Natural History Note

Secondary Sexual Trait Melanization in "Black" Scavenger Flies: Nutritional Plasticity and Its Evolution

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abstract: The black scavenger fly Sepsis thoracica exhibits polyphenic development resulting in alternate small black and large amber male morphs. Although the behavior, ecology, and physiology of both morphs are being scrutinized, the evolutionary origins of the nutritional polyphenism remain poorly understood. I here use a comparative approach to study variation in the degree of melanization of the forefemur—a secondary sexual trait. Melanization showed nutritional plasticity in all species, and character mapping suggests polyphenic development to represent the ancestral character state that was lost repeatedly. That is, interspecific variation among the studied species is mainly caused by the loss and not the gain of polyphenic development. Coevolution between male melanization and mating system differences further implicates sexual selection in the evolution of male melanization. These findings highlight the usefulness of comparative and natural history data in shedding new light on the evolution of phenotypic variation.

Keywords: coloration, sexual dichromatism, developmental plasticity, melanism, Diptera, Sepsidae.

Introduction

Animals feature a spectacular variety of coloration patterns (Darwin 1872; Wallace 1877) often attributed to sexual selection (Selz et al. 2016; Girard et al. 2018; Cooney et al. 2019). However, coloration also has important ecological functions, such as predator avoidance, and allows organisms to better deal with abiotic stressors, such as heat or UV radiation (Berry and Willmer 1986; True 2003; Caro 2017; Cuthill et al. 2017). Coloration is thus subject to multiple, often antagonistic selection pressures. At the same time, the production of pigments can be physiologically costly, leading to physiological constraints and trade-offs (Wilson et al. 2001; Zera and Harshman 2001; Debecker et al. 2015). Such context-dependent fitness consequences can favor the evo-

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lution of polyphenisms—that is, the capacity of a single genotype to produce discrete phenotypes in response to environmental variation (West-Eberhard 2003). Polyphenisms in coloration caused by social conditions (Sword 1999), substrate coloration (Noor et al. 2008), season (Hazel 2002), or the presence of predators (McCollum and Van Buskirk 1996; Ahlgren et al. 2013) are relatively common. Yet there are few systems in which the physiological and genetic basis, the ecological and behavioral causes and consequences, and the evolutionary history of the polyphenism are well characterized. This hampers our understanding of how color polyphenisms arise and, once in existence, continue to evolve. By studying intra- and interspecific variation in cuticular melanization across species of "black" scavenger flies, I here investigate the evolutionary history of a polyphenism and the coevolution between melanization and mating system differentiation.

From a developmental perspective, the evolution of polyphenisms has often been linked to the modification (or exaggeration) of an ancestrally plastic response (West-Eberhard 2003; Moczek et al. 2011). For instance, the nutritional polyphenism in horn length in male dung beetles is thought to have evolved from a weak ancestral plastic response. That is, selection on horn length first led to an increase in the slope of an ancestral linear scaling relationship, which was followed by the emergence of body size thresholds, leading to a sigmoidal scaling relationship (e.g., Emlen and Nijhout 2000). A role of ancestral plasticity in the evolution of polyphenisms has also been demonstrated in other species and traits, with close relatives of polyphenic species maintaining a weaker plastic response, suggesting that the polyphenism evolved through the exaggeration of ancestral plasticity (Levis et al. 2018; Casasa et al. 2020). Similar patterns have been shown to act in the evolution of polyphenic coloration through artificial selection (e.g., Suzuki and Nijhout 2006; van der Burg et al. 2020). Yet while a

number of studies demonstrate that the exaggeration of an ancestral response can fuel intra- and interspecific diversity, it remains unclear how often, how much, and by which mechanisms ancestral plasticity contributes to intra- and interspecific diversity.

Black scavenger flies (Diptera: Sepsidae)—as their name might suggest—are not particularly well known for eccentric coloration. However, recent studies document surprising intraspecific variability in the degree of cuticular melanization in *Sepsis thoracica* (Busso et al. 2017; Busso and Blanckenhorn 2018a, 2018b, 2018c). While females of this species are predominantly black across their entire body irrespective of their size, males exhibit a strongly sigmoidal relationship between the degree of cuticular melanization and size. This threshold separates small black males (collectively referred to as the "obsidian" morph) from large orange "amber" males, with few "intermediates" in between (Busso et al. 2017). This morphological variation further corresponds to male behavior. Small obsidian males are less likely to show aggressive behavior toward a large amber male than toward another obsidian male, and intermediate males have reduced mating success compared with both other morphs, suggesting disruptive sexual selection on the degree of male melanization and size (Busso and Blanckenhorn 2018a, 2018b).

The intraspecific variation in coloration is particularly intriguing because it is closely tied to the production of melanin—a critical component of the phenoloxidase pathway that mediates insect cellular immune defense and wound healing (Cerenius and Soderhall 2004; Schmid-Hempel 2005; Nakhleh et al. 2017) as well as the cuticular melanization of insects (Wilson et al. 2001; Armitage and Siva-Jothy 2005). Because large *S. thoracica* males had lower (pro)phenoloxidase levels than the smaller and darker males and females, previous studies are consistent with trade-offs between size, reproductive success, and immunity (Busso et al. 2017). Taken together, these findings suggest the male polyphenism to be an intriguing paradigm for scrutinizing interrelations between natural and sexual selection, alternative reproductive tactics, and the action of potential trade-offs between sexual signaling and immunity (Busso et al. 2017; Busso and Blanckenhorn 2018a, 2018b, 2018c). However, the evolutionary origin of this polyphenism remains largely unexplored. Because *S. thoracica* is phylogenetically nested between clades that contain species previously described as "black and shiny" (*S. cynipsea*; Parker 1972) or "entirely black" (*S. fulgens*; Roy et al. 2018), the male polyphenism is likely to constitute a recent autapomorphy. This suggests that the evolution of a polyphenism fueled interspecific diversity. Yet the presence and extent of genetic and plastic variation in species other than *S. thoracica* have not been investigated. It therefore remains unclear whether nutritional plasticity in melanization is limited to *S. thoracica*, when and

how the male polyphenism evolved, and whether the exaggeration of an ancestral plastic response caused interspecific variation in melanization.

Focusing on the degree of melanization of the forefemur, a secondary sexual trait under sexual selection (Busso and Blanckenhorn 2018b; Baur et al. 2020), I investigate the evolutionary history of the male polyphenism described so far only in *S. thoracica*. By quantifying species divergence and nutritional plasticity in the degree of melanization in 12 closely related species, I test whether *S. thoracica* is indeed the only species with polyphenic melanization or whether this is a more widespread phenomenon. At the same time, I assess the potential to which the exaggeration of an ancestral plastic response may have played a role in the evolution of polyphenic melanization and the genesis of phenotypic variation. In the latter case, I expected close relatives of *S. thoracica* to show moderate levels of plasticity and for the sigmoidal reaction norm (i.e., polyphenic development) to be derived from a (quasi)linear reaction norm. Furthermore, I tested whether the association between reduced melanization and mating success found in *S. thoracica* extends to the macroevolutionary scale by contrasting average melanization of the forefemur with previously documented variation in mating systems and reproductive behavior among taxa (Puniamoorthy et al. 2012a, 2012b; Rohner et al. 2016, 2018; Rohner and Blanckenhorn 2018; Blanckenhorn et al. 2020). If reduced male melanization increases mating success, the degree of melanism is expected to coevolve with the degree of male-biased sexual size dimorphism—a proxy for the degree of sexual selection and male-male aggressive behavior in these species (see Blanckenhorn et al. 2020, 2021).

Material and Methods

Laboratory Rearing

To quantify sexual dimorphism, sex-specific nutritional plasticity, and species differences, 12 closely related sepsid taxa (including North American and European populations of *Sepsis neocynipsea* and *S. punctum*, as these species show stark intraspecific variation in morphology and behavior) were reared under controlled laboratory conditions. Most individuals stem from a resource manipulation experiment described in Rohner and Blanckenhorn (2018) with the exception of *S. fulgens*, which was reared at a later stage but under identical laboratory conditions. In brief, eggs were collected from outbred laboratory cultures (200–300 individuals) and haphazardly distributed among plastic containers with varying amounts of homogenized cow dung. All offspring were subsequently incubated at constant 18°C. After adult eclosion of all individuals, 30–50 individuals of all sizes per species and sex were selected for morphometric measurements.

Quantification of Forefemur Melanization

The right front legs were removed from the thorax and mounted on a glass slide with Euparal and were imaged with a Leica DFC490 camera mounted on a Leica MZ12 microscope. All images were taken in one sitting under standardized light conditions and camera settings to keep coloration comparable across specimens. Images were then analyzed using the custom ImageJ (Schneider et al. 2012) script from Busso and Blanckenhorn (2018a). This script first measures the number of pixels of the forefemur and then quantifies how many of them fall below a certain brightness threshold in the YUV color space. Pixels with a V value (i.e., brightness) higher than 163 were defined as melanic, and the proportion of these melanic pixels in the full femur estimates the overall degree of melanism (i.e., coloration), the measure used for further analysis. In total, measurements were acquired for 979 individuals.

Sex-Specific Nutritional Plasticity

Previous studies used forefemur area as an estimate of body size (e.g., Busso et al. 2017). Because several species diverged in male forefemur size allometries (putatively driven by selection on their forefemur morphology; Rohner and Blanckenhorn 2018), the length of the hind tibia was used as a proxy for body size instead.

The shape of the reaction norm between coloration and size varied strongly among species. To identify the statistical model that best fitted these relationships, linear, curvilinear, and, where deemed appropriate, sigmoidal models (three and five parameters) were fitted with and without sex differences for each species. Akaike's information criterion (AIC) was then used to identify the most appropriate model. In cases where several models fit the data equally well (ΔAIC < 2), the model with fewer parameters was selected. To trace the evolutionary history of male plasticity, all species were assigned to three progressing categories—linear, curvilinear (i.e., quadratic), and sigmoidal—on the basis of the model with the lowest AIC. The shape of the reaction norm was then mapped onto the phylogeny using maximum parsimony, as implemented in Mesquite (ver. 3; Maddison and Maddison 2008).

Coevolution between Mating Systems and Femur Coloration

Within the genus *Sepsis*, mating systems evolve rapidly. In most species, mating systems appear dominated by female choice and sexual conflict or are characterized by elaborate pre-, peri-, and postcopulatory courtship by males (Puniamoorthy et al. 2009). However, in several species and even populations within species, intense sexual selection on male body size led to the evolution of male-biased sexual size di-

morphism (Rohner et al. 2016). These shifts in overall sexual size dimorphism coincide with the presence of aggressive male-male interactions and territoriality, exaggerated male morphology and allometry, and reduced investment into copulatory courtship (Puniamoorthy et al. 2012a, 2012b; Rohner et al. 2016, 2018; Rohner and Blanckenhorn 2018; Blanckenhorn et al. 2020, 2021). This variation here allows for testing of whether the evolution of more intense sexual selection on males and territorial behavior coevolve with nutritional plasticity and sexual dimorphism in melanization.

To compare the degree of cuticular melanization and sexual dichromatism across species, the analysis was restricted to individuals that were reared with ad lib. access to larval nutrition (>3 g per individual). This ensures that species are compared across the same (i.e., common garden) environment. Phylogenetic linear models (phylogenetic generalized least squares [PGLS], as implemented in the R package caper; Freckleton et al. 2002) incorporating the relationships among taxa (Zhao et al. 2013) were used to test for a relationship between each taxon's sexual size dimorphism index (see Rohner and Blanckenhorn 2018) and average male and female coloration. All analyses were conducted in R (ver. 3.6.3; R Core Team 2020) unless stated otherwise.
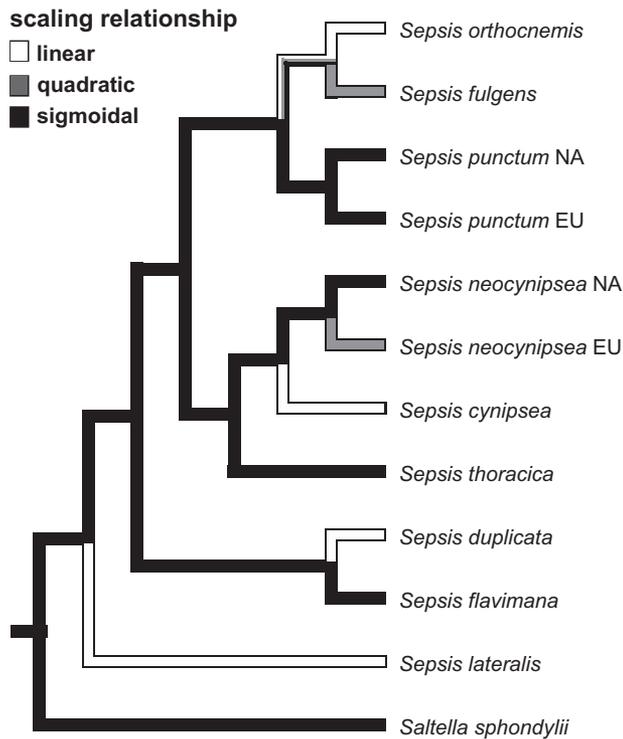
Results

Sexual Dichromatism and Sex-Specific Nutritional Plasticity in Femur Coloration

As previously documented (Busso and Blanckenhorn 2018b), *Sepsis thoracica* showed a strongly sigmoidal sex-specific relationship between cuticular melanization and body size (fig. 1). However, the degree of melanization in the forefemur varied strongly among and within the other investigated species as well, suggesting previously overlooked intraspecific variation. Large individuals were generally less melanic than small conspecifics in all species (in all cases, the intercept-only models had the highest AIC; table S1; tables S1, S2 are available online), but the shape of the relationship between melanism and size differed qualitatively among taxa, ranging from linear to curvilinear to sigmoidal (table S2). A complex sex-specific relationship similar to the one in *S. thoracica* was found in *Saltella sphondylii*.

Interestingly, character mapping suggests that the male polyphenism in *S. thoracica* did not recently evolve from a weaker ancestral response but that a sigmoid relationship represents the ancestral character state that was secondarily lost multiple times in the *Sepsis* clade (fig. 2). That is, species differences in the presence of the polyphenism are driven not by the gain but by the loss of polyphenic development. These results indicate that nutritional plasticity is evolutionarily labile and that the patterns previously documented in *S. thoracica* are not a recent autapomorphy.
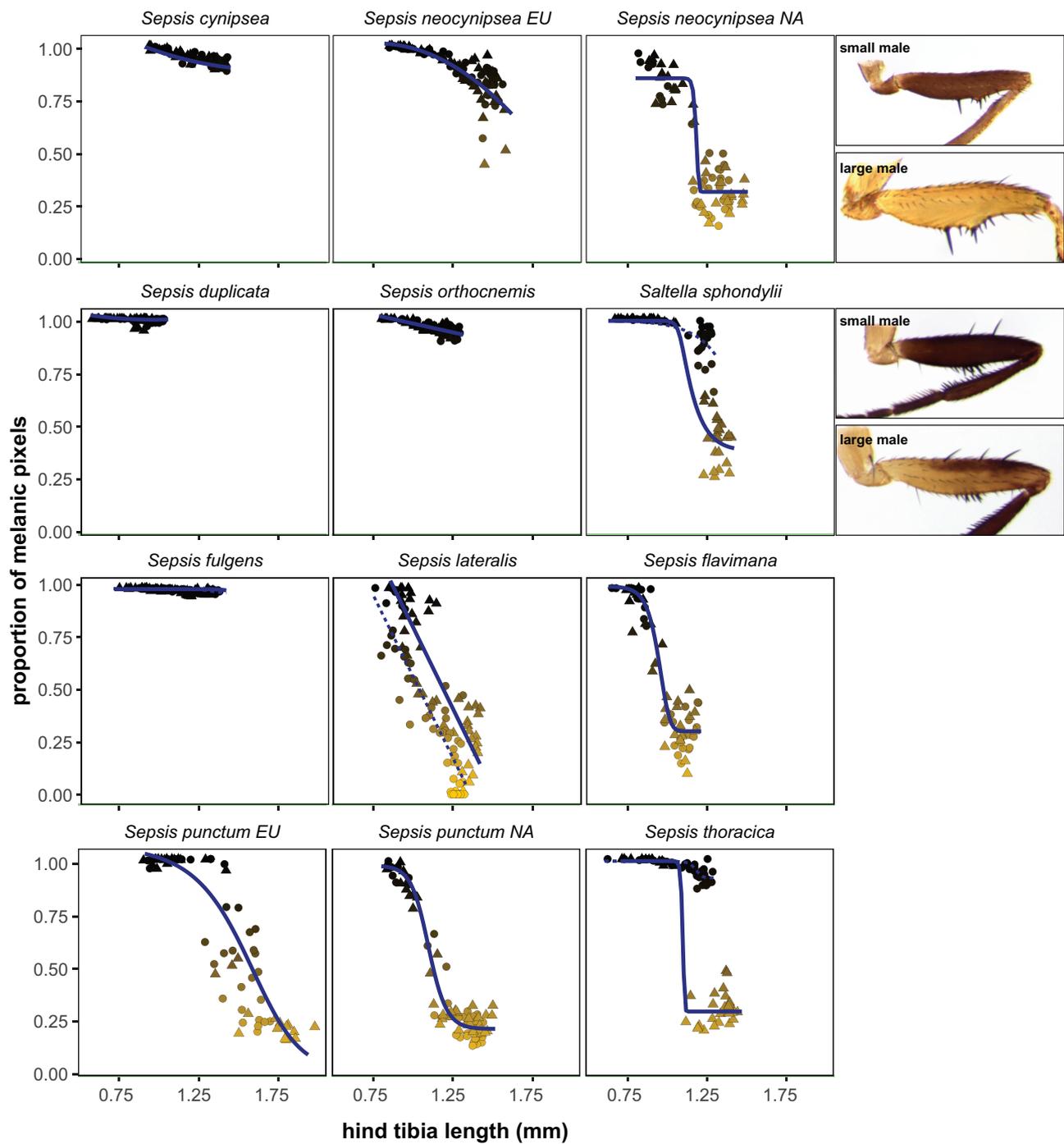
Figure 1: Nutritional plasticity in 12 closely related species of sepsid flies. Colors depict the level of melanism as a proportion. The pictures show examples of forefemur morphology and coloration for small and large males of *Sepsis neocynipsea* (*top*) and *Saltella sphondylii* (*bottom*). Males are denoted with triangles, females with circles. Blue lines indicate the fit of the simplest model with the lowest Akaike's information criterion (see tables S1, S2). In cases where reaction norms are sex specific, the relationship found in females is indicated with a dashed line. Data underlying this figure have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.q2bvq83kg; Rohner 2021). EU = European; NA = North American.

scaling relationship
☐ linear
▨ quadratic
■ sigmoidal

Sepsis orthocnemis

Sepsis fulgens

Sepsis punctum NA

Sepsis punctum EU

Sepsis neocynipsea NA

Sepsis neocynipsea EU

Sepsis cynipsea

Sepsis thoracica

Sepsis duplicata

Sepsis flavimana

Sepsis lateralis

Saltella sphondylii

Figure 2: Ancestral state reconstruction based on maximum parsimony suggests sigmoidal scaling relationships to be the ancestral character state. EU = European; NA = North American.

Sexual dichromatism (as assessed by the degree of cuticular melanization) in the forefemur was weak to absent in most species (fig. 3*A*). However, sexual dichromatism was present in *S. lateralis* and the European population of *S. punctum* and was particularly pronounced in *S. thoracica* and *S. sphondylii*. Weak melanization was present in females of several species, indicating that reduced melanism is not a phenomenon limited only to males (fig. 3). Maximum likelihood–based ancestral state reconstruction of melanization shows a strong correlation between male and female pigmentation (fig. 3*B*; PGLS: $r = 0.70$, $P = .011$).

Coevolution of Mating System and Femur Coloration

Large amber males were previously shown to have the highest mating success, suggesting that coloration is under sexual selection (Busso and Blanckenhorn 2018*b*). I therefore tested the hypothesis that mating system shifts and corresponding changes in male interactions and female choice coevolve with the average degree of melanization across species. Taxa that evolved more male-biased sexual size dimorphism also evolved less melanized forefemora in males (PGLS: $F_{1,10} = 12.98$, $P = .005$). This suggests an evolutionary link between cuticular melanism in males and more

intense sexual selection or territorial behavior (both of which coincide with male-biased sexual size dimorphism; Blanckenhorn et al. 2020). In contrast, there was no relationship between sexual dimorphism in body size and cuticular melanization in females (PGLS: $F_{1,10} = 3.05$, $P = .111$).

Discussion

Studying nutritional plasticity of the sexually exaggerated forefemur in a phylogenetic context revealed three salient results. First, black scavenger flies are not as melanic as their vernacular name suggests. Melanization not only varied strongly across species but also showed strong and often sex-specific nutritional plasticity within species (fig. 1). Second, although previously available data suggested the polyphenism in *Sepsis thoracica* to be an autapomorphy, sigmoidal reaction norms were the most common form of nutritional plasticity, and character mapping suggests nonlinear relationships to be the ancestral character state (fig. 2). This suggests that species differences in forefemur melanization are driven not by the gain but by the loss of polyphenic development. Interspecific diversity in melanization is thus generated by the canalization—as opposed to the exaggeration—of an ancestrally plastic response. Third, the evolution of male-biased sexual size dimorphism is associated with a decrease in male melanization, suggesting a link between sexual selection and reduced melanization. Below, I discuss the implications of these results for our understanding of the evolution of polyphenisms, the putative mechanisms underlying sigmoid relationships in melanization more generally, and the adaptive significance of variation in cuticular melanization in sepsids.

*Evolution of Nutritional Plasticity
in Cuticular Melanization*

Sepsids feature widespread and previously overlooked variation in the developmental plasticity of cuticular melanization (but see early taxonomic literature [e.g., Meigen 1826; Duda 1926; Hennig 1949] as well as faunistic work [e.g., Rohner and Bächli 2016]). The general decrease in melanization with size (fig. 1) could broadly be categorized into three progressing types—linear, curvilinear (quadratic), and sigmoidal—of which the sigmoidal type was, surprisingly, the most common. On the basis of character mapping, the sigmoid relationship appears to be ancestral, having evolved early in the evolutionary history of the clade, followed by subsequent and repeated losses within *Sepsis* (fig. 2). Although more (basal) sepsids should be investigated to confirm these findings, the polyphenism in male *S. thoracica* thus appears to be ancestral. At least on the phenotypic level, the large inter- and intraspecific variation observed in this clade is thus generated by the loss of

A) sexual dichromatism
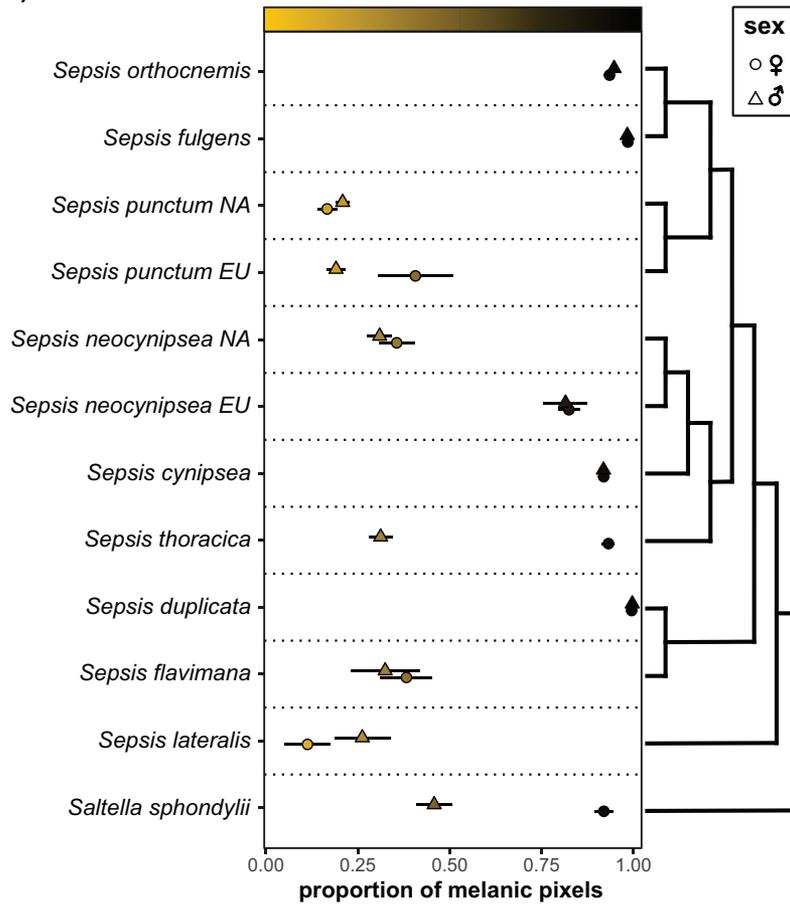
B) ancestral state reconstruction
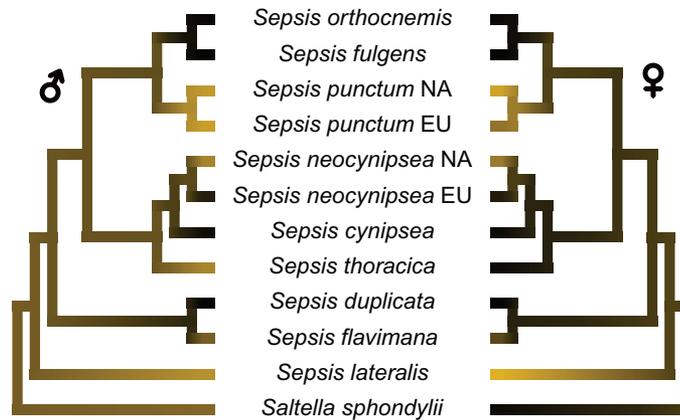
Figure 3: *A*, Sex-specific melanization of the forefemur varies strongly among closely related species of sepsids. Bars show 95% confidence intervals. *B*, Ancestral state reconstruction for forefemur coloration indicates strong correlation between male and female coloration. EU = European; NA = North American.

polyphenic melanization in some species and not the recent exaggeration of an ancestral form of plasticity. These losses appear to be frequent, as even very closely related taxa varied in the presence of a sigmoid relationship (i.e., a polyphenism). For instance, while North American *S. neocynipsea* showed a pronounced sigmoid relationship, the conspecific European population showed a curvilinear decrease of melanization with size. The closely related sister species *S. cynipsea*, on the other hand, shows a much weaker linear response, with predominantly black individuals. As all three taxa hybridize in the laboratory (Giesen et al. 2018) and given the phylogenetic context (fig. 2), this highlights that intraspecific variation in melanization was generated by the relatively fast loss of plasticity.

Given these data, not only the sigmoid relationships but also the corresponding ultimately bimodal distribution of phenotypes are likely plesiomorphic. This may indicate that the complex interactions between behavior, immunity, and sexual selection found in *S. thoracica* (Busso et al. 2017; Busso and Blanckenhorn 2018a, 2018b, 2018c) may extend to other species in this clade. As species and sexes differ in their degree of melanization (fig. 3), different species and sexes may have evolved different solutions to the proposed physiological trade-off between immunity and sexual signaling. In addition, the comparative angle taken here further suggests that *S. thoracica* stands out because of the presence of strong sex-specific nutritional plasticity in the degree of forefemur melanization (fig. 3). Future studies should thus center not only on the evolution of male polyphenism (an ancestral state) but also on the mechanisms mediating sexual dichromatism (a derived state) and the resolution of between-sex genetic correlations.

Proximate Determinants of Sigmoid Relationships

Polyphenic development is typically characterized by threshold traits that generate distinct morphs with little or no overlap (Nijhout 1999). Physiologically, such polyphenisms are often determined by a developmental switch point at which critical hormone titers elicit the development of alternative morphs (Nijhout 1999). Such switch points can cause segmented, discontinuous, or sigmoidal reaction norms and are likely to underpin phenotypic variation in *S. thoracica*. The progression from sigmoidal to curvilinear to linear relationships documented above could be explained by interspecific variation in the shape of the threshold function and the position of such a developmental switch relative to body size (as in dung beetle horns; Moczek and Nijhout 2003). That is, depending on whether a population's maximum size is smaller than, roughly equal to, or larger than the inflection point, the same underlying developmental switch may alternately manifest in a linear, curvilinear, or fully sigmoidal reaction norm (fig. 4). Evolution of how
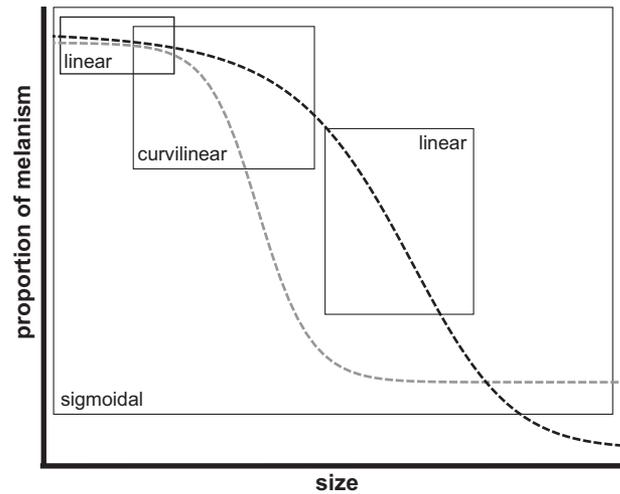
Figure 4: Hypothetical scenario for the evolution and development of different types of nutritionally plastic responses. The black dashed line indicates the sigmoid relationship found in European *Sepsis punctum*. Depending on the focal body size range, the same underlying function could generate linear, curvilinear, or sigmoid relationships (as indicated by the boxes). Evolution of the shape of the sigmoid (indicated by the relationship found in the North American population of the same species; gray dashed line) could then further diversify the relationship between coloration and body size.

the threshold impacts the shape of the scaling relationship (i.e., the parameters of the sigmoid) could then further diversify the relationship between coloration and body size, as appears to be the case in the two populations of *S. punctum* (fig. 4).

However, melanization may not necessitate a developmental switch to cause a bimodal phenotypic distribution. This is because no matter how much more melanin an entirely black obsidian male deposits in its cuticle, it will still appear black. Likewise, a large amber male cannot produce less melanin than none. If variation in size and melanization becomes sufficiently large, any allometric relationship must reach an asymptote close to either or both extremes. The curvilinear and sigmoidal relationships documented here could thus be caused by a continuous decrease in pigmentation with size that hits a boundary at one or both extreme values of the distribution, respectively. In agreement with this alternative hypothesis, small black males have higher (pro)phenoloxidase activity than larger but equally heavily melanized males (Busso et al. 2017). That is, even though small black males invest more into melanin synthesis, this does not result in even darker pigmentation, possibly because adding melanin to an entirely black cuticle has no further discernible phenotypic effect with respect to visual appearance. Whether variation in melanization is driven by a developmental switch therefore

remains to be investigated, as this heavily influences putative trade-offs and determines how phenotypic variation is generated and how nutritional plasticity evolves.

Adaptive Significance of Coloration in Sepsids

Sexual selection is thought to be among the primary drivers in the evolution of striking color variation in animals (Selz et al. 2016; Girard et al. 2018; Cooney et al. 2019). Correspondingly, Busso and Blanckenhorn (2018b) found directional as well as disruptive sexual selection on body size and the degree of melanization in *S. thoracica* and concluded that this complex fitness landscape likely shapes and maintains the male polyphenism. In agreement with this hypothesis, the data presented here show that species with relatively larger males, stronger selection on male secondary sexual morphology, and more intense male-male competition (including territorial behavior; summarized in Blanckenhorn et al. 2020, 2021) evolved a corresponding reduction in melanism. This is consistent with a role of melanization in sexual selection—via female preference, male competition, or, most likely, a combination of both—thus extending the presumed underlying intraspecific mechanisms to ultimately result in macroevolutionary divergence. Future research will be necessary to test whether mechanisms similar to the ones acting in *S. thoracica* are responsible for the maintenance of polyphenic melanization in other species and populations.

Conclusions

Black scavenger flies showcase stark intra- and interspecific variation in melanization. Surprisingly, the sigmoid relationship that generates two distinct male morphs in *S. thoracica* is also present in other species and—under the most parsimonious scenario—represents the ancestral character state. This indicates that the loss of polyphenic development fuels interspecific diversity and begs the question of why the sigmoid relationship is maintained in male *S. thoracica* while it is lost in females and several other species. Furthermore, cuticular melanization in males coevolves with the degree of sexual size dimorphism and reproductive behavior, suggesting a link between sexual selection and cuticular melanization and extending the intraspecific mechanism previously documented in *S. thoracica* to the macroevolutionary scale. This opens promising avenues for future comparative work on the (co)evolution of reproductive tactics, sexual signaling, and immunity, as well as the physiological and genetic mechanisms underlying phenotypic plasticity.

Acknowledgments

Data and Code Availability

The raw data are available in tables S1 and S2 and have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.q2bvq83kg; Rohner 2021).

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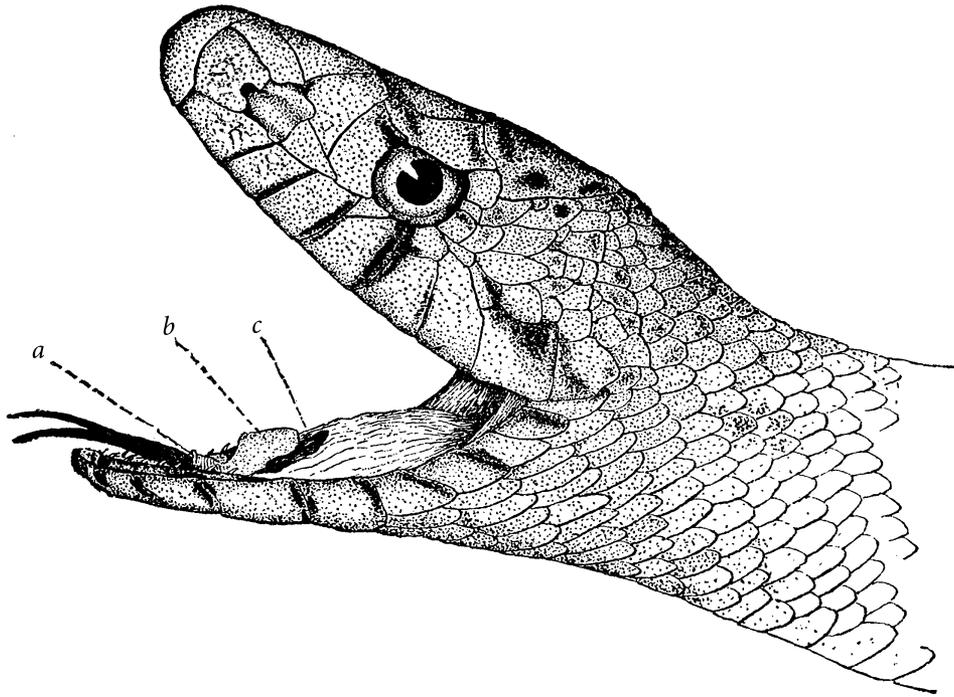
"The accompanying figures, drawn from nature by my friend Dr. R. W. Shufeldt, U. S. Army, represents the mouth-parts of *Pityophis sayi* var. *bellona*. The tongue-sheath is represented at *a*; the epiglottis at *b*; and the rima-glotidis (aperture of the windpipe) at *c*." From "On the Character and Function of the Epiglottis in the Bull-Snake (*Pityophis*)" by Charles A. White (*The American Naturalist*, 1884, 18:19–21).