could be produced by the co-option of the eye pigments biosynthesis pathway. First we inferred the evolutionary history of this trait based on its presence or absence in embryos of 34 species. We found that the ancestral state of the trait in Gerromorpha was yellow and that six independent lineages evolved bright red colour, while two lineages lost the colour. Using RNAi and in situ hybridisation on homologous genes from the pteridine and ommochrome biosynthesis pathways, we described the genetic pathway involved in the production of pigments in eyes and extra-ocular tissues in *Limnogonus franciscanus* embryos. After that, we performed a screening of three genes from this pathway in five other species with different extra-ocular colours and patterns. We discovered that the same pathway was recruited once to produce the diversity of patterns in Gerromorpha. Furthermore, we identified by UPLC-HRMS that xanthopterin and erythropterin pigments produce the variability of colours and patterns in different species. Our next step aimed to understand how the recruitment of a conserved pathway could produce this striking diversity of colour patterns. Using RNA-Seq technology and bioinformatics tools, we identified 167 transcription factors that are co-expressed in eyes, antennas and legs of embryos in *Limnogonus franciscanus*. These proteins might be involved in the regulation of genes responsible for the different colour patterns. We have started an RNAi screen of these transcription factors. This project is still ongoing but in this thesis I will present the preliminary results and conclusions.

Finally, I had the opportunity to participate in two important collaborative projects led by the team. I annotated genes in the first genome sequence for a Gerromorpha (*Gerris buenoi*). The manual annotation of this genome uncovered a number of local gene duplications and expansions of gene families known for their importance in a variety of processes associated with morphological and physiological adaptations to a water surface lifestyle. I also worked on the most actualized phylogeny from Gerromorpha prepared with transcriptomic data from 100 species. This new phylogeny enabled us to reconstruct ancestral traits that were important for the adaptation of Gerromorpha to water surface.

In conclusion, the pigmentation of semi-aquatic bugs during the embryonic stage is a good model to understand the co-option of pre-existing genetic pathways and underlying the question of how a conserved pathway could be regulated to produce diverse morphological phenotypes.

Keywords: co-option, diversification, pigments, pteridine network, phylogenomic.

## Résumé

Comprendre comment la réutilisation de voies génétiques préexistantes contrôle la biodiversité est un défi majeur de la biologie évolutive. La diversité de couleur embryonnaire des Gerromorphes (punaises semi-aquatiques) est un excellent modèle pour atteindre cet objectif. Afin d'explorer cette question, je présente une synthèse sur les différentes fonctions de la coloration dans la nature, ainsi que sur les mécanismes de synthèse de ces couleurs. J'introduis également le concept de co-option et comment les traits de pigmentation ont été utilisés pour comprendre le recrutement évolutif de gènes ou de voies préexistantes dans la production de la variabilité.

Le but de ce doctorat était de comprendre les bases génétiques de la diversification de la pigmentation extra-oculaire chez les embryons des Gerromorphes. La plupart des punaises semi-aquatiques présentent une variabilité de pattern de couleur jaune ou/et rouge dans les pattes et les antennes au stade embryonnaire. La couleur rouge observée dans les appendices étant similaire à celle présente dans les yeux, nous avons émis l'hypothèse que les couleurs extra-oculaires pouvaient être produites par la co-option des voies de synthèse des pigments des yeux. Nous avons d'abord déterminé l'histoire évolutive de ce trait à partir de sa présence ou de son absence dans les embryons de 34 espèces. Nous avons découvert que l'état ancestral du trait chez les Gerromorphes était le jaune, que six lignées indépendantes ont acquis la couleur rouge, alors que deux autres lignées ont totalement perdu toute pigmentation extra-oculaire. Grâce à l'analyse génétique par interférence ARN et hybridation *in situ*, nous avons identifié les voies impliquées dans la pigmentation des yeux et des organes extra-oculaires dans l'espèce *Limnogonus franciscanus*. Nous avons ensuite testé par interférence ARN (ARNi) et hybridation *in situ* trois gènes de la voie ptéridine dans cinq autres espèces de Gerromorphes présentant des colorations extra-oculaires différentes. Les résultats suggèrent que la même voie a été recrutée une seule fois pour produire la diversité de pattern. De plus, grâce à une analyse chimique par ultra-chromatographie couplée à de la spectrométrie de masse, nous avons identifié que la xanthoptérine et l'érythroptérine sont les deux pigments responsables de la couleur chez différentes espèces. Nous nous sommes aussi demandé comment le recrutement d'une seule et même voie conservée pouvait produire une telle diversité de pattern. En utilisant la technologie de transcriptomique du RNA-seq, nous avons identifié 167 facteurs de transcription co-exprimés dans les yeux, les antennes et les pattes des

embryons de *Limnogonus franciscanus*. Ces protéines pourraient intervenir dans la régulation des gènes impliqués dans la formation des patterns de couleur embryonnaire. Nous avons initié un crible ARNi de ces facteurs de transcription. Ce projet est toujours en cours et nous présenterons dans cette thèse les résultats et conclusions préliminaires.

Enfin, j'ai eu l'opportunité de participer à deux importants projets collaboratifs de l'équipe. J'ai annoté plusieurs gènes sur le premier génome de Gerromorpha (*Gerris buenoi*). L'annotation manuelle de ce génome a permis de découvrir un certain nombre de duplications de gènes locaux et d'expansions de familles de gènes connues pour leur importance dans une variété de processus associés à des adaptations morphologiques et physiologiques afin de pouvoir vivre à la surface de l'eau. J'ai également travaillé sur la phylogénie la plus actualisée des Gerromorpha préparée avec des données transcriptomiques de 100 espèces. Cette nouvelle phylogénie nous a permis de reconstituer des traits ancestraux importants pour l'adaptation des Gerromorphes à la surface de l'eau. .

En conclusion, la pigmentation des punaises semi-aquatiques au stade embryonnaire est un bon modèle pour comprendre la co-option des voies génétiques et la question sous-jacente de la façon dont une voie conservée pourrait être réglémentée pour produire divers phénotypes.

Mots-clés: co-option, diversification, pigments, voie ptéridine, phylogénomique.



## Foreword

I am laboratory professor since 2007 in the Genetics Department of the University of Panama (See CV in the annexes). I had the opportunity to obtain four years Panamanian fellowship to obtain a PhD in the Functional Genomics Institute of Lyon, with Abderrahman Khila and François Bonneton as advisors.

At the beginning I started my thesis as part of the Convergenomix project granted by ANR. This consortium was trying to identify the types of genomic changes that underlie convergent phenotypic evolution by studying three distant animal groups: Aselidea isopods, rodents and Gerromorpha.

The aim of The Convergenomix project is to understand the genomic causes of convergent phenotypic evolution and to provide molecular and bioinformatics methods, focusing on gene sequences, on gene expression levels, and gene expression timelines.

My collaboration as part of the thesis project was to understand the genetic basis underlying the transition from freshwater to marine habitat and the convergent evolution of this trait in Gerromorpha. Gerromorpha or semi-aquatic bugs, which initially specialized to live on the surfaces of fresh water zones, have been successful in colonizing marine habitats, including the open ocean (ANDERSEN 1982). Ancestral character reconstruction revealed that out of the 18 known marine lineages, 14 transitions were completed independently from each other in the group. The other 140 genera remained in fresh water zones. The marine versus freshwater preference covers both micro- and macro-evolutionary scales (ANDERSEN 1999). Figure 1 in the annexes shows examples of marine vs. freshwater habitat preference and the lineages that were sampled as part of the project. As is shown in Annexes Figure 1, *Mesovelgia* is one of the genera that have representative species in marine and freshwater habitats. We designed an experiment with the species *Mesovelgia mulsanti* that consisted in increasing gradually the salinity concentration until obtaining a population with the maximum tolerance. After one year I could maintain several populations, the increasing range of salinity was from 0% to 1%. Samples of females, males, nymphs and embryos were taken and are ready to be used for differential expression analysis. Sadly, because of the difficulty to keep alive some of the salinity species in the laboratory and my limited bioinformatics knowledge at that moment, my advisors proposed me to change the convergent project to studying the genetic basis of pigmentation during the embryonic stage of Gerromorpha. All that time was useful to learn

more about bioinformatics tools and evolutionary concepts as convergence, homology and parallelism, that until that moment I was unfamiliar with.

As mentioned above, I came thanks to a fellowship granted by the government of my country. The principal aim of that fellowship was to acquire knowledge in bioinformatics and Next Generation Sequences (NGS) analysis. Luckily, I was part of different projects in the laboratory where I could improve my knowledge in those areas. For example, I participated in the gene annotation of *Gerris buenoi* genome. The bioinformatics skills acquired, also were useful to make a contribution in the reconstruction of a new phylogeny of Gerromorpha group.



## Objective of the thesis

The principal objective of my thesis was to understand the genetic basis of pigmentation during embryonic development in the Gerromorpha. Most of work previously performed to understand the genetic bases of colour diversification was focused on the adult stage on Holometabolous insects (Bodo et al. 2017, P.J. Wittkopp, P. Beldade 2009 and Gautier M. et al. 2018). The extra-ocular pigmentation in semi-aquatic insects is a new model system to understand the evolution of colour and diversification. Therefore, in chapter one, I present an introduction about the nature of colours, I explain the evolutionary history of the trait and how we could test our hypothesis of eye pigment pathway co-option.

In chapter two, I describe a transcriptomic analysis of different pigmented organs from the species *Limnogonus franciscanus* to identify transcription factors that could be involved in the regulation of the production of the different colours and patterns.

Chapter three explains different studies where I was involved during my time as a PhD student: The *Gerris buenoi* genome from Gerromorpha group, the reconstruction of a novel and more complete Gerromorpha phylogeny and in the study of sexually antagonistic coevolution in a genus of water striders called *Rhagovelia*. Finally, I discuss the results obtained throughout the doctoral work, along with brief suggestions for future directions and perspectives.

# CHAPTER 1

## General introduction and literature review

To understand the genetic basis of extra-ocular colour during the embryonic development of semi-aquatic bugs (Gerromorpha), I consider necessary to first introduce what the sources of biological colours in nature are. Also, it is important to discuss what we know about extra-ocular colour, not only in Gerromorpha but also in the entire order of Hemiptera. Additionally, other subjects covered in this introduction are co-option as evolutionary process to produce diversity and how pigmentation has been used as a trait to study genetic recruitment.

The evolution of colour in nature is one of the most exiting fields in biology. In this chapter, I discuss about biological mechanisms that have been discovered to produce the diversity of colour and colour patterns. Even if my thesis is centred in pigmentation, I found important to provide a brief overview and examples of how structural colours are produced by light interference.

Until now, little is known about the pigmentation, colours and colour patterns in Hemiptera embryos. Given the scarce information, I gathered all the information that I could access to infer about the embryonic extra-ocular colour and their historical evolution.

Genetic co-option, the re-use of pre-existing genes and/or pathways, even if it is an important evolutionary process to produce phenotypic diversity, is not really well understood. The third section of the introduction is a summary of several examples where recruitment of genes for pigment pathways might have generated diversification. Lastly, I introduce Gerromorpha, an emerging model that has been used in the recent years in the field of evolution and developmental.

## **Colour in nature**

During the 19th century, naturalists were interested in classifying and understanding the function of colouration and colour patterns on animals and plants and made a correlation between this diversity and natural selection (WALLACE 1878; BATES 1879; POULTON 1890; THAYER AND THAYER 1909; CARO 2017).

Today, we know that colouration mediates the relationship between an organism and its environment in important ways, including social signalling, antipredator defence, parasitic exploitation, thermoregulation, and protection against UV, microbes, and abrasion (CUTHILL *et al.* 2017).

Two main sources are known to produce colours in nature: structural colours (schemochromes) and pigments (chemochromes).

Structural colour, which is the colour produced by the organization of micro- or nano-structures (keratin, melanin, chitin or guanine) with the interaction of air and light scattering (BAGNARA T.T. *et al.* 2006; DOUCET *et al.* 2006; SUN *et al.* 2013) have been well documented in animals and plants. Examples include the blue colouration in many amphibians, the iridescent colour pattern in wings of flies or butterflies and the “white” colour in some plants (VIGNERON *et al.* 2005; BAGNARA T.T. *et al.* 2006; SHEVTSOVA *et al.* 2011).

In biology, pigment is defined as any material that produces colour in plant or animal cells, and in the result of selective absorption (BANDARANAYAKE 2006). Chemochromes are normally products of complex biosynthetic pathways involving numerous enzymes (BANDARANAYAKE 2006). Pigments can be classified according to their origin as natural, synthetic or inorganic pigments. Also, natural pigments can be classified by their structural characteristics (DELGADO-VARGAS *et al.* 2000). Delgado-Vargas and collaborators, made a review of characteristics and biosynthesis of pigments in nature. Moreover, they presented the function of six of the groups most important pigment in nature and indicated examples of organisms where can be identified, this information is summarised in Table 1.

### *Pigment present in insects*

Insects are the most diverse and abundant group among the animal kingdom with 1 to 10 million species (MORGAN 2010). The physiology of insects is important for adaptation and competition with other species, due to their ability to synthesize or acquire an extremely diverse array of compounds for defence, attack or communication, which includes a great

diversity of secondary metabolites (EISNER *et al.* 1994). Colouration is an important trait to enhance the adaptation and competitiveness of insects in nature and plays several roles in many biological activities, such as camouflage, mimicry, aposematism or warning, selection for sex, and communication by signalling (ALCOCK 1998). Furthermore, pigments are known to have function in insect vision (SHOUP 1966).

The diversity of colour observed in insects is due either to schromochromes (VUKUSIC *et al.* 1999) or chemochromes (SHAMIM *et al.* 2014), or to both (RUTOWSKI *et al.* 2005). Understanding the biosynthesis and biochemistry of pigments has been the subject of many studies, especially for those that are widespread in nature, such as melanins and carotenoids (DELGADO-VARGAS *et al.* 2000).

Another manner to classify pigments in insects is based on their origin, with those that can be synthesised endogenously by the insects, such as anthraquinones, aphins, pterins, tetrapyrroles, ommochromes, melanins and papiliochromes; and those that are sequestered from their host plants, the anti-oxidative carotenoids and water-soluble flavonoids (SHAMIM *et al.* 2014). Many endogenous pigments may have a large range of colours and can be identified in different groups, for example melanins (Figure 1). Others pigments are restricted to a family, such as the papiliochromes in Papilionidae (Lepidoptera) or aphins in Aphididae (Hemiptera) (Figure 1).

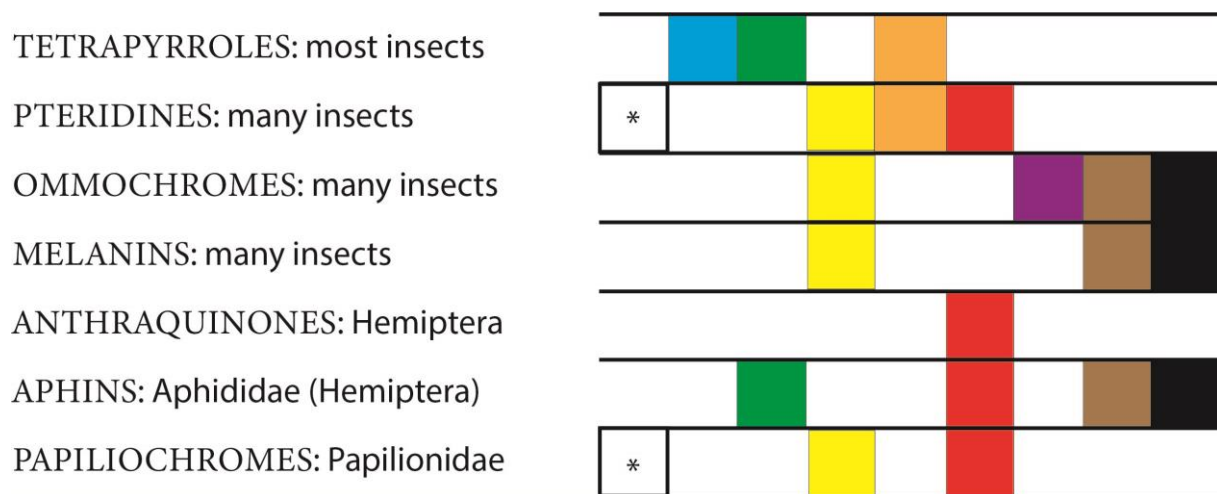


Figure 1. Endogenous pigments in insects. Certain pigments synthesized by insects and the variability of colour that are identified. Based on (SHAMIM *et al.* 2014). The boxes represent the colours observed in each pigment. (\*) when is colourless.

Table 1. Principal function of natural pigments. Major groups of organic pigments groups in nature and there function. Based on (DELGADO-VARGAS *et al.* 2000).

Structural characteristic	Group of pigments	Pigments	Organisms	Function
<b>Tetrapyrrole derivatives</b>	Chlorophylls and heme colors	Phytochrome	Algae, plants	Signal-transducing photoreceptorion
		Heme group: hemoglobin and myoglobin	Animals	Transport and storage of oxygen
		Chlorophyll	Algae, plants	Photosynthetic process
		Bilins	Invertebrates, cyanobacteries and some algae	Integuments of many invertebrates, accessory pigments in light harvesting
<b>Isoprenoid derivatives</b>	Carotenoids and iridoids	Carotenoids	photosynthetic and nonphotosynthetic organisms	Required in photosynthesis and colour of fruits, vegetables, fungi, flowers, also present in birds, insects, crustaceans, and trout.



		Iridoids	Plants	Not particularly important as colorants
<b>N-heterocyclic compounds different from tetrapyrroles</b>	Purines, pterins, flavins, phenazines, phenoxazines, and betalains	Purines	Living organisms, plants	Structural components in DNA and RNA
		Pterins	Mammals, birds, fish, insects, bacteria	Colour in some insects, in vertebrate eyes, human urine, and bacteria. Some pterins are growth factors (Ex. folic acid)
<b>Benzopyran derivatives (oxygenated heterocyclic compounds)</b>	Anthocyanins and other flavonoid pigments	Anthocyanins	Vascular plants	Attract pollinators. Antioxidant
		Flavonoid pigments: aurones, chalcones and flavonols and flavanones		Contribute to the pollinating process together with anthocyanins. Photoprotection, defence mechanism in plants
<b>Quinones</b>	Benzoquinone, naphthoquinone,		Plants, algae and Echinodermata	Echinodermata family, contribute to

	anthraquinone		family	the pigmentation of spines, shell, ovaries, and eggs. Bronze colour in <i>Polyporus rutilans</i> (fungus) and bacteria.
<b>Melanins</b>	Eumelanins	Black, gray, and brown colorations of animals, plants, and microorganisms	Vertebrate and invertebrate animals	Photoprotection
	Phaemelanins		Mammals and birds	Photoprotection
	Allomelanins		seeds, spores, and fungi, and esclerotins in arthropods	

## **Biology and evolution of extra-ocular colour in Hemiptera embryos**

### *Evolution of Hemiptera.*

Hemiptera is a hemimetabolous order of insects with unique morphological adaptations for piercing and sucking liquids from plants or other insects, due to modification of the mouthpart into elongated rostrum and other parts such as the maxilla and mandibles into long point stylets (GOULA AND MATA 2015). Bugs can be found in terrestrial and freshwater habitats; some are even marines. More than 100,000 species of hemipterans have been described in 104 families worldwide, and this number corresponds to about 10% of known insects (BARNARD 2011; JOHNSON *et al.* 2018).

Traditionally, hemipterans were divided in two groups, “Homoptera” and “Heteroptera” based on morphological classification (MYERS AND CHINA 2009). After molecular analysis, Homoptera was divided into three groups (Cicadomorpha, Fulgoromorpha, Sternorrhyncha), while Heteroptera is a monophyletic group (SONG *et al.* 2012). Recent molecular data divide the Hemiptera order into five groups: Sternorrhyncha, Cicadomorpha, Fulgoromorpha, Coleorrhyncha, and Heteroptera. Auchenorrhyncha (suborder of the Hemiptera containing most of the familiar members called the “Homoptera”) is paraphyletic, with Cicadomorpha forming the sister group to (Fulgoromorpha + Coleorrhyncha). Within Heteroptera, all infraorders were recovered as monophyletic, as previously shown (Figure 2.) (LI *et al.* 2017).

The oldest Homoptera fossil was registered since the Early Permian, a period marked with an expansion of Mesophytic gymnosperm groups, around 298.9 million years ago (Mya). A rapid diversification of homopterans occurred during the Late Permian and a considerable extinction took place at the Permo-Triassic boundary (SHCHERBAKOV 2000). Moreover, the oldest Heteroptera reported is a water bug *Arlecoris louisi*, from the earliest Middle Triassic of France (approximately 240 Mya) ((SHCHERBAKOV 2010). The true bugs became even more numerous and diverse during the Mesozoic period, especially in the Late Jurassic and Early Cretaceous (YURI *et al.* 2011).

Currently, Heteroptera species have been a focus of interest because of their potential as biological pest control agents. For example, the flower bugs of the genus *Orius* have been used to decrease the population of bean thrips (*Megalurothrips usitatus*, Thysanoptera: Thripidae) on red beans (WANG 2001). On the other hand, stink bugs such as *Halyomorpha halys* and *Homalodisca vitripennis* are considered as pests for the damage caused to crops while *Rhodnius* is a vector of Chagas disease (DIAS *et al.* 2002; GRANDGIRARD *et al.* 2008; PANIZZI 2015). Conversely, hemipterans can also be used as ecological and environmental indicators in forest ecosystems (M. L. MOIR 2007).

### *Phenotypic variability in eggs of Hemiptera*

Most of the scientific works done on the embryonic stages of Hemiptera have been directed to morphological comparisons for establishing classification of the different groups (SOUTHWOOD 1956; COBBEN 1968; PANIZZI AND GRAZIA 2015). Studies based on detailed description of embryonic stages and a few mutants were also performed with *Oncopeltus fasciatus* in the early seventies (BUTT 1949; SPRINGER 1964; LAWRENCE 1970; LAWRENCE 1973). The advent of developmental genetics in the early eighties pushed aside most of the studies with insects, including hemipterans. It was not until the evo-devo revolution of the late 1990s and the development of protocols in molecular tools, that the Hemiptera start to be used as a models to understand the molecular mechanisms regulating the evolution of specific traits (CHIPMAN 2017). Also, transcriptomic data and complete genomes of several species have been recently published (ARMISÉN *et al.* 2018; PANFILIO AND ANGELINI 2018; PANFILIO *et al.* 2019)

In order to study the evolution of colouration in the embryos of Hemiptera, I performed a bibliographic research to obtain information concerning the colouration of the eggs, description of embryonic development and ecological information on different groups (Table 2).

### *Oviposition sites*

Southwood (SOUTHWOOD 1956) classified Hemiptera eggs in four classes depending on the place where they are laid : 1) exposed, 2) semi-exposed, 3) Embedded in dead plants 4) Embedded or associated with living plants. As an example of class 1, females of Pentatomomorpha lay their embryos on the surface of different substrates (V. MATESCO 2009). Also, *Adomerus variegatus* and *Canthophorus niveimarginatus* (Pentatomorpha: Cydnidae) females lay a cluster of eggs on the ground and show a maternal care during embryogenesis and first nymphal stage (FILIPPI *et al.* 2009; MUKAI *et al.* 2010). The Belostomatidae (Nepomorpha) females lay embryos on the dorsum of the males (ICHIKAWA 1988; RODRIGUES AND MOREIRA 2005). The species *Dysdercus albofasciatus* (Pyrrhocoridae) is an example of class 2. The eggs are found semi- exposed in cracks or between litters (STADLER *et al.* 1987). As example of class 3 and 4, the glassy-winged sharpshooter (*Homalodisca vitripennis*) is considered a pest because the females lays her eggs in groups just under the epidermis layer of leaves of holly, sunflower and citrus (AL-WAHAIBI AND MORSE 2009). In the specific cases of aquatic (Heteroptera: Nepomorpha) and semi-aquatic (Heteroptera: Gerromorpha) bugs, females lay eggs embedded in plant tissues, on the surface

of submerged plants or stones attached by a gelatinous substance or with a pedicel (ANDERSEN 1982; PAPACEK 2001).

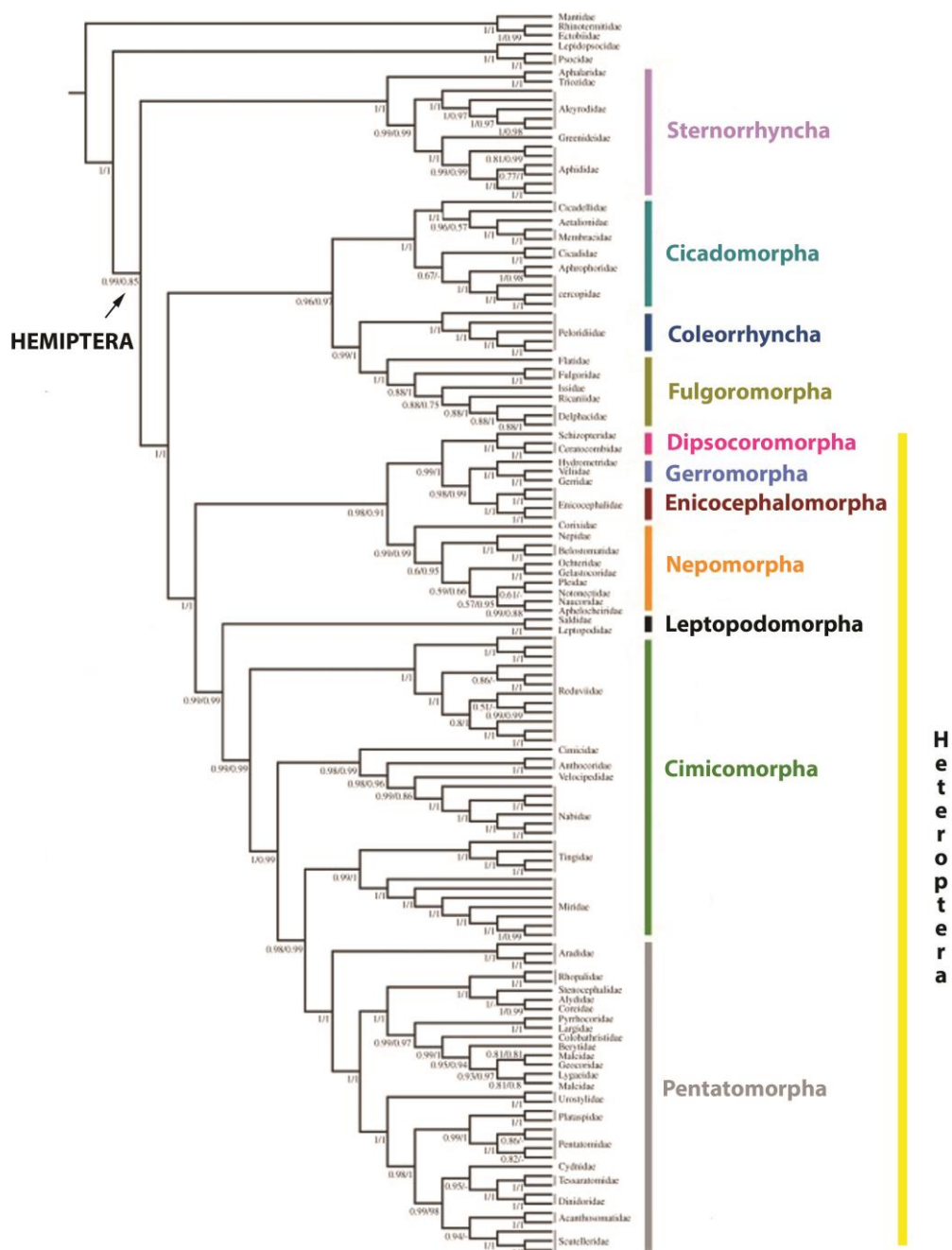


Figure 2. Molecular phylogeny of Hemiptera based in mitochondrial genome sequences. Phylogeny of Hemiptera as inferred from PhyloBayes analyses of the PCGRNA and PCG12RNA datasets under the CAT þ GTR mixture model (modified from (LI *et al.* 2017)).

Table 1. Bibliographic review of Hemiptera embryos. Summary of colouration of eggs and embryos in different species of hemipterans, with additional information about the sites where the eggs are laid and possible predators during the embryonic development. Nd: Not determined. \*: transparent chorion.

Infraorder	Family	Species	Egg-laying site	Colour of eggs *	Description of embryonic development	Egg parasites	References
<b>Cicadomorpha</b>	Cercopidae	<i>Callitettix versicolor</i>	Laboratory condition: eggs inserted singly in filter paper	Milky white when laid and became brown	Nd	Nd	(CHEN AND LIANG 2012)
	Membracidae	<i>Guayaquila projecta</i>		White when laid. The chorion was translucent and smooth	Nd	Nd	(M.A. LINARES <i>et al.</i> 2010)
	Membracidae	<i>Sextius virescens</i>		Yellowish-white	Nd	Nd	(KITCHING 1974)
	Membracidae	<i>Ceresa nigripectus</i>	Stems of alfalfa plants. Inserted and hidden.		Nd	Nd	(PEREZ GROSSO <i>et al.</i> 2014)
	Cicadellidae	<i>Homalodisca vitripennis</i>	Below the epidermis of leaves as a cluster	White-yellowish	yes	<i>Gonatocerus ashmeadi</i> and <i>Gonatocerus triguttatus</i>	(AL-WAHAIBI AND MORSE 2009)
	Cicadellidae	<i>Kolla paulula</i>	Inserted in host plant tissue.	White eggs; transparent chorion	Nd	Hymenoptera: Mymaridae and Trichogrammatidae	(TRIAPITSYN AND SHIH 2014)
<b>Fulgoroidea</b>	Dictyopharidae	<i>Phylloscelis pallescens</i>		Cream white: chorion translucent	Nd	Nd	(MCPHERSON AND WILSON 1995)

Delphacidae	<i>Megamelus bellicus</i>	Into the parenchyma of the petiole	Milky white when laid, turning yellowish white before hatching. Chorion translucent, smooth.	Nd		<i>Aprostocetus</i> ( <i>Ootetrastichus</i> ) sp	(A. J. SOSA <i>et al.</i> 2005; MARIANI <i>et al.</i> 2007)
Dictyopharidae	<i>Nersia Florens</i>		Pale yellowish *	Nd	Nd		(MCPHERSON AND WILSON 1995)
Dictyopharidae	<i>Dictyophara europaea</i>	Soil surface. Female cover the eggs with soil particles	Yellowish-green *	Nd	Nd		(KRSTIĆ <i>et al.</i> 2016)
Dictyopharidae	<i>Taosa (C.) longula</i>	Inserted in the petioles.	Milky white *	Nd	Nd		(LENICOV <i>et al.</i> 2012)
Ricaniidae	<i>Ricania speculum</i>	Inserted into the woody tissue of small twigs of different host plants.	Milky white	Nd		<i>Aprostocetus</i> ( <i>Ootetrastichus</i> ) <i>crino</i> (Hymenoptera Eulophidae), <i>Chaetostricha similis</i> , (Hymenoptera Trichogrammatidae) and <i>Polynema sp.</i> (Hymenoptera Mymaridae)	(ROSSI AND LUCCHI 2015; S. LAUDONIA <i>et al.</i> 2017)

<b>Sternorrhyncha</b>	Psyllidae	<i>Retroacizzia mopani</i>	Randomly laid on both the adaxial and abaxial surfaces of mature green and senescent leaves.	Egg clusters appeared to the naked eye as black spots on the leaflet. However, under the light microscope, the eggs were black with smooth shiny grey spots closely spaced near the basal end of the egg.	Nd	Nd	(OPPONG <i>et al.</i> 2009)
	Triozidae	<i>Bactericera lyrata</i>	In upper and lower sides of leaves	Freshly laid eggs whitish, later becoming orange-yellow, with brownish pedicel	Nd	Nd	(SELJAK AND MALENOVSKÝ 2014)
	Aleyrodidae	<i>Aleurocanthus woglumi</i>	Laid in spiral and fixed, through a stalk, in the inferior face of the leaf	yellow-clear becoming yellow-darkness when close to the appearance of the nymphs.	Nd	Nd	(RONCHI-TELES <i>et al.</i> 2009)
	Psyllidae	<i>Acizzia jamatonica</i>	On abaxial surfaces and edges of leaflets	yellow	Nd	Nd	(WHEELER AND HOEBEKE 2009)
<b>Nepomorpha</b>	Corixidae	<i>Sigara (Tropocorixa) santiagiensi</i>		Yellow. Surface ornamented by irregular interlocking smooth hexagons	Nd	Nd	(KONOPKO 2014)
	Nepidae	<i>Laccotrephe s</i>	Under the sand grains in bunches	Nd	Nd	Nd	(HINTON 1981)



Nepidae	<i>Ranatra</i>	Inside the soft parts of floating vegetation of the fresh water habitats	White (from internet pictures)	Nd	Nd	(HINTON 1961; AKILAN <i>et al.</i> 2017))	
Belostomatidae	<i>Lethocerus medius</i>	In the back of males	White when laid and then become dark	Nd	Nd	Christine L. Goforth, from the University of Arizona (video)	
Notonectidae		In plant tissues or on the surface of submerged plants or stones.	White (from internet pictures)	Nd	Nd	(PAPACEK 2001)	
Corixidae		Attached and embedded in cement by a disc or pedicel on the substratum.	Nd	Nd	Nd		
Pleidae		In slits in the tissues of submerged aquatic plants.	Nd	Nd	Nd		
Helotrephidae		On the surface of water plant leaves in clusters.	Nd	Nd	Nd		
Aphelocheiridae		On the surface of various objects on the bottom, on the cuticle of crustaceans, and shells of Bivalvia.	Nd	Nd	Nd		
Gelastocoridae		On the surface of various objects.	Nd	Nd	Nd		
Ochteridae		On the surface of sandy grains, plants, or roots of plants.	Nd	Nd	Nd		
<b>Dipsocoro</b>	Ceratocombid	<i>Ceratocomb</i>	Inserted in leaf bud of	Nd	Nd	Nd	(LATTIN 2000)

<b>morpha</b>	ae	<i>us uagans</i>	sphagnum			
<b>Gerromorpha</b>	Mesovelidae	<i>Mesovelia mulsanti</i>		Yellow *	Nd	Nd
	Velidae	<i>Oiovelia cunucunum ana</i>		White,*	Nd	Nd
	Velidae	<i>Paravelia conata</i>		White *	Nd	Nd
	Velidae	<i>Rhagovelia antilleana</i>		Yellow *	Nd	Nd
	Gerridae	<i>Gerris buenoi</i>		Yellow *	Nd	Nd
	Gerridae	<i>Limnopus dissortis</i>		Yellow, dark chorion	Nd	<i>Tiphodytes gerriphagus</i>
	Gerridae	<i>Limnogonus franciscanus</i>		Yellow with red *	yes	Nd
	Gerridae	<i>Neogerris magnus</i>		Yellow with red *	Nd	Nd
<b>Leptopodomorpha</b>			Inserted into the stems of plants near the soil surface or into an algal layer	Nd	Nd	Nd
<b>Cimicomorpha</b>	Miridae	<i>Helopeltis antonii</i>	Embedded in plant tissue. Operculum and extra-chorionic processes exposed.	White and after two days change to orange with a distinct dark orange band at the distal region.	yes	Nd

	Miridae	<i>Lygus lineolaris</i>	Inserted into plant tissue with only the cap exposed.	Eggs creamy white *	Nd	mymarid wasp <i>Anaphes ovijentatus</i>	(DIXON 1989; ALLEN 2013) <a href="http://jenny.tfrec.wsu.edu/opm/displaySpecies.php?pn=180">http://jenny.tfrec.wsu.edu/opm/displaySpecies.php?pn=180</a>
	Reduviidae	<i>Rhynocoris albopilosus</i>	Arranged in lines along the stem of the plant.	Egg body yellowish to light brown.	yes	Nd	(KWADJO <i>et al.</i> 2008)
	Miridae	<i>Lygus hesperus</i>	Inserted in plants	Red pigmentation of the distal antennal segments and eyes was first observed.	yes	<i>Anaphes iole</i> (Hymenoptera: Mymaridae)	(CONTI <i>et al.</i> 1997; COOPER AND SPURGEON 2012)
	Miridae	<i>Helopeltis bradyi</i>	Inserted in fruit.	White, shiny	Nd	Nd	(MELINA <i>et al.</i> 2016)
	Tingidae	<i>Leptoyptha hospita</i>	Inserted along leaf margins and petioles, although some were laid singly close to the leaf midrib.	Yellowish	Nd	Nd	(ZHANG <i>et al.</i> 2011)
	Tingidae	<i>Tanybyrsa cumberi</i>	Inserts eggs into <i>Astelia</i> leaves.	Yellowish	Nd	Eggs are parasitised by a mymarid wasp, at present unidentified.	(M. MAY 1977)
<b>Pentatomorpha</b>	Pentatomidae	<i>Edessa medita</i>	Deposited vertically, with the posterior pole of embryo fixed to the substratum by a female substance	Light green *	yes	Nd	(MATESCO <i>et al.</i> 2009)

Pentatomidae	Genus <i>Euschistus</i>	Deposited vertically, with the posterior pole of embryo fixed to the substratum, and, in clusters, attached to each other and to the substratum through an adhesive material secreted by the female. Attached to the surface by an unknown structure.	Before hatching, eggs milky-white in <i>E. hansii</i> and <i>E. picticornis</i> to yellow in <i>E. convergens</i> *	yes	Nd	(RODRIGUES AND MOREIRA 2005; MATESCO <i>et al.</i> 2009)
Pentatomidae	Genus <i>Chinavia</i>		Chorion surface reticulated, light brown. A light brown colour of the eggs is due to a pigmented chorion.	yes	Nd	
Pentatomidae	<i>Chinavia musiva</i>		Egg green *. With the development of the embryo, the egg becomes dark green and with dark red eyes, two median red strips.	yes	Nd	
Pentatomidae	<i>Grazia tincta</i>		Pigmented chorion white with two red round bands	Nd	Nd	
Pentatomidae	<i>Pallantia macunaim</i>		Light brown to greenish*	Nd	Nd	
Coreidae	<i>Holymenia clavigera</i>		Gold colour just after deposition, turning to brown later in the development.*	yes	Nd	

	Coreidae	<i>Anisoscelis foliacea marginella</i>	Attached to the surface by an unknown structure.	Gold colour just after deposition, turning to brown later in the development*.	Nd	Nd	(RODRIGUES AND MOREIRA 2005)
	Cydnidae	<i>Adomerus variegatus</i>	In shallow cracks on the soil surface	White with red eyes after 14 days*	Nd	Nd	(MUKAI <i>et al.</i> 2010)
	Cydnidae	<i>Canthophorus niveimarginatus</i>	On the ground, under stones or in a slight depression in the soil. Maternal care behaviour.	Creamy colour eggs gradually turned a deep pink	Nd	Nd	(FILIPPI <i>et al.</i> 2009)
	Pyrrhocoridae	<i>Dysdercus albofasciatus</i>	Wet places, cracks in the soil or between litter	Initially ivory colour, after 48 hours the embryo become red.	Nd	yes	(STADLER <i>et al.</i> 1987)
<b>Pentatomorpha</b>	Lygaeoidea	<i>Anochrostomus formosus</i>	Laboratory condition: on wet wipes, arranged in aligned masses and sometimes under the sunflower seeds.	Light orange when deposited, turning red with the development of the embryo.	yes	Nd	(CERVANTES-PEREDO AND ELIZALDE-AMELCO 2007)
<b>Pentatomorpha</b>	Lygaeoidea	<i>Lygaeus reclinatus reclinatus</i>	Laboratory condition: on the wet wipes.	Yellowish white when deposited and later orange.	yes	Nd	
<b>Pentatomorpha</b>	Lygaeoidea	<i>Oncopeltus sexmaculatus</i>	Laboratory condition: on wet wipes, within fruits or under sunflower seeds.	Pale yellow when deposited, becoming reddish with the development of the embryo.	yes	Nd	

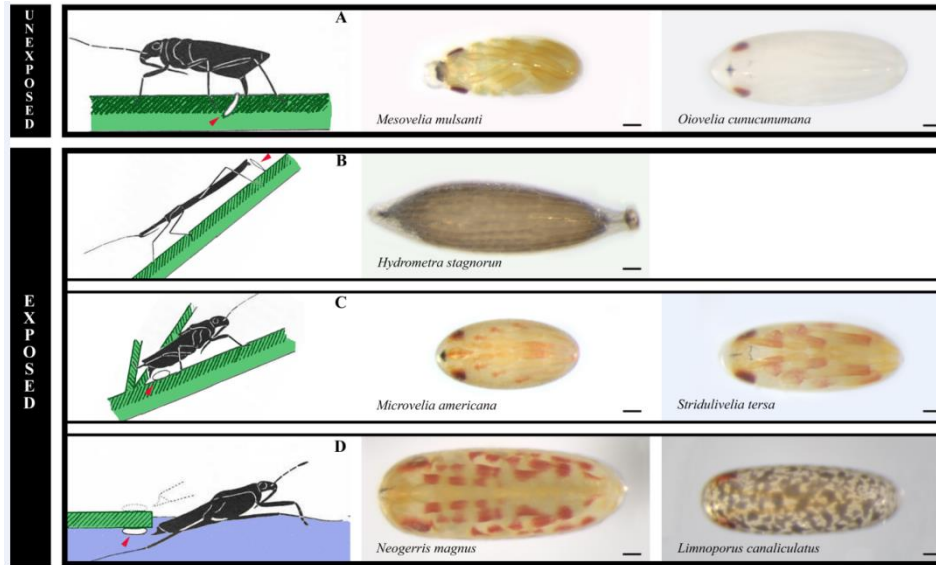


Figure 3. Correlation of the modes of oviposition in semiaquatic bugs (Gerromorpha) with the colour of the chorion and embryo. A. Egg unexposed, embedded in substrate (*Mesovelia mulsanti* and *Oiovelia cunucunumana*), the embryos have transparent chorion without red pigmentation in the appendage. Exposed embryos: B. Egg deposited superficially and upright on substrate (*Hydrometra stagnorum*), C. Egg deposited superficially (but sheltered), examples: *Microvelia americana*, *Stridulivelia tersa*. D. Egg deposited superficially, either on upper or lower side of floating object (*Neogerris magnus*, *Limnoporus canaliculatus*). The embryos that are exposed present dark chorion or red extra-ocular pigments. (Based on: (ANDERSEN 1982b))

### *Colour evolution in Hemiptera eggs*

The different ways that Gerromorpha females lay eggs, depending on the species, could be correlated with the presence or absence of the extra-ocular pigmentation during the embryonic stage. In species with faint or without extra-ocular colour, such as *Mesovelia mulsante*, *Oiovelia cunucunumana* or *Paravelia*, the embryos are found embedded in the substrate (Figure 3A). Instead, embryos with yellow-red colour in the body with transparent chorion are attached with a gelatinous substance and exposed in the water, as is the case of our biological model, *Limnogonus franciscanus* or *Neogerris magnus* (Figure 3B). Also, in some species (*Gerris buenoi* and *Hydrometra*) eggs are exposed but the chorion has a dark colour (Figure 3C). In several species of Pentatomorpha, Lygaeoidea and Pyrrhocoridae families, the eggs that are exposed or semi-exposed and have either a red extra-ocular colour or a dark chorion (STADLER *et al.* 1987; RODRIGUES AND MOREIRA 2005; CERVANTES-PEREDO AND ELIZALDE-AMELCO 2007; V. MATESCO 2009). Overall, the red colour present in the appendages of embryos or the dark chorion could be a strategy to avoid predators either by aposematism or camouflage, while species that are embedded in substrates do not need to produce colours to avoid predation.

I wondered if the presence of yellow and/or red colours in the eggs or embryos is common between the different groups of bugs. Hemipterans developed different strategies to lay eggs

and the presence or absence of colours in the eggs is documented in a few species (KWADJO *et al.* 2008; AL-WAHAIBI AND MORSE 2009; P.L. SAROJ *et al.* 2016). This lack of information might be attributed either to the insertion of eggs into substrates or to the opacity of the chorion. I prepared a cladogram using the most recent phylogeny of Hemiptera (LI *et al.* 2017) (Figure 4). Most of the pictures were found in the literature and in web pages of people interested by hemipterans eggs.

This bibliographical analysis revealed that in the previously called suborder “Homoptera”, eggs are coloured uniformly with tones that can be totally white, cream, brown or yellow-orange and a transparent chorion (Figure 4, Table 2). In the monophyletic suborder Heteroptera, the colour of the eggs, embryos and chorion is more diverse. I could identify 1) eggs without extra-ocular colours with transparent chorion, 2) eggs with dark chorion and 3) eggs with yellow/red extra-ocular colour (Figure 4, Table 2).

This brief overview suggests that yellow could be the ancestral colour of embryos in Hemiptera and that the acquisition of red colouration was specific to the sub-order Heteroptera (Figure 4). Also, most of the basal species of Hemiptera lay their eggs inside substrates or hide them after deposition. By contrast, the majority of eggs from Heteroptera species are exposed, suggesting that the red embryonic colour and opaque chorion may be an adaptation to avoid predators as aposematic signal or camouflage.

Of course, more intensive work should be done to establish relation between the colouration of the eggs, chorion and extra-ocular colour of embryos. In addition, whether the colouration present in the basal species and in the derived species is due to the same pigments is another question that needs to be clarified. In addition, the loss of extra-ocular colour in some Gerromorpha (personal observation) and Nepomorpha species (webpage photo), shows that the extra-ocular colour could have been gained or lost several times during evolution.

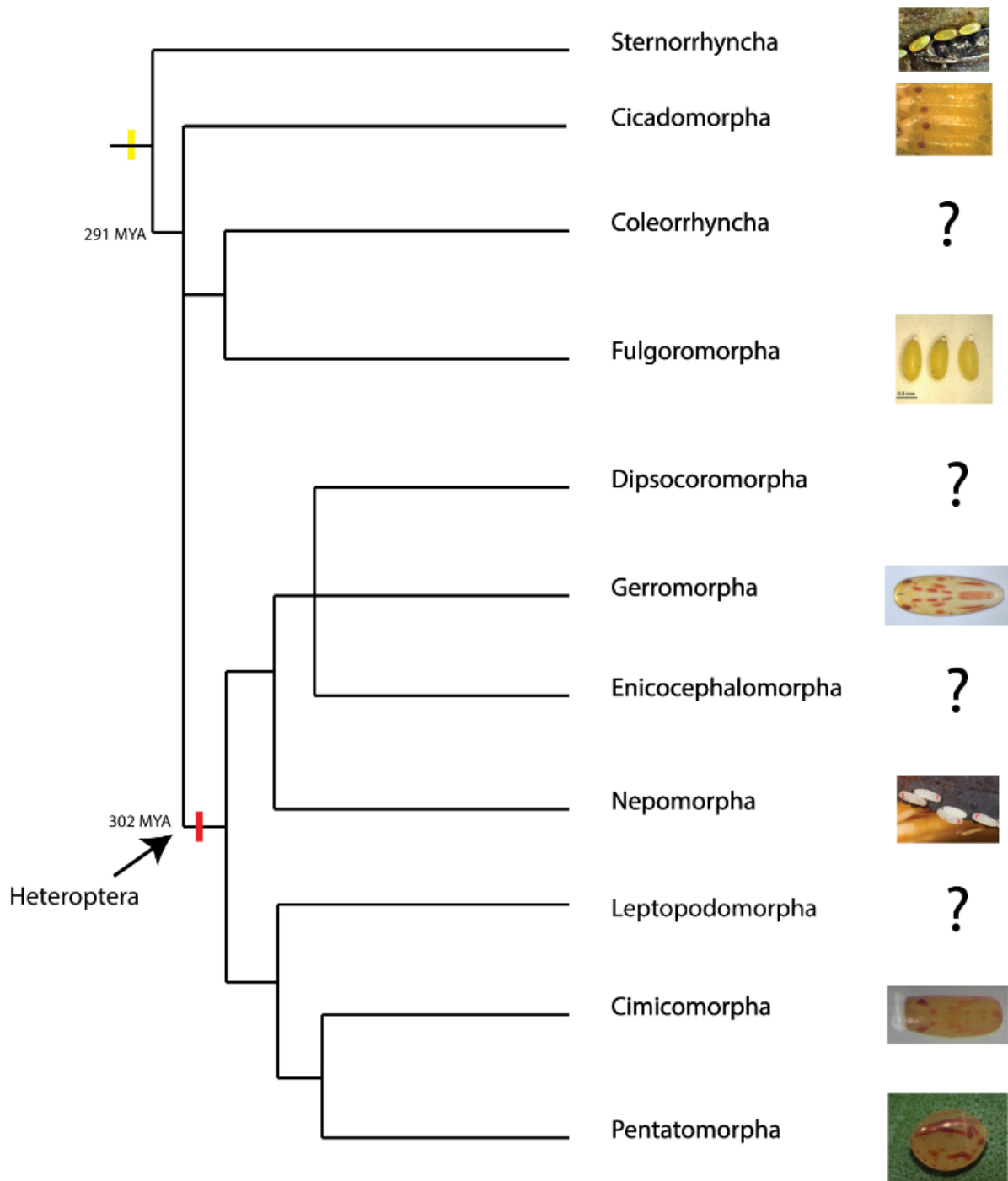


Figure 4. Evolution of extra-ocular colouration in embryos of Hemiptera. Embryos from the basal suborder “Homoptera” (show this paraphyletic group in the figure) are yellow; no evidence of red extra-ocular eggs could be identified. The red extra-ocular colour was found in the monophyletic suborder of Heteroptera. In Nepomorpha, we found images of embryos with or without extra-ocular colour, suggesting a possible loss of this character in some species of this group. The question marks indicate a lack of information.

### Some examples of genetic co-option using pigmentation as a trail.

Understanding how novel traits arise and contribute to species diversification has long been a major question in biology. Recent advances in evo-devo established co-option of existing genes and networks as one of the mechanism involved in the evolution of morphological and



physiological novelties (TRUE AND CARROLL 2002; VOPALENSKY AND KOZMIK 2009; GLASSFORD *et al.* 2015; MARTINSON *et al.* 2017; HU *et al.* 2018).

Pigmentation is a trait that has been used in evo-devo field to try to understand the evolutionary events underlying diversification. Hereafter, I provide a summary of studies where genetic co-option underlies the evolution of phenotype diversity.

### *Alternative pigments in plants by the co-option of MYB gene*

The myeloblastosis protein (MYB) DNA-binding domain defines a class of transcriptional regulators found in animals and plants. In animal, the three proteins - *c-MYB*, *A-MYB* and *B-MYB* play roles in cell proliferation (LIPSICK 1996). In plants, MYB gene family expanded dramatically with hundreds of genes in *Arabidopsis*, maize and soybeans (MARTIN AND PAZ-ARES 1997; DU *et al.* 2012a; DU *et al.* 2012b). MYB genes control various processes such as responses to biotic and abiotic stresses, development, differentiation, metabolism and defence (AMBAWAT *et al.* 2013).

In plants the MYB proteins contain two imperfect MYB repeats corresponding to the R2R3 Myb repeats of the three repeat R1R2R3 in animal MYB proteins. The R2R3-MYB proteins interact with two other large gene families, the basic helix-loop-helix (bHLH) and WD40-repeat (WDR), all together known as the MBW complex (RAMSAY AND GLOVER 2005; FELLER *et al.* 2011). The MBW complex is well characterized in regulating differences in anthocyanin pigment intensity.

Flavonoids represent a large class of secondary plant metabolites, of which anthocyanins are the most conspicuous class, due to the wide range of colours resulting from their synthesis. Synthesis of anthocyanins in petals is undoubtedly intended to attract pollinators, whereas anthocyanin synthesis in seeds and fruits might aid in seed dispersal. Betalains are red and yellow pigments that are produced in some families of the Caryophyllales order. Betalains are mutually exclusive with the anthocyanins and they appear to provide all of the functions of anthocyanins, pigmenting flowers and fruits and responding to the same environmental stimuli (HATLESTAD *et al.* 2015; LLOYD *et al.* 2017) (Figure 5A). Anthocyanins and betalains are chemically unrelated, while the precursors for the synthesis of all flavonoids, including anthocyanins, are ultimately phenylalanine, betalains compounds derive from tyrosine (TANAKA *et al.* 2008).

Until a few years ago it was thought that the regulation of betalains was achieved by similar conserved anthocyanin MBW complex. Hatlestad and collaborators have shown that a

previously uncharacterized anthocyanin MYB-like protein, *Beta vulgaris* MYB1 (BvMYB1), regulates the betalain pathway in beets. BvMYB1 is a typical protein R2R3-MYB containing the R2 and R3 MYB domains and a C-terminal activation domain. Phylogenetic analysis using the R2R3 domains showed that BvMYB1 forms a monophyletic clade with dicot anthocyanin MYBs (HATLESTAD *et al.* 2015) (Figure 5B).

However, BvMYB1 has lost the ability to interact with bHLH members of the MBW complex. Furthermore, BvMYB1 cannot up-regulate the anthocyanin pathway in *Arabidopsis*, and the *Arabidopsis* PAP MYBs cannot regulate betalains in beet. Hatlestad and collaborators established that BvMYB1 and anthocyanin MYBs probably derived from a common ancestor. The BvMYB1 gene acquired new transcriptional targets, allowing it to regulate betalain biosynthesis (Figure 5C). In conclusion, the mutual exclusion of both pigment suggest that after the co-option of BvMYB1, some Caryophyllales plants kept one of the biosynthesis gene pathway to avoid redundancy between pigments.

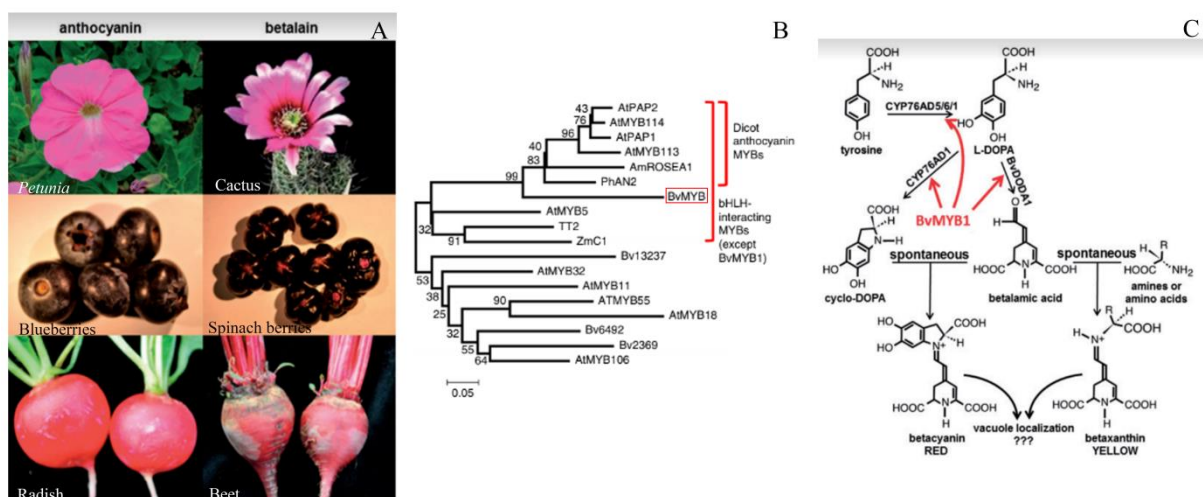


Figure 5. Evolutionary origin of BvMYB1 and betalain pathway. A) The six left panels are representative flowers, fruits and roots, with the far left panels pigmented with anthocyanins and the right with betalains. B) Neighbor-joining tree of R2R3 MYB domains of BvMYB1 and other MYBs. Scale represents amino acid changes per position. BvMYB1 clusters with dicot anthocyanin MYBs within the larger bHLHinteracting cluster. *Beta vulgaris* is a dicot. C) The betalain pathway is, with the enzymatic steps regulated by BvMYB1 indicated with arrows. Extracted from (HATLESTAD *et al.* 2015; LLOYD *et al.* 2017).

### *Co-option of CYP2J19 by possible cis- regulatory changes and gene duplication in birds*

Carotenoid ketolation is an enzymatic reaction that converts yellow dietary carotenoids (xanthophylls) to red C4 ketocarotenoid pigments such as astaxanthin or canthaxanthin (BRITTON 1995). The ketarocatenoid reaction is an important key innovation in avian evolution and colour diversification (PRAGER AND ANDERSSON 2010; THOMAS *et al.* 2014).

*CYP2J19* is the gene encoding the ketolase avian enzyme involved in the production of ketocarotenoid pigment (LOPES *et al.* 2016; MUNDY *et al.* 2016)

The *CYP2J19* is expressed in retina of all canaries but only in the feather follicles of red canaries and not in the yellow ones (Figure 6A) (LOPES *et al.* 2016). This result suggested that the differential expression of *CYP2J19* in the skin of red birds to produce the ketocarotenoid pigments could be due to *cis*- regulatory differences. Furthermore, this is not the only way that this gene evolved to generate diversification in birds. The wild-type zebra finch has a red beak due to ketocarotenoids, while the yellowbeak mutant (mutation only described for captive birds) does not contain these pigments (MUNDY *et al.* 2016). The *CYP2J19A* gene is involved in the production of ketocarotenoids in eyes, while the gene *CYP2J19B* is expressed in both eyes and beak, suggesting that after gene duplication, one of the paralog was co-opted to produce red pigments in the beak without losing its function in the eyes (Figure 6B). Additionally, in the yellowbeak birds the *CYP2J19* contains one mutation (*CYP2J19<sup>yb</sup>*) that prevents the ketarocatenoid reaction in the beak (MUNDY *et al.* 2016).

Today we know that the ancestral function of *CYP2J19* was colour vision, specifically in the production of ketocarotenoids in red retinal oil droplets of the archelosaurs (archosaurs and turtles) (TWYMAN *et al.* 2016; TWYMAN *et al.* 2018), showing that red extra-ocular pigmentation was recruited later during the evolution of some birds and turtles.

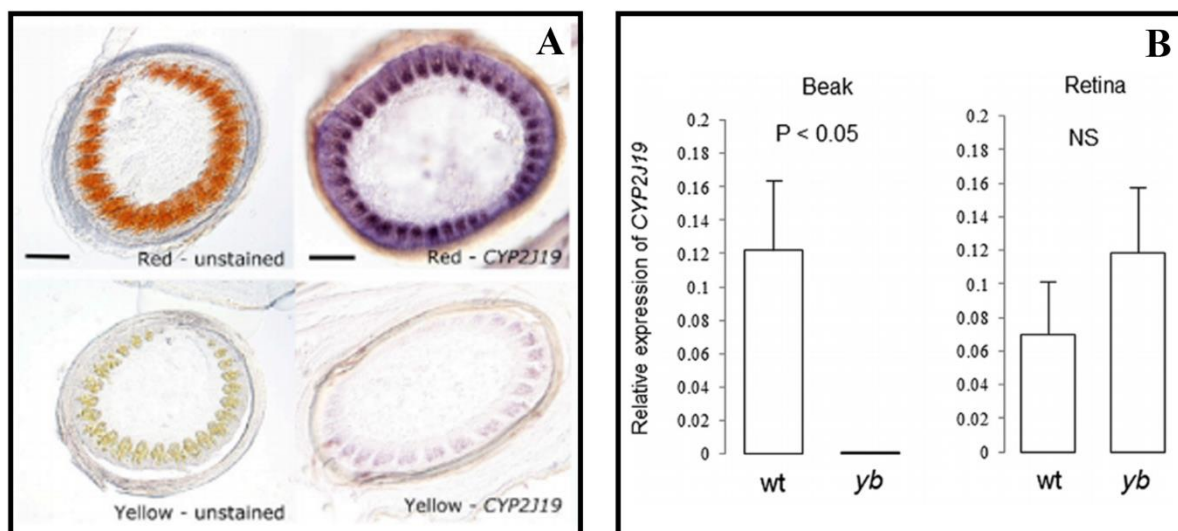


Figure 6. Expression of the gene *CYP2J19* in red feathers and red beaks of birds. A) Unstained sections and in situ hybridization of regenerating feather follicles (10 days post-pluck) of red and yellow canaries. Reddish-orange ketocarotenoid pigmentation is evident in the developing barb ridges of red canary feather follicles, whereas fainter yellow carotenoid-based pigmentation is evident in the yellow feather. B) Expression level of *CYP2J19* using qPCR with primers that amplify all copies (*CYP2J19A*, *CYP2J19B*, and *CYP2J19yb*) in the beak and retina of wildtype (wt) and yellowbeak (yb) zebra finch. Extracted from (LOPES *et al.* 2016; MUNDY *et al.* 2016).

### *Co-option of developmental regulatory genes and circuits*

The evolution of some morphological novelties is correlated with the co-option of regulatory circuits or sets of regulatory genes (BRAKEFIELD *et al.* 1996; BELDADE *et al.* 2002; ÖZSU AND MONTEIRO 2017; DESHMUKH *et al.* 2018).

In butterflies, several genes implicated in the colour patterns of wingeyespot have been identified and it is clear that many developmental genes have been co-opted to produce phenotypic diversity. However, a debate still exists to explain the interaction between these genes and how they were recruited (KEYS *et al.* 1999; MONTEIRO AND PODLAHA 2009; OLIVER *et al.* 2012a; CALLIER 2018). Previous research suggested that nymphalid butterfly eyespots originated once via the co-option of pre-existing gene networks (OLIVER *et al.* 2012b; OLIVER *et al.* 2014). Today, the twelve genes that have been associated with eyespots development in several species are involved in different gene regulatory pathways and/or circuits (WEATHERBEE *et al.* 1999; MONTEIRO *et al.* 2006; SAENKO *et al.* 2011). Recent investigation using RNA-Seq differential expression analysis predicted the association of at least two different pathways involved in the production of eyespots and different colour patterns (ÖZSU AND MONTEIRO 2017).

However, a number of hypotheses have arisen in relation to the interpretation of those findings. Developmental regulatory genes may have been involved either by co-option of pre-existing regulatory pathways, followed by modifications (OLIVER *et al.* 2012a) or by the co-option of individual genes from independent regulatory networks that shaped the new regulatory pathway.

## **Gerromorpha**

Gerromorpha also known as semi-aquatic bugs is a cosmopolitan group of insects that live on the surface of water (ANDERSEN 1982). In the recent years, the semi-aquatics bugs have been used as models in the evo-devo field to study the genetic basis of morphological innovations (SANTOS *et al.* 2017; FINET *et al.* 2018) (KHILA *et al.* 2009; REFKI *et al.* 2014; ARMISEN *et al.* 2015; CRUMIÈRE *et al.* 2016) and evolution of sexual traits (TOUBIANA AND KHILA 2019). Gerromorpha is a good model lineage to understand the genetic basis of ecological and developmental processes and their evolution.

The most recent phylogeny was built using parsimony analysis based on 64 morphological characters and three DNA markers (Figure 7) (DAMGAARD 2008). In this phylogeny, only four out of eight families were well supported (Mesoveliidae, Hebridae, Hydrometridae and Gerridae). Several relationships were not resolved, as for example the sister group relationship

between Gerridae and (Haloveliinae + Microveliinae) or the relation between “Gerroidea” and the “Hydrometroidea” super-families (Figure 7). The aim of our team and collaborators was to build a new phylogeny in order to resolve the paraphyletic classification of some clades and to have a better framework to understand the evolution of different traits.

In the last chapter of this thesis, I will also present the sequencing and manual annotation of the *Gerris buenoi* (*G. buenoi*) genome; the first water strider genome to be sequenced and published (ARMISÉN *et al.* 2018).

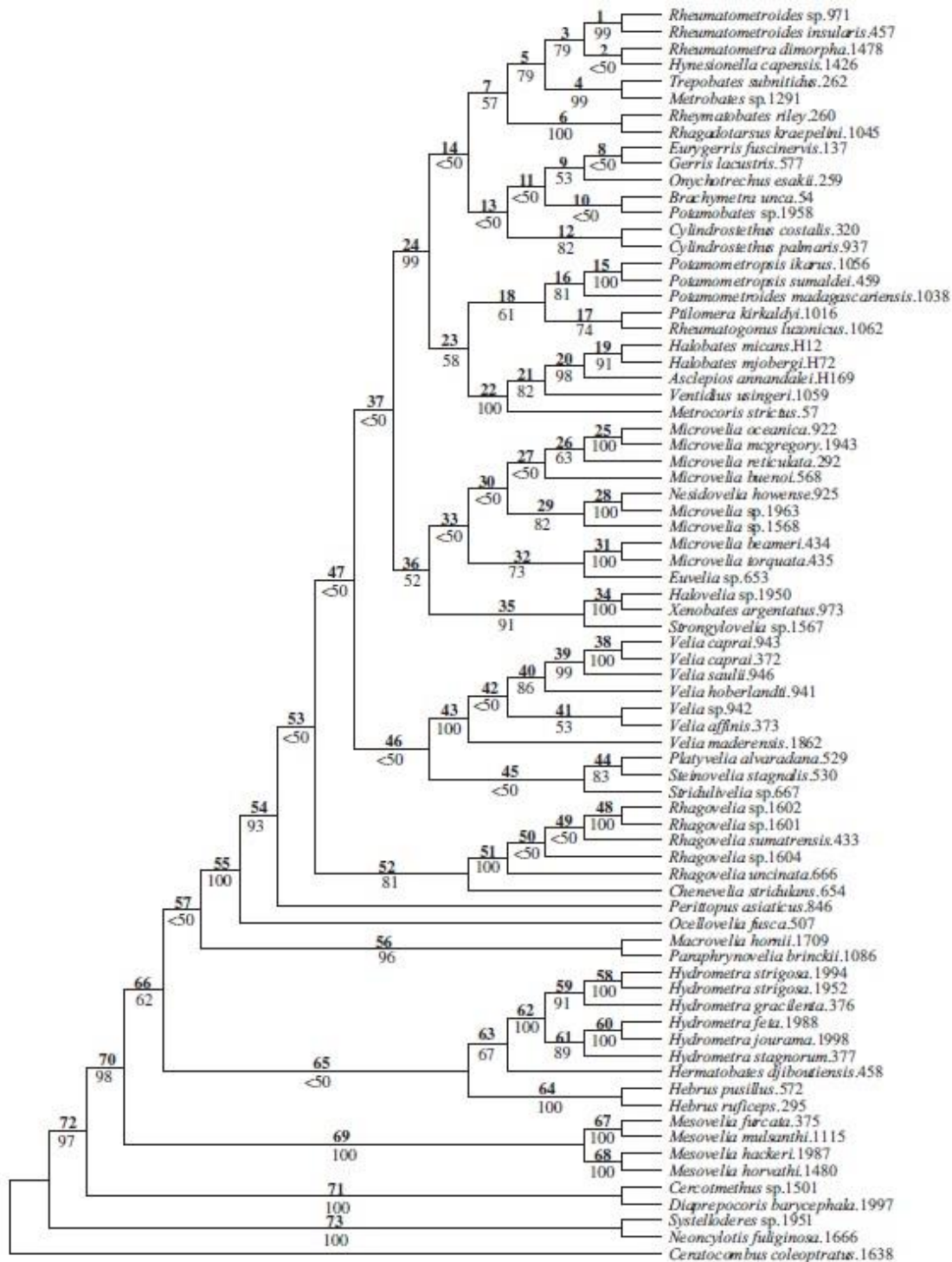


Figure 7. Phylogeny of the semiaquatic bugs. Single most parsimonious tree resulting from a simultaneous phylogenetic analysis of all morphological and molecular data and with gaps scored as a fifth character state. Numbers in bold above branches refer to Bremer support, and numbers below branches refer to bootstrap support values.



## CHAPTER 2



## **Co-option of the pteridine biosynthesis pathway underlies the diversification of embryonic colours in water striders**

### **Synopsis of the paper:**

Animal colours and colour patterns in nature have an important influence on fitness. In general, coloration related-traits are involved in sexual selection, UV protection and signalling communication. Colour-related traits are well spread in nature but our understanding of how they originate and diversify remains limited. A first important step to understand colour diversity is to study the genetic and developmental basis of colouration and the evolutionary mechanisms at the origin of their emergence across lineages.

Genetic basis of colour has been widely studied at the adult stage in different groups of insects but, we know little about colour patterns in the embryo. Here we study a new trait consisting of colouration that is specific to the embryo and absent from post embryonic stages in semi-aquatic bugs (Gerromorpha). By combining developmental genetics with chemical and phylogenetic analyses across a broad sample of species, we uncovered the mechanisms underlying the emergence and diversification of embryonic colours in this group of insects.

### **Summary of results:**

We demonstrate that the entire pteridine biosynthesis pathway, which ancestrally produces red pigment in the eyes, has been recruited during embryogenesis in various extra-ocular tissues including antennae and legs. In addition, we discovered that this co-option is common to all water striders and initially resulted in the production of yellow extra-ocular colour.

Subsequently, six lineages evolved bright red colour and two lineages independently lost all extra-ocular colour. Despite the high diversity in colours and colour patterns, we show that the underlying biosynthesis pathway remained stable throughout the 200 million years of Gerromorpha evolutionary time. Finally, we identified erythropterin, xanthopterin and chrysopterin as the pigments responsible for these colours in the embryo. These findings demonstrate how novel traits can emerge through the activation of a complete pathway in new developmental contexts.

### **Conclusion:**

Our results confirm the complete co-option of ancestral pathway through Gerromorpha group to produce diversification during the embryonic stage. Additionally, we propose colouration

trait in Gerromorpha as an excellent model to understand how the same pathway is regulated to produce phenotypic variability.

**Author contribution:**

Aidamalia Vargas-Lowman, first author: extraction of RNA, gene cloning, reconstruction of ancestral states and analysis, in situ hybridization, RNA interference, design of the study, analysis of the data and redaction of the paper, with major contribution in terms of results, methodology, discussion and figure-making.



# Coooption of the pteridine biosynthesis pathway underlies the diversification of embryonic colors in water striders

Aidamalia Vargas-Lowman<sup>a</sup>, David Armisen<sup>a</sup>, Carla Fernanda Burguez Floriano<sup>b</sup>, Isabelle da Rocha Silva Cordeiro<sup>b</sup>, Séverine Viala<sup>a</sup>, Mathilde Bouchet<sup>a</sup>, Marie Bernard<sup>a</sup>, Augustin Le Bouquin<sup>a,c</sup>, M. Emilia Santos<sup>a,1</sup>, Alexandra Berlioz-Barbier<sup>d</sup>, Arnaud Salvador<sup>e</sup>, Felipe Ferraz Figueiredo Moreira<sup>b</sup>, François Bonneton<sup>a,2</sup>, and Abderrahman Khila<sup>a,2</sup>

<sup>a</sup>Institut de Génomique Fonctionnelle de Lyon, Université Lyon, CNRS UMR 5242, Ecole Normale Supérieure de Lyon, Université Claude Bernard Lyon 1, 46 allée d'Italie F-69364 Lyon, France; <sup>b</sup>Laboratório de Biodiversidade Entomológica, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21040-360 Rio de Janeiro, Brazil; <sup>c</sup>Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON M5S 3B2, Canada; <sup>d</sup>Centre Commun de Spectrométrie de Masse (CCSM), Institut de Chimie et de Biochimie Moléculaires et Supramoléculaires (ICBMS), CNRS UMR 5246, Université Lyon, Université Claude Bernard Lyon 1, 69622 Villeurbanne, France; and <sup>e</sup>Université de Lyon, Université Claude Bernard Lyon 1, Institut des Sciences Analytiques, CNRS UMR 5280, F-69100 Villeurbanne, France

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Naturalists have been fascinated for centuries by animal colors and color patterns. While widely studied at the adult stage, we know little about color patterns in the embryo. Here, we study a trait consisting of coloration that is specific to the embryo and absent from postembryonic stages in water striders (Gerromorpha). By combining developmental genetics with chemical and phylogenetic analyses across a broad sample of species, we uncovered the mechanisms underlying the emergence and diversification of embryonic colors in this group of insects. We show that the pteridine biosynthesis pathway, which ancestrally produces red pigment in the eyes, has been recruited during embryogenesis in various extraocular tissues including antennae and legs. In addition, we discovered that this cooption is common to all water striders and initially resulted in the production of yellow extraocular color. Subsequently, 6 lineages evolved bright red color and 2 lineages lost the color independently. Despite the high diversity in colors and color patterns, we show that the underlying biosynthesis pathway remained stable throughout the 200 million years of Gerromorpha evolutionary time. Finally, we identified erythropterin and xanthopterin as the pigments responsible for these colors in the embryo of various species. These findings demonstrate how traits can emerge through the activation of a biosynthesis pathway in new developmental contexts.

pigmentation | pteridine pathway | diversification | cooption | water striders

Animal colors and color patterns have fascinated naturalists for centuries (1, 2). A large body of empirical work established the importance of color-related traits for fitness, demonstrating that such traits are frequent targets of both natural and sexual selection (3–5). Color can be important for survivorship as a protective shield against ultraviolet radiation and through its role in thermoregulation (6, 7) or by acting as a warning signal or in camouflage, thus decreasing predation risk (2, 8–11). Furthermore, color traits are important for mating success through their role in conspecific recognition and mate choice (5, 12, 13). As such, color-related traits offer powerful models for studying the general rules of phenotypic evolution (8, 14).

While color-related traits are ubiquitous in nature, our understanding of how they originate and diversify remains limited. A first important step to understand color diversity is to study the genetic and developmental basis of coloration and the evolutionary mechanisms at the origin of their emergence across lineages. It has been suggested that color-related traits have the potential to constitute important models for studying the evolutionary dynamics of developmental pathways and their relationship with phenotypic evolution (15). This is facilitated by the relative

simplicity of color patterns and the ease of their quantification and experimental manipulation (16). Color traits are often products of gene networks including multiple components of pigment biosynthesis pathways (15). The evolution of novel color-related phenotypes is usually explained by the recruitment of such networks into new developmental contexts. Whether this recruitment happens through several independent cooptions of single genes or through one major event involving an entire gene network is unclear (17–22). It is therefore important to broaden our sampling of traits and lineages to better understand the mechanisms underlying the emergence and diversification of novel phenotypes.

In this work, we describe novel color patterns in the embryos of the semiaquatic bugs, also known as water striders (Heteroptera, Gerromorpha). An original feature of this coloration trait is that it

## Significance

Understanding how existing genomic content can be reused to generate new phenotypes is important for understanding how species diversify. Here, we address this question by studying the origin of a phenotype consisting of bright coloration in the embryos of water striders. We found that the pteridine biosynthesis pathway, originally active in the eyes, has been coopted in the embryo to produce various colors in the antennae and legs. The coopted pathway remained stable for over 200 million years, yet resulted in a striking diversification of colors and color patterns during the evolution of water striders. This work demonstrates how the activation of a complete pathway in new developmental contexts can drive the evolution of novelty and fuel species diversification.

Author contributions: F.B. and A.K. designed research; A.V.-L., D.A., M. Bouchet, M. Bernard, A.L.B., E.S., A.B.-B., F.B., and A.K. performed research; C.F., I.C., S.V., F.M., and A.K. contributed new reagents/analytic tools; A.V.-L., D.A., A.S., F.B., and A.K. analyzed data; and A.V.-L., F.B., and A.K. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: Sequence data have been deposited in GenBank, <https://www.ncbi.nlm.nih.gov/genbank> (accession nos. MK480204–MK480218 and MK883710).

<sup>1</sup>Present address: Department of Zoology, University Museum of Zoology Cambridge, University of Cambridge, Cambridge CB2 3EJ, United Kingdom.

<sup>2</sup>To whom correspondence may be addressed. Email: francois.bonneton@ens-lyon.fr or abderrahman.khila@ens-lyon.fr.

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is zygotically produced by embryonic epithelia and not the eggshell. We sampled 34 species and uncovered a striking diversity of colors ranging from faint yellow to bright red and manifested in highly variable spatial patterns across this species sample. While the function of these color patterns is unknown, the fact that they are readily visible through the transparent chorion suggests a role in warning. We demonstrated that the core of the eye pteridine biosynthesis pathway has been activated in extraocular tissues resulting in the production of various colors in embryonic legs and antennae. Phylogenetic reconstruction revealed that the underlying pathway remained highly stable throughout evolution despite the striking diversification of color and color patterns across the Gerromorpha. Finally, we show that the cooption of the pteridine pathway to produce yellow color is common to all Gerromorpha and that the shift from yellow to red evolved independently in at least 6 distinct lineages.

**Results**

**Development and Variation of Extraocular Color Patterns in the Embryos of Water Striders.** We discovered a striking diversity of colors and color patterns in the embryos of 34 species of water striders (Fig. 1). Like other Hemiptera, all species of the Gerromorpha have dark red eyes (23, 24) (arrowheads in Fig. 1). In addition to the eyes, these embryos exhibit various patterns of color in the appendages, the thorax, and various parts of the abdomen (Fig. 1). We hereafter refer to these patterns as extraocular color. We found

that variation between different species ranges from presence or absence of extraocular color, various shades of yellow to bright red, to differences in the spatial color patterns (Fig. 1). Furthermore, we found variation in the timing of color production such that some species acquire the extraocular color before and others after the eyes do (SI Appendix, Fig. S1). The developmental timing of coloration was studied in detail in *Limnogonus franciscanus* embryos where eye color appears first at the 40th hour of development (30% of embryogenesis), followed by color in the legs (45 h, 32% of embryogenesis), and finally in the antennae (50 h, 35% of embryogenesis) (SI Appendix, Fig. S2). Also, this color is visible only during the embryonic stage in some species while in others it persists until the first nymphal instar (SI Appendix, Fig. S2F). Finally, *L. franciscanus* embryos incubated at 19 °C, 27 °C, or 30 °C showed identical eye and extraocular colors (SI Appendix, Fig. S3). This result is consistent with similar experiments in the rice stink bugs (25) and suggests that the color is not plastic but under the control of an unknown developmental genetic mechanism.

**Evolutionary History of Embryonic Extraocular Color.** In order to understand the evolutionary history of extraocular color acquisition and diversification in the embryos of water striders, we reconstructed the phylogenetic relationships between 34 species representing 4 of 8 Gerromorpha families (26, 27). We then reconstructed the ancestral state of presence/absence, yellow, and red extraocular color on this phylogeny (Fig. 2 and SI Appendix, Table

EVOLUTION

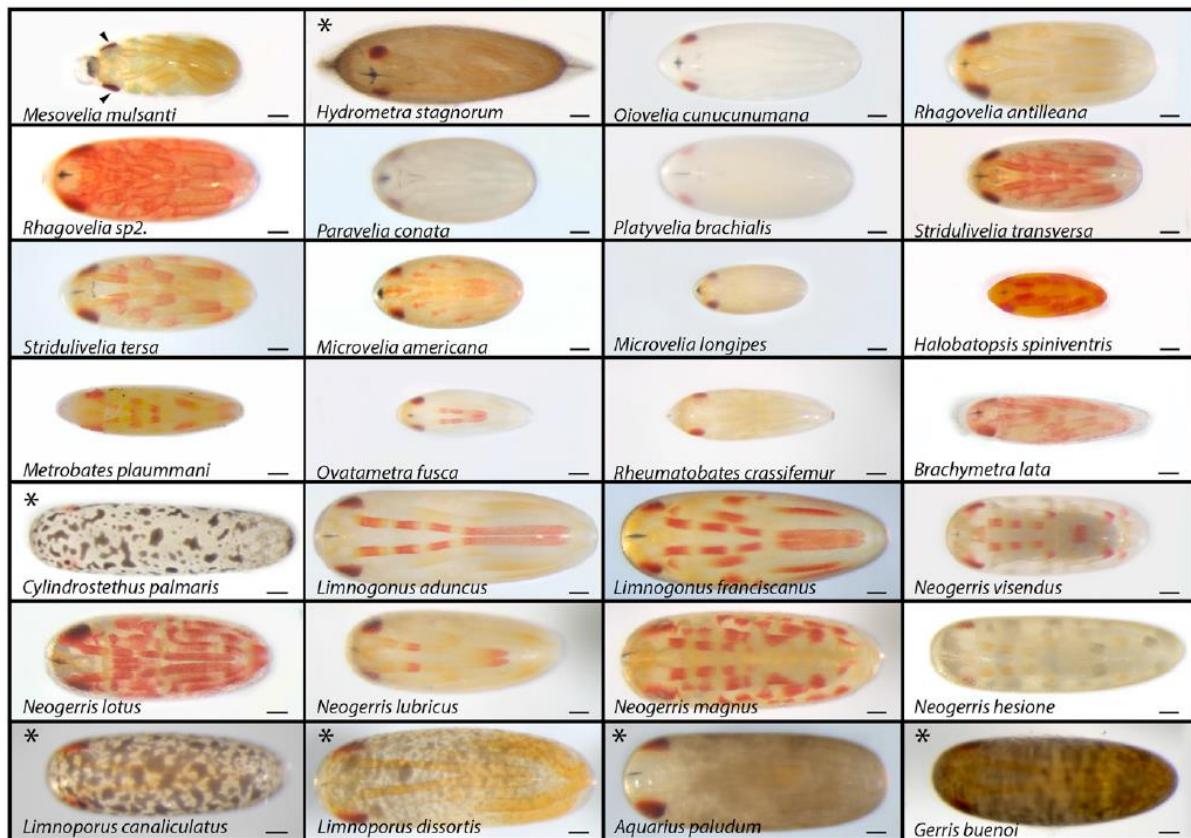
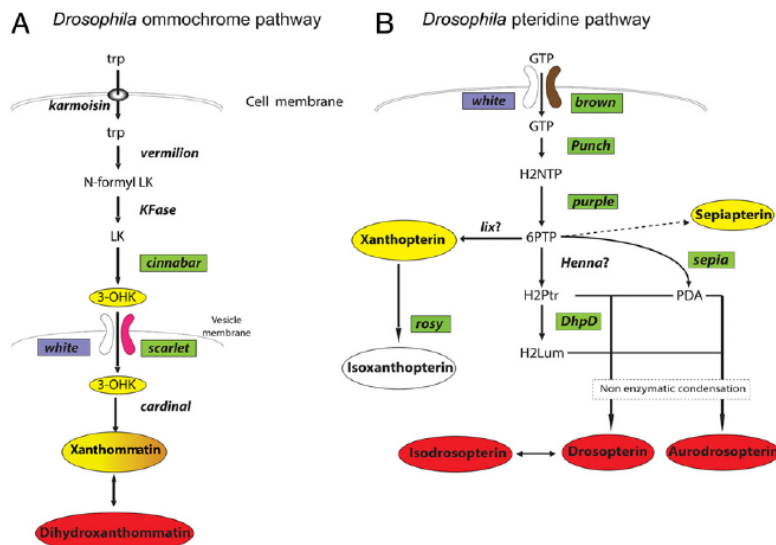


Fig. 1. Diversity of color patterns in the embryos of water striders. Species names are indicated under each image. All embryos are shown in ventral view. Embryos are wild type after completion of katatrepsis, a process where the embryo turns within the egg before dorsal closure, and formation of *ruptor* or *ov* (egg burster or hatching line). Arrowheads indicate the eyes. Asterisks indicate species with dark eggshell. (Scale bars: 100 μm.)







**Fig. 3.** Biosynthetic pathways for pigmentation in *Drosophila*. Ommochromes (A) (data from refs. 63 and 64) and pteridines pigments pathways of *Drosophila melanogaster* adult eyes (B) (data from ref. 65). Homologous genes studied in the water strider *L. franciscanus* are boxed in green. Note that the *white* gene (purple boxes) is common to both pathways. Note that, in *Drosophila*, the heterodimer white-scarlet is inserted within the membrane of pigment vesicles, while the heterodimer white-brown is inserted within the cell membrane.

into pigments with various colors ranging from yellow to red, or brown to black (29) (Fig. 3A), whereas pteridine pathway converts GTP into yellow, orange, or red pigments (30) (Fig. 3B). The ABC transporter White is common to both ommochrome and pteridine pathways (Figs. 3 and 4A and E), and null mutations in the fly *white* gene result in the complete loss of pigmentation in the eyes (31, 32). In the embryos of the water strider *L. franciscanus*, *white* mRNA expression prefigures the color pattern both within and outside the eyes (Fig. 4F), and *white* silencing using RNA interference (RNAi) depletes all colors from the entire embryo (Fig. 4G and H). This result suggests that embryonic extraocular color could originate from the extraocular activation of eye pigment biosynthesis pathways.

To determine whether ommochrome or pteridine, or both, are responsible for the extraocular pigment, we tested the role of specific genes from each pathway. For ommochromes, we selected *cinnabar* (*cn*) that encodes an enzyme and *scarlet* (*st*) that encodes an ABC transporter (Fig. 4A). The expression of *cinnabar* is confined in the eyes (Fig. 4I), and inactivation of this gene by RNAi resulted in embryos where the eyes became bright red but where the extraocular coloration remained unaffected (Fig. 4J and K). Unlike *cinnabar*, the expression pattern of *scarlet* prefigured the red coloration in the eyes and in the extraocular stripes (Fig. 4L). Surprisingly, inactivation of *scarlet* by RNAi caused the exact same phenotype as *cn* in that embryos lost dark color in the eyes but retained the extraocular coloration (Fig. 4M and N). Controls for this experiment confirmed that RNAi depleted efficiently *scarlet* mRNA (ref. 33 and SI Appendix, Fig. S6), demonstrating that *scarlet* is not required for extraocular color production despite its expression there. These results show that ommochrome pigments are responsible for dark coloration in the eye but not for the extraocular coloration in *L. franciscanus* embryos.

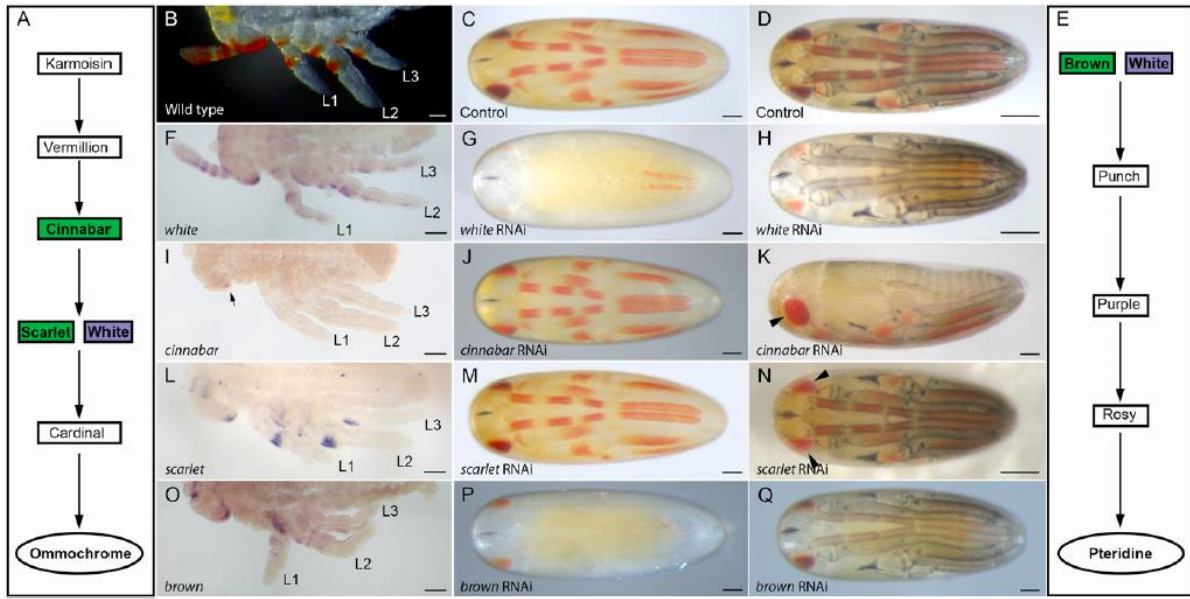
In *Drosophila*, the *brown* gene encodes an ABC transporter that forms a heterodimer with *white*, both of which are required for pteridine synthesis in the eyes (34) (Figs. 3B and 4E). We found that the expression of *brown* mRNA prefigures both ocular and extraocular color patterns (Fig. 4O) and that RNAi against *brown* resulted in the complete loss of red color both in the eyes and in extraocular tissues (Fig. 4P and Q). This phenotype is similar to

the effect of *white* RNAi in both eyes and extraocular tissues (Fig. 4G and H). Sequence alignment and molecular phylogeny of the 3 transporters *white*, *brown*, and *scarlet* revealed that these 3 genes occur in single copy in the genome of the water strider *Geris buenoi* (35), and their sequences cluster with their homologs from various insect species (SI Appendix, Fig. S7). Altogether, these results show that, in *L. franciscanus* embryos, the extraocular color is produced by pteridine pigment biosynthesis and that eye color is produced by the combination of pteridine and ommochrome pigments.

**Recruitment of Eye Pteridine Pigment Biosynthesis Pathway.** The pteridine pathways of flies and hemipterans share the same early steps consisting of 2 ABC transporters and the 3 first successive enzymatic reactions (Fig. 3B and SI Appendix, Fig. S8). The subsequent reactions diverge and lead to the production of distinct colored pigments in each of these 2 insect lineages (Fig. 3B and SI Appendix, Fig. S8) (31). To determine the extent to which the pteridine pathway has been recruited to generate red patches in *L. franciscanus* embryos, we analyzed the roles of the first 2 enzymes, Punch and Purple, known to convert GTP into eye pigment precursors in both flies and hemipterans (Figs. 3 and 5A and SI Appendix, Fig. S8). Both *punch* and *purple* mRNA expression patterns prefigured the pattern of pigment distribution in *L. franciscanus* embryos (compare Fig. 5B, D, and F). RNAi against both genes yielded identical results consisting of the elimination of extraocular pigment (compare Fig. 5C, E, and G). *punch* and *purple* RNAi-treated embryos had darker eyes, compared to controls, indicating that the eyes of these embryos lost the red pteridine and retained the dark ommochrome pigments (Fig. 5C, E, and G). This result demonstrates that the transporters and the initial enzymatic reactions of the pteridine pathway responsible for producing red pigment in the eye have been recruited to produce extraocular pigments in *L. franciscanus* embryos.

To further complete the analysis of pteridine biosynthesis pathway recruitment in *Limnogonus* extraocular tissues, we analyzed the terminal enzymes necessary for the conversion of colorless precursors into colorful pigments in both flies and hemipterans (Fig. 3B and SI Appendix, Fig. S8). We failed to detect any expression of *sepia* or *DhpD* (SI Appendix, Fig. S9), 2 genes that are necessary



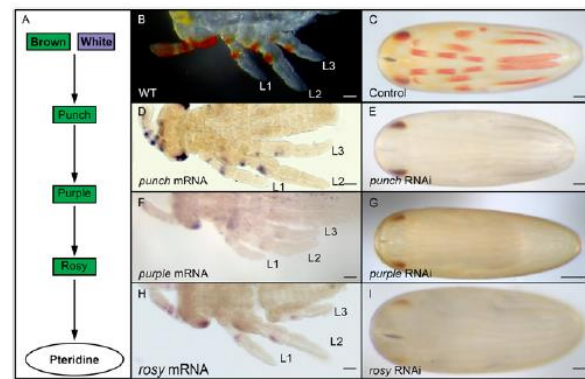


**Fig. 4.** Expression and function of genes from ommochrome and pteridine pathways in *L. franciscanus* embryos. For in situ hybridizations, dissected embryos are shown in ventral view. Embryos obtained after RNAi were not dissected and are also shown in ventral view (except K: lateral view) at 2 different stages: 74 h (C, G, J, M, and P) or 135 h after egg-laying (D, H, K, N, and Q). Simplified ommochrome (A) and pteridine (E) pathways indicating the genes analyzed in this figure (colored boxes). (B) Wild-type embryo (dissected) just after katatrepsis showing the natural color pattern. (C and D) Control RNAi embryos. (F, L, and O) Expression of *white* (*w*), *scarlet* (*st*), and *brown* (*bw*) in the eyes, antennae, and legs. (I) Expression of *cinnabar* (*cn*) in the eye (black arrow). Loss of extraocular pigmentation after the inactivation of *w* (G and H); similar results were observed in *bw* RNAi (P and Q). *cn* (J) and *st* RNAi (M), 74 h after egg-laying. *cn* (K) and *st* (N) RNAi embryos, 135 h after egg-laying, have bright red eyes, but the extraocular pattern is unchanged. (Scale bars: 100 µm; with the exception of D, H, and N, 200 µm.)

for the production of the red aurodrospterin and drosopterin in flies (Fig. 3B) (36–39). Accordingly, RNAi against these 2 genes did not affect coloration either in the eyes or in extraocular tissues (SI Appendix, Fig. S9). In contrast, we found that the mRNA expression pattern of *rosy*, a terminal enzyme required for the production of both the yellow xanthopterin and the red erythropterin pigments in the Hemiptera, prefigures the pattern of pigment accumulation both within and outside the eyes (Fig. 5H). Inactivation of *rosy* using RNAi again abolished color from the extraocular tissues and left the embryos with dark eyes (Fig. 5I). Altogether, these results show that the pteridine biosynthesis pathway is necessary for pigment production both in the eyes and in extraocular organs. This suggests that the pteridine pigment biosynthesis pathway has been coopted from the eyes to the appendages and resulted in the acquisition of extraocular color during evolution.

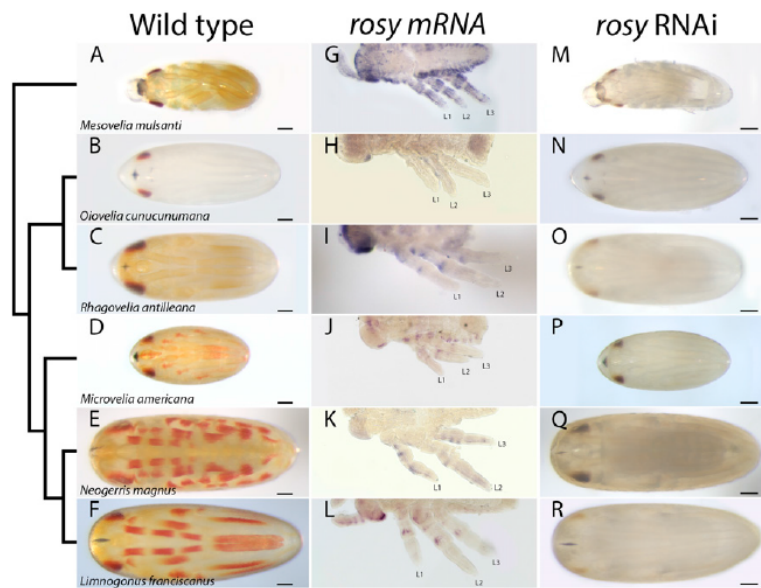
**Single Pathway but Divergent Colors and Color Patterns.** Our genetic analysis in *L. franciscanus* raises the question of whether the diversity of extraocular coloration observed in other Gerrromorpha originates from the recruitment of the same pteridine network. We therefore determined the genetic basis of pigment production in 5 additional species selected based on their phylogenetic position and the differences in their colors and color patterns (Fig. 6). We first focused on *rosy* because it encodes a xanthine dehydrogenase (40) known to produce pigments ranging from yellow to orange and red in the Hemiptera (36, 41) (SI Appendix, Fig. S8). In a species with no extraocular pigmentation, such as *Oiovelia cunucunumana* (Fig. 6B), *rosy* is expressed only in the eyes (Fig. 6H). Consequently, the inactivation of *rosy* in this species depleted the bright red pteridines resulting in dark eyes (Fig. 6N). In all other species with extraocular pigmentation, either yellow or red (Fig. 6A and C–F), the expression of *rosy* prefigured the color patterns regardless of the shade or the spatial distribution of the color (Fig. 6

G and I–L). Accordingly, the inactivation of *rosy* in these species also resulted in dark eyes and the complete loss of extraocular pigmentation (Fig. 6 M and O–R). We obtained similar results with 2 additional genes positioned upstream of the pathway, namely the ABC transporter *white* and the GTP cyclohydrolase-I *punch* (SI Appendix, Fig. S10). These results show that, despite the



**Fig. 5.** Expression and function of the genes encoding for the pteridine pathway enzymes in *L. franciscanus* embryos. (A) Simplified pteridine pathway showing the genes analyzed in this figure (colored boxes). (B) Ventral view of a dissected wild-type (WT) *L. franciscanus* embryo just after katatrepsis showing the natural color pattern. (C) Control RNAi embryo, ~74 h after egg-laying. Expression of *punch* (D), *purple* (F), and *rosy* (H) in eyes and extraocular tissues. *punch* (E), *purple* (G), and *rosy* (I) RNAi results in the loss of red color both in the eyes (dark eyes) and in the extraocular tissues. (Scale bars: 100 µm; except G, 200 µm.)





**Fig. 6.** Expression and function of *rosy* across Gerromorpha. (Left) Phylogenetic relationships between the 6 studied species. All embryos are shown in ventral view. (A–F) WT embryos showing the natural color pattern. (G–L) Expression of *rosy* (*ry*) mRNA. (M–R) Effect of *ry* RNAi on pigmentation across this species sample. (Scale bars: 100  $\mu$ m.)

high diversity observed, the extraocular pigmentation in the embryos of Gerromorpha originates from the same pteridine biosynthesis pathway.

**Identification of the Pigments in Gerromorpha Embryos.** Pteridine pigments belong to the family of aromatic compounds composed of the fusion of pyrimidine and pyrazine rings (28, 42, 43). Among these, the Hemiptera are known to produce erythropterin, which is red, in addition to sepiapterin, xanthopterin, and chrysopterin, all of which are yellow (SI Appendix, Fig. S8 and Table S4). Using hydrophilic interaction liquid chromatography (HILIC) coupled to high-resolution mass spectrometry (HRMS; see details in *Material and Methods*), we detected xanthopterin (retention time 6.7 min) and erythropterin (retention time 8.1 min) in the embryos of *L. franciscanus* (Fig. 7 and SI Appendix, Table S5). However, we only detected traces of chrysopterine and failed to detect any sepiapterin, pterin, or isoxantholumazine (violapterin) in these samples (SI Appendix, Table S6; see *Material and Methods* for more detail). Specific mass spectrometry fragments are reported in Fig. 7 B and C and SI Appendix, Table S5. The absence of sepiapterin, whose production is independent from the Rosy enzyme, is consistent with our *rosy* RNAi, which depletes all extraocular pigments. This result, together with the absence of pterin and isoxantholumazine, suggests that the parallel terminal branch of the pathway identified in Hemiptera (SI Appendix, Fig. S8) is not activated in the embryos of *L. franciscanus*.

We then asked whether the same pigments were responsible for the coloration observed in the embryos of 2 additional species. Again, both xanthopterin and erythropterin were detected in the embryos of *Limnoporus dissortis*, a species with yellow extraocular pattern (Fig. 1 and SI Appendix, Table S6). By contrast, only erythropterin could be found in *Paravelia conata*, which has no extraocular coloration (Fig. 1 and SI Appendix, Table S6). This result suggests that all of the xanthopterin is converted into the red pigment in the eyes of *P. conata*. We conclude that the red color of legs and antennae is due to the synthesis of erythropterin and the yellow color is likely due to xanthopterin. The congruence between genetic and chemical analyses strengthens the conclusion that a single

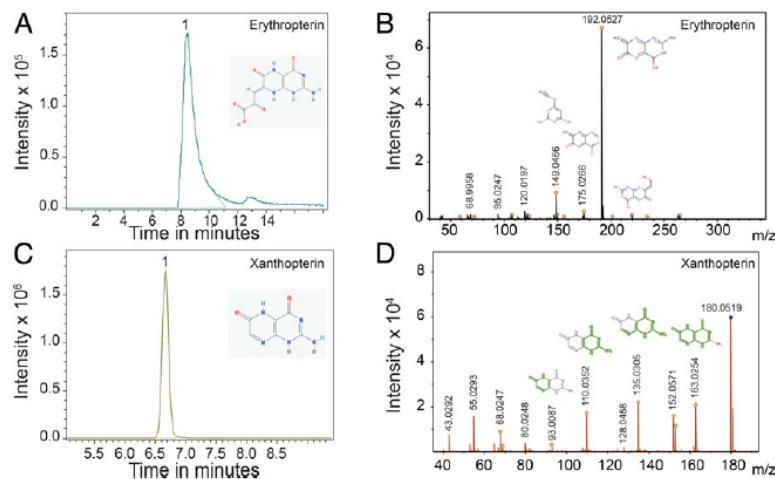
pteridine pathway is at the root of color diversification in the embryos of the Gerromorpha.

#### Discussion

Embryonic coloration in insects, unlike adult coloration, has received very little attention (22). Here, we present an extraocular coloration that is specific to the body of the embryo and absent from postembryonic stages in the semiaquatic bugs. We describe the evolutionary history and the developmental genetic mechanism underlying the diversity in embryonic colors and color patterns. We demonstrate that the cooption of the pteridine biosynthesis pathway, which produces red visual pigment in the eye, is at the origin of embryonic extraocular color. In addition, we discovered that this cooption occurred before the emergence of the Gerromorpha and initially resulted in the production of yellow extraocular color. Subsequently, 6 independent events occurred where distinct lineages evolved bright red color, in addition to the ancestral yellow state. Despite the high diversity in colors and color patterns, we show that the underlying core biosynthesis pathway remained stable throughout the 200 million years of gerromorphan evolutionary time. Finally, we identified erythropterin and xanthopterin as the main pigments responsible for these colors.

**Pteridine Biosynthesis Pathway in the Gerromorpha.** In insects, different classes of compounds (tetrapyrroles, melanins, pteridines, ommochromes, anthraquinones) contain endogenous pigments of yellow and/or red color (28). Our genetic analysis demonstrates that pteridines and ommochromes are both present in the eye, as is common in other insects (44). By contrast, only pteridines are responsible for extraocular colors present in embryonic appendages. In the Gerromorpha, we found that the production of both yellow and red pigments is *rosy* dependent, which excludes sepiapterin as a putative yellow pigment. Our RNAi results also show that *sepia* and *DhpD* are not involved in pigment production, thus demonstrating that the red pteridines found in *Drosophila* eyes are not responsible for the red color in water striders. Chemical analysis of *L. franciscanus* embryos confirmed these conclusions and revealed the presence of xanthopterin and





**Fig. 7.** Identification of erythropterin and xanthopterin from *L. franciscanus* embryos using UHPLC-high-resolution mass spectrometry (HRMS). HILIC chromatograms, and MS/MS (tandem mass spectrometry) spectrums for erythropterin (A and B) and xanthopterin (C and D). Samples consisted of pteridines extracted from whole embryos of *L. franciscanus*. The chemical structure of each pigment is shown on chromatograms (A and C). For details, see *SI Appendix, Supplementary Materials* and Tables S3 and S4.

erythropterin. Xanthopterin pigment is known to produce yellow coloration and erythropterin can produce both yellow and red, depending on concentration, in a wide variety of hemipteran species (39, 41, 45–47) (*SI Appendix, Table S4*). We failed to detect other pteridine pigments that are usually present in the Hemiptera, such as sepiapterine, pterin, and isoxantholumazine. This indicates that the pathway leading to pigment production in the embryo of the Gerromorpha is linear, unlike adult pteridine pathways, which have 2 terminal branches in *Drosophila* eyes (Fig. 3B) and in other hemipterans (*SI Appendix, Fig. S8*). We therefore conclude that the red color results from the accumulation of erythropterin and that the yellow color may be due to either erythropterin itself (depending on pH and concentration) or to its precursor xanthopterin. Therefore, the yellow color may originate from a single pigment or a combination of pigments.

**Recruitment of Pteridine Pathway from the Eye to Extraocular Tissues.** RNAi knockdown experiments demonstrated that pteridine and ommochrome pathways produce red and dark pigments in the eyes, respectively. However, only the pteridine pigments are present outside the eyes, indicating that the production of various shades of extraocular yellow to red is due to the recruitment of this pathway. The linear nature of the reactions that culminate into pigment production, from transporters to the terminal enzymes, requires all components of this network to be expressed simultaneously for the color to be produced. In the embryos of water striders, the genes that are necessary for pteridine biosynthesis in the eyes are also active in extraocular tissues across the phylogeny. This indicates that the pathway has been activated as a whole outside the eye and remained stable since.

Surprisingly, at least 1 member of the ommochrome pathway, namely the ABC transporter *scarlet*, which is not required for extraocular pigment production, is expressed in a pattern that prefigures the extraocular color pattern. This puzzling result suggests that the 3 ABC transporters, *scarlet*, *white*, and *brown*, may have a shared regulation. If true, we can speculate that the cooption of *white* and *brown* as members of the pteridine pathway resulted in the activation of *scarlet*, through the redeployment of an eye-specific upstream regulator outside the eye, although *scarlet* itself is not needed to produce extraocular pigments. Such hitchhiking effect provides a window to better understand the process of whole

pathway cooption and the developmental genetic constraints associated with it (48, 49).

**Same Pathway but Various Colors.** Our phylogenetic reconstruction revealed that the ancestral state of the trait for water striders was yellow extraocular color with no red. This indicates that the recruitment of eye pteridine pigments into the appendages predated the origin of the Gerromorpha over 200 million years ago. Inferring the date and the number of gains of this cooption would require reconstructing the ancestral state in the order Hemiptera. Despite the scarcity of information in the literature, we know that embryos with red and/or yellow extraocular colors are found in some other infraorders of Heteroptera, such as Pentatomomorpha and Cimicomorpha, some of which could be produced through the pteridine pathway (50–52). The cooption of pteridines may therefore be as old as 300 million years.

Our sample presents high variation in terms of presence or absence of extraocular color, various shades of yellow and red, and various spatial color patterns. Interestingly, the core pathway underlying this diversity remained unchanged throughout the evolutionary history of the Gerromorpha (Figs. 2 and 6). Two independent lineages lost the extraocular color, through the loss of expression of members of the pathway, and 6 lineages evolved bright red color independently (Fig. 2). A possible explanation for these 6 convergence events would be the repeated cooption of the last (unknown) enzyme of the pathway that converts xanthopterin into erythropterin. An alternative explanation could be that the various color shades from yellow to red result from differences in the concentration of Erythropterin (39, 41, 53). In favor of this latter hypothesis, perturbation of pigment production through *white* RNAi in 2 species with yellow extraocular color, namely *Limnoporus dissortis* and *Mesovelia mulsanti* (*SI Appendix, Fig. S11*), resulted in patches of red color. This result suggests that species with yellow extraocular pigment possess the entire genetic machinery to produce the red pigment and lends support to the hypothesis that the same pigment could be responsible for both yellow and red.

**Perspectives on the Ecological Role and Spatial Regulation of Embryonic Color Patterns.** It is widely established that colors can play crucial ecological roles in survivorship and mate recognition (4, 5). Yellow and red colors, such as those expressed in the

embryos of the Gerromorpha, are frequently associated with aposematism in insects (54). Water striders' eggs, being glued to various aquatic substrates, are vulnerable to predation (by other aquatic Heteroptera, fisher spiders, fishes, frogs, birds, mites) and parasitism by parasitic wasps (23, 27). In the eggs of Hemiptera, the chorion is frequently colored with black melanin (55). Interestingly in the Gerromorpha, when the extraocular tissues are red, the chorion is invariably transparent, allowing the extraocular colors to be exposed (Fig. 1). This suggests that the exposure of color patterns have been favored throughout evolution, possibly due to a signaling function. As embryogenesis duration is longer in the Gerromorpha (6–14 d) (23), compared to other insects, such as *Drosophila* (1 d) or *Tribolium castaneum* (3 d) (56), it is possible that coloration increases survivorship by allowing the embryos to avoid predation through signaling unpalatability (54). Our ability to manipulate these color patterns provides a good system to test these hypotheses in the future.

Another striking aspect of embryonic coloration in the Gerromorpha is the high variation in spatial patterns across species. The origin of this variation probably lies in differences of the spatial regulation of this pathway. This system paves the way for a metamodel (15) to better understand the regulatory mechanism underlying pathway cooption to generate novel phenotypes, and the ecological forces favoring these phenotypes in nature.

## Material and Methods

**Animal Collection and Rearing.** Water striders were collected in different locations listed in *SI Appendix, Table S7* (27). Animals were kept at 27 °C with a 14-h light/10-h dark cycle in containers with tap water and fed on frozen crickets. A piece of floating Styrofoam was supplied to the females for egg-laying, which were then employed in subsequent experiments.

**Gene Cloning.** Total RNA from *M. mulsanti*, *O. cunucunumana*, *Rhagovelia antilleana*, *Microvelia americana*, *Neogerris magnus*, and *L. franciscanus* were extracted from embryos, nymphs and adults. First-strand cDNA synthesis was then performed for each species, using total RNA as a template, according to instructions outlined in the Invitrogen cDNA synthesis kit. *white*, *brown*, *scarlet*, *purple*, *punch*, *sepia*, *DhpD*, or *rosy* were cloned using PCR. The primers and GenBank accession numbers of the gene sequences are in *SI Appendix, Table S8*.

**Embryo Collection and Dissection.** Embryos were collected, treated with bleach diluted 1/4 in water, and then washed with 0.05% PTW (1 × PBS; 0.05% Tween-20). Pictures of whole embryos around midembryogenesis were captured with AxioCam ICc 5 Zeiss camera. For in situ hybridization, embryos of various early stages were dissected out of the chorion, cleaned from yolk as much as possible, and kept briefly in PTW 0.05% on ice until fixation.

**In Situ Hybridization.** Dissected embryos were fixed in 200 μL of 4% paraformaldehyde + 20 μL dimethyl sulfoxide and 600 μL of heptane for 20 min at room temperature, then washed several times in cold methanol. Embryos were then rehydrated in decreasing concentrations of methanol in 0.05% PTW and washed in 0.05% PTW and 0.3% PBT (1 × PBS; 0.3% Triton X-100) 3 times each. Embryos were washed twice with 1% PBT, transferred to 1:1 1% PBT /hybridization solution (50% formamide; 5% dextran sulfate; 100 μg/mL yeast tRNA; 1 × salts). The composition for 100 mL of 10× salts is as follows: 17.5 g of sodium chloride, 1.21 g of Tris-base, 0.71 g of monosodium phosphate, 0.71 g of sodium phosphate dibasic, 0.2 g of Ficoll 400, 0.2 g of polyvinylpyrrolidone, 10 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA), and 0.2 g of bovine serum albumin (BSA) (pH 6.8). Embryos were prehybridized for 1 h at 63 °C, followed by the addition of a Dig-labeled RNA probe overnight at 63 °C. Embryos were then transferred gradually from hybridization solution to 0.3% PBT through consecutive washes with

3:1, 1:1, and 1:3 (prewarmed hybridization solution: 0.3% PBT gradient). In situ Pro VSI robot was programmed to make all previous steps. A blocking step was performed manually with PAT (1 × PBS; 1% Triton X-100; 1% BSA) at room temperature followed by incubation with anti-dig antibody coupled with alkaline phosphatase for 2 h at room temperature. Embryos were washed several times in 0.3% PBT then in 0.05% PTW before color reaction is conducted with nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) in AP buffer (0.1 M Tris pH 9.5; 0.05 M MgCl<sub>2</sub>; 0.1 M NaCl; 0.1% Tween-20). Photos were captured with DM 6000 Leica microscope.

**Parental RNAi.** Parental RNA interference employs delivery of double-stranded RNA of the gene of interest to the embryos through injecting their mothers. This technique specifically depletes gene activity resulting in developmental phenotypes. Knockdown of each gene using parental RNAi was conducted following the protocol described in refs. 57 and 58. Control RNAi was conducted by injecting buffer injection 1 × (1 M NaH<sub>2</sub>PO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 6.8, 2.5 M KCl). Template for in vitro transcription to produce double-stranded RNA for each gene was prepared using the T7-tagged primers (*SI Appendix, Table S9*). Number of females injected and counts for phenocopy individuals obtained are shown in *SI Appendix, Table S10*.

**Reconstruction of Ancestral Character State.** The matrix data were built based on 3 different categories depending on the extraocular colors: 1) absence of yellow or red, 2) presence of yellow or red, and 3) presence of both yellow and red (Fig. 2 and *SI Appendix, Figs. S4 and S5 and Table S1*).

Reconstruction of the ancestral state for extraocular colors was performed using the ace (Ancestral Character Estimation) function from the APE package version 5.2 (59) in Rstudio version 1.1447 using a maximum likelihood method adapted to discrete characters and represented using phytools (60). The simplest model, "ER," with equal transition rates on extraocular colors, was the best with likelihood ratio test ( $P = 0.58$  to yellow ancestral reconstruction and  $P = 0.41$  to red ancestral reconstruction for comparison ER; failure of convergence of ARD model). The pies for ancestral nodes represent marginal ancestral states likelihoods (*SI Appendix, Fig. S5 and Tables S2 and S3*).

**Pteridine Pigment Extraction and Identification.** Pteridines were extracted using a modified Robson et al. method (61). Briefly, 1,200 whole post-katatrepsis (a process in some insects through which the embryo turns 180° and positions itself at the ventral side of the yolk) colored embryos were grinded in liquid nitrogen and sonicated for 10 min. As 6-biopterin was never detected in our samples, it was also added as internal standard (Sigma Aldrich). Erythropterin was synthesized by the Compound Library and Custom Synthesis of "Institut de Chimie et de Biochimie Moléculaires et Supramoléculaires" (see details in *SI Appendix, Supplementary Methods*) Other standards used were as follows: sepiapterin (Sigma Aldrich) and xanthopterin (BOCSCI Inc). Specific MS fragments are reported in Fig. 7 B and C and *SI Appendix, Table S5*.

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## CHAPTER 3

## **Spatial regulation of colour patterns in *Limnogonus franciscanus* embryos.**

**Author:** Aidamalia Vargas-Lowman<sup>1</sup>, Kahina Saker<sup>1</sup>, François Bonneton<sup>1</sup>, Abderrahman Khila<sup>1</sup>.

### **Affiliation:**

1-Developmental genomics and evolution team; official affiliation from IGFL web site (no accent): Institut de Genomique Fonctionnelle de Lyon, Univ Lyon, CNRS UMR 5242, Ecole Normale Supérieure de Lyon, Université Claude Bernard Lyon 1, 46 allée d'Italie F- 69364 Lyon, France.

### **Abstract:**

Determining the regulatory processes underlying effector genes of morphological trait is essential to understand the evolution and maintenance of phenotypic diversification. Here, we showed a preliminary study to identify transcriptional regulators involved in the recruitment of the pteridin network in semi-aquatic embryos. We performed RNA sequencing, comparative transcriptomics, and gene function assays to identify possible transcription factors involved in the production and regulation of pteridine pigments in eyes and appendages. A total of 135 transcription factors were found to be in common between pigmented organs. From the 80 genes tested, we failed, so far, to identify any that were involved in the spatial regulation of colour patterns in the appendages of *L. franciscanus* embryos.

**Key words:** Colour, transcription factors, pteridine pathway, diversification, Gerromorpha, co-option, semi-aquatic insects, embryos.

## Introduction

Conservation in coding regions across species indicates that evolutionary novelties may arise through combinatorial processes, such as changes in gene regulation and the co-option of novel genes into existing regulatory gene networks (SANETRA *et al.* 2005). Studies in butterflies suggest that not more than four genes (*optix*, *wntA*, *cortex* and *doublesex*) play causative roles in the formation of wing colour patterns (REED *et al.* 2011; MARTIN *et al.* 2012; KUNTE *et al.* 2014; NADEAU *et al.* 2016). These genes are characterized as “adaptive hotspots,” which repeatedly drive morphological evolution across different lineages (PAPA *et al.* 2008; MARTIN AND ORGOGOZO 2013). However, pigmentation traits in *Drosophila* have tended to be caused by differences in *cis*-regulatory elements of the pigmentation enzymes themselves (ORDWAY *et al.* 2014).

In Gerromorpha (semi-aquatic bugs bugs), embryos have red-dark colour in the eyes, while the appendages and abdominal parts can be yellow, yellow-red or colourless, depending on the species (Figure 1). We have shown that the colour diversification of semi-aquatics embryos is produced by the co-option of the same pteridine pathway

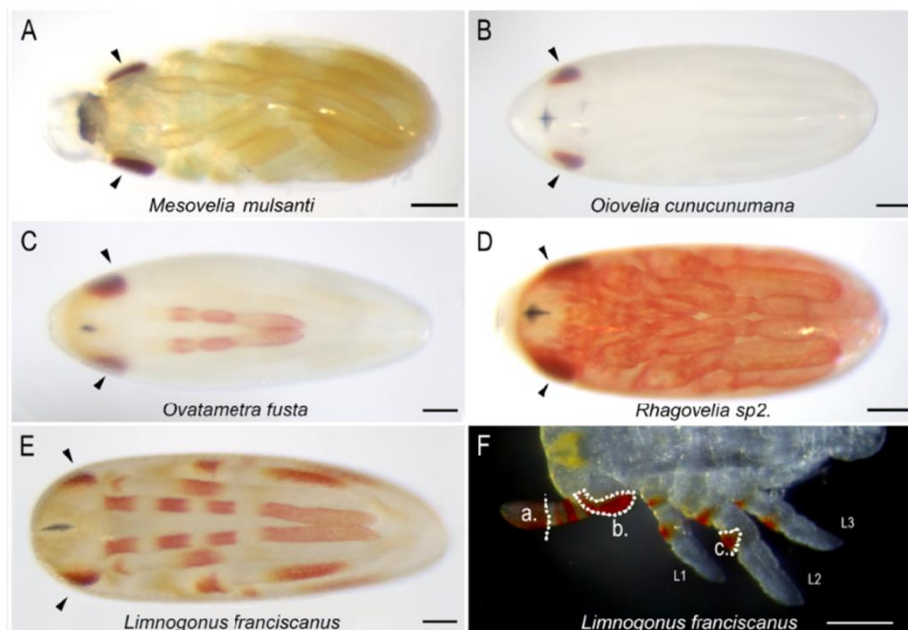


Figure 1. Diversity of colour and patterns in Gerromorpha embryos. A-E) different species of semi-aquatic bugs xx hours after lay. F) *Limnogonus franciscanus* embryo without chorion, 64 hours after laying. Dotted lines represent the tissues that were dissected. A. distal part of the antennae, b. eye and c. second spot of the second leg. Arrow heads signalling the eyes. Scale bar 100  $\mu$ .

involved in the production of red pigments in the eye (VARGAS-LOWMAN *et al.* 2019). Additionally, this co-option is common to all Gerromorpha and the shift from yellow to red

evolved independently in at least six distinct lineages. These results suggest that the diversification of spatial patterns is due to changes in the regulation of the genes acting in the pteridine pathway. We propose that the regulatory genes that are involved in the production of pigments in the eye were recruited to control the diverse patterns found in the extra-ocular tissues of Gerromorpha embryos. If this hypothesis is true, some of the transcription factors that regulate pteridine genes in the eyes should also be expressed in legs and antennae.

To test this hypothesis, we used next-generation sequencing and comparative transcriptomics approach to identify the set of transcription factors associated with pigmentation in the embryos of *Limnogonus franciscanus*. We then used RNAi to test the role of these genes in embryonic colouration. In this study, I present and discuss the preliminary results of this screen.

## Methods

### *Animal husbandry*

Wild-type *Limnogonus franciscanus* adults were collected in Puerto Rico and established in the laboratory. Stocks were reared in climate controlled room at 27 °C on a 14 L: 10 D photoperiod in containers with tap water. Adults were fed with frozen crickets. A piece of floating Styrofoam was supplied to the females for egg laying. The embryos collected from *L. franciscanus* females were then used to do dissection of different organs.

### *Embryos dissection*

The embryos were collected and incubated at 27°C until katatrepsis developmental stage (about 64 h after laying); at this stage the eyes and appendages of the embryos are red pigmented. The embryos were treated with 25% bleach, washed several times with PTW 0.05% (1 × PBS; 0.05% Tween-20) to be dissected in the same solution. The initial approach achieved was a differential expression analysis between two developmental stages of *Limnogonus franciscanus*: 1) in young embryos, before the colours appear (30 h after laying) (Supplementary Figure 1); and 2) when colours were evident (katatrepsis stage) (Figure 1f). Tissues were selected according to the presence of red colours: eyes, distal segment of the antennae and the second red spot of the second leg (Figure 1F). Samples of each tissue were dissected using home-made scalpels, collected into an Eppendorf tube with PBS buffer containing RNase inhibitor and frozen immediately in liquid nitrogen. The tissues were sent

to GenomeEast Plateforme (France) to be sequenced. Three separate libraries were made for each type of tissue to represent biological replicates. Dissection of eye, antennae and leg were performed in random order to avoid any gene expression biases caused by the operation. After the differential expression analysis between the two different developmental stages, we could observe that the genes involved in the pathway were already expressed in the young embryos (data not shown). For this reason, we decide to change the approach to analyse genes that were expressed between pigmented tissues.

#### *Library preparation and cDNA sequencing*

Full length cDNA were generated from tissues using the SMART-Seq v4 Ultra Low Input RNA kit for Sequencing (Takara Bio Europe, Saint Germain en Laye, France) according to manufacturer's instructions with 13 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Six hundred ng of pre-amplified cDNA were then used as input for Tn5 transposon tagmentation by the Nextera XT kit (Illumina, Paris, France) followed by 12 cycles of library amplification. After purification using Agencourt AMPure XP beads (Beckman-Coulter, Villepinte, France), the size and concentration of library DNA were assessed on a Fragment Analyzer (library activity > 6 nM). Libraries were then sequenced on a HiSeq 4000 as paired-end 100 bp reads following Illumina's instructions. After sequencing, adaptors were removed from sequences and initial quality check reports for raw reads were provided by GenomeEast Plateforme.

#### *Transcriptomic assembly*

We used a transcriptomic approach (RNAseq) to identify transcription factors expressed in common between the different pigmented tissues (eye, antennae and leg). We first assembled the reads of each tissue independently. Read filtering, cleaning and adaptor removal was done using Trimmomatic version 0.32 with sliding window of three base pairs with a minimum quality of 15 (BOLGER *et al.* 2014). Any base pair at the beginning or end of the read whose quality would be under 15 was removed and only reads with a minimum length of 50bp were kept. The assembly of these reads was conducted with Trinity r20140717, the reads were normalized to a maximum coverage of 50 and the path reinforcement distance was set to 50 (GRABHERR *et al.* 2011). Gene ontology (GO) annotation was conducted using Blast2GO Blast2GO version 4.0.7 (CONESA *et al.* 2005). BlastX searches were performed against the invertebrate non-redundant protein NCBI database using default parameters whilst setting the



e-value to  $1.0 \times 10^{-20}$  and the number of hits to 5 (PRUITT *et al.* 2005). The mapping and annotation steps were done using default settings. Additionally, we re-constructed a reference transcriptome from *L. franciscanus* adults and embryos using a “de novo assembly” approach (HAAS *et al.* 2013). A quantitative measure for the assessment of the reference transcriptome assembly was conducted using BUSCO v3 to obtain the percentage of single-copy orthologues (SIMÃO *et al.* 2015)

#### *Identifying genes potentially involved in pigmentation*

Genes expressed in common between the different tissues were identified using RSEM software for estimating transcript abundance (LI AND DEWEY 2011). All the genes that were expressed in common between eye, antennae and leg were kept. Next, we performed a filtering using the term ‘transcription’ on the GO annotation data base. Using this method, we identified 167 transcripts in our dataset.

#### *Parental RNAi*

Double stranded RNA (dsRNA) was produced for 80 of 167 transcripts labelled as transcription factors (KHILA *et al.* 2009; REFKI *et al.* 2014). Control RNAi was conducted by injecting buffer injection 1X (NaH<sub>2</sub>PO<sub>4</sub>/ NaH<sub>2</sub>PO<sub>4</sub> 1M pH 6.8, KCL 2.5M). Template for in vitro transcription to produce ds-RNA for each gene was prepared using the T7-tagged primers (Supplementary Table 2). Pictures were captured with SteREO Discovery.V12 Zeiss stereomicroscope using the Zen Pro 2011 software.

## **Results**

#### *Transcriptome assembly*

Around 500 million paired end reads (~100 bp) were generated from 18 different mRNA libraries. These libraries consisted of three biological replicates of micro-dissected tissue from eye, distal segment of the antennae and the middle of the second leg at two different stages. The first stage was 30 h after egg-laying, when there is no sign of colour, and the second stage was during katatrepsis will full colouration. (Figure 1F). The pigmentation process starts a couple of hours before katatrepsis (39h after laying), first in the eye, then on the rest of tissues. In this manner we ensure that all the dissected tissues were pigmented. A “de novo assembly” transcriptome reference was performed using reads from adults and

embryos of different developmental stages. A total of 106515 contigs were assembled with N50 value of 1409 bp. Median contig length was 417 bp. We used **Benchmarking Universal Single-Copy Orthologs (BUSCO)** analysis to assess the completeness of the transcriptome assembly. The number of complete BUSCO Arthropoda copy detected was 92.0% (91.4% singles and 0.6% duplicated), with 1.8% and 6.2% orthologous genes fragmented and missing, respectively.

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#### *Transcription factors in common between red organs*

In a previous study, we found that the extra-ocular pigments were recruited from the same pteridine pathway that produces the pigmentation in the eye (Vargas-Lowman et. al. under review). From this observation, we proposed that the genes that are involved in the regulation of the extra-ocular colour pattern in embryos of *L. franciscanus* were co-opted from the eye to extra-ocular organs. To test this hypothesis, we searched for transcription factors expressed in common between three pigmented tissues (the eye, the distal segment of the antennae and the second spot of the second leg). First, we selected all the 12160 transcripts expressed in the eye. Secondly, we selected the transcripts that were expressed in antennae and leg together with eye, independently. A total of 10528 transcripts were expressed in common between the three pigmented tissues. From these transcripts, 9714 had GO annotation. Finally, 167 transcripts labelled as “transcription” in the description from the GO annotation database were identified as being in common between the three organs (Figure 2). From these 167 transcripts, 135 are homologous to known genes in *D. melanogaster*, mostly involved in segmentation, embryonic development and morphogenesis (THURMOND *et al.* 2018), three genes where unknown to *D. melanogaster*.

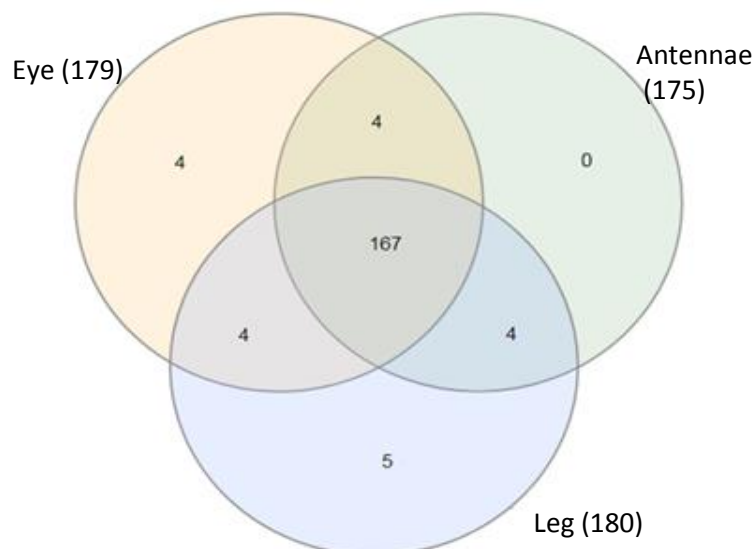


Figure. 2. Data base analysis to obtain the 167 transcription factors expressed between three red pigmented tissues. Venn Diagram made with (HEBERLE *et al.* 2015).

### *Preliminary results of transcription factor screening.*

In order to test whether these 135 genes were associated with the regulation of patterning colouration of *L. franciscanus* embryos, we used RNAi to deplete functions of these candidate genes in the embryos. At the time of writing (first week of July 2019), we analysed the function of 78 genes that correspond to 57% of our candidates. Ten genes caused disruption of the early development, before pigmentation starts. Another group of 37 genes did not show any obvious phenocopy after RNAi. Finally, phenocopies were observed for 31 genes (Fig.3). Unfortunately, this preliminary analysis did not yet allowed us to identify any gene related with the production of pigments in *L. franciscanus* embryos (Supplementary Table 1). As expected, most of the genes with clear phenotypes affected the normal development of antennae and legs, with segmentation and/or patterning effects (Figure 3 and Supplementary Table 1).

### **Discussion and perspectives**

To identify the transcriptional regulators involved in the recruitment of the pteridin network in Gerromorpha embryos, we generated the transcriptomic data from three red organs of *Limnogonus franciscanus* embryos, when no sign yet of colour (early embryos) and in katatrepsis stages. The expression of 167 transcripts was shared between the three dissected tissues corresponding to 135 annotated genes in *D. melanogaster*. In addition, from the 167 transcripts, the 98% of the transcripts were expressed in the two different developmental stages. If our hypothesis of *trans* mechanism regulation were true, then we would expect to identify a gene which inactivation should remove the colour in eyes and appendages. The functional tests of 78 genes yield negative results with regard to the production of pigments or regulation of the pattern of colour. However, most of the phenotypes observed are related with the development of appendages or eyes (Supplementary Table 1). These results suggest that we are targeting the expected organs. Also, most of the targeted genes are involved in development, morphogenesis or segmentation, which correlates well with phenotypes previously described in *D. melanogaster*. This confirms the conservation of regulatory genes between distant lineages (BONINI *et al.* 1993; TAVSANLI *et al.* 2004).

If the regulation of colour patterns is due to *trans* regulatory mechanism, we can still hope that the TFs involved in this process are among the 45 genes that are to be tested. Furthermore, for ten genes, the inactivation resulted in the early death of the embryos, well before the appearance of pigmentation. It is thus possible that some of those genes are pleiotropic, with essential functions during early development and a later role in the production of pteridines pigments at later stages. One way to test this idea could be to repeat the inactivation of the genes using lower concentration of double-stranded RNAs in order to obtain less severe effects at early stages, therefore allowing observation of later stages with putative colour defects. Conversely, a significant number of genes showed no phenocopy after RNAi, even though they are expressed in the three organs. We could test those genes using higher concentration of double-stranded RNAs in the hope to obtain effects on colouration. Another possible explanation of our preliminary negative results could be that the TFs involved in the production of pteridine pigments are expressed before the stage where the tissues were sampled. However, this is unlikely because the colouration in eyes and appendages is a continuous process until the embryos reach nymphal stages (Vargas-Lowman et. al. under review). Therefore, the genes involved should continue to be expressed.

In the recent years, differential expression approaches have been useful to identify TFs involved in the regulation of morphological, physiological or developmental processes (ARMISEN *et al.* 2015; SANTOS *et al.* 2017; XUE *et al.* 2017). It is now possible to have access to Gerromorpha species that lost the extra-ocular colours during evolution (*Oiovelia* or *Paravelia*). A differential expression analysis could be achieved between the same organs of pigmented species (*L. franciscanus*) and non-pigmented species (*Oiovelia*, *Paravelia*), with the aim to identify differentially expressed genes whose pattern correlate with the state of pigment.

In conclusion, this study aimed to identify a gene or genes involved in the regulation of effector genes of the pteridine pathway in Gerromorpha embryos. The main idea of this project was to perform a functional analysis of transcription factors expressed in common between three different red pigmented organs. Any of the genes tested until the moment where related with changes in the coloration or the eyes or any of the other red organs. In the future, another approach should be tested as for example, the study of the expression of the genes and analysis the function of the remainder of candidate.

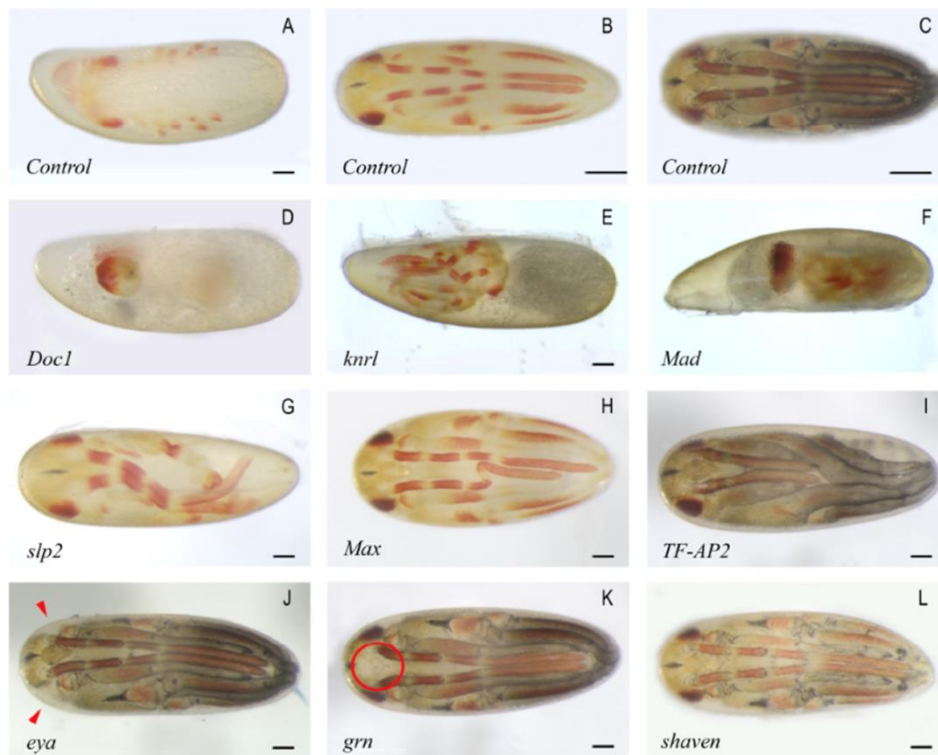


Figure 3. Samples of the different phenotypes identified during the screening process. Most of the phenotypes correspond to morphological and segmentation effects. A-C are control: A) *Limnogonus franciscanus* embryos at 48 hours after laying, B) *L. franciscanus* embryos at 74 hours after laying and C) *L. franciscanus* embryos at 130 hours after laying. D-F) genes that showed segmentation effects. G-I) genes showing effects in appendages. J. RNAi *eya absent* (*eya*) phenotype, red arrowhead point out the total absent of the ocular globe. K) RNAi *grain* (*grn*), absent of the feeding organ and L) RNAi *shaven* loss of most of bristles.

## Supplementary information

Query	Name	Symbol	FlyBase_ID	<i>L. franciscanus</i> _phenocopy		
c17538_g1_i1	<i>knirps-like</i>	<i>knrl</i>	FBgn0001323	Severe segmentation effect. Abdominal segments are reduced and fused.	5	21
c20475_g1_i1	<i>Cyclin H</i>	<i>CycH</i>	FBgn0022936	Severe segmentation effect. Head normal, <b>antennae</b> and <b>legs</b> shorter or not well developed.	7	15
c22037_g2_i1	<i>cubitus interruptus</i>	<i>ci</i>	FBgn0004859	Severe: embryos with malformations, "Not body". Moderate: Some of them could follow the development but couldn't hatch.	2	17
c224_g1_i1	<i>mothers against decapentaplegic homolog 3</i>	<i>Mad</i>	FBgn0011648	Severe segmentation effect, the embryonic development is stopped before katatrepsis.	2	6
c28948_g1_i3	<i>Transcription factor AP-2</i>	<i>TfAP-2</i>	FBgn0261953	Severe segmentation defects and reduced appendage growth.	24	24
c29962_g1_i2	<i>transcription factor kayak isoform X1</i>	<i>kay</i>	FBgn0001297	Malformation, several defects in development	57	72
c30834_g1_i2	<i>zinc finger protein</i>	<i>ush</i>	FBgn0003963	Abdominal body segments and <b>legs</b> lack. Distinct boundaries and have begun to fuse. However, antennae and head appear to be morphologically normal.	83	147
c33220_g1_i1	<i>marionette</i>	<i>mrn</i>	FBgn0261109	Segmentation effects and <b>legs antennae</b> developmental defects.	5	15
c42479_g1_i1	<i>forkhead box protein O isoform X1</i>	<i>bun</i>	FBgn0259176	<b>Head, antennae and legs</b> like a mass, without segmentation.	7	51
c8605_g1_i1	<i>transcription factor Sox-3-like</i>	<i>SoxN</i>	FBgn0029123	Embryos malformed	5	35
c24229_g1_i1	<i>Dorsocross1</i>	<i>Doc1</i>	FBgn0028789	Severe effect in segmentation. Early arrest of embryogenesis	25	37
c24807_g1_i1	<i>groucho</i>	<i>gro</i>	FBgn0001139	Early arrest of embryogenesis.	112	141
c32503_g1_i1	<i>crooked legs</i>	<i>crol</i>	FBgn0020309	Defect in the development. Embryos dye during katatrepsis.	2	33
c32610_g1_i1	<i>DNA-binding protein Ewg</i>	<i>Zasp52</i>	FBgn0265991	Embryos cannot follow development after katatrepsis, effect in <b>appendages</b> .	12	32
c29659_g1_i3	<i>transcriptional enhancer factor TEF-1 isoform X1_scalloped</i>	<i>sd</i>	FBgn0003345	Severe segmentation problems. Embryogenesis stops before katatrepsis.	52	68
c35263_g8_i1	<i>homeobox protein extradenticle isoform X1</i>	<i>exd</i>	FBgn0000611	Early arrest of embryogenesis. Only head is complete, without <b>legs</b> or <b>antennae</b> .	58	61

c12474_g1_i1	<i>cropped</i>	<i>crp</i>	FBgn0001994	The proximal segment of the <b>antennae</b> appeared normal, but distal segments showed a truncation giving an effect of being broken.	27	44
c30266_g1_i1	<i>ajuba LIM protein</i>	<i>jub</i>	FBgn0030530	<b>Antennae and legs</b> developmental defects	3	13
c31792_g2_i1	<i>pointed</i>	<i>pnt</i>	FBgn0003118	Deformation in head and <b>legs</b> . Legs with "hook" shape. Can't hatch.	21	31
c32834_g1_i1	<i>(3)73Ah - 53 F</i>	<i>(3)73 Ah</i>	FBgn0002283	<b>Legs</b> shorter than normal or <b>legs</b> in "hook" shape. The phenotype starts to be evident until the total melanisation before hatching. Legs thinner with defects.	2	10
c34990_g6_i2	<i>ecdysone-induced protein 74EF isoform X1</i>	<i>Eip74 EF</i>	FBgn0000567	Normal development, defect in <b>legs</b> . Can't hatch.	117	153
c35763_g1_i4	<i>protein max isoform X1</i>	<i>Max</i>	FBgn0017578	<b>Antennae and legs</b> truncated, some of them can hatch.	34	76
c6517_g1_i1	<i>Fork head domain transcription factor slp2</i>	<i>slp2</i>	FBgn0004567	Severe defect in <b>antennae and legs</b> . Antennae and legs thinner, abdomen fused.	69	72
c13474_g1_i1	<i>grain</i>	<i>grn</i>	FBgn0001138	Third pair of <b>legs</b> longer than normal, "mouth" part absent. Can't hatch	79	79
c20788_g1_i1	<i>Shaven</i>	<i>shaven</i>	FBgn0005561	Effect in bristles, Normal development, died after hatching.	25	29
c31948_g1_i3	<i>Eyes absent</i>	<i>eya</i>	FBgn0000320	Moderate: The <b>eyes</b> are absent. Severe: the head is absent with segmentation problem or cannot develop.	33	33
c31671_g1_i2	<i>midline</i>	<i>mid</i>	FBgn0261963	duplication of feeding part and <b>legs</b> truncated	15	18
c6433_g1_i1	<i>pituitary homeobox 2</i>	<i>pitx2</i>	FBgn0020912	normal development but cannot hatch	10	31
c35161_g1_i1	<i>anterior open</i>	<i>aop</i>	FBgn0000097	segmentation problem	5	6
c29766_g1_i3	<i>dachshund</i>	<i>dac</i>	FBgn0005677	<b>legs</b> short/truncated	9	11
c52959_g1_i1	<i>medea</i>	<i>Med</i>	FBgn0011655	Development stops before katrepsis. Segmentation problem	12	12

**Supplementary Table 1.** Transcription factor genes tested with evident phenotypes.

Oligo name	Sequence
slbo-G1_F_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTT-CTT-AGT-AAA-GAG-CTC-TCC-TT
slbo-G1_R_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAG-AAT-AAC-AGC-CTA-TCC-TTA-TAA-C
slbo-G4_F_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATA-TCA-GAA-GAT-ACA-CTG-AAC-TCA-C
slbo-G4_R_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAG-GCT-GAA-TTT-CTG-AAC-TCT-T
slbo-G4_F_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-GAA-AGA-AAA-TGA-CCG-TCT-T
slbo-G4_R_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-AGT-GAG-AAA-GTA-CTC-GAG-ATA-A
E2F7_F_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAC-TCT-TAC-TAA-TGG-CAC-TAT-TGA-G
E2F7_R_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTT-CAT-TTT-CAC-GCT-CTT-CTG-ATA-T
E2F7_F_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GGC-TGC-GTA-TCC-AAT-TTT-AAA-TAT-C
E2F7_R_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-ATA-GTG-AAC-TCT-GAG-TTT-CAA-G
Eip74EF_F_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATT-ACT-TCA-AGA-CAG-AGA-ATA-CTG-C
Eip74EF_R_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-CTC-AAC-TAT-ATC-TTT-GGG-TAC
Hfs_F_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GCT-GAA-AAT-CAT-CTG-GAT-AAA-GTA-C
Hfs_R_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTG-TTG-GTA-ATT-CGT-ATT-TCA-AGT-C
Hfs_F_P2	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TCT-ATA-TTG-TCC-AAC-ATC-AGA-TGG
Hfs_R_P2	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAA-GTG-TAC-TCA-GTC-AAT-CAG-TAT-A
Mad_F_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GCC-TAC-TAT-GAA-TTA-AAC-TGT-AGA-G
Mad_R_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAG-ATT-GAA-CAA-ATA-TAG-CAG-AGT-C
so_F_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TCA-TAT-CTC-TCC-ACT-ATT-TCT-CTT-C
so_R_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATT-TCA-TGA-CAA-TTA-CAG-TCG-G
slp2_F_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-GGT-TAA-AAT-GGA-AGG-AGG-TAC
slp2_R_P1_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTC-ATG-ATG-TAT-TCA-TAG-ATT-CCG
slp2_F_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAC-TAA-CTT-CCC-ATA-TTA-CAG-AGA-T
slp2_R_P2_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GGA-TAA-AGA-GGA-TAT-CCA-AGT-GG
Sox3_F_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AGA-ACA-TCC-CGA-TTA-TAA-GTA-TCG
Sox3_R_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTA-AGA-ACC-GTT-CAT-GTA-AGA-C



<b>pannier_F_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-TTA-TGA-TTA-TCC-AGC-ACC-TG
<b>pannier_R_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TGC-TTT-CTC-CTG-TTT-AGG-TC
<b>pannier_F_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TTT-GTT-GTT-CCA-ATT-GTG-GTA-C
<b>pannier_R_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAA-ATG-GCA-AAT-TCA-GTG-ATG-G
<b>FoxK_F_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AAT-GTC-TGA-AGC-TAA-GCT-CAT-A
<b>FoxK_R_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCT-ACC-ATA-TAC-TTC-TCT-GAA-ACA-T
<b>FoxK_F_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AGA-CAC-TAT-CTA-TAC-TAG-TCC-CTT-T
<b>FoxK_R_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-GAA-TAT-GGC-GGC-TTA-GAA-TC
<b>E-spl-m<sup>Δ</sup>?-HLH_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATA-TTG-AAC-ATC-ATC-AAC-CCA-TCT-C
<b>E-spl-m<sup>Δ</sup>?-HLH_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCA-AGT-ATT-TTG-AAA-CTT-CGT-TGG
<b>lozenge_F_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTG-AAT-CTG-TAC-CAT-ACC-AAA-CTT
<b>lozenge_R_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAT-TGA-TAG-TTA-CAT-TGT-TAG-GAC-C
<b>lozenge_F_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-TTA-CTT-TGA-CGA-TAA-CAG-TAG-C
<b>lozenge_R_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TGT-CTG-TAA-CTT-GAG-GTT-TGT-A
<b>scalop_F_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-CAC-ATA-TAC-AAG-TTC-TTG-CAA-G
<b>scalop_R_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTG-ACT-TAC-TTC-CAG-AGT-AAG-TTC
<b>scalop_F_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTG-TAG-ATG-TTC-GTC-AGA-TTT-ATG
<b>scalop_R_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AGC-GAT-GGA-TTC-TGT-ATA-CAA-ATC
<b>ush_F_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-AAG-AAA-GAA-ACA-TCA-CCG-ATG
<b>ush_R_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-TCT-CTT-ATG-AGC-TAT-AAA-GGT-G
<b>Xfin_F_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-ACA-TGG-ATT-TGT-TCA-AGG-TAC
<b>Xfin_R_P1_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTT-TGT-TCT-GGT-GTA-GGA-TTA-TAA-G
<b>Xfin_F_P2_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTG-ACT-ATA-AAA-CTG-ATC-AGA-AGA-C
<b>Doc1_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TTC-TTT-TCC-ACC-GAG-TTA-AAC-TTA-C
<b>Doc1_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TCT-CTG-AAT-CCT-TTT-GCA-AAT-G
<b>E2f1_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCT-AGA-GGC-ATT-GTG-GAT-TTA-AAT-T
<b>E2f1_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAT-AAT-CAT-TCT-GGG-CTT-TTC-TAA-G

<b>eagler_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CGT-AGG-AGT-AAT-TGG-TTT-AAA-ATC-C
<b>eagler_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ACA-CAT-CCT-AGG-ATC-TAG-AGA-TAA-C
<b>jub_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTC-TAA-GCT-CAG-TTA-GTC-TAA-GC
<b>jub_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAT-TAT-CTA-TGT-AGC-TTG-TTC-TAG-G
<b>vri_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ACA-AGA-AAG-ACG-AGA-ACT-ACT-G
<b>vri_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAC-TGT-ATA-ATA-CTG-TTG-AAC-TGA-G
<b>knrl_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AAA-TCC-ACT-GTC-TAC-TTC-AAG-AG
<b>knrl_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATT-GAG-GTA-TAT-GAG-ATT-GTG-GTG
<b>lola_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AAG-CTC-TCT-GTG-AAT-ACA-TGT-AC
<b>Lola_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-ACA-TAA-GAT-ACA-TTG-TCA-GAG-G
<b>mrn_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GCC-TAA-ACT-TCC-TAT-TTC-AAC-TTA-G
<b>mrn_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TTT-CTG-TAA-ACA-GTC-CTA-GAA-GTG
<b>luna_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TCA-AAC-CTG-TCT-CAA-GTC-TC
<b>luna_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAG-ATG-TGA-TGA-CTT-AGT-GTA-GAC
<b>pnt_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TTC-AAT-GGA-TAT-GCC-TAC-TCA-A
<b>pnt_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAT-AAA-ATG-TCA-CCA-GTG-TAA-GG
<b>(3)73Ah_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-GTA-AAG-GTT-ATC-TCA-TCG-ACG
<b>(3)73Ah_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-CCT-CTT-CCA-TTT-CGT-TGT-TTT-G
<b>Rbf_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAG-ACT-TCT-AAT-GAC-TCC-CTT-AAA
<b>Rbf_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CGA-CAA-ATA-ATA-GAA-CTT-CCT-GAA-G
<b>Nrd1_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTT-TAC-CCA-TCT-GAG-CAT-TAT-ATC
<b>Nrd1_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAC-TGG-TAA-TAT-GGT-GAA-GTT-ATC
<b>TfAP-2_F</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-TTT-GGT-TAT-GTA-TGT-GAG-ACT-G
<b>TfAP-2_R</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAC-ATT-CTG-GAT-AGC-TGT-GA
<b>Bigmax_F</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAA-GTT-GAA-GGA-AGA-AGA-GGA-AA
<b>Bigmax_R_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-CAA-CTA-TAC-TCT-GAA-ACA-CTT-G
<b>bip2_F_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-CCA-ATA-GAC-TTG-TAT-GAA-TCA-C

<b>bip2_R_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTT-TTC-TTC-TTT-GTC-TTT-GAC-CTT-C
<b>ewg_F_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TGA-TGA-AAT-ATT-CTA-CTG-GTC-GAG
<b>ewg_R</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTG-AAG-AGA-TCT-GGT-ATA-TAG-CTC
<b>grn_F</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-AGT-TCC-TCA-CAG-AGT-AGT-TAT-C
<b>grn_R</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-TTG-TCC-GTT-CAT-CTT-GTA-GTA-G
<b>gro_F</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTA-GGA-TTA-AAG-CAG-AAC-TAA-CTT-C
<b>gro_R</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTA-GAG-AGA-ATA-TCT-GAG-AGG-AGA-A
<b>lwr_F</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GCC-TCT-ACA-AGC-TTA-GAA-TGA-T
<b>lwr_R</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTC-GTT-GAG-GAG-GTC-TTG-AAT-A
<b>mat1_F</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-AAG-AAG-TAA-TTT-CAG-AGC-TCA-G
<b>mat1_R</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-TCT-ATA-AGT-TCG-TCC-AGT-TC
<b>nub_F</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAA-TCA-GGC-TCT-CTT-GAA-TCC-TAA
<b>nub_R_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTC-GAT-ATT-GTA-GTT-TGA-GAG-AAG
<b>Glass_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AAA-CCT-TTG-CAG-AAT-ATG-TGG-TAA-G
<b>Glass_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AGT-GTT-GAG-GAA-TCA-GAG-AAA-G
<b>Shaven_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTA-CGA-TAC-TAT-CAATAT-CTC-CTC-T
<b>Shaven_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCT-GTA-CTA-TCC-TGT-ATA-CTA-GGA
<b>Scargot_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATA-ACT-CTG-AGC-CTG-AGA-ATT-T
<b>Scargot_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-AAG-GAG-AGA-AAG-ATT-TAG-GC
<b>eya_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAC-ATC-TAA-TCA-CAT-TTC-AGA-CCT-T
<b>eya_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTA-CCA-TTG-TTA-CTA-CTT-GAA-GAA-G
<b>CG11247_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-GAT-AAC-AAA-TGT-AAC-GAT-GGA-C
<b>CG11247_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTT-GTT-AAG-AGT-TTC-AAA-TCC-ACA-G
<b>CG12769_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TTG-AAT-GTG-ACA-TAT-GTC-TGG-T
<b>CG12769_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAG-AGG-GTG-ATG-TAA-AAG-AAC-AAA-G
<b>MTF-1_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCT-GAA-GAA-AAT-GAT-TAC-CCT-CTT
<b>MTF-1_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTA-TGT-CCT-TCC-

	ATC-AAT-AAG-TAT-G
<b>stc_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GGA-ACT-ACA-CAA-TAT-CCT-CAT-AGA-T
<b>Stc_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GCT-GTT-AGA-TCT-TCG-ATA-ATC-ATT-G
<b>sim_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAA-TGT-ACA-TAT-CAG-AAA-CAG-CGT-C
<b>Sim_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAT-CTG-TTA-TAG-GGA-ACT-GTT-TTA-C
<b>mid_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-AAA-TTC-CAT-GAG-TTA-GGA-ACA-G
<b>mid_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-GGT-AAG-TTT-GAC-TTT-TTC-GAA-G
<b>Tnks_F_T7__</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTC-AAG-TAA-CTC-AAT-ACC-TCA-TAG-A
<b>Tnks_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CGA-TAC-AGT-TAT-TTT-CTA-CGG-CAA-A
<b>c59383_g1_i1_F_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATC-TGC-TCA-TTT-CAG-TCA-GTT
<b>c59383_g1_i1_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAT-GAC-GGT-GAG-TAT-ATC-GAT
<b>pitx2_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-TTT-GCC-AGA-GAG-GAA-TTA-G
<b>pitx2_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTC-TGG-TCT-AGT-ATA-CAG-CTC-ATA-T
<b>Meics_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GGA-AAC-GAT-ACT-AAA-TCT-AAG-TGC
<b>Meics_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAT-TTC-ATC-TTG-TAG-ATC-TAT-GTC-G
<b>CG1965_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTA-TCA-CCA-CAG-AAG-ATG-ATA-TAG-T
<b>CG1965_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAC-CAC-CAT-TCT-CTT-TAT-CTC-TAG-A
<b>Ssb-c31a_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATC-TTC-TCA-TTG-CAT-ACT-AGT-CC
<b>Ssb-c31a_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TCT-GAC-TTT-AAG-GAA-ACG-TTT-G
<b>aop_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAA-TTT-CTT-TTC-TAC-ATC-AAG-ACA-G
<b>aop_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAA-TTC-ATC-GAC-AAA-TGA-TTC-TTC-T
<b>dac_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-GAG-GAT-ATT-GTA-AAA-CAC-TTG-G
<b>dac_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATT-ATC-TAC-AGC-CAA-TTT-TAG-AAG-G
<b>Crte_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-GAA-ATA-TAT-CAG-CTA-CAC-ACA-T
<b>Crte_R_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTG-CTA-TGC-CAT-TGT-TAT-AAT-TTA-C
<b>c20131_F_T7_</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AAT-ATG-ATG-TTC-TTG-TTC-ACG-TAG
<b>c20131_R_T7</b>	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-TTT-GGG-AAA-CAA-

	ATA-CTC-TTC-T
lethal_(2)_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-TAT-AAA-ACT-TCT-CTC-TGG-GGA-T
lethal(2)_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTA-TCA-CAT-TAG-GTA-TAA-GTT-GTG-G
maf-S_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AAA-ATG-CAA-GGA-AGC-TTG-AC
maf-S_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAT-TTG-GCA-TAG-AGG-CAA-TCT-ATT-T
c36900_F_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAG-TAA-TTT-TTG-ACT-GTT-CTC-TTT-C
c36900_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATA-ATT-TTA-AAC-AGA-AAA-ACC-ACC-C
Net_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAA-CCA-CTG-AGT-AAG-ATA-GAA-GA
Net_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCC-TAC-TCA-ATG-TTA-AGA-TGT-ATG
ftz-f1_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAA-CCT-AAA-GTC-TCT-CCT-ATG-ATA-C
ftz-f1_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GGA-GGA-ACT-TAA-GGC-AAA-TAT-AAT-C
Sp1_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAC-GGA-AAT-CAA-ACA-GGA-AC
Sp1_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GTT-GTT-AGA-GTT-AGA-GCT-AGA-TTT-G
Chi_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GAG-ATA-TAC-GAT-AGG-TAG-GAC-ATT-A
Chi_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTG-AGG-TAG-TTT-AAA-GTA-GAA-TTG-G
Gem_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCA-AGA-ATT-ACT-TTC-ACC-TCT-ACA-G
Gem_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCT-ACA-TAT-ATC-GAC-AAT-TCC-GTA-AG
Med_F_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-ATA-CCC-TTC-TGC-TTA-TAT-TAA-GGT-G
Med_R_T7_	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAA-ATG-TAC-CTC-TAT-CCA-ACA-AG
Mybbp_F_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-GGA-GAA-ATC-AGA-TAC-TAT-CAA-CAA-G
Mybbp_R_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CAT-GAC-TGA-ATG-TAC-TAT-AGA-TGA-C
NC2alpha_F_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-AGG-CTG-TAC-CAG-TTA-TAA-TTT-CTA-G
NC2alpha_R_T7	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CTA-AAT-CAG-TAT-CAG-AGT-TGT-AGT-C

**Supplementary Table 2.** T7-tagged primers used for synthesis of ds-RNA for each gene.

## **CHAPTER 4**

### **Collaborations on side-projects**

**The genome of the water strider *Gerris buenoi* reveals expansions of gene repertoires associated with adaptations to life on the water**

Paper published in *BMC Genomics*

Co-author

**Escalation and constraints of antagonistic armaments in water striders**

Paper published in *Frontier in Ecology and Evolution*

Co-author

## **The genome of the water strider *Gerris buenoi* reveals expansions of gene repertoires associated with adaptations to life on the water**

### **Synopsis of the paper:**

Gerromorpha (semi-aquatic bugs) is a monophyletic group of insects that live in the water-air interface of streams, lakes, ponds, marshes, and the open ocean. Originally, Gerromorpha were adapted to inhabit humid and terrestrial environments. This shift in habitat exposed the semi-aquatic bugs to new selective pressures compared to their terrestrial ancestors. For example, major challenges of this life style include how to remain afloat, how to generate efficient thrust on the fluid substrate for locomotion, and how to obtain food in this ecosystem. In recent years, Gerromorpha have become an excellent model system to understand the developmental genetic and evolutionary processes underlying the adaptation and diversification to new ecological opportunities, but studies of these mechanisms at the genomic level are hindered by the lack of a representative genome. In this work, we report the genome of the water strider *Gerris buenoi*, the first sequenced member of the infraorder Gerromorpha. *G. buenoi* is part of the family Gerridae, and has been previously used as a model to study sexual selection and developmental genetics. Particularly, we were focused on the manual annotation and analyses of processes involved in phenotypic adaptations to life on water.

### **Summary of results:**

The size of the *G. buenoi* genome is approximately 1,000 Mb, and this sequencing effort has recovered 20,949 predicted protein-coding genes. Manual annotation uncovered a number of local (tandem and proximal) gene duplications and expansions of gene families known for their importance in a variety of processes associated with morphological and physiological adaptations to a water surface lifestyle. These expansions may affect key processes associated with growth, vision, desiccation resistance, detoxification, olfaction and epigenetic regulation. Strikingly, the *G. buenoi* genome contains three insulin receptors, suggesting key changes in the rewiring and function of the insulin pathway. Other genomic changes affecting opsin genes may be associated with wavelength sensitivity shifts in opsins, which is likely to be key in facilitating specific adaptations in vision for diverse water habitats.

### **Conclusions:**



In conclusion, local gene duplications might have played an important role during the evolution of water striders. Moreover, the sequencing of the *G. buenoi* genome now provides us the opportunity to pursue exciting research opportunities to further understand the genomic underpinnings of traits associated with the adaptive body plan and life history of water striders.

Personal contribution:

Aidamalia Vargas-Lowman: manual curation of genome annotation: fix gene feature coordinates, isoform annotation and functional annotation by inference.

RESEARCH ARTICLE

Open Access



# The genome of the water strider *Gerris buenoi* reveals expansions of gene repertoires associated with adaptations to life on the water

David Armisen<sup>1\*</sup>, Rajendhran Rajakumar<sup>2</sup>, Markus Friedrich<sup>3</sup>, Joshua B. Benoit<sup>4</sup>, Hugh M. Robertson<sup>5</sup>, Kristen A. Panfilio<sup>6,7</sup>, Seung-Joon Ahn<sup>8,9</sup>, Monica F. Poelchau<sup>10</sup>, Hsu Chao<sup>11</sup>, Huyen Dinh<sup>11</sup>, Harsha Vardhan Doddapaneni<sup>11</sup>, Shannon Dugan<sup>11</sup>, Richard A. Gibbs<sup>11</sup>, Daniel S. T. Hughes<sup>11</sup>, Yi Han<sup>11</sup>, Sandra L. Lee<sup>11</sup>, Shwetha C. Murali<sup>12</sup>, Donna M. Muzny<sup>11</sup>, Jiaxin Qu<sup>11</sup>, Kim C. Worley<sup>11</sup>, Monica Munoz-Torres<sup>13</sup>, Ehab Abouheif<sup>14</sup>, François Bonneton<sup>1</sup>, Travis Chen<sup>14</sup>, Li-Mei Chiang<sup>10</sup>, Christopher P. Childers<sup>10</sup>, Andrew G. Cridge<sup>15</sup>, Antonin J. J. Crumière<sup>1</sup>, Amelie Decaras<sup>1</sup>, Elise M. Didion<sup>4</sup>, Elizabeth J. Duncan<sup>15,16</sup>, Elena N. Elpidina<sup>17</sup>, Marie-Julie Favé<sup>14</sup>, Cédric Finet<sup>1</sup>, Chris G. C. Jacobs<sup>18,19</sup>, Alys M. Cheatle Jarvela<sup>20</sup>, Emily C. Jennings<sup>4</sup>, Jeffery W. Jones<sup>3</sup>, Maryna P. Lesoway<sup>14,21,22</sup>, Mackenzie R. Lovegrove<sup>15</sup>, Alexander Martynov<sup>22</sup>, Brenda Oppert<sup>23</sup>, Angelica Lillo-Ouachour<sup>14</sup>, Arjuna Rajakumar<sup>14</sup>, Peter Nagui Refki<sup>1,24</sup>, Andrew J. Rosendale<sup>4</sup>, Maria Emilia Santos<sup>1</sup>, William Toubiana<sup>1</sup>, Maurijn van der Zee<sup>18</sup>, Iris M. Vargas Jentsch<sup>6</sup>, Aidamalia Vargas Lowman<sup>1</sup>, Severine Viala<sup>1</sup>, Stephen Richards<sup>11\*</sup> and Abderrahman Khila<sup>1\*</sup>

## Abstract

**Background:** Having conquered water surfaces worldwide, the semi-aquatic bugs occupy ponds, streams, lakes, mangroves, and even open oceans. The diversity of this group has inspired a range of scientific studies from ecology and evolution to developmental genetics and hydrodynamics of fluid locomotion. However, the lack of a representative water strider genome hinders our ability to more thoroughly investigate the molecular mechanisms underlying the processes of adaptation and diversification within this group.

(Continued on next page)

\* Correspondence: davidarmisen@gmail.com; stephen@bcm.edu; abderrahman.khila@ens-lyon.fr

<sup>1</sup>Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Claude Bernard Lyon 1, CNRS UMR 5242, Ecole Normale Supérieure de Lyon 46, allée d'Italie, 69364 Lyon Cedex 07, France

<sup>11</sup>Human Genome Sequencing Center, Department of Human and Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA

Full list of author information is available at the end of the article



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**Results:** Here we report the sequencing and manual annotation of the *Gerris buenoi* (*G. buenoi*) genome; the first water strider genome to be sequenced thus far. The size of the *G. buenoi* genome is approximately 1,000 Mb, and this sequencing effort has recovered 20,949 predicted protein-coding genes. Manual annotation uncovered a number of local (tandem and proximal) gene duplications and expansions of gene families known for their importance in a variety of processes associated with morphological and physiological adaptations to a water surface lifestyle. These expansions may affect key processes associated with growth, vision, desiccation resistance, detoxification, olfaction and epigenetic regulation. Strikingly, the *G. buenoi* genome contains three insulin receptors, suggesting key changes in the rewiring and function of the insulin pathway. Other genomic changes affecting with opsin genes may be associated with wavelength sensitivity shifts in opsins, which is likely to be key in facilitating specific adaptations in vision for diverse water habitats.

**Conclusions:** Our findings suggest that local gene duplications might have played an important role during the evolution of water striders. Along with these findings, the sequencing of the *G. buenoi* genome now provides us the opportunity to pursue exciting research opportunities to further understand the genomic underpinnings of traits associated with the extreme body plan and life history of water striders.

**Keywords:** Water striders, Genome sequence, Water surface locomotion, Evolution, Adaptation,

## Background

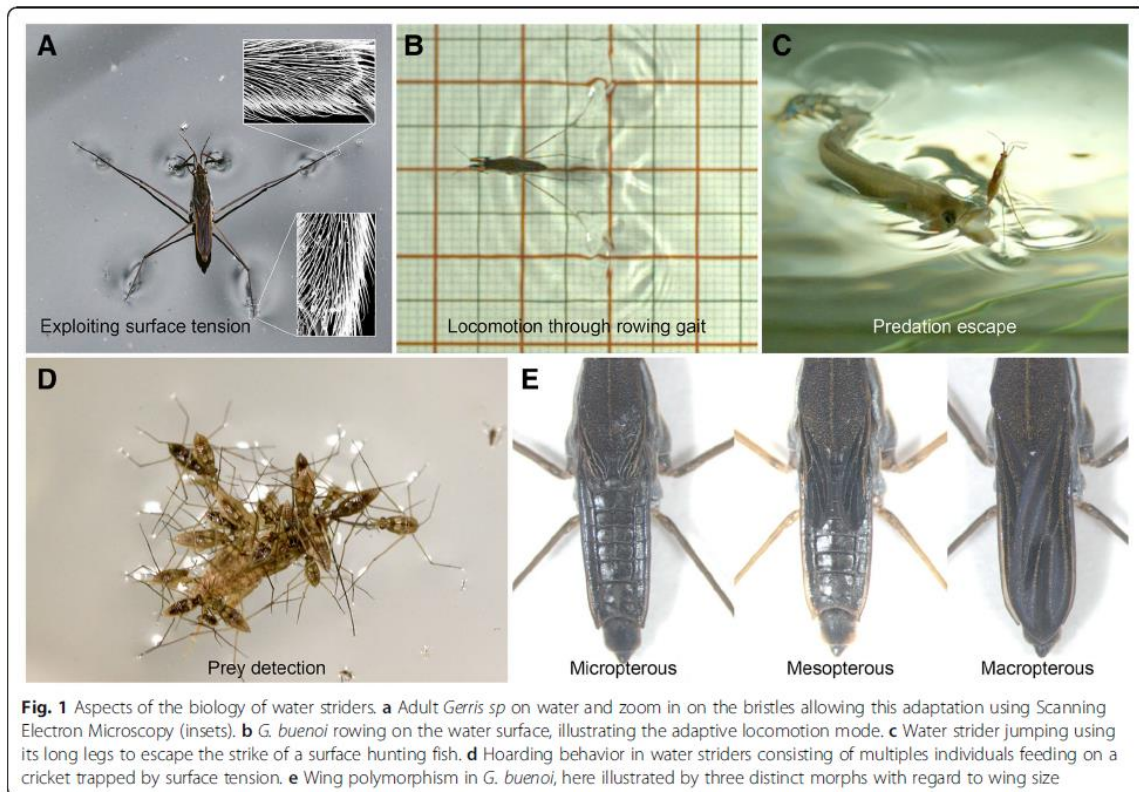
The semi-aquatic bugs (Gerromorpha) are a monophyletic group of predatory heteropteran insects characterized by their ability to live at the water-air interface [1–4]. Over 200 million years ago, the ancestor of the Gerromorpha transitioned from terrestrial habitats to the water surface, leading to a radiation that has generated over 2,000 species classified into eight families [1]. Phylogenetic reconstructions suggest that the ancestral habitat of the Gerromorpha was either humid and terrestrial or marginally aquatic [1, 5, 6]. Water striders subsequently became true water surface dwellers and colonized a diverse array of niches, including streams, lakes, ponds, marshes, and the open ocean [1, 7, 8]. The invasion of these new habitats provided access to resources previously underutilized by insects and made the Gerromorpha the dominant group of insects at water surfaces [1]. This novel specialized life style makes the Gerromorpha an exquisite model system to study how new ecological opportunities can drive adaptation and species diversification [2, 9–11].

This shift in habitat exposed the Gerromorpha to new selective pressures compared to their terrestrial ancestors. The Gerromorpha face two primary challenges unique among insects: how to remain afloat and how to generate efficient thrust on the fluid substrate for locomotion [2, 3, 12]. Due to their specific arrangement and density, the bristles covering the legs of water striders are adapted to keep them afloat by acting as non-wetting structures, which exploit water surface tension by trapping air between the leg and water (Fig. 1a) [2, 3, 12, 13]. Furthermore, locomotion is made possible through evolutionary changes in the morphology and biomechanical adaptations associated with patterns of leg movement (Fig. 1b) [2, 3, 12, 13]. Two distinct modes of locomotion are employed by distinct species: an ancestral mode using a tripod gait with alternating leg

movements, and a derived mode using a rowing gait through a simultaneous sculling motion of the pair of middle legs (Fig. 1b) [2, 12]. The rowing mode is characteristic of the Gerridae and some Veliidae and is associated with a derived body plan where the middle legs are the longest (Fig. 1a–b) [2, 12]. The evolutionary trajectory of this group is also thought to have been shaped by the novel predator-prey interactions (Fig. 1c and d) associated with their water surface life history. Following the invasion of water surfaces, other adaptations have emerged, including: (1) the adaptation of their visual system to the surface-underwater environment; (2) the evolution of wing polymorphisms associated with dispersal strategies and habitat quality (Fig. 1e) [14], and changes in cuticle composition that optimized water exchange and homeostasis associated with living on water.

While we are starting to uncover the developmental genetic and evolutionary processes underlying the adaptation of water striders to the requirements of water surface locomotion, predator-prey, and sexual interactions [2, 15–19], studies of these mechanisms at the genomic level are hampered by the lack of a representative genome. Here we report the genome of the water strider *G. buenoi*, the first sequenced member of the infraorder Gerromorpha. *G. buenoi* is part of the family Gerridae, and has been previously used as a model to study sexual selection and developmental genetics [15, 20–22]. Moreover, *G. buenoi* can easily breed in laboratory conditions and is closely related to several other *Gerris* species used as models for the study of water-walking hydrodynamics, salinity tolerance, and sexual conflict. With a particular focus on manual annotation and analyses of processes involved in phenotypic adaptations to life on water, our analysis of the *G. buenoi* genome suggests that the genomic basis of water surface invasion might be, at least in part, underpinned by clustered gene family expansions and tandem gene duplications.





**Fig. 1** Aspects of the biology of water striders. **a** Adult *Gerris* sp on water and zoom in on the bristles allowing this adaptation using Scanning Electron Microscopy (insets). **b** *G. buenoi* rowing on the water surface, illustrating the adaptive locomotion mode. **c** Water strider jumping using its long legs to escape the strike of a surface hunting fish. **d** Hoarding behavior in water striders consisting of multiples individuals feeding on a cricket trapped by surface tension. **e** Wing polymorphism in *G. buenoi*, here illustrated by three distinct morphs with regard to wing size

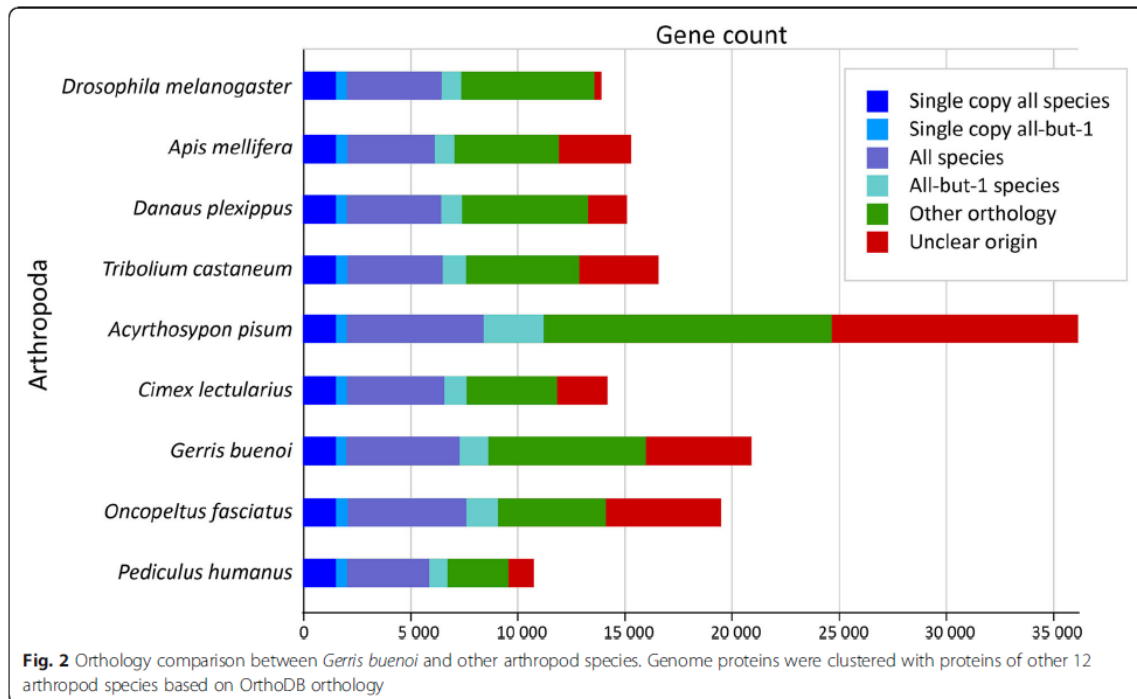
## Results and discussion

### General features of the *G. buenoi* genome

The draft assembly of *G. buenoi* genome comprises 1,000,194,699 bp (GC content: 32.46%) in 20,268 scaffolds and 304,909 contigs (N50 length is 344,118 and 3812 bp, respectively). The assembly recovers ~87% of the genome size estimated at ~1.15 GB based on k-mer analysis. The *G. buenoi* genome is organized into 18 autosomal chromosomes with a XX/X0 sex determination system [23]. The MAKER automatic annotation pipeline predicted 20,949 protein-coding genes, which is greater than the 16,398 isogroups previously annotated in the transcriptome of the closely related species *Limnoporus dissortis* (PRJNA289202) [18, 24], as well as the 14,220 genes in the bed bug *Cimex lectularius* genome [25] and the 19,616 genes in the genome of the milkweed bug *Oncopeltus fasciatus* [26]. The final *G. buenoi* official gene set (OGS) 1.0 includes 1,277 manually annotated genes, including 1,378 mRNAs and 15 pseudogenes, representing development, growth, immunity, cuticle formation as well as olfaction and detoxification pathways genes, amongst others (see Additional file 1). Using OrthoDB [27, 28], we found that ~75% of *G. buenoi* genes have at least one orthologue in other arthropod

species (Fig. 2). We then used benchmarking sets of universal single-copy orthologs (BUSCOs) [29, 30] to assess the completeness of the assembly. A total of 85.4% of BUSCOs were found complete and 12.3% were fragmented.

In addition to BUSCOs, we used Hox and Iroquois Complex (Iro-C) gene clusters as indicators of draft genome quality and as an opportunity to assess synteny among species. The Hox cluster is conserved across the Bilateria [31], and the Iro-C is found throughout the Insecta [25, 32]. In *G. buenoi*, we were able to find and annotate gene models for all ten Hox genes (Additional file 1: Table S3). While linkage of the highly conserved central class genes *Sex combs reduced*, *fushi tarazu*, and *Antennapedia* occurred in the expected order and with the expected transcriptional orientation, the linked models of *proboscipedia* and *zerknüllt (zen)* occur in opposite transcriptional orientations (head-to-head, rather than both 3' to 5'). Inversion of the divergent *zen* locus is not new in the Insecta [33], but was not observed in the hemipteran *C. lectularius*, in which the complete Hox cluster was fully assembled [25]. Future genomic data will help to determine whether such a microinversion within the Hox cluster is conserved within the



hemipteran family Gerridae. Assembly limitations are also evident in our Hox cluster analysis. For example, the complete gene model for *labial* is present but split across scaffolds, while only partial gene models could be created for *Ultrabithorax* and *Abdominal-B*. Furthermore, while there are clear single-copy orthologues of members of the small Iroquois complex, *iroquois* and *mirror*, they are not linked in the current assembly (Additional file 1: Table S3). However, both genes are located near the ends of their scaffolds, and direct concatenation of the scaffolds (5'-Scaffold451-3', 3'-Scaffold2206-5') would correctly reconstruct this cluster: (1) with both genes in the 5'-to-3' transcriptional orientation along the (+) DNA strand, (2) with no predicted intervening genes within the cluster, and (3) with a total cluster size of 308 Kb, which is fairly comparable with that of other recently sequenced hemipterans in which the Iro-C cluster linkage was recovered (391 Kb in *C. lectularius* [25] and 403 Kb in *O. fasciatus* [26]). Lastly, building on the automated BUSCO assessment for presence of expected genes, we examined genes associated with autophagy processes, which are highly conserved among insects, and all required genes are present within the genome (Additional file 2). Therefore, along with the Hox and Iroquois Complex (Iro-C) gene cluster analyses, the presence of a complete set of required autophagy genes suggest good gene representation and supports further analysis.

#### Adaptation to water surface locomotion

One of the most important morphological adaptations that enabled water striders to conquer water surfaces is the change in shape, density, and arrangement of the bristles that span the contact surface between their legs and the fluid substrate. These bristles, by trapping air, act as non-wetting structures, forming a cushion between the legs and the water surface (Fig. 1a) [2, 3, 12, 13]. QTL studies in flies uncovered dozens of candidate genes and regions linked to variation in bristle density and morphology [34]. In the *G. buenoi* genome we were able to annotate 90 out of 120 genes known to be involved in bristle development [34, 35] (Additional file 1: Table S4). Among these, we found a single duplication, the gene *Beadex* (*Bx*). A similar duplication found in *C. lectularius* and *H. halys* suggest that the *Bx* duplication may have predated the separation of these lineages and the radiation of Gerromorpha, although a broader phylogenetic sampling is needed to strengthen this conclusion. In *Drosophila*, *Bx* is involved in neural development by controlling the activation of *achaete-scute* complex genes [36] and mutants of *Bx* have extra sensory organs [36]. Based on this, it is reasonable to speculate that duplication of *Beadex* might have been exploited by water striders and subsequently linked to changes in bristle pattern and density. This possibility opens up new research avenues to further understand the adaptation of water striders to living on the water surface.



### A new duplication in the Insulin Receptor gene family in the Gerromorpha

The insulin signaling pathway coordinates hormonal and nutritional signals in animals [37–39]. This facilitates the complex regulation of several fundamental molecular and cellular processes including transcription, translation, cell stress, autophagy, and physiological states, such as aging and starvation [39–42]. The action of insulin signaling is mediated through the Insulin Receptor (InR), a transmembrane receptor of the tyrosine kinase class [43]. While vertebrates possess one copy of the InR [44], arthropods generally possess either one or two copies, although the highly duplicated *Daphnia pulex* genome [45] contains four copies [46]. Interestingly, the *G. buenoi* genome contains three distinct InR copies. Further sequence examination using in-house transcriptome databases of multiple Gerromorpha species confirmed that this additional copy is common to all of them, indicating that it was present in the common ancestor of the group (Fig. 3). In addition, cloning of the three InR sequences using PCR indicates that these sequences originate from three distinct coding genes that are actively transcribed in this group of insects. Comparative protein sequence analysis revealed that the three InR copies possess all the characteristic domains found in InR in both vertebrates and invertebrates (Fig. 3a). Together, these results validate the presence of three InR copies in Gerromorpha, an exceptional situation amongst Arthropoda.

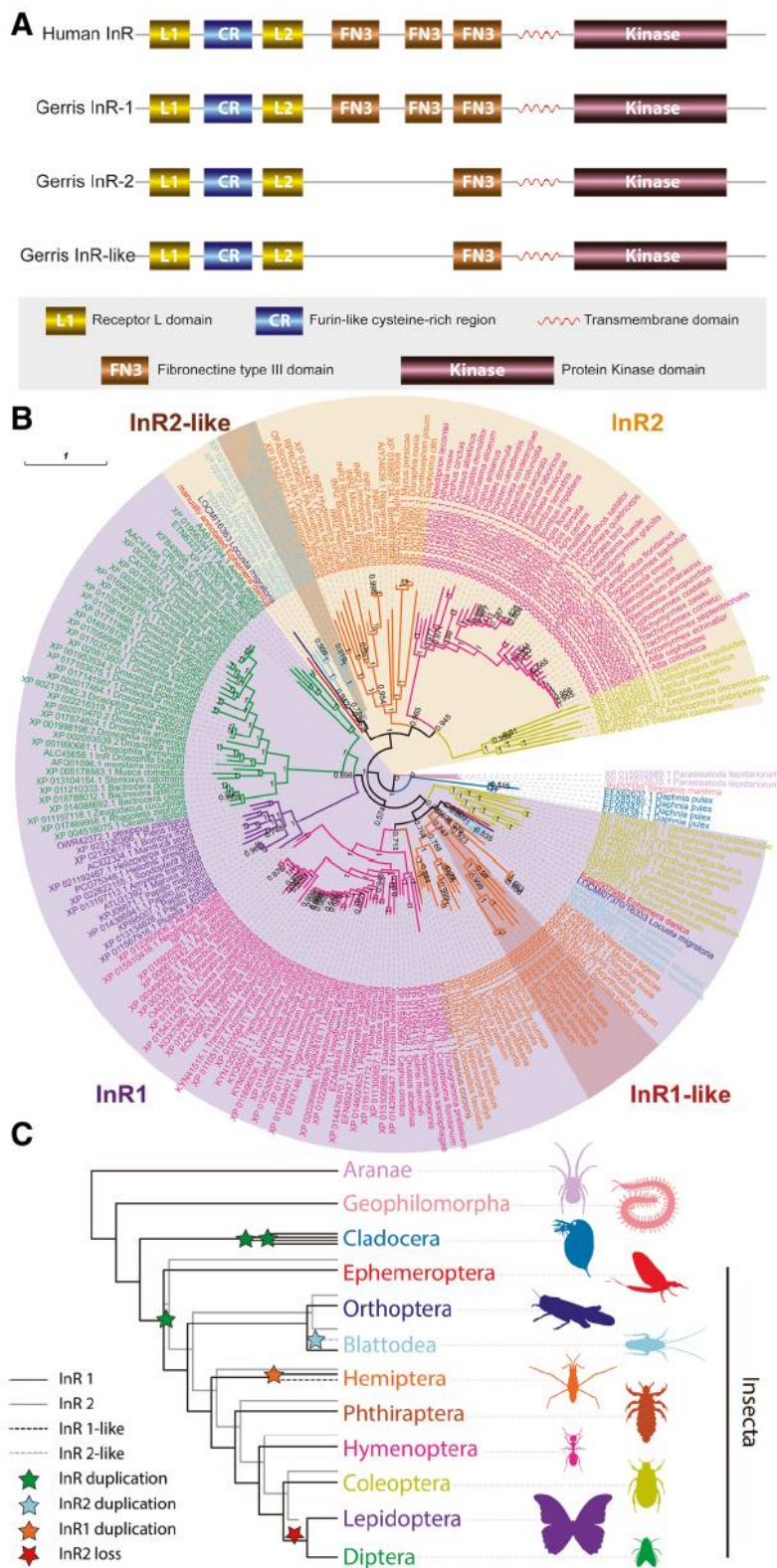
While this manuscript is under evaluation, an independent study reported the presence of a third InR gene in Blattodea [47]. To determine: (1) the origin of the three InR copies in the *G. buenoi* genome; and (2) whether the third copy in Gerromorpha and Blattodea share a common ancestor, we performed a phylogenetic reconstruction that included the sequences of eight Gerromorpha (three InR copies), four Blattodea (three InR copies), *Daphnia* (four copies) and an additional sample of 126 Arthropoda, all of which possess either one or two InR copies (see Additional files 3 and 4). The four InR duplicates of *Daphnia* were all lineage-specific and together formed a sister group to those found in insects. Within insects, this analysis clustered two InR copies into distinct InR1 and InR2 clusters (Fig. 3b). Furthermore, gerromorphan InR1 and InR2 copies clustered with bed bug and milkweed bug InR1 and InR2, respectively, while the Gerromorpha-restricted copy clustered alone (Fig. 3b; Additional file 1: Figure S1). These data suggest that the new InR copy, which we designated InR1-like, most likely originated from the InR1 gene in the common ancestor of the Gerromorpha. In contrast, the third InR copy in Blattodea clustered with InR2, suggesting an independent origin of novel InR copies in Gerromorpha, which we therefore would suggest be

designated InR2-like. A closer examination of the organization of the genomic locus of the InR1-like gene in *G. buenoi* revealed that this copy is intronless. This observation, together with the phylogenetic reconstruction, suggests that InR1-like is a retrocopy of InR1 that may have originated through RNA-based duplication [48]. In addition, our analysis suggests two independent losses of InR2. InR2 is lost among the parasitoid wasps yet retained in other wasps, and InR2 is also lost in the common ancestor of Diptera and Lepidoptera. Taken together, our current phylogenetic reconstruction demonstrates that: (1) InR was duplicated at the base of insects, generating InR1 and InR2; (2) InR1 was subsequently duplicated within the Gerromorpha, while InR2 was duplicated at the common ancestor of Blattodea; (3) InR2 was independently lost in the common ancestor of Lepidoptera and Diptera as well as among the parasitoid wasps, while other wasps have retained it.

In insects, the insulin signaling pathway has been implicated in the developmental regulation of complex nutrient-dependent growth phenotypes such as beetle horns and wing polyphenisms in plant hoppers, as well as morphological caste differentiation in social termites and bees [49–52]. In the particular case of wing polymorphism in *G. buenoi* [1, 14, 52], our analysis found no DNA methylation signature, as previously found in wing polyphenic ants and aphids [53–57], but rather an increased number of histone clusters and a unique duplication of the histone methyltransferase *grappa* (see Additional file 1: Supplementary Data). Taken together, it will be of interest to test the functional significance of the new InR copy in relation to wing polyphenism, as well as more generally how it may be potentially involved in appendage plasticity, either independent of, or alongside, epigenetic processes. Moreover, a comparative functional approach between the novel InR genes in Gerromorpha and Blattodea will shed light on the role independent insulin receptor duplications have played in functional convergence and/or diversification.

### A lineage-specific expansion and possible sensitivity shifts of long wavelength sensitive opsins

Visual ecology at the air-water interface and the exceptionally specialized visual system of water striders has drawn considerable interest [58, 59]. Consisting of over 900 ommatidia, the prominent compound eyes of water striders are involved in prey localization, mating partner pursuit, predator evasion and dispersal by flight [60–62]. Realization of the first three tasks is associated with dorsal-ventral differences in the photoreceptor organization of the eye [63, 64], and polarized light-sensitivity [65] (see Additional file 1: Supplementary Data). Each water strider ommatidium contains six outer and two inner. Recent work has produced evidence of at least two types of ommatidia,



**Fig. 3** Characterization of the three copies of the Insulin Receptor in *Gerris buenoi*. **a** Protein domain comparison between the three InRs of *G. buenoi* and the Human InR. **b** InR phylogenetic relationship amongst Insecta. Branch support numbers at branches. A non-circular version included in Additional file 1: Figure S1. **c** Simplified Arthropoda phylogeny based on [115] depicting InR duplications and loss events



with outer photoreceptors that are sensitive to either green (~ 530 nm) or blue (~ 470–490 nm) wavelengths [66], but the wavelength specificity of the two inner photoreceptor cells is still unknown. At the molecular level, the wavelength specificity of photoreceptor subtypes is mostly determined by the expression of paralogous opsins (light sensitive G-protein coupled receptor proteins), which differ in their wavelength absorption maxima. Interestingly, our genomic analysis of opsin diversity in *G. buenoi* uncovered 8 opsin homologs. Among these, we uncovered three arthropod non-retinal opsins (c-opsin, Arthropsin and Rh7 opsin) (see Additional file 1: Supplementary Data) in addition to five retinal opsins (Fig. 4a; Additional file 1: Figure S2). One of these five retinal opsins was identified as a member of the UV-sensitive opsin subfamily and the other four were identified as tandem, clustered members of the long wavelength sensitive (LWS) opsin subfamily (Fig. 4b).

Surprisingly, both genomic and transcriptomic searches in *G. buenoi* and other water strider species failed to detect sequence evidence of homologs of the otherwise deeply conserved blue-sensitive opsin subfamily (Fig. 4b; Additional file 1: Table S5) [67]. Although the apparent lack of blue opsin in *G. buenoi* was unexpected given the presence of blue sensitive photoreceptors [66], it was consistent with the lack of blue opsin sequence evidence in the available genomes and transcriptomes of other heteropteran species including *Halyomorpha halys*, *Oncopeltus fasciatus*, *Cimex lectularius*, and *Rhodnius prolixus*. Blue opsin, however, is present in other hemipteran clades, including Cicadomorpha (*Nephotettix cincticeps*) and Sternorrhyncha (*Pachypsylla venusta*) (Fig. 4b). Based on the currently available sample of hemipteran species, these data suggest that the blue-sensitive opsin subfamily was lost early in the last common ancestor of the Heteroptera (Fig. 4b and Additional file 1: Table S5). This raises the question of which compensatory events explain the presence of blue sensitive photoreceptors in water striders.

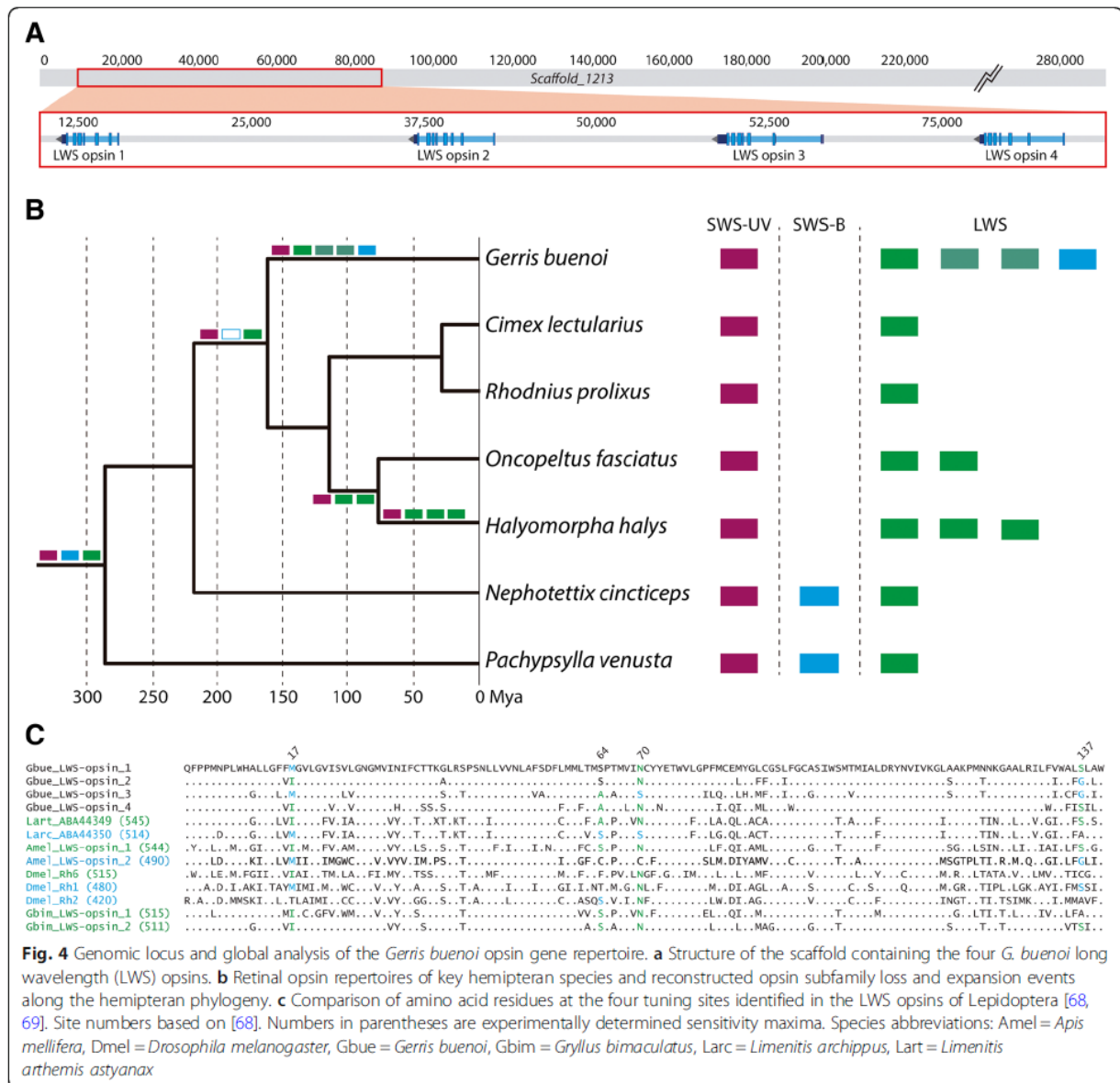
Studies in butterflies and beetles produced evidence of blue sensitivity shifts in both UV- and LWS-opsin homologs following gene duplication [68–70]. In butterflies, molecular evolutionary studies have implicated amino acid residue differences at four protein sequence sites in sensitivity shifts from green to blue: Ile17Met, Ala64Ser, Asn70Ser, and Ser137Ala [68, 69] (Fig. 4c; Additional file 1: Figure S2 and Supplementary Data). Based on sequence information from physiologically characterized LWS opsins in other insect orders and the degree of amino acid residue conservation at these sites in a sample of 114 LWS opsin homologs from 54 species representing 12 insect orders (Additional file 1: Supplementary Data and Additional file 5), we could identify *G. buenoi* LWS opsin 3 as a high confidence candidate for a blue-shifted paralog, followed by *G. buenoi* LWS opsin

1 and 2. Moreover, the *G. buenoi* LWS opsin 4 paralog matches all of the butterfly green-sensitive amino acid residue states, thus favoring this paralog as green-sensitive (Fig. 4). These conclusions are further backed by the fact that water striders lack ocelli, which implies that all four paralogs are expressed in photoreceptors of the compound eye. Overall, it is most likely that the differential expression of the highly diverged *G. buenoi* LWS opsin paralogs accounts for the presence of both blue- and green-sensitive peripheral photoreceptors in water striders. Moreover, given that the outer blue photoreceptors have been specifically implicated in the detection of contrast differences in water striders [66], it is tempting to speculate that the deployment of blue-shifted LWS opsins is a convergent characteristic of a fast-tracking visual system, similar to visual systems in dipteran species that also feature open rhabdomeres, neural superposition, and polarized light-sensitivity.

#### Expansion of cuticle gene repertoires

Desiccation resistance is essential to the colonization of terrestrial habitats by arthropods [71]. However, contrary to most insects, the Gerromorpha spend their entire life cycle in contact with water and exhibit poor desiccation resistance [1]. Cuticle proteins and aquaporins are essential for desiccation resistance through regulation of water loss and rehydration [72–75]. Unexpectedly in the *G. buenoi* genome, most members of cuticular and aquaporin protein families are present in similar numbers compared to other hemipterans (Additional file 1: Table S6 and Figure S3; Additional files 6 and 7). We identified 155 putative cuticle proteins belonging to five cuticular families: CPR (identified by Rebers and Riddiford Consensus region), CPAP1 and CPAP3 (Cuticular Proteins of Low-Complexity with Alanine residues), CPF (identified by a conserved region of about 44 amino acids), and TWDL (Tweedle) [76, 77] (Additional file 1: Table S6). Interestingly, almost half of them are arranged in clusters, indicative of local duplication events (Additional file 1: Table S7). Moreover, while most insect species, including other hemipterans, have only three TWDL genes, we found that the TWDL family in *G. buenoi* has been expanded to ten genes (Additional file 1: Figure S4). This expansion of the TWDL family is similar to that observed in some Diptera that possess *Drosophila*-specific and mosquito-specific TWDL expansions [77, 78]. Mutations in the *Drosophila TwdlD* are known to alter body shape [78]. Given the high diversification in body sizes and shapes in association with various aquatic habitats in the Gerromorpha in general [1, 2] and the Gerridae in particular [79, 80], it is possible that the expansion of the TWDL gene family is linked to this diversification. Therefore, a functional analysis of TWDL genes and comparative analysis with other hemipterans will provide





**Fig. 4** Genomic locus and global analysis of the *Gerris buenoi* opsin gene repertoire. **a** Structure of the scaffold containing the four *G. buenoi* long wavelength (LWS) opsins. **b** Retinal opsin repertoires of key hemipteran species and reconstructed opsin subfamily loss and expansion events along the hemipteran phylogeny. **c** Comparison of amino acid residues at the four tuning sites identified in the LWS opsins of Lepidoptera [68, 69]. Site numbers based on [68]. Numbers in parentheses are experimentally determined sensitivity maxima. Species abbreviations: Ame1 = *Apis mellifera*, Dmel = *Drosophila melanogaster*, Gbue = *Gerris buenoi*, Gbin = *Gryllus bimaculatus*, Larc = *Limenitis archippus*, Lart = *Limenitis arthemis astyanax*

important insights into the evolutionary origins and functional significance of TWDL expansion in *G. buenoi*.

**Prey detection in water surface environments**

Unlike many closely related species that feed on plant sap or animal blood, *G. buenoi* feeds on various arthropods trapped by surface tension (Fig. 1d), thus making their diet highly variable. Chemoreceptors play a crucial role for prey detection and selection, in addition to vibrational and visual signals. We annotated the three families of chemoreceptors that mediate most of the sensitivity and specificity of chemoperception in insects: odorant receptors (ORs; Additional file 1: Figure S5A and

Additional file 8), gustatory receptors (GRs; Additional file 1: Figure S5B and Additional file 8) and ionotropic receptors (IRs; Additional file 1: Figure S5C and Additional file 8) (e.g. [81, 82]). Interestingly, we found an increase in the number of chemosensory genes in *G. buenoi* (Additional file 1: Table S8). First, the OR family is expanded, with a total of 155 OR proteins. This expansion is the result of lineage-specific “blooms” of particular gene subfamilies, including expansions of the 4, 8, 9, 13, 13, 16, 18, and 44 subfamilies (Additional file 1: Figure S5A and Supplementary Data). Second, the GR family is also fairly large (Additional file 1: Figure S5B), but the expansions here are primarily the result of extensive alternative splicing, such that 60 genes encode

135 GR proteins (Additional file 1: Table S8). These GRs include six genes encoding proteins related to the carbon dioxide receptors of flies, three related to sugar receptors, and one related to the fructose receptor (Additional file 1: Figure S5B). The remaining GRs include several highly divergent proteins, as well as four blooms, the largest of which comprises 80 proteins (Additional file 1: Figure S5B and Supplementary Data). By analogy with *D. melanogaster*, most of these proteins are likely to be “bitter” receptors, although some might be involved in perception of cuticular hydrocarbons and other molecules. Finally, the IR family is expanded to 45 proteins. In contrast with the OR/GR families, where the only orthologs across four heteropterans (*Rhodnius prolixus*, *Cimex lectularius*, *Oncopeltus fasciatus* and *Gerris buenoi*) and *Drosophila* are the single OrCo and fructose receptors, the IR family has single orthologs in each species. This is not restricted to only the highly conserved co-receptors (IR8a, 25a, and 76b) but also includes receptors implicated in sensing amino acids, temperature, and humidity (Ir21a, 40a, 68a, and 93a). As is common in other insects the amine-sensing IR41a lineage is expanded to four genes, while the acid-sensing IR75 lineage is highly expanded to 24 genes, and like the other heteropterans there are nine more highly divergent IRs (Additional file 1: Figure S5C and Supplementary Data).

We hypothesize that the high number of ORs may be linked to prey detection mediated by odor molecules at the air-water interface, although functional analysis will be needed to test this. As *G. buenoi* are faced with prey that have fallen on the water surface, and therefore individuals exhibit more of a scavenger strategy as compared to a hunter strategy, this expansion of ORs may enhance their ability to evaluate palatability. As toxic molecules are often perceived as bitter, the GR expansion might provide a complex bitter taste system to detect and even discriminate between molecules of different toxicities [83]. Finally, expansion of the IR family could be linked with prey detection as well as pheromone detection of water-soluble hydrophilic acids and amines, many of which are common chemosensory signals for aquatic species [84, 85].

#### Detoxification pathways

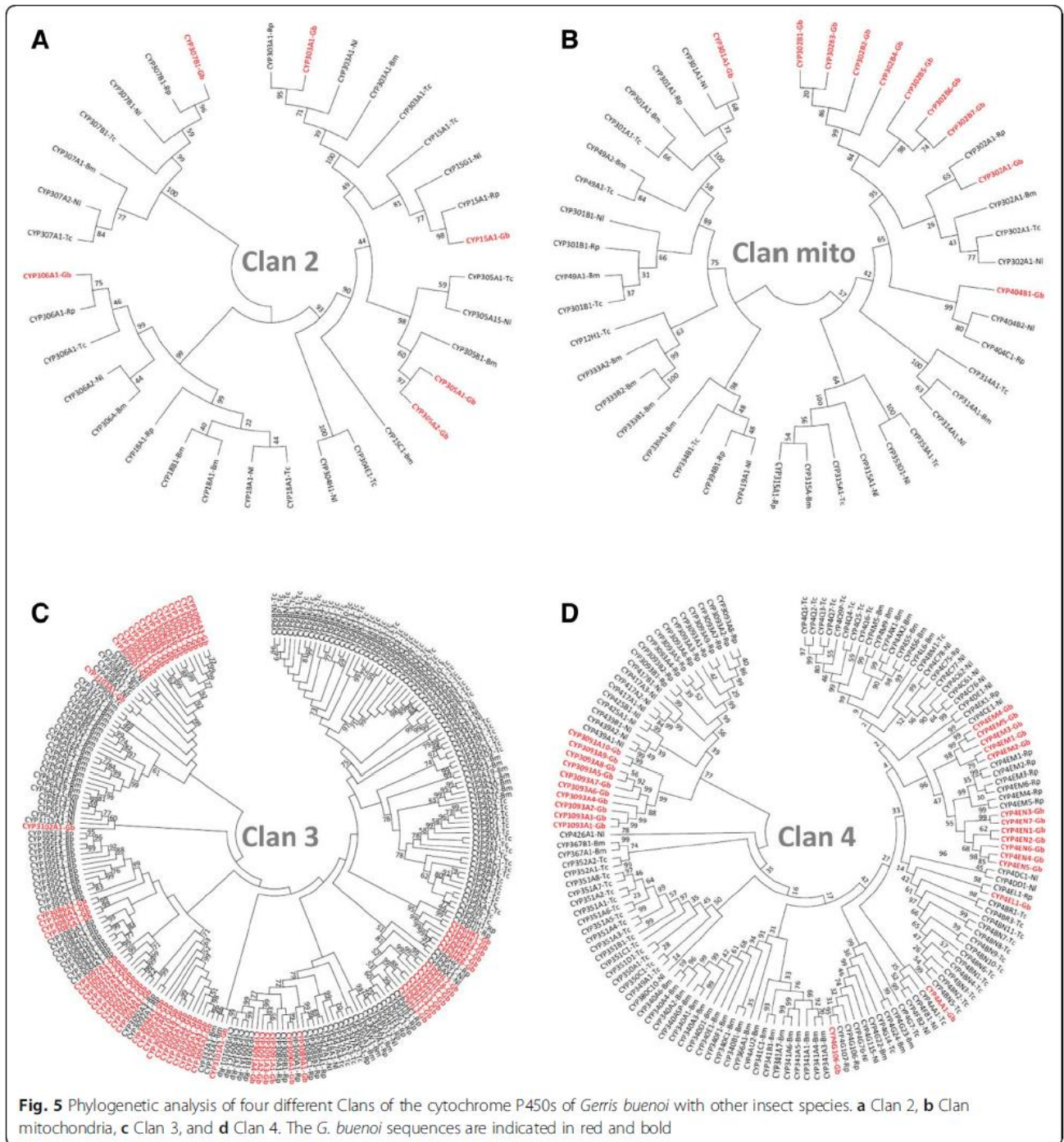
Water striders can be exposed to various toxic compounds found in the water, including those generated by pesticides, insecticides, and from other human activities as well as those found in their prey. Insect cytochrome P450 (CYP) proteins play a role in metabolic detoxification of xenobiotics including insecticides [86, 87]. They are also known to be responsible for the synthesis and degradation of endogenous molecules, such as ecdysteroids [88] and juvenile hormone [89]. The insect CYPs, one of the oldest and largest gene families in insects, underwent a high degree of diversification after multiple instances of

gene duplication, which may have enhanced a species' adaptive range [90]. In addition to CYP proteins we have also surveyed the presence of UDP-glycosyltransferase (UGT) genes in *G. buenoi*. UGTs are important for xenobiotic detoxification and the regulation of endobiotics in insects [91]. UGTs catalyse the conjugation of a range of small hydrophobic compounds to produce water-soluble glycosides that can be easily excreted in a number of insects [92, 93].

We annotated and analyzed a total of 103 CYP genes (Additional file 1: Table S9, Additional files 3 and 9) and 28 putative UGT genes, including several partial sequences due to genomic gaps (Additional file 1: Table S10). Ten more CYP fragments were found, but they were not included in this analysis due to their short lengths (<250 aa). This is the largest number of CYP genes among the hemipteran and other species' genomes in which CYPomes were annotated: *O. fasciatus* (58 CYPs), *R. prolixus* (88 CYPs) and *N. lugens* (68 CYPs) [26, 94, 95], *D. melanogaster* (85), *A. mellifera* (45), and *B. mori* (86) (Additional file 1: Table S9). Indeed, the *G. buenoi* CYP protein family size is only exceeded by that of *T. castaneum* (131 proteins). CYP genes fall into one of the four distinct subfamilies: Clan 2 (6 genes), Clan mito (62 genes), Clan 3 (25 genes) and Clan 4 (10 genes) (Fig. 5; see Additional file 1: Supplementary Data). Similarly, the number of UGT genes is also higher than that of *O. fasciatus* (1) [26], *C. lectularius* (7) [25], *D. melanogaster* (11), *A. mellifera* (6) and *B. mori* (14) [96], and identical to *T. castaneum* (28) [96].

Interestingly, both CYP and UGT gene family expansions seem to be closely linked with tandem duplication events. In the particular case of *G. buenoi* CYPs, the Clan 2 and Clan mito have undergone relatively little gene expansion (Fig. 5a and b). However, an exceptional gene expansion is observed in the mitochondrial Clan of the *G. buenoi* CYPs, where seven CYP302Bs form a lineage-specific cluster (Fig. 5b). The Clan 3 and Clan 4 are highly expanded in insects such as *T. castaneum*, *B. mori*, *R. prolixus*, and *N. lugens*, as well as in *G. buenoi*, of which 45% (28/62 CYP genes) might have been generated by tandem gene duplications (Fig. 5c and d). On the other hand, ten UGT genes are clustered on Scaffold1549, suggesting gene duplication events may have produced this large gene cluster (Additional file 1: Figure S6). In addition, multiple UGT genes are linked within Scaffold1323, Scaffold3228, and Scaffold2126. A consensus Maximum-likelihood tree (Additional file 1: Figure S7) based on the conserved C-terminal half of the deduced amino acid sequences from *G. buenoi* UGTs supports the conclusion that genes clustered within the genome derive from recent tandem duplications.





**Fig. 5** Phylogenetic analysis of four different Clans of the cytochrome P450s of *Gerris buenoi* with other insect species. **a** Clan 2, **b** Clan mitochondria, **c** Clan 3, and **d** Clan 4. The *G. buenoi* sequences are indicated in red and bold

Overall, our phylogenetic analysis revealed the conservation of CYPs and UGTs across insects, and the possibility for expansions via lineage-specific gene duplication. We hypothesize that this expansion may have been important in order to diversify the xenobiotic detoxification range and the regulation of endobiotics during the terrestrial-to-water surface transition.

**Conclusions**

The sequencing of the *G. buenoi* genome provides a unique opportunity to understand the molecular mechanisms underlying initial adaptations to water surface life and the subsequent diversification that followed. In particular, gene duplication is known to drive the evolution of adaptations and evolutionary innovations in a variety of lineages including water striders [80, 97–99]. The *G.*

*buanoi* genome revealed a number of clustered duplications in genes that can be linked to processes associated with the specialized life style of water striders. Some are shared with closely related Hemiptera, for example, the duplicated factor *Beadex* is an activator of the *Achaete/Scute* complex known to play an important role in bristle development. Other genes and gene family duplications are particularly rare, such as that found with the insulin receptors, which are known in other insects to be involved in a range of processes including wing development, growth, as well as a number of life history traits including reproduction [49, 52, 100]. The functional significance of the duplication of the histone methyltransferase *grappa* and histone cluster duplications remains unknown, yet opens up new avenues for investigation into the relationship between epigenetics and phenotypic plasticity. Expansions in the cuticle protein families involved in desiccation resistance or genes repertoires involved in xenobiotic detoxification and endobiotic regulation pathways may have played an important role during water surface specialization [78, 101]. Furthermore, the expansion of the opsin gene family and possible light sensitivity shifts are also likely associated with particularities of polarized light detection within the aquatic environment in which *G. buanoi* specializes. The impact of these duplications on the ability of water striders to function efficiently in water surface habitats remains to be experimentally tested. *G. buanoi*, which is now emerging as a tractable experimental model, offers a range of experimental tools to test these hypotheses. More generally, the *G. buanoi* genome provides a good opportunity to further understand the molecular and developmental genetic basis underlying adaptive radiations and diversification upon the conquest of new ecological habitats.

## Methods

### Animal collection and rearing

Adult *G. buanoi* individuals were collected from a pond in Toronto, Ontario, Canada. *G. buanoi* were kept in aquaria at 25 °C with a 14-h light/10-h dark cycle and fed on live crickets. Pieces of floating Styrofoam were regularly supplied to female water striders to lay eggs. The colony was inbred following a sib-sib mating protocol for six generations prior to DNA/RNA extraction.

### DNA and total RNA extraction

Genomic DNA was isolated from adults using Qiagen Genome Tip 20 (Qiagen Inc., Valencia CA). The 180 and 500 bp paired-end libraries as well as the 3 kb mate-pair library were made from eight adult males. The 8 kb mate-pair library was made from eight adult females. Total RNA was isolated from 39 embryos, three first instar nymphs, one second instar nymph, one third instar nymph, one fourth instar nymph, one fifth instar nymph,

one adult male and one adult female. RNA was extracted using a Trizol protocol (Invitrogen).

### Genome sequencing and assembly

Genomic DNA was sequenced using HiSeq2500 Illumina technology. 180 and 500 bp paired-end and 3 and 10 kb mate-pair libraries were constructed and 100 bp reads were sequenced. Estimated coverage was 28.6×, 7.3×, 21×, 17×, 72.9× respectively for each library. Sequenced reads were assembled in draft assembly using ALLPATHS-LG [102] and automatically annotated using custom MAKER2 annotation pipeline [103]. (More details can be found in Additional file 1: Supplementary Data). Expected genome size was calculated counting from Kmer based methods and using Jellyfish 2.2.3 and perl scripts from [104].

### Community curation of the *G. buanoi* genome

International groups within the i5k initiative have collaborated on manual curation of *G. buanoi* automatic annotation. These curators selected genes or gene families based on their own research interests and manually curated MAKER-predicted gene set GBUE\_v0.5.3 at the i5k Workspace@NAL [105] resulting in the non-redundant Official Gene Set OGSv1.0 [106].

### Assessing genome assembly and annotation completeness with BUSCOs

Genome assembly completeness was assessed using BUSCO [29]. The Arthropoda gene set of 2675 single copy genes was used to test *G. buanoi* predicted genes.

### Orthology analyses

OrthoDB8 (<http://orthodb.org/>) was used to find orthologues of *G. buanoi* (OGS 1.0) on 76 arthropod species. Proteins on each species were categorised using custom Perl scripts according to the number of hits on other eight arthropod species: *Drosophila melanogaster*, *Danaus plexippus*, *Tribolium castaneum*, *Apis mellifera*, *Acyrtosiphon pisum*, *Cimex lectularius*, *Pediculus humanus* and *Daphnia pulex*.

### Insulin receptors phylogeny

Sequences were retrieved from 'nr' database by sequence similarity using BLASTp with search restricted to Insecta (taxid:50557). Each *G. buanoi* InR sequence was individually blasted and best 250 hits were recovered. A total of 304 unique id sequences were retrieved. Additionally, we recovered the genes annotated by Kremer et al. [47] as well as *Caenorhabditis elegans* insulin receptor homolog AAC47715.1 as outgroup. We performed a preliminary analysis aligning the sequences with Clustal Omega [107–109] and building a simple phylogeny using MrBayes



[110] (one chain, 100,000 generations). Based on that preliminary phylogeny, we selected a single isoform for each InR gene (Additional file 4). Final InR phylogeny tree was estimated aligning the sequences with MAFFT [111] using E-INS-i iterative method and using MrBayes (four chains, for 1,000,000 generations). Final phylogeny include InR sequences from (copy number in parenthesis):

*Acromyrmex echinator* (2), *Acyrtosiphon pisum* (2), *Aedes aegypti* (1), *Aedes albopictus* (1), *Aethina tumida* (2), *Agrilus planipennis* (2), *Amyelois transitella* (1), *Anopheles darlingi* (1), *Anopheles gambiae* (1), *Anopheles sinensis* (1), *Anoplophora glabripennis* (2), *Aphis citricidus* (2), *Apis cerana* (2), *Apis dorsata* (2), *Apis florea* (1), *Apis mellifera* (2), *Aquarius paludum* (3), *Athalia rosae* (2), *Atta cephalotes* (2), *Atta colombica* (2), *Bactrocera dorsalis* (1), *Bactrocera latifrons* (1), *Bactrocera oleae* (1), *Bemisia tabaci* (2), *Blattella germanica* (3), *Bombus impatiens* (2), *Bombus terrestris* (2), *Bombyx mori* (1), *Caenorhabditis elegans* (1), *Camponotus floridanus* (2), *Cephus cinctus* (2), *Ceratina calcarata* (2), *Ceratitis capitata* (1), *Ceratosolen solmsi marchali* (1), *Cimex lectularius* (2), *Clunio marinus* (1), *Copidosoma floridanum* (1), *Cryptotermes secundus* (3), *Cyphomyrmex costatus* (2), *Danaus plexippus* (1), *Daphnia pulex* (4), *Dendroctonus ponderosae* (1), *Diachasma alloeum* (2), *Diaphorina citri* (1), *Dinoponera quadricaps* (2), *Diuraphis noxia* (2), *Drosophila ananassae* (1), *Drosophila arizonae* (1), *Drosophila biarmipes* (1), *Drosophila bipunctinata* (1), *Drosophila busckii* (1), *Drosophila elegans* (1), *Drosophila erecta* (1), *Drosophila eugracilis* (1), *Drosophila ficusphila* (1), *Drosophila grimshawi* (1), *Drosophila kikkawai* (1), *Drosophila melanogaster* (1), *Drosophila miranda* (1), *Drosophila mojavensis* (1), *Drosophila obscura* (1), *Drosophila persimilis* (1), *Drosophila pseudoobscura* (1), *Drosophila rhopaloa* (1), *Drosophila sechellia* (1), *Drosophila serrata* (1), *Drosophila simulans* (1), *Drosophila sukuzii* (1), *Drosophila takahashii* (1), *Drosophila virilis* (1), *Drosophila willistoni* (1), *Drosophila yakuba* (1), *Dufourea novaeangliae* (2), *Ephemera danica* (2), *Eufriesea mexicana* (2), *Fopius arisanus* (2), *Gerris buenoi* (3), *Glossina morsitans morsitans* (1), *Habropoda laboriosa* (2), *Halyomorpha halys* (2), *Harpegnathos saltator* (2), *Hebrus sp* (3), *Helicoverpa armigera* (1), *Heliopsis virescens* (1), *Hydrometra cumata* (3), *Lasius niger* (2), *Leptinotarsa decemlineata* (2), *Limnopus dissortis* (3), *Linepithema humile* (2), *Locusta migratoria* (2), *Macrotermes natalensis* (3), *Manduca sexta* (1), *Maruca vitrata* (1), *Megachile rotundata* (2), *Melipona quadrifasciata* (1), *Mesovelia furcata* (3), *Microplitis demolitor* (2), *Microvelia longipes* (3), *Monochamus alternatus* (1), *Monomorium pharaonis* (2), *Musca domestica* (1), *Myzus persicae* (2), *Nasonia vitripennis* (1), *Neodiprion lecontei* (2), *Nicrophorus*

*vespilloides* (2), *Nilaparvata lugens* (2), *Oncopeltus fasciatus* (2), *Onthophagus nigriventris* (1), *Onthophagus taurus* (2), *Ooceraea biroii* (2), *Orussus abietinus* (2), *Oryctes borbonicus* (1), *Papilio machaon* (2), *Papilio polytes* (1), *Papilio xuthus* (1), *Parasteatoda tepidariorum* (2), *Pediculus humanus corporis* (1), *Pieris rapae* (1), *Plutella xylostella* (1), *Pogonomymex barbatus* (2), *Polistes canadensis* (2), *Polistes dominula* (2), *Pseudomyrmex gracilis* (2), *Rhagoletis zephyria* (1), *Rhagoletia antilleana* (3), *Rhodnius prolixus* (2), *Solenopsis invicta* (2), *Spodoptera litura* (1), *Stomoxys calcitrans* (1), *Strigamia maritima* (1), *Trachymyrmex cornetzi* (2), *Trachymyrmex septentrionalis* (2), *Trachymyrmex zeteki* (2), *Tribolium castaneum* (2), *Trichogramma pretiosum* (1), *Trichomalopsis sarcophagae* (1), *Vollenhovia emeryi* (2), *Wasmannia auropunctata* (2), *Zeugodacus cucurbitae* (1), and *Zootermopsis nevadensis* (3).

#### Cytochrome P450 proteins phylogeny

CYPs phylogenetic analysis was performed using Maximum-Likelihood method and the trees were generated by MEGA 6. The phylogenetic trees were generated by MEGA 6 with Maximum-Likelihood method using the amino acid sequences from *Gerris buenoi* (Gb), *Rhodnius prolixus* (Rp), *Nilaparvata lugens* (Nl), *Bombyx mori* (Bm) and *Tribolium castaneum* (Tc). All nodes have significant bootstrap support based on 1000 replicates.

#### Additional files

**Additional file 1:** Supplementary Online Information. Additional files 10 and 11. (ZIP 18100 kb)

**Additional file 2:** List of annotated autophagy genes in *Gerris buenoi*. (XLSX 12 kb)

**Additional file 3:** Accession number and gene name of 225 arthropod genes used to reconstruct Insulin receptors phylogeny (highlighted). (XLSX 91 kb)

**Additional file 4:** Nucleotide sequences of Insulin receptors genes annotated in Gerromorpha (*Gerris buenoi*, *Aquarius paludum*, *Limnopus dissortis*, *Rhagoletia antilleana*, *Microvelia longipes*, *Mesovelia furcata*, *Hydrometra cumata* and *Hebrus sp.*). (DOCX 40kb)

**Additional file 5:** SNPs summary at position 17, 64, 70 and 137 of 114 LWS opsin homologs from 54 species representing 12 insect orders. (XLSX 26 kb)

**Additional file 6:** List of annotated aquaporin genes in *Gerris buenoi* including gene names and closest identity. (XLSX 58 kb)

**Additional file 7:** List of annotated aquaporin genes in *Gerris buenoi* including gene names and closest identity. (XLSX 15 kb)

**Additional file 8:** Protein sequences of annotated chemoreceptors in *Gerris buenoi*: 155 Or, 135 Gr and 45 IR genes. (DOCX 83 kb)

**Additional file 9:** List of cytochrome P450 (CYP) genes annotated from the *Gerris buenoi* genome. (XLSX 14 kb)

**Additional file 10:** *Gerris buenoi* serosins nucleotide and protein sequences. (DOCX 12 kb)

**Additional file 11:** List of antioxidant enzyme genes identified in the *Gerris buenoi* genome. (XLSX 13 kb)

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### Availability of data and materials

The datasets generated and/or analysed during the current study are available in GenBank BioProject PRJNA203045. Genome assembly can be found at [113], gene annotation can be found at [114], and official gene set can be found at [106].

### Image rights

The authors acquired all images in this manuscript.

### Authors' contributions

DA, AK, and SR conceived, managed, and coordinated the project; SD-P, DMM, RAG and SR managed the i5K project; SLL led the submissions team; HVD, HC led the library team; YH, HD led sequencing team; JQ, SCM, DSTH, SR and KCW assembled the genome; DSTH and SR did the automated annotation; DA, RR, MF, JBB, HMR, KAP, S-JA, MM-T, EA, FB, TC, CPC, AGC, AJJC, AD, EMD, ED, ENE, M-JF, CGCJ, AJ, ECI, JWJ, MPL, ML, AM, BO, AL-O, AR, PNR, AJR, MES, WT, MvdZ, IMVJ, AVL and SV contributed to manual curation; LMC, CPC, MMT and MFP performed curation quality control and generated the OGS; EA, M-JF contributed to statistical analyses of wing development and polyphenism genes; IMVJ and KAP performed associated phylogenetic and synteny analyses of Wnt and homeodomain transcription factor gene clusters; DA performed InR phylogeny and InR analyses; RR contributed with Insulin/TOR signaling genes analyses and InR analyses; MF performed opsins analyses; JBB analyzed aquaporins, cuticle genes and antioxidant proteins; HMR carried out chemoreceptors analyses; AJ, KAP and IMVJ performed homeobox gene and Wnt signaling pathway analyses; S-JA analyzed UGTs detoxification genes; EA conducted wing polyphenism analyses; FB performed nuclear receptors and bHLH-PAS proteins analyses; AGC carried out early developmental genes analyses; ED conducted histone genes and histone modification machinery; ENE, AM and BO performed cysteine peptidase analyses; CF contributed to analysis of Beadex duplication; CGCJ and MvdZ performed immune genes analyses; RR, MF, JBB, HMR, KAP, S-JA, EA, FB, AGC, ED, ENE, CF, AJ, BO, AM, AL-O, MvdZ and IMVJ contributed to report writing; DA and AK wrote the manuscript; DA, AK, RR, FB, MF, JBB, HMR, KAP and SR edited the manuscript; DA and AK organized the Additional file 1. All authors approved the final manuscript.

### Ethics approval

The bugs were collected in road side ditches near Toronto and no permission was required.

### Consent for publication

Not applicable.

### Competing interests

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### Author details

<sup>1</sup>Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Claude Bernard Lyon 1, CNRS UMR 5242, Ecole Normale Supérieure de Lyon 46, allée d'Italie, 69364 Lyon Cedex 07, France. <sup>2</sup>Department of Molecular Genetics & Microbiology and UF Genetics Institute, University of Florida, 2033 Mowry Road, Gainesville, FL 32610-3610, USA. <sup>3</sup>Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA. <sup>4</sup>Department of Biological Sciences, McMicken College of Arts and Sciences, University of Cincinnati, 318 College Drive, Cincinnati, OH 45221-0006, USA. <sup>5</sup>Department of Entomology, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA. <sup>6</sup>Institute for Zoology: Developmental Biology, University of Cologne, Zùlpicher Str. 47b, 50674 Cologne, Germany. <sup>7</sup>School of Life Sciences, University of Warwick, Gibbet Hill Campus, Coventry CV4 7AL, UK. <sup>8</sup>USDA-ARS Horticultural Crops Research Unit, 3420 NW Orchard Avenue, Corvallis, OR 97330, USA. <sup>9</sup>Department of Crop and Soil Science, Oregon State University, 3050 SW Campus Way, Corvallis, OR 97331, USA. <sup>10</sup>USDA Agricultural Research Service, National Agricultural Library, Beltsville, MD 20705, USA. <sup>11</sup>Human Genome Sequencing Center, Department of Human and Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA. <sup>12</sup>Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195, USA. <sup>13</sup>Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA. <sup>14</sup>Department of Biology, McGill University, 1205 Avenue Docteur Penfield Avenue, Montréal, Québec H3A 1B1, Canada. <sup>15</sup>Laboratory for Evolution and Development, Department of Biochemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand. <sup>16</sup>School of Biology, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK. <sup>17</sup>A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119991, Russia. <sup>18</sup>Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, Netherlands. <sup>19</sup>Max Planck Institute for Chemical Ecology, Hans-Knöll Strasse 8, 07745 Jena, Germany. <sup>20</sup>Department of Entomology, University of Maryland, College Park, MD 20742, USA. <sup>21</sup>Smithsonian Tropical Research Institute, Apartado Postal 0843-03092, Balboa Ancon, Panama City, Panama. <sup>22</sup>Center of Life Sciences, Skolkovo Institute of Science and Technology, Skolkovo 143025, Russia. <sup>23</sup>USDA ARS Center for Grain and Animal Health Research, 1515 College Ave., Manhattan KS-66502, USA. <sup>24</sup>Department of Evolutionary Genetics, Max-Planck-Institut für Evolutionsbiologie, August-Thienemann-StraÙe 2, 24306 Plön, Germany.

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## **Escalation and constraints of antagonistic armaments in water striders**

### **Synopsis of the paper:**

Sexual conflict may result in the escalating coevolution of sexually antagonistic traits. However, our understanding of the evolutionary dynamics of antagonistic traits and their role in association with sex-specific escalation remains limited. Here we study sexually antagonistic coevolution in a genus of water striders called *Rhagovelia*. We identified a set of male grasping traits and female anti-grasping traits used during pre-mating struggles and show that natural variation of these traits is associated with variation in mating performance in the direction expected for antagonistic co-evolution. Phylogenetic mapping detected signals of escalation of these sexually antagonistic traits suggesting an ongoing arms race. Moreover, their escalation appears to be constrained by a trade-off with dispersal through flight in both sexes. Altogether our results highlight how sexual interactions may have shaped sex-specific antagonistic traits and how constraints imposed by natural selection may have influenced their evolution.

### **Summary of results:**

The genus *Rhagovelia* have winged and wingless morphs in both sexes. Additionally, differences in morphology between the sexes were identified. Behavioural observations of sexual interactions suggest that *Rhagovelia* females evolved morph-specific strategies to reduce mating frequency. The study of the behaviour and mating rate in the species *Rhagovelia antilleana* shows that the struggles produced during the pre-mating interaction are indicative of sexual conflict over mating rate. The sex-specific modifications observed might be the result of antagonistic interaction between the sexes in this species. Analysis made in winged and wingless morphs between females and males indicated that the presence of wings favours the development of the pronotum projection and constrains the narrowing of the abdomen. These constraints may have driven the evolution of alternative sex-specific and morph-specific strategies to escape fitness costs due to frequent mating in females. In males, the winged morphs typically have the thinnest rear-leg femurs and were less successful in mating, contrary to wingless morphs that have the largest rear-leg femurs. Finally, the ancestral reconstruction of arms race indicated a strong phylogenetic signal where males of the *Rhagovelia* genus evolved an increasing number of secondary sexual traits. This pattern indicates substantial escalation of conflict in males. Altogether, these patterns of armament of the two sexes suggest an on-going arms race in the *Rhagovelia* genus.

### **Conclusion:**

Our results indicate how sexual interactions can drive antagonistic coevolution between males and females and how certain developmental processes can constrain this escalation.

Personal contribution:

Aidamalia Vargas-Lowman (third author): extraction of RNA of *Rhagovelia* species, assembly of sequences that were used to build the phylogeny and ancestral reconstruction.



# Escalation and Morphological Constraints of Antagonistic Armaments in Water Striders

## OPEN ACCESS

Antonin Jean Johan Crumière<sup>1†</sup>, David Armisén<sup>1†</sup>, Aïdamalia Vargas-Lowman<sup>1</sup>, Martha Kubarakos<sup>1†</sup>, Felipe Ferraz Figueiredo Moreira<sup>2</sup> and Abderrahman Khila<sup>1\*</sup>

### Edited by:

Jingchun Li,  
University of Colorado Boulder,  
United States

### Reviewed by:

Jessica Goodheart,  
University of California, Santa Barbara,  
United States  
Luigi Caputi,  
Stazione Zoologica Anton Dohrn, Italy

### \*Correspondence:

Abderrahman Khila  
abderrahman.khila@ens-lyon.fr

### †Present Address:

Antonin Jean Johan Crumière,  
Section for Ecology and Evolution,  
Department of Biology, University of  
Copenhagen, Copenhagen, Denmark  
David Armisén,  
INRA-Université Clermont-Auvergne,  
UMR 1095 GDEC, Clermont-Ferrand,  
France

Martha Kubarakos,  
Department of Pharmaceutical  
Sciences, University of Toronto,  
Toronto, ON, Canada

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<sup>1</sup> Institut de Génomique Fonctionnelle de Lyon, CNRS UMR 5242, Ecole Normale Supérieure de Lyon, Université de Lyon, Université Claude Bernard Lyon 1, Lyon, France, <sup>2</sup> Laboratório de Biodiversidade Entomológica, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

Sexual conflict may result in the escalating coevolution of sexually antagonistic traits. However, our understanding of the evolutionary dynamics of antagonistic traits and their role in association with sex-specific escalation remains limited. Here we study sexually antagonistic coevolution in a genus of water striders called *Rhagovelia*. We identified a set of male grasping traits and female anti-grasping traits used during pre-mating struggles and show that natural variation of these traits is associated with variation in mating performance in the direction expected for antagonistic coevolution. Phylogenetic mapping detected signal of escalation of these sexually antagonistic traits suggesting an ongoing arms race. Moreover, their escalation appears to be influenced by a trade-off with dispersal through flight in both sexes. Altogether our results highlight how sexual interactions and natural selection may have shaped sex-specific antagonistic trait coevolution.

**Keywords:** sexual conflict, antagonistic armaments, coevolution, water striders, sexual dimorphism

## INTRODUCTION

The evolutionary interests of males and females in reproductive interactions often diverge, leading to the coevolution of sexually antagonistic traits that are favored in one sex at a fitness cost to the other (Arnqvist and Rowe, 2005). Females possess a limited number of eggs and pay increasing costs for multiple mating (Rowe et al., 1994; Arnqvist and Rowe, 2005). On the other hand, males are favored to mate repeatedly because increased mating rate in males results in increased fitness (Rowe et al., 1994; Arnqvist and Rowe, 2005). These opposing interests during mating interactions cause sexual conflict over mating rate (Arnqvist and Rowe, 2005). In this specific case, sexual antagonism drives the evolution of armaments whereby grasping traits allowing males to persist and increase their mating success are often matched by the evolution of anti-grasping traits in females improving their ability to resist and thus escape fitness costs associated with superfluous mating events (Weigensberg and Fairbairn, 1996; Arnqvist and Rowe, 2002b). The repeated evolution of such sex-specific armaments can lead to episodes of escalation and ultimately arms race (Arnqvist and Rowe, 1995, 2002a; Pennell and Morrow, 2013). Sex chromosome evolution (Dean et al., 2012; Pennell and Morrow, 2013), sex biased gene expression (Snook et al., 2010; Mank et al., 2013; Mank, 2017) and intra- and inter-locus sexual conflict (Rowe and Day, 2006; Bonduriansky and Chenoweth, 2009; Crumière and Khila, 2019), are expected to drive the divergence of the sexes, often resulting in striking cases of sexual dimorphism (Rowe and Day, 2006; Bonduriansky and Chenoweth, 2009).

The dynamics of morphological co-evolution of sexually antagonistic traits has attracted much attention (Arnqvist and Rowe, 2002a; Bergsten and Miller, 2007; Kuntner et al., 2009) and water striders have been widely used as a model to study sexual dimorphism and antagonistic co-evolution in the context of sexual conflict (Arnqvist, 1992; Arnqvist and Rowe, 1995; Weigensberg and Fairbairn, 1996). Despite the rich diversity of this group of semiaquatic insects, the vast majority of these studies have focused on the Gerridae family (Insecta, Heteroptera, Gerromorpha, Veliidae) (Arnqvist, 1992; Arnqvist and Rowe, 1995, 2002a; Weigensberg and Fairbairn, 1996). Here, we investigated the evolution of sexual dimorphism in the tropical genus *Rhagovelia* from the Veliidae family (Insecta, Heteroptera, Gerromorpha, Veliidae) (Andersen, 1982; Polhemus, 1997; Moreira and Ribeiro, 2009). In this genus, sexual dimorphism is particularly dramatic with both sexes often bearing morphological traits reminiscent to those usually found in species with strong sexual conflict (Andersen, 1982; Polhemus, 1997; Arnqvist and Rowe, 2005; Moreira and Ribeiro, 2009). However, the process of sexual selection; i.e., male competition, mate choice or sexual conflict over mating rate, driving sexual dimorphism in *Rhagovelia* is unknown (Andersson, 1994; Arnqvist and Rowe, 2005). Through morphological and behavioral observations, we identified a large set of potentially sexually antagonistic armaments in the *Rhagovelia* genus and tested their role during sexual interactions in a representative species called *Rhagovelia antilleana* (Andersen, 1982; Polhemus, 1997; Moreira and Ribeiro, 2009). We then tested how variation in sexually antagonistic traits affects male and female success during sexual interactions. We show that this variation is associated with trade-offs with flight capability and egg storage. Finally, we used phylogenetic reconstruction of male and female secondary sexual traits to understand how antagonistic coevolution and diversification may have shaped the divergence of the sexes in this lineage and assess the extent of evolutionary arms race.

## RESULTS

### Sexual Dimorphism in *Rhagovelia*

Sexual dimorphism in the water strider genus *Rhagovelia* ranges from subtle to spectacular differences in morphology between the sexes (Figures 1A–D'; Polhemus, 1997; Moreira and Ribeiro, 2009). *Rhagovelia* populations contain both winged and wingless morphs (Polhemus, 1997; Moreira and Ribeiro, 2009). In our *R. antilleana* lab population, winged females were present at 27.5% and wingless females at 72.5% while winged males were present at 18.75% and wingless males at 81.25%. In both male morphs, the foreleg tarsi are equipped with prominent, hook-like, combs that are absent in the females (Figures 1E,E'; Polhemus, 1997; Moreira and Ribeiro, 2009). The rearlegs of males possess several rows of spikes of different sizes along the trochanter, femur, and tibia (Figures 1F,F'–G,G'; Polhemus, 1997; Moreira and Ribeiro, 2009). In addition, the femurs of the males' rearlegs are very thick compared to those of the females (Figures 1C,D,E,C',D',F'; Polhemus, 1997; Moreira and Ribeiro, 2009). Staining for actin fibers revealed that the width of the

femur is entirely occupied by muscles (Figure S1), suggesting that thicker femurs have the potential to apply stronger grip force. In females, winged morphs possess a prominent spike-like projection of the pronotum (Figure 1H'; Polhemus, 1997) that is not found in wingless females (Figure 1C') or winged males (Figure 1H; Polhemus, 1997). Wingless females have a narrow-shaped abdomen (Figure 2B) that can be even more pronounced in some species such as *Rhagovelia obesa* (Figure 2C). We have never observed winged females with narrow abdomen or wingless females with pronotum projection indicating that these two traits are mutually exclusive (Figures 2A,B). This observation suggests that morph-specific strategies to reduce mating frequency have evolved in *Rhagovelia* females.

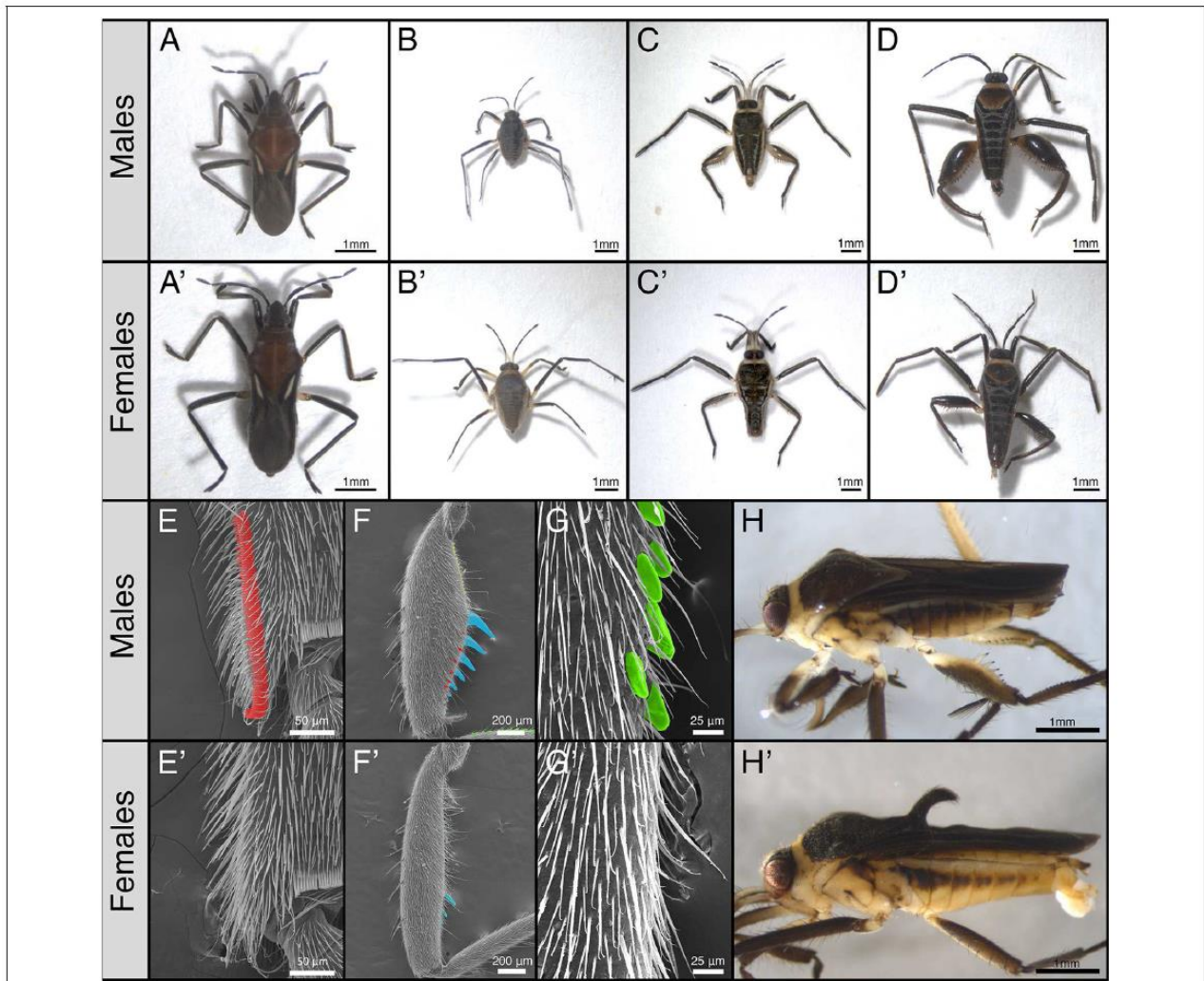
### Mating Behavior in *Rhagovelia antilleana*

Pre-mating interactions in *R. antilleana* include vigorous struggles between males and females, characteristic of sexual conflict over mating rate (Arnqvist and Rowe, 1995, 2005; Khila et al., 2012). Behavioral quantification revealed that both winged and wingless males approach females preferably on the side and the back by grasping female's mid- and rearlegs (Figure S2A). We did not detect a significant male preference for winged or wingless morphs or any differences in the frequency of mating events related to the overall numbers of pre-mating interactions across female morphs (Figures S2B–D). When mounted, males use the sex combs to clasp the pronotum of the female and tighten the grip on her legs using their modified rearlegs (Supplementary Video 1). Females vigorously shake their body and perform repeated somersaults, which frequently result in rejecting the male (Supplementary Video 2). The struggles are vigorous and their duration is typically  $0.68 \pm 0.99$  s in average, but can vary between fractions of a second ( $<0.15$  s) to several seconds (9.75 s) (Figure S2E). These pre-mating struggles are indicative of sexual conflict over mating rate and the sex-specific modifications we observed might be the result of antagonistic interaction between the sexes in this species.

### Effect of Natural Variation in Male and Female Antagonistic Traits on Mating Rate

Artificially generated variation in sexually antagonistic traits is known to result in variation in mating success (Khila et al., 2012). Here, we wanted to test whether natural variation in male persistence and female resistance traits is also associated with variation in mating success. In *R. antilleana*, only winged females have the pronotum projection (Figure 2A) and only wingless females have narrow abdomen (Figure 2B), whereas in males there is considerable natural variation in the degree of elaboration of the rear-legs (Figures 2D–F). To determine the extent to which this variation affects individual performance during pre-mating struggles, we used a tournament design (Figure S3; Ivy and Sakaluk, 2007) composed of 10 replicates with 8 males and 8 females each for a total of 80 males and 80 females. This experiment separated males and females based on the outcome of premating struggles into successful, intermediate, and unsuccessful individuals (Figure 3 and Figure S3). Successful males are those who copulate after a premating struggle and successful females are those who

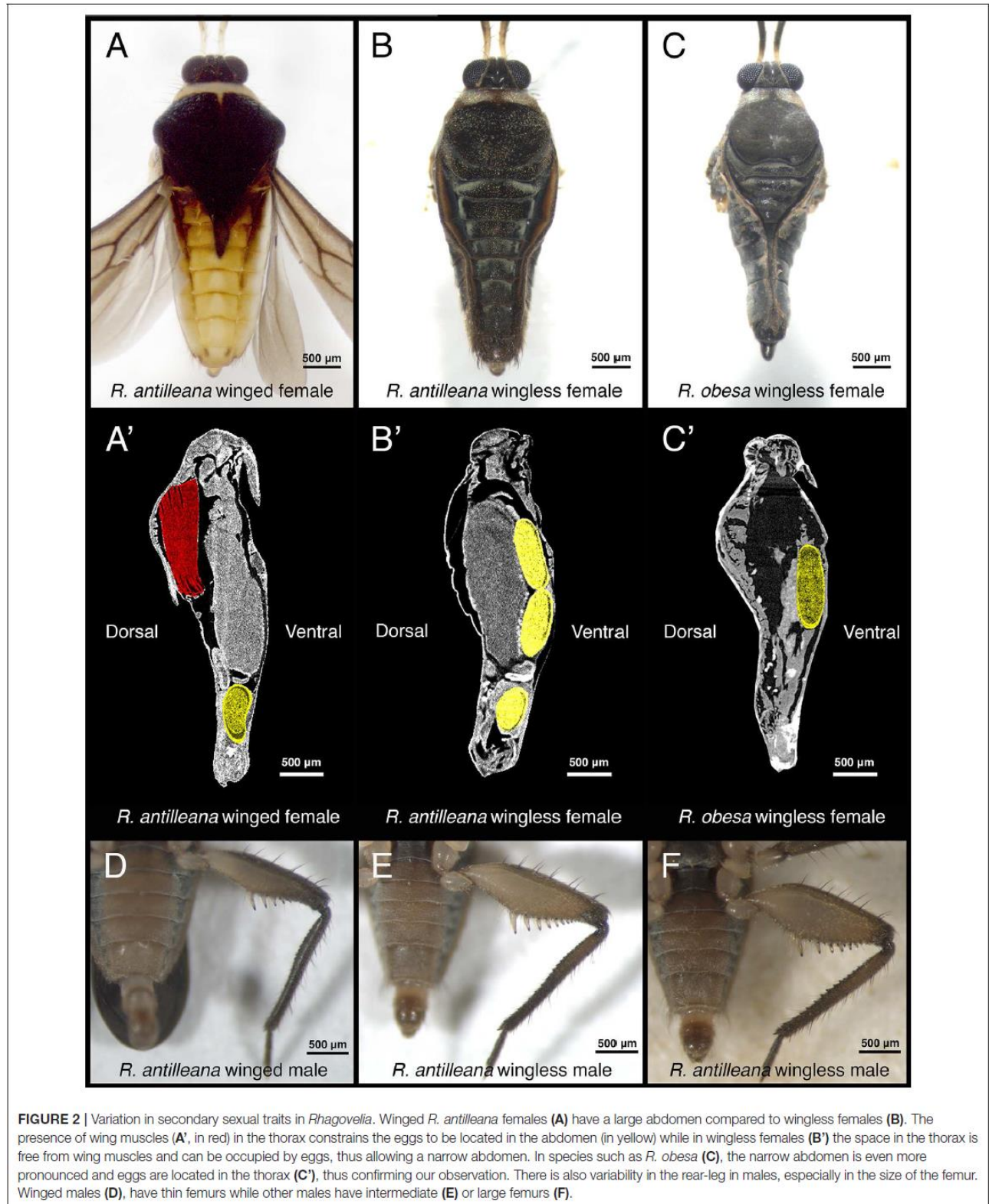




**FIGURE 1 |** Sexual dimorphism and secondary sexual traits in the genus *Rhagovelia*. The outgroup *Oiovelia cunucunumana* (A,A') does not show strong dimorphism. In the genus *Rhagovelia*, sexual dimorphism can be subtle as in *Rhagovelia plumbea* (B,B') or more extreme as in *Rhagovelia antilleana* (C,C') or *Rhagovelia* sp.2 (D,D'). This dimorphism affects secondary sexual traits such as the sex combs (in red) present in male fore-legs (E) and absent in females (E'); the presence of spikes (in blue, yellow and red) and the larger femur of the rear-leg femur in males (F) compared to females that only have some small femur spikes (F'); and the presence of spikes (in green) along the rear-leg tibia in males (G) that are also absent in females (G'). Females possess secondary sexual traits such as a narrow abdomen (C') and a pronotum projection (H') that are absent in males (C,H).

reject males after pre-mating struggle (Figure S3). We found that successful males have significantly more rear-leg tibia spikes and thicker rear-leg femurs compared to unsuccessful males (respectively Wilcoxon test:  $p = 0.045^*$ ; Student  $t$ -test:  $p = 0.02^*$ ; Figures 3A,B and Figure S4), however body size, number of spikes on rear-leg femur, and the length of rear-leg tibia and femur did not significantly differ between the two groups (Figures 3C–F and Figure S4). This result highlights the importance of the rear-legs for increasing male mating frequency and therefore, to some extents, male fitness (Andersson, 1994; Danielsson, 2000; Arnqvist and Rowe, 2005). In females, our data indicated that individuals with a pronotum projection

were significantly more efficient in rejecting males than females with narrow abdomen (Cochran-Mantel-Haenszel chi-squared test: 23,45;  $df = 2$ ;  $p$ -value:  $8.089e-06^{***}$ ; Figure 3G). To further test the validity of this conclusion, we conducted an experiment where the performance of 30 wingless females (narrow abdomen and absence of pronotum projection) was compared to that of 18 winged females (wide abdomen) from which we amputated the pronotum projection. We found that winged females with amputated pronotum projection were less efficient at rejecting males, which resulted in higher mating frequency (Figures 3G,H). This result indicates that in the absence of the pronotum projection, narrow abdomen increases



**FIGURE 2** | Variation in secondary sexual traits in *Rhagovelia*. Winged *R. antilleana* females (**A**) have a large abdomen compared to wingless females (**B**). The presence of wing muscles (**A'**, in red) in the thorax constrains the eggs to be located in the abdomen (in yellow) while in wingless females (**B'**) the space in the thorax is free from wing muscles and can be occupied by eggs, thus allowing a narrow abdomen. In species such as *R. obesa* (**C**), the narrow abdomen is even more pronounced and eggs are located in the thorax (**C'**), thus confirming our observation. There is also variability in the rear-leg in males, especially in the size of the femur. Winged males (**D**), have thin femurs while other males have intermediate (**E**) or large femurs (**F**).



female's ability to resist harassing males. Therefore, alternative morph-specific strategies evolved in the females possibly under selection by male harassment. Altogether, these experiments suggest that natural variation in sexually antagonistic traits is associated with variation in the ability of both sexes to control mating rate.

### Armament Escalation Is Influenced by Flight Capability in *Rhagovelia*

Because natural variation in antagonistic traits can result in variation in the performance of males and females during pre-mating struggles, we investigated whether life history traits might influence this variation. Many studies described the presence of trade-offs between flight capability and certain morphological or physiological traits, such as fertility, in a number of insects (Fairbairn and Desranleau, 1987; Langelotto et al., 2000; Simmons and Emlen, 2006; Karlsson and Johansson, 2008). We therefore tested whether wing polymorphism in *R. antilleana* affects variation in male's and female's sexually antagonistic traits. The pronotum projection, a sexually antagonistic trait that increases the ability of females to resist male harassment, only develops in winged female morphs (Figure 1H'). The presence of the wings is accompanied by the presence of flight muscles, which fill most of the space in the thorax (Figure 2A'; Fairbairn and Desranleau, 1987; Kaitala, 1988). These winged females position their developing eggs mostly in the abdomen (Figure 2A'). In wingless females of both *R. antilleana* and *R. obesa*, we found that the absence of flight muscles provides a space in the thorax that is occupied by the developing eggs (Figures 2B',C'). Furthermore, we found that wingless females contained a significantly higher number of developing eggs than winged females, consistent with the trade-off between flight and fertility (mean of 4.15 eggs  $\pm$  2.96 for  $n = 20$  wingless females vs. mean of 1.35 eggs  $\pm$  2.35 for  $n = 20$  winged females; Student *t*-test: *p*-value 0.002094\*\*; Figures 2A'-C') (Fairbairn and Desranleau, 1987; Fairbairn, 1988; Roff and Fairbairn, 1991). These data suggest that the presence of wings is associated with the development of the pronotum projection and constrains the narrowing of the abdomen (Figure S5). These morphological constraints may have driven the evolution of alternative sex-specific and morph-specific strategies to escape fitness costs due to frequent mating in females. In males, we observed that winged morphs typically have the thinnest rear-leg femurs (Figure 2D) and were under-represented in the successful group of our tournament set-up, contrary to wingless morphs that have the largest rear-leg femurs (Cochran-Mantel-Haenszel chi-squared test: 10,957; degree of freedom: 2; *p*-value: 0.004177\*\*; Figure 3I). Altogether, our observations highlight a trade-off between dispersal and mating success in both males and females.

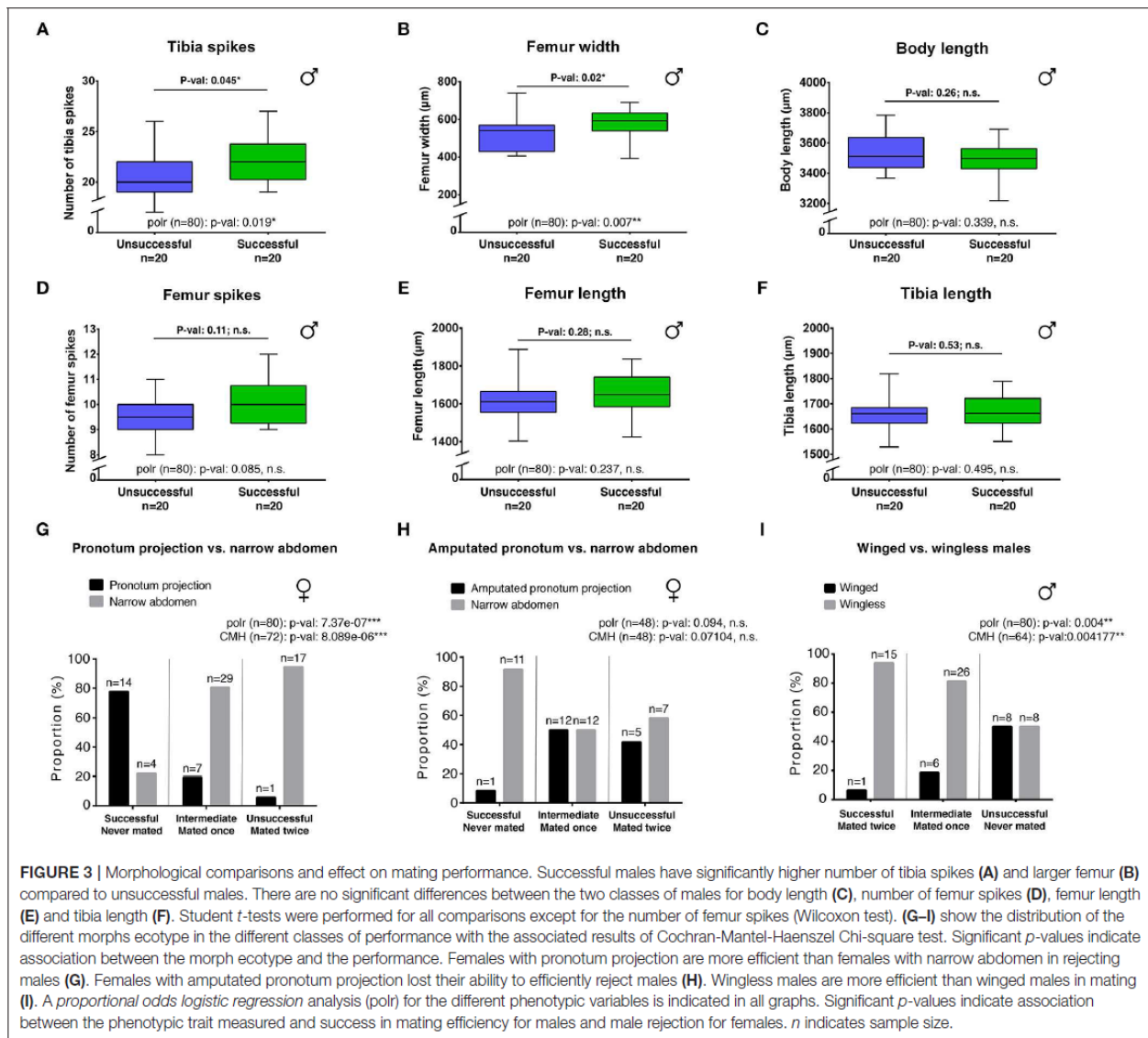
### Signs of Evolutionary Arms Race Between the Sexes in the *Rhagovelia* Genus

Coevolution of the sexes due to antagonistic interactions could lead to escalation and arms race that deeply shape the evolutionary trajectory of lineages in nature (Arnqvist and Rowe, 1995, 2002a,b; Bergsten and Miller, 2007; Kuntner et al., 2009).

We therefore tested the evolution of armament and presence of arms race in the *Rhagovelia* genus by analyzing phylogenetic patterns of correlation of male and female phenotypic complexity in terms of secondary sexually antagonistic traits (Arnqvist and Rowe, 2002a; Bergsten and Miller, 2007; Kuntner et al., 2009). We mapped eight male specific traits and two female specific traits on a phylogeny comprising a sample of 13 species to determine the patterns of phenotypic complexity between the sexes (Tables S1, S2, Figure 4, and Figures S6, S7). Our reconstruction showed a strong phylogenetic signal where males of the *Rhagovelia* genus evolved an increasing number of secondary sexual traits (Figure 4). This pattern indicates substantial escalation of conflict in males (Arnqvist and Rowe, 2002a,b; Bergsten and Miller, 2007; Kuntner et al., 2009). In females, we detected the evolution of anti-grasping traits in a clade where males are the most armed (Figure 4). However, these armaments do not show phylogenetic signal in females and we did not detect correlation between male and female phenotypic complexities (correlation test with phylogenetic independent contrast; rho: 0.28; *p*-value: 0.37, n.s.). Based on our samples, these results suggest an on-going arms race between males and females in the *Rhagovelia* genus and more specifically in *R. antilleana* and *R. obesa* (Arnqvist and Rowe, 2002a,b; Bergsten and Miller, 2007; Kuntner et al., 2009).

## DISCUSSION

We have shown that sexual conflict over mating rate generates sexual dimorphism via the presence of secondary sexual traits in the *Rhagovelia* genus. Males have evolved a set of persistence grasping traits aimed at increasing mating frequency, whereas females evolved a set of anti-grasping traits aimed at decreasing mating frequency. Natural variation in these antagonistic traits directly impacts mating frequency and therefore influences the fitness of both sexes. *Rhagovelia* males possess the sex combs as grasping traits similar to other insects (Spieth, 1952; Markow et al., 1996; Polak et al., 2004; Chesebro et al., 2009; Tanaka et al., 2011). An additional male set of grasping traits consists of elaborations in the rearlegs, which are used as clasps that neutralize female's legs (Supplementary Video 1). Our analysis revealed that variation in number of spines on the tibia and in the width of the femurs results in variation in male mating success. While these two traits have shown significant importance, the legs possess a complete set of secondary sexual modifications that likely act in synergy altogether and increase male mating frequency. Females on the other hand, possess morph-specific modifications consisting of a narrow abdomen in wingless morphs and a pronotum projection in winged morphs. Both of these traits increase the chances for the female to reject harassing males. The projection forms a barrier and increases the distance between the abdomen of the male and the female (Supplementary Video 2), likely making it easier for the female to reject the male. Our experiments also showed that females with narrow abdomen are better at rejecting males than females to which we removed the pronotum projection and that have a wide abdomen. Because males use their rearlegs

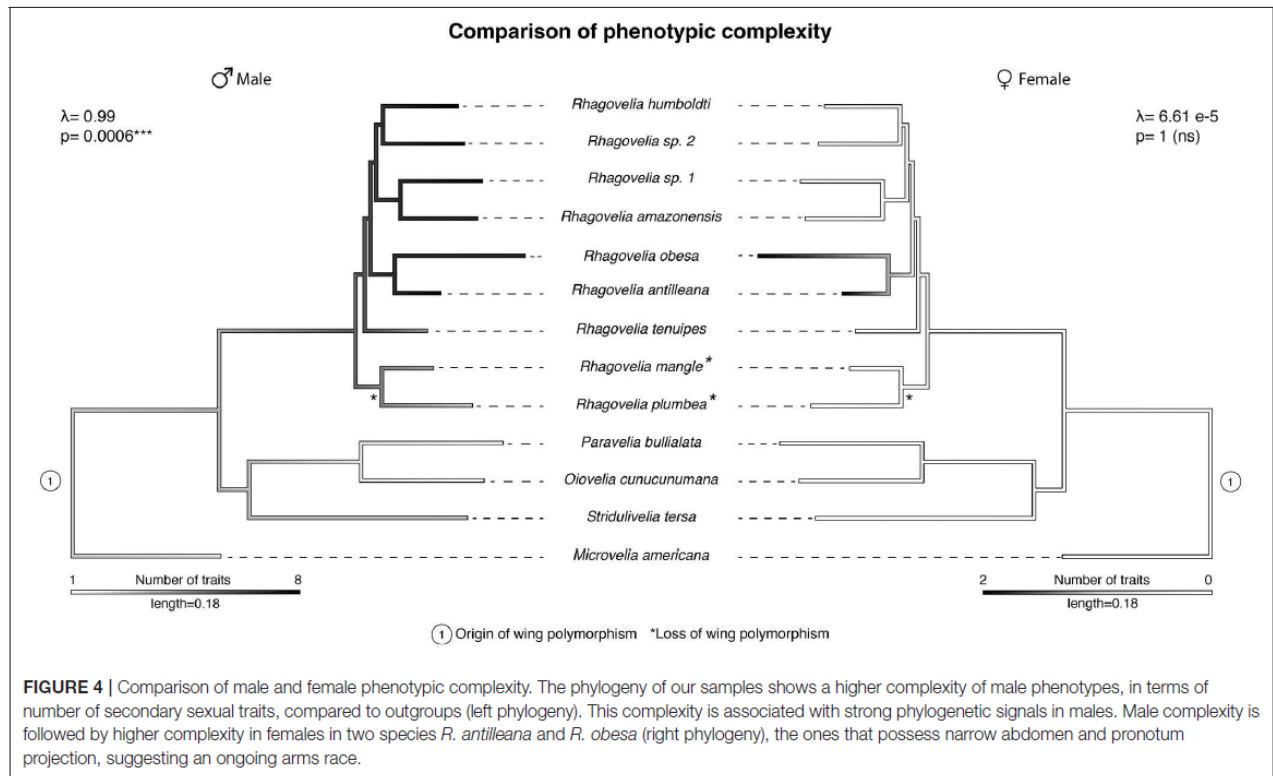


**FIGURE 3 |** Morphological comparisons and effect on mating performance. Successful males have significantly higher number of tibia spikes (A) and larger femur (B) compared to unsuccessful males. There are no significant differences between the two classes of males for body length (C), number of femur spikes (D), femur length (E) and tibia length (F). Student *t*-tests were performed for all comparisons except for the number of femur spikes (Wilcoxon test). (G–I) show the distribution of the different morphs ecotype in the different classes of performance with the associated results of Cochran-Mantel-Haenszel Chi-square test. Significant *p*-values indicate association between the morph ecotype and the performance. Females with pronotum projection are more efficient than females with narrow abdomen in rejecting mating males (G). Females with amputated pronotum projection lost their ability to efficiently reject males (H). Wingless males are more efficient than winged males in mating (I). A proportional odds logistic regression analysis (polr) for the different phenotypic variables is indicated in all graphs. Significant *p*-values indicate association between the phenotypic trait measured and success in mating efficiency for males and male rejection for females. *n* indicates sample size.

as clamps on the legs of the female, it is possible that the narrowing of the abdomen allows the female to slide out of this grip. Altogether our observations indicate a role of these sex-specific phenotypes in male persistence and female resistance (Arnqvist and Rowe, 2002a,b).

Our results also indicated that some life history traits such as fertility and dispersal influence sexually antagonistic armaments in both sexes (Fairbairn and Desranleau, 1987; Fairbairn, 1988; Roff and Fairbairn, 1991). Dispersal, manifested by the presence of wings (Andersen, 1982; Spence, 1989; Fairbairn and King, 2009), is associated with variation in the expression of sexually antagonistic traits in both sexes. Variation in the elaboration of these traits in turn influences the ability of the sexes to control mating frequency. Therefore, there is a tradeoff between

dispersal and the expression of sexually antagonistic traits in *Rhagovelia*. In many wing-dimorphic Gerrids, winged morphs are larger, less reproductively active, and mate and lay eggs at reduced rates compared with wingless morphs (Roff and Fairbairn, 1991; Fairbairn and Preziosi, 1996). In the case of *R. antilleana* winged females, the presence of wings is associated with the extension of the pronotum, which suggests that the development of this antagonistic structure might be contingent on the activation of the wing development program, although this hypothesis remains to be experimentally tested. This structure is an efficient female resistance trait that allows winged females to reduce mating events and potentially increase their fitness. Inversely, winged males develop relatively simple antagonistic traits associated with a low mating success but with



the dispersal capability. In wingless morphs, males have evolved more elaborated secondary sexual traits that are associated with increased in mating success and the narrow abdomen of females allows male rejection but at a lower efficiency compared to winged females. These differences in mating success correlate with the higher egg numbers observed in wingless females that could potentially need to replenish sperm more often than winged females. However, how the decision to develop into winged or wingless morphs and whether sexual conflict affects this decision in *Rhagovelia* is unknown. *Rhagovelia* is a tropical genus and populations occur in large groups that can reach hundreds of individuals, suggesting that male harassment is frequent (Ditrich and Papacek, 2010). It is possible that under these conditions, winged morphs are favored to allow for dispersal and occupation of niches with lower population densities. However, winged morphs may not be favored in other contexts because of the trade-offs we observed between the presence of wings, the number of eggs produced by females, and the low success in pre-mating interactions we observed for males. It is more likely that trade-offs, between mating frequency, dispersal, and fertility are associated with differences in benefits and costs that maintain the variation of these traits and have shaped phenotypic evolution in this group of insects (Roff and Fairbairn, 1991).

Our study also highlights that antagonistic sexual interactions and morphological constraints might have driven evolutionary escalation in the *Rhagovelia* genus, similar to the dynamics

observed in many species of the Gerridae (Arnqvist and Rowe, 2002a,b). Our analysis has shown that, across this genus, males have evolved an arsenal of traits across different species while females only evolved two morph-specific armaments (i.e., narrow abdomen and pronotum projection) in only two species from a single clade in our sample. These species, *R. antilleana* and *R. obesa* both belong to the *collaris* complex in the genus *Rhagovelia* (Polhemus, 1997). This complex of 44 species, out of the 396 *Rhagovelia* species identified, is characterized by the presence of pronotum projection in winged females and narrow abdomen in wingless females (Polhemus, 1997; Polhemus and Chordas, 2010). This suggests that sexual antagonism had strongly impacted the evolution of the genus. In addition to morphological modifications, the females have evolved complex behavior among their resistance arsenal through shaking and vigorous somersaults. In our study we have conducted behavioral observation in one species and our arms race hypothesis is based on morphological traits only. It would be interesting to expand the analysis to the difference in female resistance behavior across *Rhagovelia* species and assess to which extant females rely on behavior rather than morphological modifications in this genus (Rowe and Arnqvist, 2002). These observations could provide important insights about how behavioral modifications could participate and influence escalation and arms race. This could also explain why females from only some species evolved morphological secondary sexual traits, and why females generally evolved less traits than males. At a broader scale, comparison



across families and therefore across different ecological niches, with Gerrids inhabiting stagnant water (Crumière et al., 2016) and *Rhavogelia* inhabiting stream water (Santos et al., 2017), would provide interesting insights about how habitats and lifestyles can influence behavioral and morphological coevolution (Perry and Rowe, 2018).

## MATERIALS AND METHODS

### Insect Sampling and Culture

Species were collected during fieldwork in the locations indicated in Table S1. Specimens from Brazil were collected under SISBIO permit # 43105-1. Specimens from other locations did not request any official authorizations. Lab populations were established for *R. antilleana*. They were kept in water tanks at 25°C, 50% humidity, 14 h of daylight and fed daily on crickets. Styrofoam floaters were provided for adult female egg laying. Adults and nymphs were raised in independent tanks to decrease nymph cannibalism.

### Imaging

Picture acquisition and observation of secondary sexual traits were performed using a SteREO Discovery V12 (Zeiss), a Stereomicroscope M205 FA (Leica) and using Scanning Electron Microscopy at Centre Technologique des Microstructures (UCBL).

### Muscle Staining

Male rear-leg femurs were dissected and opened using forceps and scalpel to remove the cuticle. Femurs were fixed with Formaldehyde 4% in PTW 1% during 20 min. Femurs were then washed in 5 successive 10-min baths of PTW 1%. The muscles were marked with 1/1,000 Phalloidine 488 (Invitrogen) for 1 h followed by 5 successive 10-min baths in PTW 1%. The femurs were mounted in 50% glycerol + Dapi.

### Analyze of Behavior During Pre-mating Struggles

Male behavior during pre-mating interactions has been analyzed through 4 different experiments. **Experiment 1:** 5 pairs of 2 males (4 pairs of 2 wingless males and 1 pair of 2 winged males) and 5 pairs of 2 females (1 winged and 1 wingless females) have been isolated during 5 days prior experiment in order to standardize mating receptivity in females and mating motivation in males. All individuals were taken from the stock population and were potentially non-virgin individuals. The experiment was performed during 5 consecutive days. Each day each pair of males were placed with a different pair of females in a common 14 × 10 cm plastic container with water, no food and no resting place. They were recorded during 10 min using a NIKON D7100 camera. We stopped trials when mating event occurred. Videos were analyzed using QuickTime software (Apple). Between each days/trials the different pairs were maintained isolated. With this set-up we obtained 5 biological replicates with 5 technical replicates for every pairs. **Experiment 2:** In this experiment we placed 1 male and 1 female from the general stock population in 8 × 6 cm plastic box containing water without food or resting

area and captured every interaction using a Phantom Miro M310 Digital High-Speed Camera and PCC Software (Vision research, Ametek) for video acquisition. We recorded interactions until we reached 20 videos per couples or until a mating event occurred. We used 3 couples (biological replicates) with 3 trials each (technical replicates) for the 4 different possible morph combinations: wingless male with wingless female, winged male with winged female, wingless male with winged female, and winged male with wingless female. **Experiment 3:** In this experiment we took males and females from the general stock population. In each trial we placed 2 males (1 winged and 1 wingless) with 1 female (winged or wingless) in a 14 × 10 cm plastic container with water, no food and no resting place and we counted the number of interactions of each male with the female during 10 min. We used 3 different females for each morph type (biological replicates) with 3 replicates for each female (technical replicates). **Experiment 4:** We took males and females from the general stock population. For each trial we placed 1 male with 1 female in a 14 × 10 cm plastic container with water, no food and no resting place and using a stopwatch with centisecond option we recorded the duration of interactions over 10-min observation or until mating event occurred. Interactions shorter than 0.15 s were too fast to take note and were not consider in the analysis of data. We used 3 couples (biological replicates) with 3 trials each (technical replicates) for the 4 different possible morph combinations: wingless male with wingless female, winged male with winged female, wingless male with winged female, and winged male with wingless female. **Data analysis:** Data collected from experiments 1 and 2 were used to analyse the way males approach females by counting and classifying the different types of approach into “anteriorly” if males approach from the front and block the female T1-legs (fore-legs), “posteriorly” if males approach from the back and block the female T3-legs (rear-legs), and from the side if the males block female T1- and T2-legs (mid-legs), T1- and T3-legs or T2- and T3 legs (Figure S2A). Data from experiments 1 and 3 were used to evaluate the interaction and mating frequencies by counting the total number of interactions and mating events and by calculating the ratio between these two variables (Figures S2B–D). Data from experiment 4 were used to quantify the duration of pre-mating interactions (Figure S2E).

### Quantification of Trait Functions

Non-virgin males and females were isolated into two different buckets (one for each sex), feed with frozen crickets, during 5 days to increase the motivation to mate. We set-up 10 tournaments between 8 males and 8 females (80 males and 80 females in total; with 22 winged females and 58 wingless females) to split them in three different categories: successful, intermediate and unsuccessful, depending the ability in copulating for males and the ability in rejecting male for females. Both males and females were introduced at the same time in a common 26 × 35 cm bucket of water without food or resting place. During the first round, for each mating we removed the couple and interrupted the mating by separating the male and the female in order to preserve the motivation to mate for the second round. The male was classified as successful and the female as unsuccessful. Inversely, the remaining males that did

not succeed to mate, despite interactions with females, were classified as unsuccessful in mating. Remaining females that were never mated, despite interactions with males, were classified as successful in rejecting. The first round goes on until we obtained 4 successful males and 4 unsuccessful females (Figure S3). For the second round, we used two different buckets. In the first bucket, we introduced, at the same time, the 4 successful males with the 4 successful females, and in the second bucket we introduced, at the same time, the 4 unsuccessful males with the 4 unsuccessful females. When mating occurred, we removed the couple from the bucket, as in the first round. The second round goes on until we obtained 2 successful males and 2 unsuccessful females per buckets. The different rounds of this experiment occurred for few minutes to several hours depending on the activity of the bugs. For each replicate males were actively harassing females. At the end, we were able to obtain the following rank for both males and females: successful for males that have mated twice and females that have never mated, intermediate for individuals that mated once, and unsuccessful for males that have never mated and for females that mated twice (Figure S3). Then, using SteREO Discovery V12 (Zeiss) with ZEN 2011 software (Zeiss) we took pictures of each individual to quantify the individual phenotypes. We measured the length and the width of the rear-leg femur, the length of the rear-leg tibia and we counted the number of spikes on the rear-leg femur and the number of spikes on the rear-leg tibia. We also recorded the presence of a pronotum projection, narrow abdomen and body length in females. To further test the role of the pronotum projection, we manually removed the pronotum projection from 18 winged females using forceps and kept these females in lab condition during 1 week to allow them to recover and assess whether they behaved normally. Then we set-up 6 new tournaments to compare the ability of these 18 winged females in rejecting males against 30 wingless females. Prior experiment, all females were kept isolated from males. All data are available in the Dryad Digital Repository (doi: 10.5061/dryad.8cq6427) (Crumière et al., 2019).

### Statistical Analysis

Cochran-Mantel-Haenszel chi-squared tests (Figures 3G–I) were performed on data of the tournament using only the replicates where both morph types were present in order to compare performance [i.e., winged vs. wingless; 9 replicates (72 females) for pronotum projection vs. narrow abdomen; 6 replicates (48 females) for amputated pronotum vs. narrow abdomen; 8 replicates (64 males) for winged vs. wingless morphs]. Proportional odds logistic regression (MASS package) (Venables and Ripley, 2002) were performed on data of the tournament using all individuals in order to analyse phenotypes related to mating performance. Shapiro tests, and Student *t*-tests or Wilcoxon tests with *fdr* correction for multiple comparisons were performed using RStudio version 1.0.153 for analysis of male preference, duration of interactions, comparison of trait values for successful and unsuccessful males, depending whether variables followed normal distributions. R Script used in this study is available in the Dryad Digital Repository (doi: 10.5061/dryad.8cq6427) (Crumière et al., 2019).

### Phylogenetic Reconstruction

Sequences were retrieved from in house transcriptome databases and from Crumière et al. (2016) for the following markers: *12S RNA*; *16S RNA*; *18S RNA*; *28S RNA*; *Cytochrome Oxidase subunit I (COI)*; *Cytochrome Oxidase subunit II (COII)*; *Cytochrome Oxidase subunit III (COIII)*; *Cytochrome b (cyt b)*; *NADH-ubiquinone oxidoreductase chain 1 (ND1)*; *Ultrabithorax (Ubx)*; *Sex combs reduced (Scr)*; *Gamma interferon inducible thiol reductase (gilt)*; *Antennapedia (Antp)*; *Distal-less (dll)*; *Doublesex (dsx)*. All these markers were submitted to GenBank and their accession numbers can be found in the Dryad Digital Repository (doi: 10.5061/dryad.8cq6427) (Crumière et al., 2019). Phylogenetic reconstruction (Figure S5) was performed using MrBayes version 3.2.6 (GTR model; one million generations; 25% burnin) and PhyML version 3.0 (GTR model with 100 bootstraps) in Geneious 7.1.9 as described in Crumière et al. (2016). Concatenation of sequence alignments and phylogenetic tree in Newick format are also available in the Dryad Digital Repository (doi: 10.5061/dryad.8cq6427) (Crumière et al., 2019).

### Assessment of Armament Escalation

We created a matrix of presence/absence of secondary sexual traits for both males and females (Table S2) in 13 species (4 outgroups and 9 *Rhagovelia* species). Based on morphological observation, we found eight traits exclusively present in males; the sex combs in the forelegs, the different rows of spines (up to 5 rows) and the shape of the different segments of rear-legs. Traits exclusively present in females include the pronotum projection and the narrow abdomen shape. Then, we mapped each sexually antagonistic trait individually and wing polymorphism on our phylogeny to infer their ancestral states (Figure S6). Finally, we performed a reconstruction using the combination of all traits, in males and females separately, to determine the pattern of phylogenetic complexity in this sample. Reconstruction of ancestral state was performed using ML reconstruction in Mesquite version 3.2 (Maddison and Maddison, 2018). Mapping of phenotypic complexity (ML reconstruction) and estimation of phylogenetic signal were performed using, respectively contMap and phylosig (package phytools, Revell, 2012). Correlation test with phylogenetic independent contrast has been conducted using the package ape (Paradis et al., 2004). These different analysis have been performed in RStudio version 1.0.153.

### Observation and Imaging of Egg Position and Egg Counting

We used CT Scan to observe egg position without disrupting internal morphology of our samples. We fixed females of *R. antilleana* ( $n = 5$  winged;  $n = 5$  wingless) and *R. obesa* ( $n = 5$  wingless) during 2 days using Bouin solution (MM France). Then, samples were washed twice with PBS 1X and were emerged 4 days in 0.3% phosphotungstic acid + 70% ethanol + 1% Tween20 solution to improve the contrast during microtomography acquisition (Metscher, 2009). Specimens were scanned using Phoenix Nanotom S (General Electrics) using the following parameters: 30 kV tensions, 200  $\mu$ A intensity, 2,400 images with time exposure of 2,000 ms and a 2.5  $\mu$ M voxel size. Three-dimensional images were reconstructed with the software



attached to the machine (data rec) and then visualized with VGStudioMax (Volume Graphics). Longitudinal pictures were acquired using this same software to assess the presence of wing muscles and the position of eggs. Further egg counting was performed after dissection of 20 winged and 20 wingless *R. antilleana* females.

## Two-Dimensional Morphometric Analyses of Body Shape

Pictures of adult females of *R. antilleana* ( $n = 10$  winged;  $n = 10$  wingless) and *R. obesa* ( $n = 9$  wingless) were acquired using SteREO Discovery V12 (Zeiss). The body outline of each individual was extracted using Adobe Illustrator CC 2017 (Adobe). The analysis of outlines of the different conditions and Principal Component Analysis were performed using the package Momocs (Bonhomme et al., 2014) in Rstudio version 1.0.153.

## CONTRIBUTION TO THE FIELD STATEMENT

Sexual conflict is ubiquitous in nature and is thought to be a potent force driving the evolution of sexually dimorphic traits. Under sexual conflict, the evolutionary interests of males and females in reproductive interactions often diverge, leading to the coevolution of sexually antagonistic traits that are favored in one sex at a fitness cost to the other. However, our understanding of the role of sexually antagonistic selection in driving the evolution of this dimorphism has been acquired through the study of some historical model systems. In this work, we studied the coevolution of males and females in the water strider genus *Rhagovelia*, to understand the evolutionary dynamics and the ecological factors that may influence the escalation of antagonistic traits between the sexes. In these insects, sexual dimorphism ranges from subtle to spectacular morphological differences between males and females; with various traits having a role during pre-mating struggles. By integrating morphological, behavioral and phylogenetic comparisons, we highlighted how sexual interactions and natural selection can influence and trigger diversification of secondary sexually antagonistic traits between males and females. Finally, our study promotes a new model system for the study of sexual conflict, which offers new perspectives for future studies.

## DATA AVAILABILITY

The data collected and resources used in this study are available on Dryad Digital Depository (doi: 10.5061/dryad.8cq6427) (Crumière et al., 2019).

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## AUTHOR CONTRIBUTIONS

AC and AK: conception and design of experiments, analysis and interpretation, and drafting manuscript. AC, DA, AV-L, MK, FM, and AK: acquisition of data, revising manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2019.00215/full#supplementary-material>

**Supplementary Video S1** | Interaction between a winged male and a winged female. The winged male attacks the female from the side by catching her by the mid- and rear-leg. Then the male climbs on the back of the female and block her mid- and rear-legs using his own rear legs as clamps. The male uses the fore-legs to catch the female pronotum, close to the head. The female resists by shaking its body to reject the male. This movie is a slow-motion video.

**Supplementary Video S2** | Interaction between wingless male and a winged female. The wingless male attacks the female by the back and climb on her while trying to block her mid- and rear-legs using its own rear-legs. The pronotum projection of the female forms a barrier increasing the distance between the male and the female thus facilitating male rejection. This movie is a slow-motion video.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## CHAPTER 5

## Discussion and perspectives

The main goal of this doctoral thesis was to understand the genetic and developmental basis underlying the diversity of extra-ocular colouration in the embryos of semi-aquatic insects. This work has established that the evolution of the extra-ocular pigmentation of Gerromorpha embryos is due to the specific co-option of one of the two eye pigment pathways, and those different events, such as maintenance and convergence, occurred during the evolution of this trait. Here, I review the main results and conclusions of the experiments, and analyse their contribution to the topic of evolution of complex morphological traits.

One of the major discoveries in evo-devo is that many genes and developmental processes are remarkably conserved throughout evolution, sometimes over large phylogenetic distances. From this discovery came the recognition that molecular co-option at the level of genes and proteins has played an important role in the production of evolutionary innovations. So far, identifying and answering the “why” of evolution for many genetic co-option events has been possible, but we have only an incomplete picture of “how” co-option acts (MCLENNAN 2008).

In this thesis, we introduce colouration of the semi-aquatic insects at the embryonic stage as a trait that can help to understand the genetic mechanism of co-option. We could identify by genetic and chemical analyses that the colours present in embryos of semi-aquatic insects are due to the presence of xanthopterin and erythropterin pigments, of the pteridine family.

In this project, we presented a phylogenetic reconstruction of the extra-ocular colouration using information obtained from 34 species of Gerromorpha from different clades. Interestingly, the yellow colour found on the appendages and abdomen was already present in the embryos in early diverging lineages of Gerromorpha, indicating that this trait was gained early, before emergence of this group. Bibliographic review presented in Chapter 1, confirms that the yellow colouration of the eggs is present in basally branching groups of Hemiptera (KITCHING 1974; MCPHERSON AND WILSON 1995; RONCHI-TELES *et al.* 2009). It would be interesting to perform a comparative analysis of embryonic pigmentation throughout the Hemiptera phylogeny. In our reconstruction, the evolution of red colouration involved at least six events of convergence and two independent losses. The literature review (Chapter 1) pointed out that the red extra-ocular colour is present in other Heteropteran groups but absent in Homoptera (Figure 4, Chapter 1) (STADLER *et al.* 1987; CERVANTES-PEREDO AND ELIZALDE-AMELCO 2007; FILIPPI *et al.* 2009). This suggests that the red colour patterns were gained in Heteroptera, with several events of convergence within this sub-order.

From the visual observation of different species of Gerromorpha embryos, we hypothesized that the red colour in appendages could be produced by the genetic co-option of the same pathway involved in the production of red colour in the eye. Pteridines are present in the eyes of many insects where they act as screening pigments, together with ommochromes (SUMMERS *et al.* 1982). The expression and functional analysis of orthologous genes from pteridine and ommochrome pathways first in *Limnogonus franciscanus*, and then in five other species revealed that pteridines were involved in the production of red colour in eyes and yellow/red colour in appendages. Interestingly, our results suggest that the network of genes from the pteridine pathway that we tested (*white, brown, punch, purple* and *rosy*) were co-opted once and were maintained without any changes during 200 million years of Gerromorpha evolutionary time. Together, our results suggest that the co-option of pteridine pigments in extra-ocular organs was produced early in the evolution of Heteroptera, given the basal position of Gerromorpha (LI *et al.* 2017). In addition, the conservation of the pteridine pathway, suggests that the diversity of colours and patterns is produced by upstream regulatory genes. We can now propose various possible scenarios to explain the different events of convergence and maintenance that occurred during the evolution of the trait. The first hypothesis involves an unknown enzyme “x” (Figure 8). Biochemical studies in Hemiptera and butterflies, showed that erythropterin (yellow-red colour) could be produced by enzymatic conversion of xanthopterin (yellow pigment) (FORREST *et al.* 1966). It might be that the gene coding for enzyme “x” was already present in the ancestral Gerromorpha embryos (yellow) to synthesize the red erythropterin in the eyes, and that the production of red in appendages could be due to regulatory changes. Moreover, the yellow species maintained the ancestral pathway without changes (Figure 8). The second proposition is based on the fact that erythropterin can change from yellow to red colour depending on its concentration. Erythropterin at low concentration is yellow and at high concentration produce red. The concentration of erythropterin changes the pH and the colour (MELBER AND SCHMIDT 1992; MELBER AND SCHMIDT 1994; MELBER AND SCHMIDT 1997). It might be that gene “x” was present in the extra-ocular organs in the ancestral Gerromorpha, but a quantitative change of expression of the gene encoding “x” produces red colour in specific cells (Figure 8). In the case of embryos without extra-ocular pigmentation, it is likely that in the ancestral state, possibly of the Homoptera or Paraneoptera groups, xanthopterin and erythropterin were absent in appendages, suggesting that the independent losses of the extra-ocular pigmentation could be explained by reversion events due to *cis* regulatory changes.

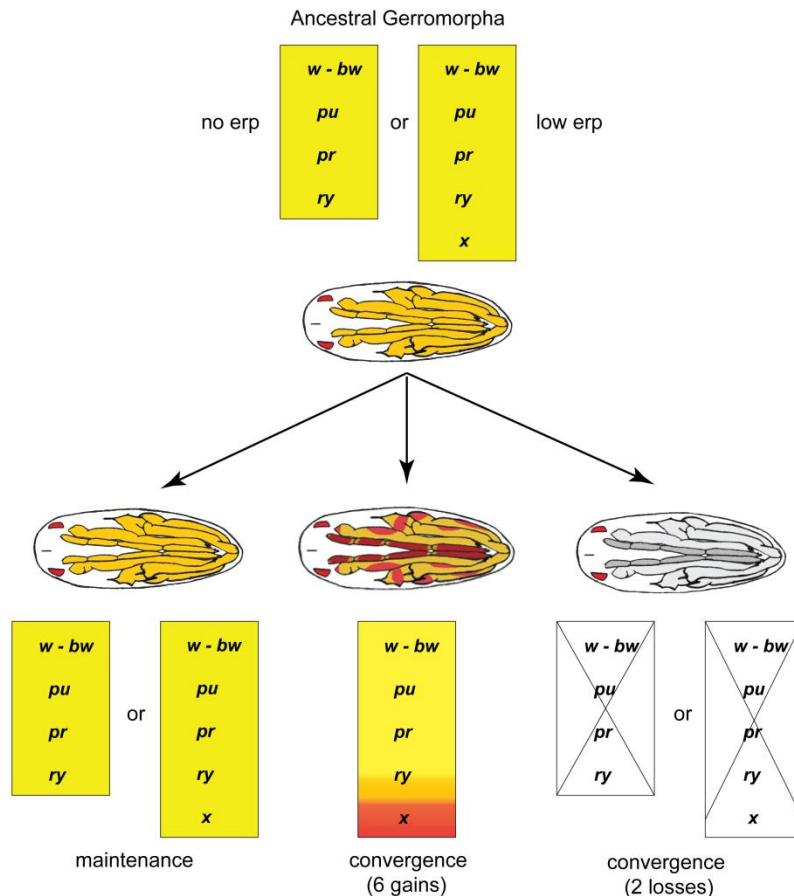


Figure 8. Hypothesis to explain the different evolutionary process of extra-ocular pigmentation in Gerromorpha embryos. The ancestral state of Gerromorpha was yellow with red eye. Hypothesis 1, the gene “x” that encoded the enzyme to produce erythropterin (red pigment) was absent in the ancestral Gerromorpha embryo (yellow embryos), then was independently gained during the evolution to produce the red colour in certain clades. Hypothesis two, the “x” gene was already present in the ancestor, but the regulation of the erythropterin concentration produced the red colour. The loss of the extra-ocular pigmentation is an example of reversion to the ancestral state anterior to Gerromorpha.erp= erythropterin, w= white, bw= brown, pu= punch, pr= purple, ry= rosy.

In conclusion in chapter two, it was established that the pigmentation in abdomen and appendages is due to the co-option of the same pathway producing pigments in the eyes. Also, we discovered that the pteridine pathway remained invariable during the 200 million years of Gerromorpha evolution. Then, how is it possible to produce different colours and patterns with the same pathway? Numerous studies have implicated regulatory variation in generating phenotypic diversity, both within populations and between species (BRADLEY *et al.* 2010; BARBOSA-MORAIS *et al.* 2012; JONES *et al.* 2012).

Chapter three is a study that aimed to understand the transcriptional regulation underlying the phenotypic variation of Gerromorpha embryos. In our study, we could establish that the genes that were involved in the production of the pigmentation of the eyes were co-opted to produce extra-ocular colours and colour patterns. We hypothesized that *trans* regulatory



changes in the pteridine pathway produce the diversity of colours and patterns. It might be possible that the ancestral state only had pteridine pigments in the eye, after *trans* regulatory changes during evolution, the yellow and later red colour appeared in extra-ocular tissues (Figure 9A). To investigate the possible *trans* regulatory mechanism involved in the evolution of extra-ocular pigments, we used RNA-seq of three red pigmented organs from *Limnogonus franciscanus* embryos to build a list of candidate transcription factors (TFs) expressed in common between these three tissues. With this experiment, we expected that after the inactivation of the genes expression using RNAi we could observe a loss of the colour in the eyes and appendages. Approximately, 60% of the genes were tested by functional assays. Until now, we failed to identify a transcription factor involved in the production of the pigmentation. I consider that the remaining 40% candidate genes should be functionally tested. In parallel, a differential expression analysis could be made between species pigmented (*Limnogonus franciscanus* for example) against species with unpigmented appendages (such as *Oiovelia* or *Paravelia*) to identify TFs involved in the regulatory mechanism of the pteridine pathway. In this experiment we expect that the transcriptional regulator involved in the yellow and red colouration should be expressed in pigmented species and absent in the tissues coming from naturally unpigmented species.

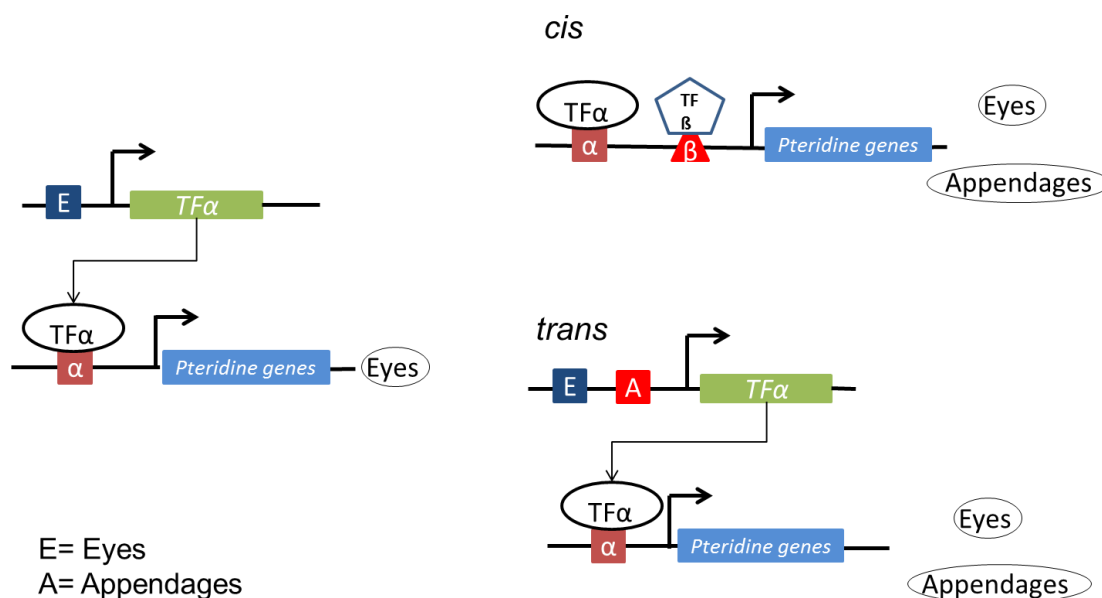
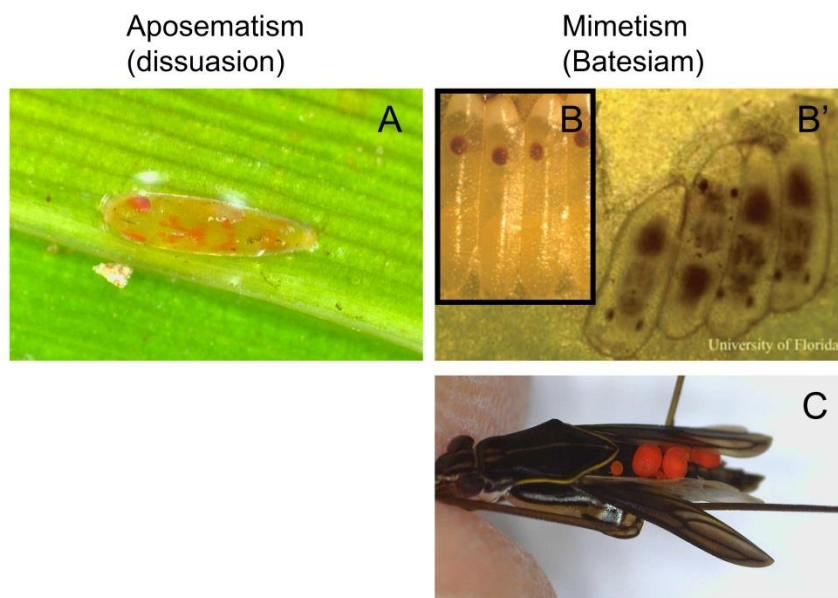


Figure 9. Hypothesis on gene regulatory evolution of the pteridine pathway. Likely, the ancestor embryo of Hemiptera was extra-ocular colourless; after mechanistic regulatory changes at level of genes regulators (*trans*) or mutations in the *cis* regulatory elements produced the co-option of the pteridine pathway in appendages.

An alternative hypothesis to explain the diversity of colour and patterns might be changes in *cis* regulatory regions of the downstream genes of pteridine pathway (*brown*, *white*, *punch*, *purple* and *rosy*). Likely, *cis* regulatory mutations occurred and produced changes in the expression of the effector genes of pteridine pigment pathway in appendages of embryos (Figure 9B). A possible way to test this hypothesis is a genome-wide search in promoter regions of pteridine effector genes already described (VARGAS-LOWMAN *et al.* 2019). With this approach, it is possible to identify motifs that correspond to consensus binding sites shared between the promoter regions of the five effector genes of the pteridine pathway. While some of the genes involved in the production of pteridine pigments have been studied from the origin of genetics (MORGAN 1910; KLEMENZ *et al.* 1987; PITTS AND ZWIEBEL 2001), there is a surprising lack of information about their regulation, even in a model system such as *Drosophila*. For this reason, finding consensus binding site sequences is the principal challenge, but several *in silico* approaches (BAILEY *et al.* 2009; KHAN *et al.* 2017) have been developed to construct positions weight matrix (PWM) and to make predictions of potential binding sites in the two genomes that are available, *Gerris buenoi* and *Microvelia longipes*.

The function of the extra-ocular pigmentation in semi-aquatic insects during the embryonic stage is another open question. In Chapter one, I made a correlation between the sites where Heteroptera females lay their embryos and the colour of the eggs. In general, species that lay embryos inserted in substract, hidden or with paternal care, are yellowish-white (KITCHING 1974; AL-WAHAIBI AND MORSE 2009) (Chapter 1, Table 2). The eggs deposited on exposed substracts are either dark or with transparent chorion and red colouration in appendages (STADLER *et al.* 1987; CERVANTES-PEREDO AND ELIZALDE-AMELCO 2007; FILIPPI *et al.* 2009) (Gerromorpha, this manuscript). Therefore, we can speculate that the embryonic colours of Gerromorpha have a protective role, either by providing invisibility (camouflage) or, on the contrary, by sending a visual warning signal (mimicry, aposematism). It is important to mention that embryonic development in the semi-aquatic bugs in average can take two weeks (ANDERSEN 1982), and that during this period the embryos are susceptible to parasitism and/or predation. Information about natural enemies of Gerromorpha embryos or Hemiptera embryos in general is very scarce. *Pseudoligosita plebeia* and *Gonatocerus* wasps parasite eggs of *Homalodisca vitripennis*, the glassy-winged sharpshooter (Hemiptera: Cicadellidae) (TIPPING AND MIZELL 2004; M LYTLE *et al.* 2012) The eggs of semi-aquatic insects are parasitized by different species of chalcidoid and proctotrupoid wasps (ANDERSEN 1982). The hypothesis of camouflage is implausible,

given the sharp contrast between the bright red and yellow colours of the embryos and the tint of the supports where they are laid (Figure 10A), which are green or brownish plants. Invisibility would require that the putative predators have a different visual perception of the colours, which is possible. However, given the very wide range of predators and parasites, this possibility is unlikely. If we assume that this pigmentation pattern is visible to all, then it may be used to warn predators against toxicity or unprofitability. Therefore, we have two hypotheses to test: aposematism and mimicry. We can speculate that the yellow/red colour could be used as an aposematism mechanism to deter predators (FABRICANT *et al.* 2013). Another possibility could be Batesian mimicry, where the red colouration in the appendages of the embryos could mimic parasitized eggs. If an egg seems to contain an insect ready to emerge, then parasitic wasps could be misled and avoid laying into this apparently already



occupied embryo (Figure 10B). This is observed in several species of Lepidoptera, where some eggs resemble to parasitized eggs (HINTON 1981). Another strategy could be to mimic unpalatable species, as it is known that some species of Gerromorpha adults are parasitized by unpalatable red mites (Khila A. personal communication) (Figure 10C).

This thesis has contributed to our understanding of the evolution and diversification of extra-ocular pigmentation in Gerromorpha, and can be used as a basis for a range of exciting studies into the origin diversification and function of morphological novel traits. We still have a list of candidate genes that should be functionally tested and to give more insights about the production of variability of pattern after co-option events.



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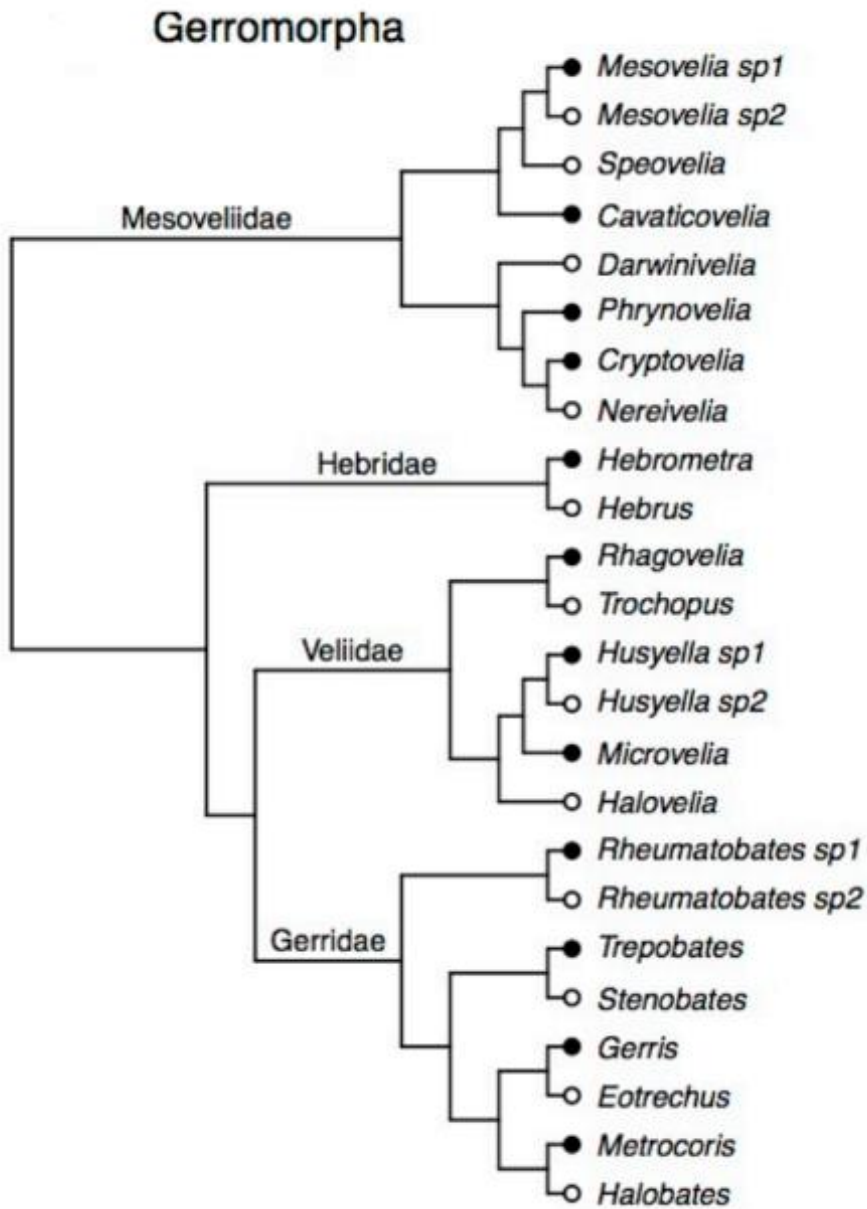
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## **Annexes**



Annexes Figure 1. Simplified tree of Gerromorpha showing 12 out of the 14 independent transitions to marine environment confirmed by Andersen (1999). The groups indicated at the leaves are those we plan to sample (white = marine, black = freshwater). Figure taken from Convergenomix 2015: Integrative study of convergent genomic evolution across three animal groups.

## **Genome-wide markers-based phylogenetic of Gerromorpha (In preparation)**

### **Synopsis of the paper:**

Gerromorpha, also known as semi-aquatic bugs, is a cosmopolitan group of insects that evolved from terrestrial niches to the surface of water. The radiation of semi-aquatic bugs has generated over 2,000 species classified into eight families. Gerromorpha are found in different niches such as streams, lakes, ponds, marshes and even the open ocean. In the recent years the semi-aquatics bugs have been used as emerging models in the evo-devo field to study the genetic basis of morphological structures and developmental processes involved in the adaptation to new environments. Also, these are prominent models to study the evolution of sexually selected traits. The most recent phylogeny of Gerromorpha was published in 2008, and was made using parsimony analysis based on morphological characters and three DNA markers (DAMGAARD 2008). In this phylogeny, the relation of some families was not resolved, and only four families were well supported (Mesoveliidae, Hebridae, Hydrometridae and Gerridae). The relationship between the sister groups Gerridae and Haloveliinae + Microveliinae is problematic. The aim of this study was to build a new phylogeny to resolve certain important clades and to have a better framework to test hypotheses about the evolution of different traits.

### **Summary of results:**

Phylogenetic reconstruction based on 72 ortholog genes of 100 species confirmed the monophyletic classification of Gerromorpha. The basal families Mesoveliidae, Hebridae, and Gerridae were monophyletic, whereas Hydrometridae is paraphyletic. In our analysis, the subfamilies Microveliinae + Haloveliinae appeared as sister to Gerridae and we propose to move it to family Gerridae. As previous works (ANDERSEN 1982; DAMGAARD 2008); the relationships among basal families (Hermatobatidae, Macroveliidae, Paraphrynoveliidae, Mesoveliidae, Hebridae, and Hydrometridae) was not clarified in our phylogeny due to the lack of samples for the Hermatobatidae, Macroveliidae, Paraphrynoveliidae families, but we established that the familie Mesoveliidae is sister to (Hebridae + Veliometra), and the three families are sister to (Hydrometra + (Veliidae + Gerridae)).

### **Conclusion:**



Our results confirm the monophyletic classification of Gerromorpha and solved some important clades presented in the previous phylogenies of Gerromorpha. Moreover, a new phylogenetic reconstruction brings us the possibility to understand the evolution and diversification of different traits related with the ability to conquer different habitats.

**Personal contribution:**

Aidamalia Vargas-Lowman: extraction of RNA of 50 percent of the species used in the construction of the phylogeny, assembly of full transcriptomes.

# AIDAMALIA VARGAS LOWMAN

304 Ave. Jean Jaurès, 69007 Lyon, France/ amaval025@gmail.com

## Experience

- Current** Assistant Professor, Department of Genetics and Molecular Biology, University of Panama
- 2004- 2007** Supervisor, Laboratorio Achotines, Inter-American Tropical Tuna Commission (IATTC)
- 1999-2004** Voluntary work and Laboratory Assistant, Smithsonian Tropical Research Institute (STRI)

## Education

- Current** PhD (last year) in Molecular and Cellular Biology, University of Lyon 1, Ecole Normale Supérieure de Lyon, France
- 2012-2014** Master degree in Genetic and Molecular Biology University of Panama, Panama
- 1999-2003** Degree (Licenciatura) in Biology, University of Panama, Panama

## Researches and Publications

“Aspectos fisiológicos y morfométricos de las especies *Strombus raninus* y *Strombus gracilior* (Mesogastropoda: Strombidae), composición bioquímica y análisis histológico en la especie *Strombus raninus*”. Degree thesis, 2003

“Estudio del polimorfismo genético de la apolipoproteína E en la población mestiza y amerindia de Panamá”. Master thesis, 2014

David Armisen, Rajendhran Rajakumar, Markus Friedrich, Joshua B. Benoit, Hugh M. Robertson, Kristen A. Panfilio, Seung-Joon Ahn, Monica F. Poelchau, Hsu Chao, Huyen Dinh, Harsha Vardhan Doddapaneni, Shannon Dugan, Richard A. Gibbs, Daniel S. T. Hughes, Yi Han, Sandra L. Lee, Shwetha C. Murali, Donna M. Muzny, Jiaxin Qu, Kim C. Worley, Monica Munoz-Torres, Ehab Abouheif, François Bonneton, Travis Chen, Li-Mei Chiang, Christopher P. Childers, Andrew G. Cridge, Antonin J. J. Crumière, Amelie Decaras, Elise M. Didion, Elizabeth J. Duncan<sup>1</sup>, Elena N. Elpidina, Marie-Julie Favé, Cédric Finet, Chris G. C. Jacobs, Alys M. Cheatle Jarvela, Emily C. Jennings, Jeffery W. Jones, Maryna P. Lesoway, Mackenzie R. Lovegrove, Alexander Martynov, Brenda Oppert, Angelica Lillico-Ouachour, Arjuna Rajakumar, Peter Nagui Refki, Andrew J. Rosendale, Maria Emilia Santos, William Toubiana, Maurijn van der Zee, Iris M. Vargas Jentsch, **Aidamalia Vargas Lowman**, Severine Viala, Stephen Richards and Abderrahman Khila<sup>1</sup>. The genome of the water strider *Gerris buenoi* reveals expansions of gene repertoires associated with adaptations to life on the water. 2018, *BMC Genomics* 19:832

Antonin Jean Johan Crumière, David Armisen, **Aidamalia Vargas-Lowman**, Martha Kubarakos, Felipe Ferraz Figueiredo Moreira and Abderrahman Khila. Escalation and Morphological Constraints of Antagonistic Armaments in Water Striders. 2019, *Frontiers in Ecology and Evolution*, section Coevolution (7):215

**Aidamalia Vargas-Lowman**, David Armisen, Carla Fernanda Burguez Floriano, Isabelle da Rocha Silva Cordeiro, Séverine Viala, Mathilde Bouchet, Marie Bernard, Augustin Le Bouquin, M. Emilia Santos, Alexandra Berlioz-Barbier, Arnaud Salvador, Felipe Ferraz Figueiredo Moreira, François Bonneton, Abderrahman Khila. Coptation of the pteridine biosynthesis pathway underlies the diversification of embryonic colors in water striders.

**Proceedings of the National Academy of Sciences** Sep 2019, 116 (38) 19046-19054; DOI: 10.1073/pnas.1908316116

## Grants

- 2002:** Elektra NORESTE/ STRI, Degree fellowship (2500 USD)

**2010:** Secretaria Nacional de Ciencia y Tecnología (SENACYT) Grant for “New Researchers” “I Convocatoria Pública para Nuevos Investigadores” for 24 months (16000 USD)  
**2015:** SENACYT/ Instituto para la Formación y Aprovechamiento del Recurso Humano (IFARHU) “Programa Nacional de Estudios de Postgrado en Ciencias Básicas” PhD fellowship for 4 years (199,439 USD)

### Presentation at scientific conferences

**2002:** 55° Gulf and Caribbean Fisheries Institute Congress in Xel-Ha, Quintana Roo, México  
**2016:** Internal Seminar, “Embryonic pigmentation as a model system to study convergent evolution in the semi-aquatic insects”. Ecole Normale Supérieure, Lyon, France.  
**2017:** Second Joint SFBD / SBDF Meeting. 26-29 April, 2017. Lyon, France  
**2018:** 7th meeting of the European Society for Evolutionary Developmental Biology (EED). June 26-29, 2018 in Galway, Ireland.  
**2018:** SPBD/SEBD/SFBD joint meeting Porto. 7-10 November, 2018.

### Scientific training course attendance

NAUI Worldwide Diving Course Standards, STRI-Panamá  
**2002:** Taller Uso Sustentable del Recurso Caracol *Strombus gigas*, Mérida, Yucatán, México  
**2004:** Voluntary work in Florida Fish and Wildlife (FWC) Stock Enhancement Research Facility (SERF) (two months). Port Manatee, Florida, EEUU  
**2006:** Taller de Fisiología y Acuicultura de Especies Pelágicas con Énfasis en Reproducción y Desarrollo de Estadios Tempranos del Atún Aleta Amarilla (*Thunnus albacares*). Inter-American Tropical Tuna Commission (IATTC) / University of Miami. Los Santos, Panama  
**2006:** II Curso Internacional de Acuicultura “Sistemas Productivos en Acuicultura: Metodologías, Avances y Proyecciones”, Universidad de Concepción, Chile  
**2009:** Diploma course of Forensic Genetic, Instituto de Medicina Legal y Ciencias Forenses, Ministerio Público, Panamá.  
**2012:** First course of Molecular Biology and Genomic, Universidad Nacional Autónoma de México, Mexico  
**2014:** Course in Biotechnology, United Nations University – Universidad de Granada – Universidad de Panamá  
**2016:** Exploring Biological Sequence Data course. 01-03 Novembre, 2016. EMBL-EBI, Hinxton.  
**2016:** Formation Philogénie Moléculaire (Lyon) 22-25 March, 2016. CNRS Formation Entreprises.  
**2016:** Anglais pour doctorants. January, 2016. Université de Lyon 3, France  
**2017:** Phylogenetic Analysis using R. 06-10 March, 2017. Paleontological Restoration and Interpretation Center – CRIP, Barcelona, Spain.

### Foreign Languages

**Spanish:** Native speaker

**English:** Good

**French:** Good

