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

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, et al.

To cite this version:
Aidamalia Vargas-Lowman, David Armisen, Carla Fernanda Burguez Floriano, Isabelle da Rocha Silva Cordeiro, Séverine Viala, et al.. Cooption of the pteridine biosynthesis pathway underlies the diversification of embryonic colors in water striders. Proceedings of the National Academy of Sciences of the United States of America, National Academy of Sciences, 2019, 116 (38), pp.19046-19054. 10.1073/pnas.1908316116. hal-03078427

HAL Id: hal-03078427
https://hal.archives-ouvertes.fr/hal-03078427

Submitted on 16 Dec 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives| 4.0 International License
Cooption of the pteridine biosynthesis pathway underlies the diversification of embryonic colors in water striders

Aidamalia Vargas-Lowman, David Armisen, Carla Fernanda Burguez Floriano, Isabelle da Rocha Silva Cordeiro, Séverine Viola, Mathilde Bouchet, Marie Bernard, Augustin Le Bouquin, M. Emilia Santos, Abderrahman Khila, Alexandra Berlioz-Barbier, Arnaud Salvador, Felipe Ferraz Figueiredo Moreira, François Bonneton, and Abderrahman Khila.*

©Institut de Génomique Fonctionnelle de Lyon, Université Lyon, CNRS UMR 5242, Ecole Normale Supérieure Lyon, Université Claude Bernard Lyon 1, 46 allée d’Italie F-69364 Lyon, France; Laboratório de Biodiversidade Entomológica, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21040-360 Rio de Janeiro, Brazil; Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON MSS 3B2, Canada; 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 Université de Lyon, Université Claude Bernard Lyon 1, Institut des Sciences Analytiques, CNRS UMR 5280, F-69100 Villeurbanne, France

Edited by Claude Desplan, New York University, New York, NY, and approved August 9, 2019 (received for review May 14, 2019)

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 (Gerrimorpha). 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 extracuticular 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 extracuticular 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 Gerrmorpha embryonic time. Finally, we identified erythroptorin 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.

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.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1908316116/-/DCSupplemental.

Published online September 4, 2019.
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

![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 ovi (egg burster or hatching line). Arrowheads indicate the eyes. Asterisks indicate species with dark eggshell. (Scale bars: 100 μm.)](https://www.pnas.org/content/116/38/19047)
S1 and Figs. S4 and S5). We obtained strong support (99%) for the yellow extraocular color as ancestral, probably predating the divergence of the Gerromorpha (Fig. 2A and SI Appendix, Figs. S4 and S5 and Tables S2 and S3). In addition, red extraocular color as a derived character was strongly supported (72%) and evolved at least 6 times independently in this sample (Fig. 2B and SI Appendix, Figs. S4 and S5 and Tables S2 and S3). This analysis also identified 2 independent events with strong support (99% and 93%) for complete loss of extraocular color (Fig. 2A and SI Appendix, Figs. S4 and S5 and Tables S2 and S3). Finally, 3 distinct lineages independently evolved dark color patches on the eggshell (Fig. 2). Interestingly, we could not find any species with red color in any of these 3 lineages, suggesting that bright red color is associated with transparent eggshell. Altogether, these findings indicate a common origin of extraocular color followed by a diversification of color shades and spatial patterns in the Gerromorpha. This rich diversity in embryonic coloration provides a suitable model to study the developmental genetic and evolutionary processes underlying the origin and diversification of color phenotypes.

**Activation of Pteridine Biosynthesis Is Responsible for the Extraocular Pigmentation in the Embryo.** Because of the striking resemblance between eye and extraocular color in the embryos of the red species (Fig. 1), we hypothesized that this coloration results from the production of eye pigment outside the eyes. Insect eye color results from the accumulation of ommochrome and/or pteridine pigments (28). The ommochrome pathway converts tryptophan

![Phylogenetic reconstruction of the ancestral state of extraocular colors in Gerromorpha.](image-url)

**Fig. 2.** Phylogenetic reconstruction of the ancestral state of extraocular colors in Gerromorpha. (A) Ancestral state reconstruction of the presence/absence of yellow in antennae and/or legs. (B) Ancestral state reconstruction of the presence/absence of red in the same appendages. Nodes are colored according to the most probable ancestral state of that node (maximum likelihood). White stars indicate losses of extraocular color, grey stars loss of red color, and black stars gains of red color. Asterisks indicate species with a dark pigmented chorion. Estimated landmark dates for the infraorder Gerromorpha (−211 mya), the superfamily Gerroidea (−191 mya) and the family Gerridae (−117 mya) (dates from timetree: ref. 62).
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 omochromes 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 extracocellular activation of eye pigment biosynthesis pathways.

To determine whether omochromes or pteridine, or both, are responsible for the extraocular pigment, we tested the role of specific genes from each pathway. For omochromes, we selected cninabar (cn) that encodes an enzyme and scarlet (st) that encodes an ABC transporter (Fig. 4I). The expression of cninabar is confined in the eyes (Fig. 4J), and inactivation of this gene by RNAi resulted in embryos where the eyes became bright red but where the extracocellular coloration remained unaffected (Fig. 4K). Unlike cninabar, the expression pattern of scarlet prefigured the red coloration in the eyes and in the extracocellular 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 extracocellular 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 extracocellular color production despite its expression there. These results show that omochromes pigments are responsible for dark coloration in the eye but not for the extracocellular 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 extracocellular color patterns (Fig. 4O) and that RNAi against brown resulted in the complete loss of red color both in the eyes and in extracocellular tissues (Fig. 4 P and Q). This phenotype is similar to the effect of white RNAi in both eyes and extraocular tissues (Fig. 4 G 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 Gerris buesnon (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 extracocellular color is produced by pteridine pigment biosynthesis and that eye color is produced by the combination of pteridine and omochromes 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 E, SI Appendix, Fig. S8). Both punch and purple mRNA expression patterns prefigured the pattern of pigment distribution in L. franciscanus embryos (compare Fig. 5 B, D, and F). RNAs against both genes yielded identical results consisting of the elimination of extracocellular pigment (compare Fig. 5 C, 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 extracocellular pigments in L. franciscanus embryos.

To further complete the analysis of pteridine biosynthesis pathway recruitment in Limnogonus extracocellular 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
for the production of the red aurodrosopeterin and drosopeterin 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 Gerromorpha 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 (36, 41) (Fig. 6). In a species with no extraocular pigmentation, such as *Oiovelia cuniculiformis* (Fig. 6B), rosy is expressed only in the eyes (Fig. 6F). 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. 6G 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 cyclodrolase-I punch (SI Appendix, Fig. S10). These results show that, despite the
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 *Limonopus 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 *sepio* 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. 6. Expression and function of ros outstanding. (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 ros (ry) mRNA. (M–R) Effect of ry RNAi on pigmentation across this species sample. (Scale bars: 100 μm.)](image-url)
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 redeplication 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 lands 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 antitileana, Microvelia americana, Neogerris magnus, and L. flavicans was 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 ICS 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 10x 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 Ficol 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), InstiProfix V5i robot was programmed to make all previous steps. A blocking step was performed manually (1× PBT (1× PBS; 0.3% Triton X-100) at room temperature followed by incubation with anti-dig antibody coupled with alkaline phosphate 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 MgCl2; 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 NaH₂PO₄/NaH₂PO₄ pH 6.8, 2.5 M KC). 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. Parental RNAi and SS and Table S5 was 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 MgCl₂; 0.1 M NaCl; 0.1% Tween-20). Photos were captured with DM 6000 Leica microscope.

Pteridine Pigment Extraction and Identification. Pteridines were extracted using a modified Robson et al. method (61). Briefly, 1,200 whole post-katatrepis (a process in some insects through which the embryo turns 180° and positions itself at the water surface side of the yolk) correctly oriented embryos were grinned in liquid nitrogen and sonicated for 10 min. As 6-bioterin 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.

ACKNOWLEDGMENTS. We thank Camille Mermet-Bouvier for help with bug rearin, Michalis Averof and Alistair McGregor for discussions, and all the members of the A.K. team for comments and helpful discussions. Thanks also go to master students Sophie Carre, Marie Nalin, Thomas Planelli, Cindy Planus, and Charlie Schaeffer for the development of pteridine extraction and the Compound Library and Custom Synthesis of the “Institut de Chimie et de Biochimie Moléculaires et Supramoléculaires” (Lyon 1) for the synthesis of erythropterin. Specimens from Brazil were collected under Sistema de Autorização e Informação em Biodiversidade (SISBIO) permit 43105.1. This work was funded by European Research Council Consolidator Grant 616346, Agence Nationale de la Recherche, France (ANR) Convergenomix, and Conselho Nacional de Desenvolvimento Científico e Tecnológico - Pesquisador Visitante Especial (CNpq-PVE) Grant 400751/2014-3 (to A.K.); Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) Grants E-26/202.203/2017 and E-26/260.149/2019 (to F.M.); a PhD Panama Secretaria Nacional de Ciencia, Tecnologia e Innovacion (SENACYT) fellowship from Panama (to A.V.-L.); and a PhD scholarship from Coordenacao de Aperfeicoamento de Pessoal de Nivel Superior–Brazil (CAPES; to I.C.).

1. T. Caro, Wallace on coloration: Contemporary perspective and unresolved insights. Trends Ecol. Evol. (Amst.) 32, 23–30 (2017).
2. A. R. Wallace, The colors of animals and plants. Am. Nat. 11, 641–662 (1877).
3. R. K. Marshall, M. Stevens, Wall lizards display conspicuous signals to conspecifics and red signals to avian predators. Behav. Ecol. 25, 1325–1337 (2014).
4. U. E. Siebeck, A. N. Parker, D. Sprenger, L. M. Mächler, G. Wallis, A species of reef fish that uses ultraviolet patterns for covert face recognition. Curr. Biol. 20, 407–410 (2010).
5. S. D. Finkbeiner, A. D. Briscoe, R. D. Reed, Warning signals are seductive: Relative contributions of color and pattern to predator avoidance and mate attraction in Heliconius butterflies. Evolution 68, 3410–3420 (2014).
6. W. B. Watt, Adaptive significance of pigment polymorphisms in collas butterflies. I. Variation of melanin pigment in relation to thermoregulation. Evolution 22, 437–458 (1968).
7. P. M. Brakefield, P. G. Willmer, The basis of thermal melanin in the ladybird Adalia bipunctata: Differences in reflectance and thermal properties between the morphs. Heredity 59, 9–14 (1985).
24. R. H. Cobben, N. M. Andersen, A. J. J. Crumière, G. Shamim, S. K. Ranjan, D. M. Pandey, R. Ramani, Biochemistry and biosynthesis of Pterin pigment granules are responsible for the warning coloration of Heteroptera. J. Chromatogr. A 1336, 94–100 (2014).

25. C. Melber, G. H. Schmidt, Identification of fluorescent compounds in certain species of Dysdercus and some of their mutants (Heteroptera, Pyrrhocoridae). Comp. Biochem. Phys. B 101, 115–133 (1992).

26. T. P. Keith et al., Structure of the functional gene for xanthine dehydrogenase (rosy locus) in Drosophila melanogaster. Genetics 116, 67–73 (1987).

27. C. Melber, G. H. Schmidt, Quantitative variations in the pteridines during the post-embryonic development of Dysdercus species (Heteroptera, Pyrrhocoridae). Comp. Biochem. Phys. B 108, 79–94 (1994).

28. A. Albert, Quantitative studies of the avidity of naturally occurring substances for trace metals. 3. Pteridines, riboflavin and purines. Biochem. J. 56, 646–654 (1953).

29. A. Albert, The transformation of purines into pteridines. Biochem. J. 65, 124–127 (1957).

30. P. E. Lederer, Les pigments des invertébrés (à l’exception des pigments respiratoires). Biol. Rev. Camb. Philos. Soc. 15, 273–306 (1940).

31. A. H. Bartel, B. W. Hudson, R. Craig, Pteridines in the milkweed bug, Oncopeltus fasciatus (Dallas). 1. Identification and localization. J. Insect. Physiol. 2, 348–354 (1958).

32. H. S. Forrest, M. Menaker, J. Alexander, Studies on the pteridines in the milkweed bug, Oncopeltus fasciatus (Dallas). J. Insect. Physiol. 12, 1411–1421 (1966).

33. R. L. Smith, H. S. Forrest, Pattern of pteridine accumulation in eggs of Pyrrhocoris apterus. J. Insect. Physiol. 15, 953–957 (1969).

34. J. M. Smith et al., Developmental constraints and evolution: A perspective from the mountain lake conference on development and evolution. Q. Rev. Biol. 60, 265–287 (1985).

35. P. Beldade, K. Koops, P. M. Brakefield, Developmental constraints versus flexibility in morphological evolution. Nature 416, 844–847 (2002).

36. W. R. Cooper, D. W. Spurgeon, Oviposition behaviors and ontogenetic embryonic characteristics of the western tarnished plant bug, Lygus hesperus. J. Insect. Sci. 12, 36 (2012).

37. D. Rodrigues, G. R. P. Moreira, Comparative description of the immature stages of two very similar leaf footed bugs, Holymenia clavigera (Herbst) and Anisoscelis folicacea marginella (Dallas) (Hemiptera, Coreidae, Anisocelidae). Rev. Bras. Entomol. 49, 7–14 (2005).

38. J. Liu, “Unravelling the molecular mechanisms Of aposematic pigmentation in oncopeltus fasciatus,” Dissertations, Wayne State University, Detroit, MI (2016).

39. C. Melber, G. H. Schmidt, Body coloration related to the deposition of pteridines in the epidermis and other organs of Dysdercus species (Insecta: Heteroptera: Pyrrhocoridae). Comp. Biochem. Phys. A 116, 17–28 (1997).

40. S. A. Fabrictiar, D. J. Kemp, J. Krajicek, Z. Bosakova, M. E. Herberstein, Mechanisms of color production in a highly variable shield-back stinkbug, tectocoris diopthalmus (Heteroptera: Scutelleridae), and why it matters. PLoS One 8, e64082 (2013).

41. P. K. Abram et al., An insect with selective control of egg coloration. Curr. Biol. 25, 2007–2011 (2015).

42. F. Bonneton, When Tribolium complements the genetics of Drosophila [in French]. Med. Sci. (Paris) 26, 297–303 (2010).

43. P. N. Refki, D. Arminiet, A. J. Crumiere, S. Viala, A. Khila, Emergence of tissue sensiti- vity to Hox protein levels underlies the evolution of an adaptive morphological trait. Dev. Biol. 392, 441–453 (2014).

44. A. Khila, E. Abouheif, L. Rowe, Evolution of a novel appendage ground plan in water striders is driven by changes in the Hox gene Ultrabithorax. PLoS Genet. 5, e1000583 (2009).

45. E. Paradis, J. Claude, K. Strimmer, APE: Analyses of phylogenetics and evolution in R. Bioinformatics 19, 53–60 (2003).

46. Q. Rev. Biol. 60, 265–287 (1985).