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

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

[a]Institut de Génomique Fonctionnelle de Lyon, Université Lyon, CNRS UMR 5242, Ecole Normale Supérieure de Lyon, Université Claude Bernard Lyon1, 46 allée d'Italie F-69364 Lyon, France; [b]Laboratório de Biodiversidade Entomológica, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, 21040-360 Rio de Janeiro, Brazil; [c]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON M5S 3B2, Canada; [d]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 Lyon1, 69622 Villeurbanne, France; and [e]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.

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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).

[1]Present address: Department of Zoology, University Museum of Zoology Cambridge, University of Cambridge, Cambridge CB2 3EJ, United Kingdom.

[2]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. S2*F*). 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

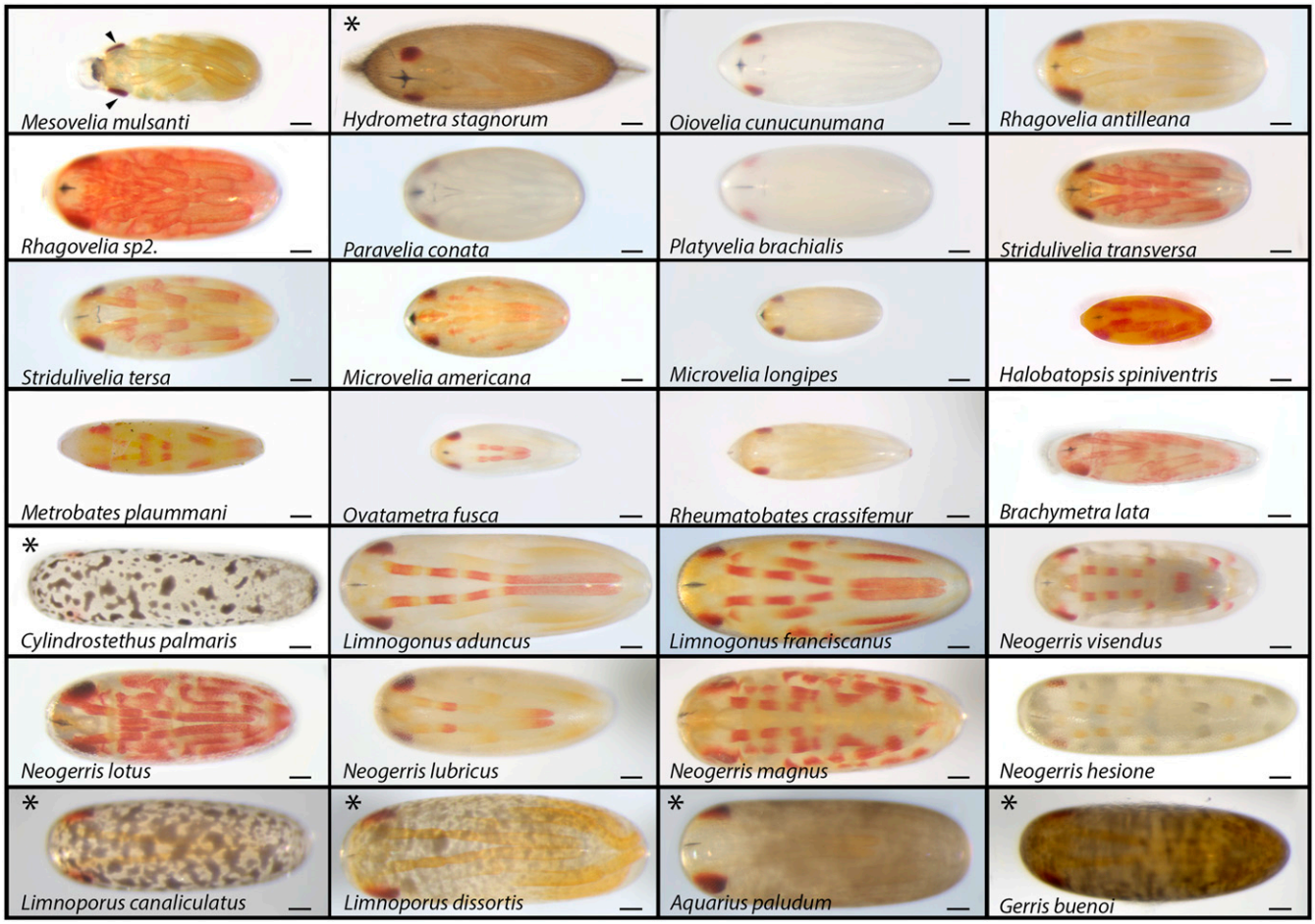

Fig. 1. Diversity of color patterns in the embryos of water striders. Species names are indicated under each image. All embryos are shown in ventral view. Embryos are wild type after completion of katatrepsis, a process where the embryo turns within the egg before dorsal closure, and formation of *ruptor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

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. 2*A* 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. 2*B* 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. 2*A* 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

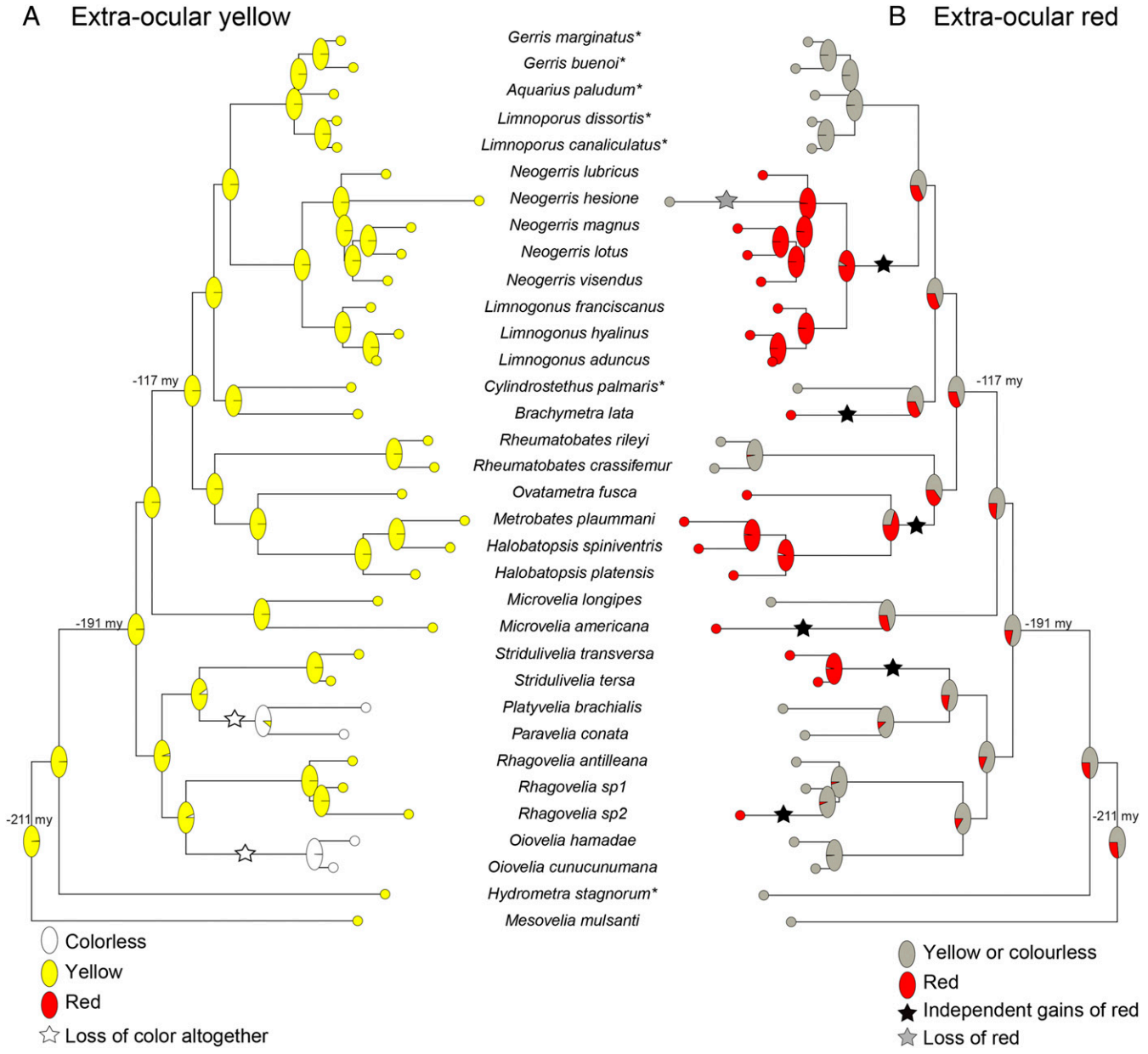

Fig. 2. Phylogenetic reconstruction of the ancestral state of extraocular colors in Gerromorpha. (*A*) Ancestral state reconstruction of the presence/absence of yellow in antennae and/or legs. (*B*) Ancestral state reconstruction of the presence/absence of red in the same appendages. Nodes are colored 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).

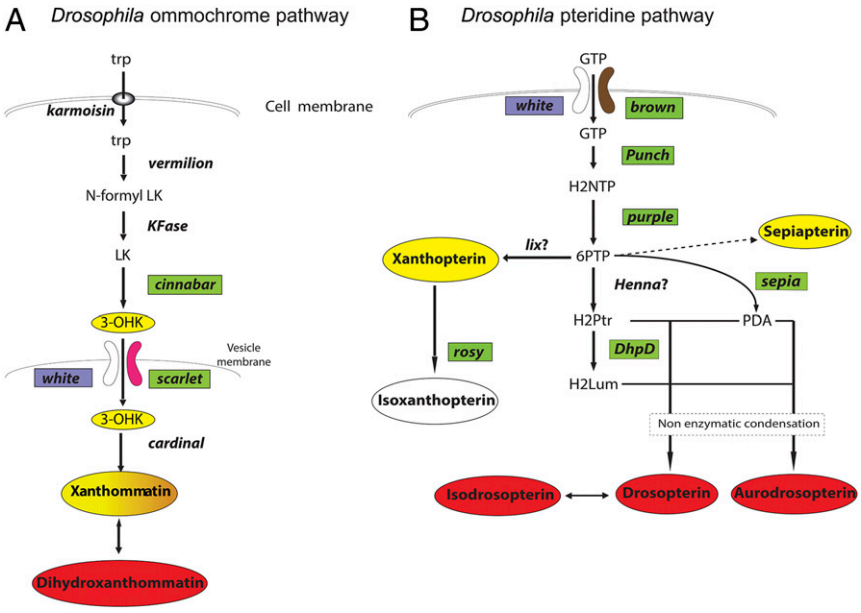

Fig. 3. Biosynthetic pathways for pigmentation in *Drosophila*. Ommochromes (*A*) (data from refs. 63 and 64) and pteridines pigments pathways of *Drosophila melanogaster* adult eyes (*B*) (data from ref. 65). Homologous genes studied in the water strider *L. franciscanus* are boxed in green. Note that 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. 3*A*), whereas pteridine pathway converts GTP into yellow, orange, or red pigments (30) (Fig. 3*B*). The ABC transporter White is common to both ommochrome and pteridine pathways (Figs. 3 and 4 *A* 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. 4*F*), and *white* silencing using RNA interference (RNAi) depletes all colors from the entire embryo (Fig. 4 *G* 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. 4*A*). The expression of *cinnabar* is confined in the eyes (Fig. 4*I*), and inactivation of this gene by RNAi resulted in embryos where the eyes became bright red but where the extraocular coloration remained unaffected (Fig. 4 *J* and *K*). Unlike *cinnabar*, the expression pattern of *scarlet* prefigured the red coloration in the eyes and in the extraocular stripes (Fig. 4*L*). 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. 4 *M* 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. 3*B* and 4*E*). We found that the expression of *brown* mRNA prefigures both ocular and extraocular color patterns (Fig. 4*O*) and that RNAi against *brown* resulted in the complete loss of red color both in the eyes and in extraocular 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 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. 3*B* 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. 3*B* 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 5*A* and *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*). RNAi against both genes yielded identical results consisting of the elimination of extraocular pigment (compare Fig. 5 *C*, *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. 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 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. 3*B* and *SI Appendix*, Fig. S8). We failed to detect any expression of *sepia* or *DhpD* (*SI Appendix*, Fig. S9), 2 genes that are necessary

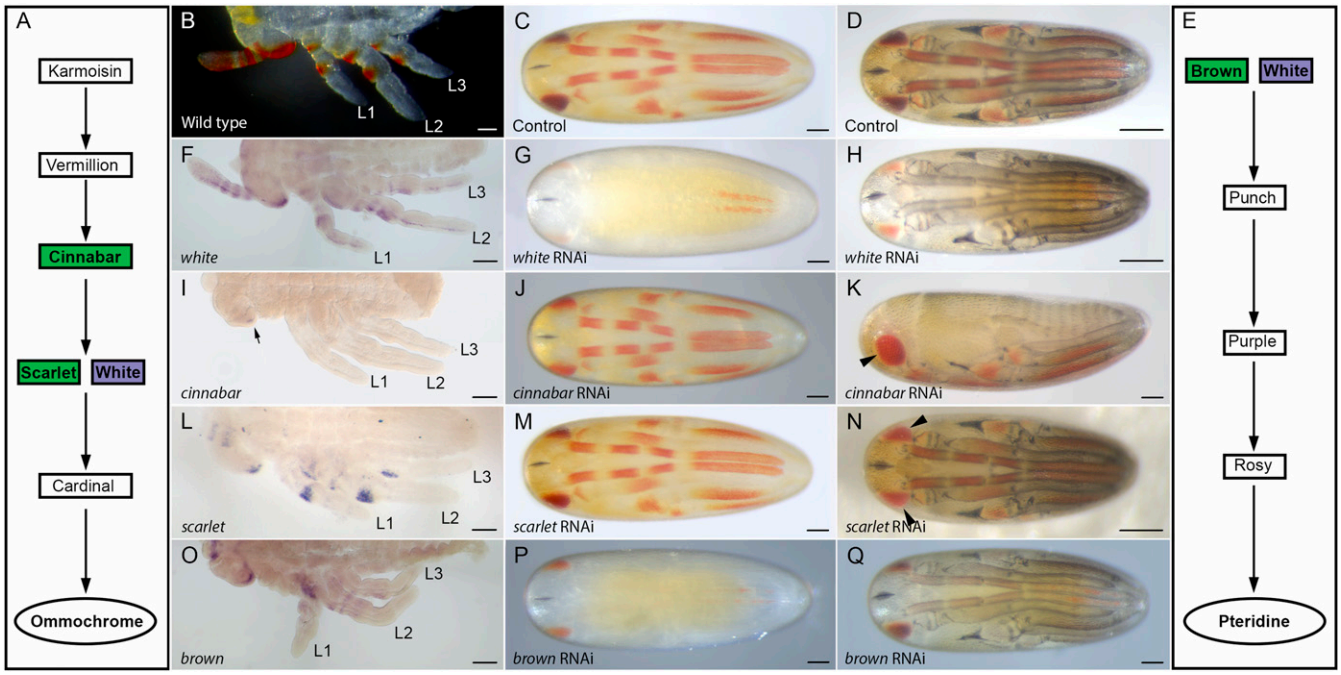

Fig. 4. Expression and function of genes from ommochrome and pteridine pathways in *L. franciscanus* embryos. For In situ hybridizations, dissected embryos are shown in ventral view. Embryos obtained after RNAi were not dissected and are also shown in ventral view (except *K*: lateral view) at 2 different 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 aurodrosopterin and drosopterin in flies (Fig. 3*B*) (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. 5*H*). Inactivation of *rosy* using RNAi again abolished color from the extraocular tissues and left the embryos with dark eyes (Fig. 5*I*). 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 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. 6*B*), *rosy* is expressed only in the eyes (Fig. 6*H*). Consequently, the inactivation of *rosy* in this species depleted the bright red pteridines resulting in dark eyes (Fig. 6*N*). In all other species with extraocular pigmentation, either yellow or red (Fig. 6 *A* 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

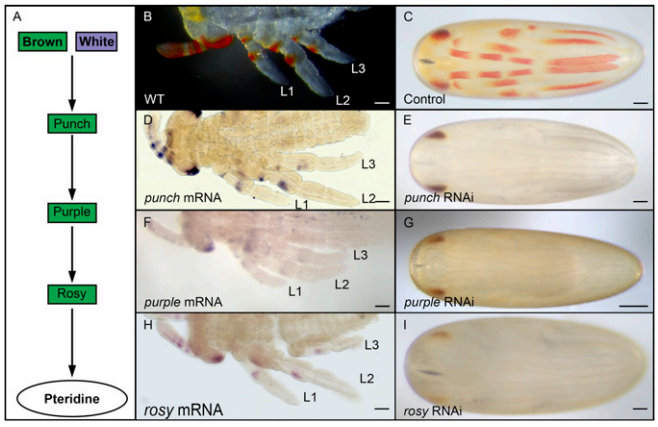

Fig. 5. Expression and function of the genes encoding for the pteridine pathway enzymes in *L. franciscanus* embryos. (*A*) Simplified pteridine pathway showing the genes analyzed in this figure (colored boxes). (*B*) Ventral view of a dissected wild-type (WT) *L. franciscanus* embryo just after katatrepsis 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.)

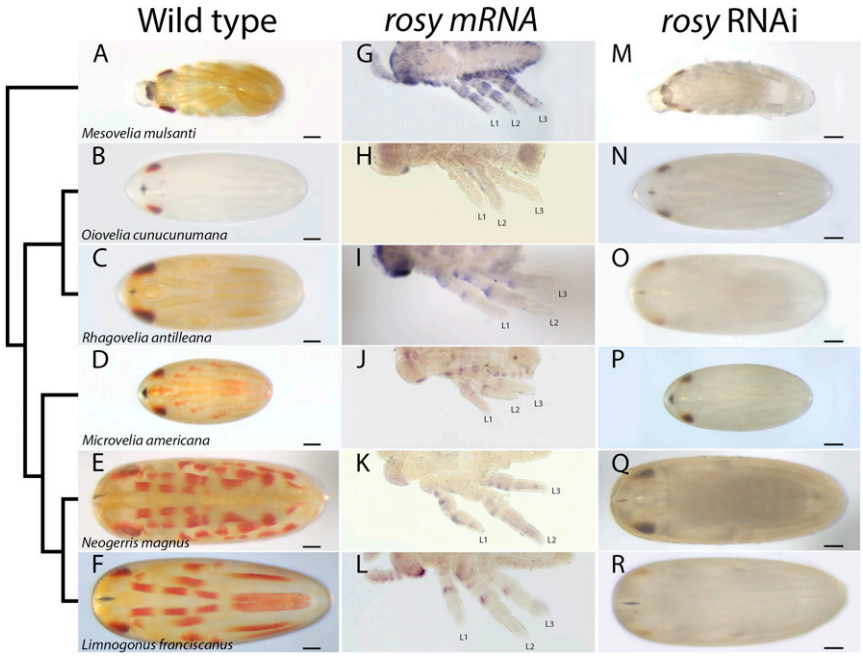

Fig. 6. Expression and function of *rosy* across Gerromorpha. (*Left*) Phylogenetic relationships between the 6 studied species. All embryos are shown in ventral view. (*A–F*) WT embryos showing the natural color pattern. (*G–L*) Expression of *rosy* (*ry*) mRNA. (*M–R*) Effect of *ry* RNAi on pigmentation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

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

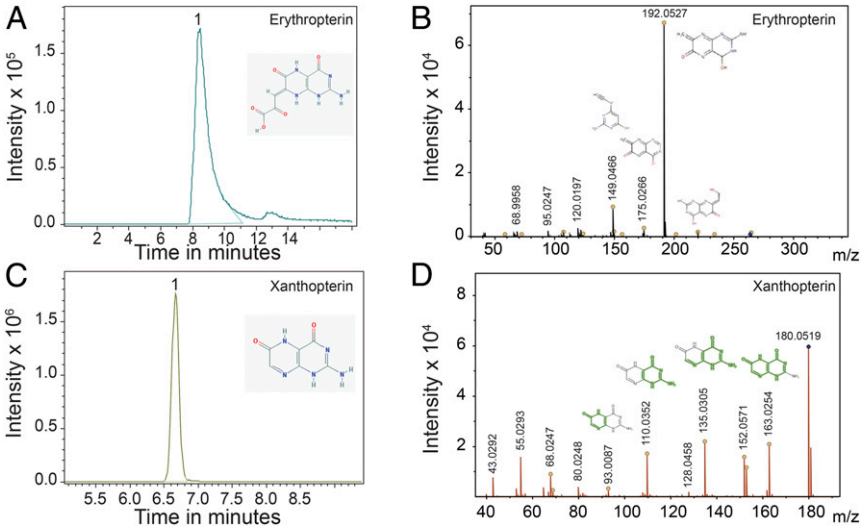

Fig. 7. Identification of eryhtropterin and xanthopterin from *L. franciscanus* embryos using UHPLC-high-resolution mass spectrometry (HRMS). HILIC chromatograms, and MS/MS (tandem mass spectrometry) spectrums for erythropterin (*A* and *B*) and xanthopterin (*C* and *D*). Samples consisted of pteridines 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. 3*B*) 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 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 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). InsituPro 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 $MgCl_2$; 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_2PO_4/NaH_2PO_4 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 radio 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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