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Healthier or bigger? Trade-off mediating male dimorphism in the black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae)

Dimorphic Sepsis thoracica males trade off health and size

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ABSTRACT

Life history trade-offs emerge when limited resources are allocated to multiple functions of an organism. Under highly competitive conditions trade-offs can result in alternative phenotypes that differ morphologically and physiologically. Such is the case in insect species that grow under high densities, where competition for resources but also the risk of disease contagion is high, prompting important adjustments in immune response and melanic cuticular pigmentation, with consequent sacrifices in other fitness-related traits. Here we evaluated potential trade-offs between total- and active phenoloxidase (PO), body size and body pigmentation in *Sepsis thoracica* black scavenger flies that show alternative male morphs differing in cuticular pigmentation and body size. As expected, small/dark (*obsidian*) males showed higher total-PO activity than larger/orange (*amber*) males. We found a negative relationship between total-PO activity and body size that was more intense in females and *obsidian* but not *amber* males, suggesting that growth and immunity are more costly for the former. At the same time, body size decreased in females and *amber* but not *obsidian* males as rearing density increased, showing that male morph is largely determined by condition-dependent plasticity rather than genes. In contrast, density did not affect PO activity, as predicted by the density-dependent prophylaxis hypothesis, which was not tested in dipterans before. Our study provides good evidence that male polymorphism results from a trade-off between body size and immune response permitting the evolution of alternative resource allocation strategies and resulting in strikingly different alternative phenotypes.

INTRODUCTION

The limiting nature of resources requires organisms to carefully distribute their investment into a variety of traits, resulting in life-history trade-offs (Zera & Harshman 2001). Trade-offs originate because the best phenotype for one task is usually not the best for other tasks (Campbell, Grime & Mackey 1991). Accordingly, organisms tend to maximize their fitness through multi-objective optimization (Abraham & Jain 2005; Shoal *et al.* 2012), which not necessarily results in only one optimal solution (Farnsworth & Niklas 1995).

Among these trade-offs, the relative resource allocation to body size and immunity is of particular interest because both traits influence fitness in different but complementary ways. Body size provides fitness benefits in almost every aspect of an organism's biology (reviewed by LaBarbera 1986; Peters 1986), while immunity provides benefits against pathogens, against which body size is no defense. Hence, organisms face the challenge to attain the benefits of larger body size (Kingsolver & Pfennig 2004; Hone & Benton 2005) while retaining the possibility to defend it against pathogens. This investment in immunity strongly compromises the investment into other traits (Zuk & Stoehr 2002; Schmid-Hempel 2003). Since maintaining an immune system is very costly (Schmid-Hempel 2003), and body size is also resource-demanding (Reichle 1968; Hone & Benton 2005), we hypothesize that there will be a trade-off between these two traits, as has been observed in some other insects (Rantala & Roff 2005) but often also not (Suwanchaichinda & Paskewitz 1998; Vainio *et al.* 2004; Córdoba-Aguilar, Jiménez-Cortés & Lanz-Mendoza 2009). These contradicting results drove us to address this relationship in our study.

The immune system of insects defends the organism against pathogens via humoral and cellular components (Schmid-Hempel 2005; Rolff & Reynolds 2009). The humoral immune response targets mainly microbial pathogens (Boman & Hultmark 1987; Morishima *et al.* 1995; Adamo 2004), while the

cellular response targets primarily foreign intrusions by fungi, nematodes, parasitoids and mites (Schmid-Hempel 2005; Siva-Jothy, Moret & Rolff 2005). An important component of the cellular response is the encapsulation of intruders, which results from the phenoloxidase (PO) cascade (Iwanaga & Lee 2005; González-Santoyo & Córdoba-Aguilar 2012). This cascade ends in the production of melanin, which is deposited around the invaders in nodules and capsules, resulting in their isolation (Schmid-Hempel 2005; Strand 2008). Interestingly, the immunity-related expression of melanin influences the cuticular coloration of insects (Aso *et al.* 1985; Wilson & Reeson 1998; Barnes & Siva-Jothy 2000; Wilson *et al.* 2001; Armitage & Siva-Jothy 2005). We therefore further expect that the trade-off between body size and immunity should result in a negative correlation between body size and cuticular melanization.

Investment in immunity is also a function of the resources acquired, which in turn strongly depends on the competitive environment in terms of population density (Agnew *et al.* 2002). Density not only affects resource acquisition but also imposes immunological costs by influencing the risk of disease contagion (Goulson & Jenny 1995; Patterson & Ruckstuhl 2013; Kappeler, Cremer & Nunn 2015). As parasite transmission is often density-dependent, animals living at higher densities are expected to invest more resources into parasite defense, a phenomenon named ‘density-dependent prophylaxis’ (Wilson & Reeson 1998) that has found support in various social and non-social arthropods including orthopterans, lepidopterans, thysanopterans, coleopterans and hymenopterans (Barnes & Siva-Jothy 2000; Wilson *et al.* 2002; Cotter, Kruuk & Wilson 2004; Stow *et al.* 2007; Turnbull *et al.* 2011; but see Wilson *et al.* 2003; González-Tokman *et al.* 2014). Because of the involvement of melanin in insect immunity through the PO cascade, increased pathogen resistance in highly dense groups is often accompanied by cuticular darkening (Wilson *et al.* 2002; Cotter *et al.* 2004). Hence, we expect that the density at which individuals develop will affect the insect’s immune response, also altering their coloration.

An excellent system to address the putative trade-off between body size and immunity is the black scavenger fly *Sepsis thoracica* (Diptera: Sepsidae). Males of this species exhibit extreme variability in size and color, with smaller males being mostly black and the largest males bright orange, while females are completely black (Pont & Meier 2002). This variation suggests a putative trade-off between size and immunity in the males but not the females. Females of this species lay their eggs into (primarily cow) dung pats from which larvae cannot escape during development. Hence these dung pats are like islands that spatio-temporally vary in intra- and interspecific competitor density depending on the number of eggs laid into them. A highly competitive environment is expected to be more costly (Zuk & Stoehr 2002; Rantala & Roff 2005). Consequently, in *S. thoracica* the correlation between body size and melanization, together with the variable intraspecific competition levels during development, presents a unique opportunity to analyze how body size and (here larval) population density influence the insects’ investment into immunity. In this study we raised individuals at different densities and measured their body size and coloration as well as the PO activity as a proxy of their immune system (González-Santoyo & Córdoba-Aguilar 2012).

MATERIAL AND METHODS

***Sepsis thoracica* maintenance and rearing**

We sampled four European *S. thoracica* populations from Asturias, Spain (43.3° N, 6.0° E), Petroia, Italy (43.23° N, 12.56° W), Prati di Stronconi, Italy (42.48° N, 12.68° W), and Borgonuovo, Italy (46.33° N, 9.44° W). Wild-caught females were brought to the laboratory and used to establish cultures of multiple (10–20) replicate iso-female lines per population that were housed in separate plastic

containers and regularly supplied with fresh cow dung, sugar, and water ad libitum. The cultures were maintained at 18 ± 1 °C under a 14:10 h light:dark cycle.

For our study we used a mix of *S. thoracica* flies from random populations and lines to homogenize any possible effects of local adaption. To raise the flies, a container with dung was placed for 24 hours inside any of the *S. thoracica* stock cultures for females to deposit eggs. This dung was incubated afterwards in a novel container to obtain experimental adult flies. For each container we assessed the density of emerged adults reflecting differences in food limitation due to larval competition. We photographed the foreleg of each individual and measured its area and coloration using a self-written code scoring the number of orange and black pixels in ImageJ. We further measured the phenoloxidase activity from 76 randomly selected individuals of both sexes encompassing the entire body size range.

Measurement of phenoloxidase activity

Melanization results from the enzymatic activation of pro-phenoloxidase (proPO) into Phenoloxidase (PO), which converts phenols to quinones that ultimately polymerize to form melanin (Söderhäll & Cerenius 1998). PO is the ready-to use enzyme that has been naturally activated by the insect (henceforth “active-PO”), but the total investment in the PO pathway includes both proPO and active-PO (henceforth “total-PO”). Consequently, active-PO refers to the amount of naturally activated enzyme when taking the measurement, while total-PO is the overall investment in the immune pathway. Active-PO can be measured directly as described below, but to estimate total-PO, proPO has to be converted into PO using chymotrypsin (González-Santoyo & Córdoba-Aguilar 2012).

Since the amount of haemolymph that can be extracted from a single fly is very limited, phenoloxidase activity was quantified from whole individual homogenates (see similar procedure in Cornet, Gandon & Rivero 2013). Flies were crushed individually in 80 µL of cold PBS buffer. Afterwards we centrifuged the individuals (5 min, 5°C, 13000 rpm) in a spin column (M1003, Mobicol) with a 10 µm pore filter (M2210, Mobicol). The clean extract was collected for subsequent analysis. For each individual we measured the protein content, the active-PO and total-PO using a spectrophotometric assay.

To measure proteins we used the bicinchoninic acid assay (BCA), for which 5 µL of fly extract were added to a microplate well containing 45 µL cold PBS. Afterwards we added 150 µL of BCA solution (BCA Protein Assay Kit 23225, Thermo Scientific). After 9 min, we measured the absorbance at 590 nm and obtained the amount of protein relative to the albumin standard of the same kit.

The rest of the extract was divided into 2 parts, one to measure total-PO and the other to measure active-PO. To measure total-PO, 20 µL of fly extract were added to a microplate well containing 7 µL of α -chymotrypsin (Sigma C7762, 2 mg/mL of ddH₂O) and left 10 min at room temperature to convert proPO into PO. We then added 20 µL of cold PBS and 133 µL of ddH₂O. Lastly, we added 10 µL of L-Dopa solution (the substrate for the PO, Sigma D9628, 2mg/mL of ddH₂O) to the mix and the colorimetric reaction was followed in a microplate reader (SpectraMax 340PC384, Molecular Devices) at 30°C. Readings were taken for 4 hours at 490 nm.

To measure active-PO, we added to 40 µL of fly extract 10 µL of protease inhibitor (Sigma P2714), to avoid endogenous enzymes from activating more proPO. Afterwards 20 µL of this mix were added to a microplate well containing 20 µL of cold PBS, and then we added 140 µL of ddH₂O. Thereafter we added the 10 µL of L-Dopa solution to the mix, and the colorimetric reaction was followed in a microplate reader as described above. Controls in both essays followed the exact same procedures,

but using cold PBS instead of fly extract (see similar procedures in Cotter *et al.* 2004; González-Santoyo *et al.* 2010).

Statistical Analyses

To analyze enzyme activity, we fitted a 5-parameter logistic regression model (Gottschalk & Dunn 2005) to the readings of the colorimetric reaction. We measured the slope (Vmax) of the reaction during the linear phase (inflection point). To control for the autonomous conversion of L-Dopa to dopaquinone we subtracted the activity of the control essays. We then divided these values by the protein amount to standardize for body size of the individuals. The reported values refer to activity present per µg of protein.

Males were subdivided into two groups based on the bimodal distribution observed for their coloration: obsidians (black and small) and amber (orange and large). The two male morphs plus the females composed the three levels of the factor *morph* in the model. Intermediate phenotypes were removed from the analyses due to their ambiguity and low number, hence the sample size for all analyses is N = 68.

To analyze the relation between body size and coloration in *S. thoracica* we fitted a 5-Parameter logistic regression model: $\text{Orangeness} = c + (d-c) / [1 + ((BS/e)^b)^f]$, where BS is body size, *d* is the estimated coloration at body size zero, *c* is the estimated Orangeness at infinite body size, *e* is the mid-range body size, *b* is the slope factor, and *f* is the asymmetry factor (Gottschalk & Dunn 2005).

To determine the effect of larval density on body size we fitted a generalized mixed effect model. The model included the factor *morph*, larval density as a continuous covariate and the possible two-way interaction between these two. We also incorporated in the model a random effect of line nested within populations, to control for their relatedness. The significance of the differences in body size between morphs was evaluated with the Tukey's 'Honest Significant Difference' method.

To analyze the influence of body size, density and morph on the immune system, we also employed generalized mixed effect models. The model included the predictors morph, body size and density, plus all the possible two-way interactions between these variables as well as the three-way interaction. We also incorporated in the model a random effect of line nested within populations. We used the same model for active and total-PO. All analyses were done using the software R Version 3.2.2 (R Core R Development Core Team 2015).

RESULTS

We found a sigmoid relationship between body size and coloration in *S. thoracica* males across all the sampled populations, featuring bimodal body size and bimodal coloration distributions (Fig. 1). The 5-parameter nonlinear regression model of these two variables fitted this relationship (Fig. 1, $R^2 = 0.924$). Female coloration overlaps with that of the obsidian males, while their body size is intermediate between the two male morphs (Fig. 2).

Body size differed between morphs (Fig 2, $X^2 = 205.309$, $df = 2$, $p < 0.001$), and also decreased with larval density overall ($X^2 = 21.918$, $df = 1$, $p < 0.001$). However, the interaction between these two variables was also significant ($X^2 = 12.872$, $df = 2$, $p = 0.002$), due to the relatively flat slope of the obsidian males relative to females and amber males (Fig. 3).

Active-PO did not vary between morphs ($X^2 = 4.169$, $df = 2$, $p = 0.244$; females = 1.413 ± 0.750 , ambers = 1.050 ± 0.402 , obsidians = 1.106 ± 0.483 (all values $\times e^{-6}$); it was also not affected by any other predictor variable (*body size*, $X^2 = 0.037$, $df = 1$, $p = 0.848$; *density*, $X^2 = 0.377$, $df = 1$, $p = 0.539$) or their two-way (*morph:body-size*, $X^2 = 3.619$, $df = 2$, $p = 0.306$; *morph:density*, $X^2 = 1.024$, $df = 2$, $p = 0.795$; *body-size:density*, $X^2 = 1.084$, $df = 1$, $p = 0.298$) or three-way interactions ($X^2 = 2.838$, $df = 2$, $p = 0.417$).

By contrast, total-PO was significantly influenced by morph, being highest for obsidian males (Fig. 4, $X^2 = 11.860$, $df = 2$, $p = 0.008$), and decreased with body size overall ($X^2 = 15.729$, $df = 1$, $p < 0.001$), although there was also an interaction in that total-PO was largely unaffected by the body size of the largest, amber males (Fig. 5, $X^2 = 14.803$, $df = 2$, $p = 0.002$). All other effects were non-significant (*density*, $X^2 = 0.005$, $df = 1$, $p = 0.943$; *morph:density*, $X^2 = 0.031$, $df = 2$, $p = 0.999$; *body-size:density*, $X^2 = 1.291$, $df = 1$, $p = 0.256$; *morph:body size:density*, $X^2 = 0.322$, $df = 2$, $p = 0.956$).

DISCUSSION

Sepsis thoracica males display a sigmoid relationship between body size and coloration, resulting in a body size threshold that divides males into two color morphs: the small and black morph, which we call obsidian, and the large and orange morph, which we call amber (Fig. 1). These two morphs differ in their total immune investment (total-PO), but not in their naturally activated immune responses (active-PO). Importantly, body size, which is inversely related to rearing density, influences the total immune investment in males and females, revealing a trade-off between the two traits in both sexes, but also across the morphs in that obsidian males focus their investment into immunity while amber males invest more in body size.

Life-history costs often allow for variation between individuals, even within the same sex, in the allocation of resources to different traits (Zuk & Stoehr 2002). In *S. thoracica*, differential allocation revealed a trade-off between immunity and body size in both males and females. In males this trade-off is further apparent between morphs, resulting in two strategies: obsidians invest more in immunity and ambers more in body size (Figs. 2, 4, 5). This trade-off between body size and immunity has been observed in other insects (Yan, Severson & Christensen 1997; Rantala & Roff 2005; Cotter *et al.* 2008), as well as in vertebrates (Leach's Storm-Petrel: Mauck *et al.* 2005; Atlantic salmon: Visse *et al.* 2015). Interestingly, we observed a limit to this trade-off in amber males at the lowest immune investment: their body size keeps increasing while their immune investment does not decrease any further (Fig. 5). It is likely that this further body size increase in ambers involves costs other than immunity. Indeed, larger individuals take longer to develop (Busso, unpublished data; Blanckenhorn, Meier & Teder 2007), which likely entails viability costs (Blanckenhorn 2000). Nevertheless, the costs of reducing their immunity may be outweighed by the benefits of this larger body size, for example, by decreasing their vulnerability to predators (Busso & Blanckenhorn, 2016), or by conferring to the amber males a higher mating success

(Busso, unpublished data). Consequently, *S. thoracica* male dimorphism reveals both resource (in terms of energy and nutrients) and viability costs of the immune system (Zuk & Stoehr 2002), and by choosing either cost each morph has adopted an alternative solution.

This body size / immunity trade-off concurs with the application of the Bateman's principle to immunity (Rolff 2002). Originally this hypothesis states that females invest more in immunity to increase their fitness through longevity, whilst males invest less to increase their fitness by augmenting mating success. Here we apply this hypothesis not to male / female differences, as seen in some insects (Nunn *et al.* 2009), but to explain differences between ambers and obsidians. The two male *S. thoracica* morphs provide an excellent opportunity for an intrasexual evaluation of this hypothesis, excluding possible confounding influences of sexually related hormones that also regulate immunity and body coloration in animals (Folstad & Karter 1992). Following the logic of Rolff's (2002) hypothesis, obsidians would invest more in immunity to increase their fitness through longevity, while ambers would invest more in body size and increase their fitness through improved mating success, resulting in alternative reproductive tactics (Busso, unpublished data). A similar situation presents itself for the damselflies *Paraphlebia zoe* and *Mnais pruinosa*, whose two male morphs also employ alternative reproductive tactics that correlate with different immune investments (Tsubaki, Hooper & Siva-Jothy 1997; Tsubaki & Hooper 2004; Ruiz-Guzmán *et al.* 2013). Hence, species with alternative reproductive tactics suggest that the Bateman's principle can explain not only differential immune investment between but also within the sexes.

Alternatively, the different immune investments between morphs could result from differences in their rearing densities (Svensson, Sinervo & Comendant 2001; Wilson & Cotter 2008). The 'density-dependent prophylaxis' hypothesis (Wilson & Reeson 1998) predicts that individuals that live at higher densities should invest more in immunity in preparing for a more stressful larval and adult environment. However, our results indicate that larval density does not directly affect investment into immunity because density negatively influenced the body size of ambers and females but not obsidians (Fig. 3). It is conceivable that increased competition at higher larval densities influences the resources individuals invest in body size and immunity, although the lacking density effect on the obsidians' body size does not support this hypothesis. Unfortunately, it is difficult to evaluate the extent of this indirect density effect (through body size) in the context of the density-dependent prophylaxis hypothesis since most other studies did not assess body size of individuals (Barnes & Siva-Jothy 2000; Wilson *et al.* 2003; González-Tokman *et al.* 2014). Future studies should evaluate the effect of density on other immune components, such as the resulting melanic encapsulation response or antibacterial lysozyme activity, which are negatively affected by rearing density in other insects (Barnes & Siva-Jothy 2000; Cotter *et al.* 2004).

Nevertheless, the higher total investment in immunity by the black obsidian morph concurs with the correlation between immunity and cuticular pigmentation observed in several insect taxa (Mitsui & Kunimi 1988; Kunimi & Yamada 1990; Reeson *et al.* 1998; Cotter *et al.* 2004; Armitage & Siva-Jothy 2005). Based on this evidence one could argue that the melanic coloration could be an honest signal to parasitoids of an immunocompetent host that has high PO activity and should consequently be avoided (Wilson *et al.* 2001). However, the fact that female *S. thoracica* are as black as the obsidians and have a total-PO activity similar to the ambers does rule out a simple relationship between PO and melanic coloration here. And in fact there are several other species that do not show higher PO activity of melanic phenotypes (Barnes & Siva-Jothy 2000; Wilson *et al.* 2002). Therefore, more evidence is needed to assess the influence and significance of immunity on cuticular coloration. In *S. thoracica*, if the cuticular melanization acts as an immunocompetence signal, it would only do so in the males.

On the other hand, the lack of differences for active-PO, which constitutes only a few percent of total-PO, indicates that all individuals show similar investment into their active immune system when lacking a pathogenic threat. These differences between the total immune investment and active immunity are due to high costs of deploying an immune response (Schmid-Hempel 2003; Verhulst, Riedstra & Wiersma 2005). Mounting an immune response involves resource costs to synthesize and maintain the PO-cascade (González-Santoyo & Córdoba-Aguilar 2012), as well as immune autoreactive costs of the cytotoxic by-products of this cascade (Schmid-Hempel 2003; González-Santoyo & Córdoba-Aguilar 2012). Consequently, individuals should minimize the activation of their immune system in the absence of pathogens, resulting in no obvious differences in their active immune system, as observed in our study.

To conclude, we found in *S. thoracica* two male morphs defined by their coloration and body size that also show differential investment in their immunity. This differential investment results from a trade-off between immunity and body size, which is also observed in the females. Besides the costs of immunity in terms of reduced body size, the amber morphs suggest further indirect viability costs of immunity owing to the longer development necessary to gather more resources to express larger body size (Blanckenhorn, Meier & Teder 2007). The coloration of the amber morph seems to result from a drastic drop of investment into immunity, hence it is possible that melanic coloration acts as a signal of the immunocompetence of males. To our knowledge, this is the first study evaluating density-dependent prophylaxis in dipterans (Wilson & Reeson 1998), although the lacking effect of larval density on both active and total-PO does not support this hypothesis. Nevertheless, *S. thoracica* is to our knowledge the first example of a trade-off between body size and immunity that strongly divides the morphospace of the males into two strikingly different morphs: ambers and obsidians. This male dimorphism supports the idea that there is not only one optimal solution to a problem (Farnsworth & Niklas 1995).

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Figure Legends:

Figure 1. Relationship between body size and melanization in *Sepsis thoracica* males, and their respective frequency distributions.

Figure 2. Differences in body size between females and the two male morphs.

Figure 3. Effect of larval density on the body size of both sexes and male morphs.

Figure 4. Differences in total-PO activity between females and the two male morphs. The activity was measured as Absorbance units/min/ μ g of protein.

Figure 5. Effect of body size in total-PO activity in females and both male morphs. The activity was measured as Absorbance units/min/ μ g of protein.