Male morph predicts investment in larval immune function in the dung beetle, 
*Onthophagus taurus*

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Investment in immunity is costly, so that resource-based trade-offs between immunity and sexually selected ornaments might be expected. The amount of resources that an individual can invest in each trait will be limited by the total resources available to them. It would therefore be informative to investigate how investment in immune function changes during growth or production of the sexual trait as resources are diverted to it. Using the dung beetle, *Onthophagus taurus*, which displays both sexual and male dimorphism in horn size, we examined changes in one measure of immune function, phenoloxidase (PO) activity, in the hemolymph of larvae prior to and during horn growth. We found that PO levels differed between small- and large-horned males throughout the final instar prior to the point where investment in horn growth was taking place. PO levels in females were intermediate to the 2 male morphs. These differences could not be accounted for by differences in condition, measured as hemolymph protein levels and weight. We suggest that the observed differences might be associated with sex- and morph-specific dimorphism in horn size, we examined changes in one measure of immune function, phenoloxidase (PO) activity, in the hemolymph of larvae prior to and during horn growth. We found that PO levels differed between small- and large-horned males throughout the final instar prior to the point where investment in horn growth was taking place. PO levels in females were intermediate to the 2 male morphs. These differences could not be accounted for by differences in condition, measured as hemolymph protein levels and weight. We suggest that the observed differences might be associated with sex- and morph-specific variation in juvenile hormone levels. Key words: condition dependence, dimorphism, immunocompetence, phenoloxidase, sexual selection, trade-offs. [Behav Ecol 19:331–337 (2008)]

Parasites are ubiquitous and costly to their hosts, with the risk of parasitism and parasitism itself constituting important selective forces in most species (e.g., Hamilton 1980; Hamilton and Zuk 1982; Moore 1984). Consequently, investment in the immune system should be a priority for most organisms. This should be especially true of juveniles, as failure to reach adulthood means complete failure to produce offspring for subsequent generations. Indeed, the importance of resistance against parasites is such that Hamilton and Zuk (1982) suggested that it could drive sexual selection. The parasite-mediated sexual selection hypothesis predicts that females should prefer males that are resistant to parasites in order to gain genetic benefits for their offspring. Males sporting bright colors or large ornaments are therefore assumed to be advertising their quality to potential mates (Hamilton and Zuk 1982). As investment in immunity is expected to be costly, there may be resource-based trade-offs between immunity and sexually selected ornaments. Therefore, the amount that an individual can invest in each trait will be limited by the resources available to them (Sheldon and Verhulst 1996). It would therefore be informative to investigate how investment in immune function changes during growth or production of the sexual trait as resources are being diverted to it.

Insects provide excellent models for examining the relationship between sexually selected traits and immune function: the invertebrate immune system is significantly simpler than that of vertebrates in that there is no acquired immunity and insects do not possess lymphocytes or immunoglobulins (Gillespie et al. 1997). Nonetheless, the insect immune system does share many fundamental characteristics with the innate immune system of vertebrates, with many of the basic factors showing remarkable homology across species (Vilmos and Kurucz 1998).

A number of previous studies on different insect species have reported positive correlations between immune function and investment in sexually selected traits, the majority using sexually mature adults (Rantala et al. 2000; Siva-Jothy 2000; Rantala and Kurtet 2003; Jacot et al. 2004; Ahtiainen et al. 2005; Simmons et al. 2005; Pomfret and Knell 2006; but see Kurtz and Sauer 1999; Jacot et al. 2005 for correlations with larval immune function). These positive correlations suggest that only high-quality individuals, that is, those in good condition with plentiful resources, can simultaneously invest in both traits. However, we are not aware of any previous studies that have examined ontogenetic changes in immune function both prior to and during maximal investment in sexually selected traits in order to address the hypothesis that trade-offs are mediated by resource availability.

Scarabaeid dung beetles are a group of organisms that display large sexual ornaments in the form of horns, which are formed from cuticular material during the final larval instar. *Onthophagus taurus* is a sexually dimorphic species in which only the males produce horns. Within the males there is a further dimorphism, with large males producing large horns (major males) and small males being either hornless or producing rudimentary horns (minor males) (Hunt and Simmons 1997; Emlen and Nijhout 1999). Each male phenotype is associated with a number of behavioral differences, which constitute alternative reproductive tactics, such that major males compete for females using their large horns as weapons whereas minor males attempt to sneak matings with females (Emlen 1997).

Horn development is facultative and depends on the attainment of a certain body size, which, in turn, depends on the nutrients available to the larva (Hunt and Simmons 1997).
Larval weight peaks during the final instar; larvae cease feeding and purge their guts in preparation for pupation (Emlen and Nijhout 1999). At this point, males larger than a critical size will become major males whereas those that have failed to reach this size will become minor males (Emlen and Nijhout 1999). During this late larval stage, growth of a number of adult structures occurs underneath the larval cuticle, including horns in males, so that they appear fully extended in the freshly molted pupa (Emlen 2000). However, prior to this point, males are not committed to either morph, and investment in these costly structures has not yet taken place.

In this study, we examined how both immune function, measured as phenoloxidase (PO) activity, and body condition, measured as body weight and hemolymph protein levels in the larvae (Cotter et al. 2004), changed during the final larval instar in females, and in males that would later become minors or majors. PO is a key enzyme in the synthesis of the melanin pigment that darkens the cuticle of many insects. Levels of this immune system enzyme have been shown to be a repeatable, heritable indicator of encapsulation ability, an insect’s key response to metazoan parasites (Cotter and Wilson 2002, but see Yang et al. 2007 who showed that these traits could be uncoupled under conditions of starvation). PO has also been implicated in resistance to microparasitic infection in a range of taxa (Rowley et al. 1990; Ourth and Renis 1993; Hagen et al. 1994; Hung and Boucias 1996; Washburn et al. 1996; Wilson et al. 2001; Cerenius and Soderhall 2004; Rantala and Roff 2007). We then asked whether variation in PO activity could be explained by differences in larval body condition or by body size, measured as pronotum width in the emerged adults. If the relationship between investment in horn growth and investment in the immune system is driven by the availability of resources, then we might expect differences between males and females, or between male morphs, to be most apparent at the time when horn growth is taking place. However, previous studies have found that levels of juvenile hormone (JH) can reduce PO activity in adult Tenebrio molitor beetles (Rolff and Siva-Jothy 2002; Rantala et al. 2003). If this result is more generally applicable to insects from other taxa, then we might expect PO activity to increase during larval development as a by-product of decreasing JH levels in preparation for the pupal molt (Chapman 1998). Furthermore, it has been suggested in O. taurus that JH is responsible for initiating the growth of horns in major males late in the final instar (Emlen and Nijhout 1999). Therefore, differences in JH titer between the morphs may result in different levels of PO activity independent of any differences in body condition.

Using O. taurus larvae, we experimentally addressed the following questions.

1. Does PO activity increase throughout the final instar?
2. Do patterns of investment in PO activity differ between the sexes or between larvae that will develop into minor males and major males?

In addition, we consider whether variation in immunity is reflective of nutrient availability by testing statistically for the effect of body condition on investment in PO activity.

**METHODS**

**Experimental populations**

*Onthophagus taurus* beetles were originally collected from fresh cattle dung from a paddock in Margaret River, southwest Western Australia. Beetles were maintained in culture for 1 week, and then females were established in individual breeding chambers: 30-cm long, 9-cm diameter sections of polyvinyl chloride piping, three-quarters filled with moist sand topped with 250 mL of fresh cow dung. *Onthophagus taurus* females dig tunnels directly under a dung pat and lay a single egg inside a ball of dung, known as a brood ball. Chambers were left at 25 °C for 1 week before being sieved and brood balls collected. Brood balls were buried en masse in moist sand in 64 containers.

**Hemolymph collection and larval staging**

Larval development occurs entirely within the brood ball; *O. taurus* go through 3 larval instars before pupation with the majority of the larval stage being spent in the third instar (egg, first and second instar ca. 1 week, third instar ca. 2 weeks; Emlen and Nijhout 1999). The third instar can be further subdivided into 5 morphologically and behaviorally distinct stages: stages 3I–3III representing active feeding and growth during which time the integument shows a transition from clear through mottled to opaque as fat body is laid down; stage 3IV is defined by cessation of feeding and gut purging, during which time larvae construct a pupal shell inside the brood ball from anal exudate and dung; stage 3V is the prepupal stage by which time larvae have completed the gut purge and no longer produce an anal exudate and the pupal shell is complete (Emlen and Nijhout 1999).

Preliminary investigation found that larvae could be assigned to each instar by the size of the head capsule, with third instars having head capsules of approximately 2 mm in width. Ten boxes of brood balls were used for the experiment, each box containing approximately 200 brood balls. Each day, a subsample of brood balls from each box was randomly chosen. Over a period of 2 weeks, from approximately 1 week after laying, brood balls were opened and third instar larvae were staged using the criteria described above (for further details see Emlen and Nijhout 1999). For each larva, the integument was cleaned with ethanol and a hemolymph sample was taken using the tip of a drawn capillary tube. Every second larva was also weighed to 4 decimal places prior to hemolymph sampling. Hemolymph samples were frozen immediately at −80 °C for later analysis. PO and protein were both measured in each hemolymph sample. Larvae were then placed back into the brood ball, which was carefully reconstructed and placed into an individual 25-mL plastic cup containing damp sand. The brood balls were returned to the incubator and checked daily until emergence, moistening the sand as necessary to ensure the brood balls did not dry out. Sex, pronotum width, and horn size (males only) were measured for all emerged adults, allowing individuals to be assigned to female, major male, or minor male morphs. Hemolymph was sampled from 300 larvae in total, 575 of which emerged as adults, comprising 180 females and 193 males. However, in some cases, there was insufficient hemolymph for the PO and protein measurements, and so 158 females and 175 males were used for the final analyses.

**PO assay**

Hemolymph PO was measured using a modified version of the method described in Cotter and Wilson (2002). In brief, 4 μL of hemolymph was added to 200 μL of ice-cold phosphate-buffered saline (pH 7.4) in a plastic Eppendorf tube and vortexed. PO activity was assayed spectrophotometrically with dopamine as a substrate. This assay involved adding 90 μL of 4 mM dopamine to 90 μL of the buffered hemolymph and incubating duplicate samples of the mixture on a temperature-controlled VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA) for 10 min at
25 °C. PO activity was expressed as the slope of the line over 10 min which is in the linear phase of the reaction.

Protein assay
Protein was measured using the BioRad protein assay kit with BSA as the protein standard. Two replicates of 5 µL of the hemolymph/phosphate-buffered saline mixtures were used to measure the protein in each sample. Absorption was measured on a temperature-controlled VERSAmax tunable microplate reader (Molecular Devices) at 600 nm.

Determining male morph
Males of this species are considered dimorphic due to the change in scaling relationship between body size and horn length that occurs between small and large males. Horn length scales linearly in both groups, but the slope is much steeper in major than minor males (Hunt and Simmons 2001). However, as horn length is a continuous variable it is not possible to objectively separate minor and major males by eye, and so a switch point function is used to determine the point at which the scaling relationship changes. Male morph was determined using the 2 switch point functions described in Kotiaho and Tomkins (2001).

\[
Y = \alpha + \beta_1 X + \beta_2 (X - X_D) D + \beta_3 D + \varepsilon,
\]

\[
X = \alpha + \beta_1 Y + \beta_2 (Y - Y_D) D + \beta_3 D + \varepsilon,
\]

where \(Y\) is horn length, \(X\) is pronotum width, and \(Y_D\) and \(X_D\) are the proposed switch points. \(D = 0\) if \(X < X_D\), \(D = 1\) if \(X \geq X_D\). \(\alpha\) is a constant, \(\beta\) is the regression coefficient, and \(\varepsilon\) is the error term. Briefly, these models provide a statistical test for the existence of dimorphic variation in a character associated with body size. Firstly, the value of \(X_D\) or \(Y_D\) that gives the highest \(R^2\) is determined by iteration, and then this value is fitted into the model to give the regression coefficients. The value \(\beta_3\) is then tested to see if it is significantly different from zero (Wilson K, unpublished code). The first switch point is determined using body size and the second using horn length. Both switch points were calculated for comparison with other values reported in the literature; however, the horn length switch point alone was used to separate males into minor and major morphs. Importantly, this statistically determined switch point coincides with a change in reproductive behavior adopted by alternative male phenotypes (Hunt and Simmons 2000).

Statistical analyses
The determination of male morph was carried out in SPlus 7. Brood balls were selected randomly and the stage of each larva noted. At this stage, we could not know the sex or morph of the larvae, and so blood samples were collected from a large number of individuals in order to ensure that sufficient individuals of each morph were represented for the statistical analyses. However, this necessarily resulted in an unbalanced design; therefore, all other analyses were carried out using linear mixed effects restricted estimate maximum likelihood models in Genstat 8, which are more robust with regard to unbalanced designs than analysis of variance procedures. In each case, the box from which a brood ball was sampled was included as a random effect and morph, third instar stage, protein, weight, pronotum width, and their interactions were included as fixed effects.

RESULTS
Determining male morph
Both body size and horn length switch points were calculated (Figure 1), but the horn length switch point alone was used to categorize the males into major or minor morphs as this resulted in fewer males being misclassified (Kotiaho and Tomkins 2001). The body size switch point was 5.16 mm \((R^2 = 0.838, \beta_3 = 0.751, P < 0.001)\), and the horn length switch point was 0.30 mm \((R^2 = 0.830, \beta_3 = 0.114, P = 0.045)\). Males were classified using the horn length switch point only.

Larval weight
Weight was measured for half of the individuals tested. A quadratic linear regression model was fitted to the weight data with morph (female, minor male, or major male) and third instar stage as main effects. There were significant main effects of stage \((F_{1,162} = 71.24, P < 0.001)\) and stage \(^2\) \((F_{1,162} = 71.67, P < 0.001)\). There was also a significant interaction between morph and stage \((F_{2,162} = 3.05, P = 0.048)\). This resulted in separate curves being fitted for each of the morphs. As shown previously (Emlen and Nijhout 1999), larval weight peaked around the third stage of the third instar then fell after the gut purge at the beginning of the fourth stage. It was at this time that differences between the morphs became apparent (Figure 2). The predicted curves for the females and minor males were quite similar with weight peaking at stage 3 then dropping to stage 5 (Figure 2). However, in the major males the maximum weight was reached at stage 4 then dropped to stage 5.

From stage 3 onward, there were significant differences between the morphs. At stage 3, females were just significantly heavier than minor males \((t_{21} = 2.13, P = 0.045)\), for
The protein levels did not differ significantly between minor males. In contrast, protein levels in the females increased at stages 2 and 3 then dropped steadily at stage 5. However, the increase in the major males was much sharper than in the minor males. Protein levels increasing to stage 2 then staying fairly constant until they increase sharply between stages 4 and 5. However, the morphs until stage 4. At this time, major males had significantly higher protein levels than both females (t_{63} = 0.32, P = 0.75). At stage 4, major males and females were significantly heavier than minor males (female vs. minor male, t_{60} = 2.14, P = 0.04; major male vs. minor male, t_{6} = 2.77, P = 0.01), though not significantly different from each other (t_{60} = 1.17, P = 0.25). At stage 5, major males were significantly heavier than minor males (t_{6} = 2.92, P = 0.02) but females were not significantly different to either major males (t_{6} = -1.62, P = 0.13) or minor males (t_{6} = 1.74, P = 0.11).

**Protein**

A cubic linear regression model was fitted to the protein data with morph and third instar stage as main effects. There was a significant main effect of stage (F_{1,326} = 19.43, P < 0.001). There were also significant interactions between morph and both stage (F_{2,326} = 5.46, P = 0.005) and stage (F_{2,326} = 9.14, P < 0.001). This again resulted in separate curves being fitted for each of the morphs. The predicted curves for the males were quite similar (Figure 3), with protein levels increasing to stage 2 then staying fairly constant until they increase sharply between stages 4 and 5. However, the increase in the major males was much sharper than in the minor males. In contrast, protein levels in the females increased at stages 2 and 3 then dropped steadily at stage 5. The protein levels did not differ significantly between the morphs until stage 4. At this time, major males had significantly higher protein levels than both females (t_{64} = 2.07, P = 0.04) and minor males (t_{64} = 2.05, P = 0.046), whereas females and minor males were not significantly different from each other (t_{67} = 0.04, P = 0.97). At stage 5, major males had significantly higher protein levels than either minor males (t_{63} = 4.67, P < 0.001) or females (t_{63} = 9.63, P < 0.001), and minor males had significantly higher protein levels than females (t_{40} = 5.00, P < 0.001).

**Hemolymph PO activity**

Figure 4 shows that PO activity increased slowly during the first 4 stages of the third instar but then increased sharply at the fifth stage. A cubic linear regression model was fitted to the PO data with morph and third instar stage as main effects. All interactions were nonsignificant and were dropped from the model. There was a significant effect of morph on hemolymph PO activity (F_{2,327} = 8.28, P < 0.001). Mean (standard error) V_{max} values for the untransformed PO data were as follows, major males V_{max} = 22.59 (0.91), females V_{max} = 21.21 (0.65), and minor males V_{max} = 18.22 (0.76). Minor males had significantly lower PO activity than major males (t_{327} = -2.27, P = 0.024). Female PO activity did not differ significantly from each other (F_{1,326} = 0.04, P = 0.876).

**Figure 2**

The relationship between larval weight and stage. Weight changes over the third instar for each morph. Quadratic curves were fitted for each morph, the bars represent the group means ±1 SE (standard error). Minimal model: weight = morph + stage + stage^2. Fitted values are plotted for each morph and stage, and the predicted curve for each morph is shown. Significant differences between the male morphs only occur during stages 4 and 5 when minor males are significantly smaller than major males.

**Figure 3**

The relationship between hemolymph protein levels and stage. Cubic curves were fitted for each morph, the bars represent the group means ±1 SE (standard error). Minimal model: protein = morph + stage + morph:stage + stage^2. Fitted values are plotted for each morph and stage, and the predicted curve for each morph is shown.

**Figure 4**

The relationship between hemolymph PO activity and stage. Cubic curves were fitted for each morph, the bars represent the group means ±1 SE (standard error). Minimal model: PO activity = morph + stage + stage^2 + stage^3. Fitted values are plotted for each morph and stage, and the predicted curve for each morph is shown.
significantly from either male morph (minor males, $t_{227} = 1.76$, $P = 0.079$; major males, $t_{227} = -1.00$, $P = 0.319$) although it was closer to major males. There was a significant effect of stage ($F_{1,227} = 3.96$, $P = 0.047$), stage$^2$ ($F_{1,227} = 5.49$, $P = 0.019$), and stage$^3$ ($F_{1,227} = 9.02$, $P = 0.003$) on hemolymph PO levels, with PO activity levels per milliliter of hemolymph generally increasing with stage throughout the third instar.

**PO activity and condition**

In order to test the hypothesis that differences in PO activity between the sexes or morphs was driven by differences in condition, the analysis for PO activity was repeated including hemolymph protein level as a covariate (Cotter et al. 2004). Hemolymph protein is correlated with body weight and so is an indicator of body condition ($r = 0.35$, $t_{251} = 6.63$, $P < 0.001$). The maximal model contained all interactions between protein, morph, and the stage terms. All the terms fell out of the model except for those included in the original analysis, namely morph, stage, stage$^2$, and stage$^3$ (comparison of models: maximal model Residual Sums of Squares (RSS) $= 230.17$, degrees of freedom $= 295$, final model RSS $= 261.79$, degrees of freedom $= 526$, $F_{1,31} = 1.31$, $P = 0.13$).

Next, the same linear regression model was fitted to the subset of PO data with weight included as a covariate. PO is also correlated with body weight ($r = 0.17$, $t_{231} = 3.04$, $P = 0.003$); however, when included in the model weight was marginally nonsignificant ($F_{1,134} = 3.74$, $P = 0.055$). However, even with weight retained in the model morph was still significant ($F_{2,134} = 3.71$, $P = 0.027$). Similarly, for the protein data, weight was not significant ($F_{1,146} = 0.21$, $P = 0.65$) whereas with weight included in the model morph was still significant ($F_{2,146} = 5.10$, $P = 0.007$).

The main effect of pronotum width was also included in the analysis of PO activity as a measure of body size. The effect was nonsignificant ($F_{1,280} = 0.40$, $P = 0.53$). However, if it was retained in the model, the main effect of morph was still significant ($F_{2,280} = 3.82$, $P = 0.023$). These analyses suggest that morph variation in PO activity is not a consequence of variation in condition and is not driven by differences in body size alone.

**DISCUSSION**

Investment in immune function is expected to be costly. For species that display exaggerated sexually selected traits, only high-quality individuals are expected to be able to invest in sexual display and immunity simultaneously (Hamilton and Zuk 1982; Sheldon and Verhulst 1996). For vertebrates, it has been suggested that this trade-off could be mediated by testosterone (Folstad and Karter 1992), but no such hormonal mechanism has been unequivocally identified for invertebrates, which lack sex-specific hormones. Instead, the trade-off is generally assumed to be mediated by resource costs (Chapman 1998); therefore, the sex differences may be due to differences in hormone profiles at this time. Despite this, not all hemolymph PO activities in females because hemolymph PO levels increased during stages 4 and 5 despite the overall reduction in protein levels.

Although minor male larvae appear to be in a poorer condition than major males during the latter stages of the final
instar, the differences in condition alone cannot account for the differences in PO activity between the 3 groups. Neither larval weight nor hemolymph protein levels were significant predictors of variation in PO activity, though the experimental manipulation of dung quantity and/or quality would be required to clarify this. An alternative hypothesis is that the patterns are driven by other physiological differences between the morphs, such as different hormone profiles during larval development.

JH remains high throughout larval development, only dropping in preparation for the pupal molt (Chapman 1998). In third instar O. taurus, ecdysteroid titer increases from larval stage 3 onwards (Emlen and Nijhout 1999). PO levels follow these changes in hormone titer, increasing as JH levels are predicted to drop from stage 3. It is possible, therefore, that PO activity is reduced by the presence of JH in the hemolymph (Rolf and Siva-Jothy 2002; Rantala et al. 2003), only increasing when JH titers fall.

Previous work has shown that there are differences in ecdysone titer between larvae during the feeding stages of the third instar, which correspond to the time period when larvae are assessing their body size in order to determine which morph they will develop into (Emlen and Nijhout 1999). Whether or not there are also differences in JH titers between the male morphs remains to be seen. However, previous work with this species has shown that the 2 morphs show different levels of susceptibility to the JH analogue, methoprene. Application of methoprene after gut purge can induce small, normally hornless males to develop horns (Emlen and Nijhout 1999; Emlen and Nijhout 2001; Moczek and Nijhout 2002), whereas application earlier in the final instar seemed to increase the threshold body size at which larvae developed horns (Emlen and Nijhout 2001). These results have recently been interpreted to reflect a morph-specific difference in the timing of the decline in JH, with JH levels predicted to drop earlier in large, major males than in smaller, minor males (Emlen and Nijhout 2001; Emlen et al. 2005). This pattern remains to be tested, but it would be consistent with our observed pattern of PO activity in major and minor males and suggests a possible role of this insect hormone in the mediation of individual immune responses. Quantification of the JH profile during this critical larval stage would elucidate the effects of JH on horn development and may shed light on the hormonal control of immune function during this time. Further investigations are clearly required to clarify the immunomodulatory role of hormones in invertebrates and the role they may play in mediating trade-offs with other traits such as sexually selected ornaments.

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