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

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Running title: Male morph and immune function in *O. taurus*
Abstract

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 activity, in the hemolymph of larvae prior to, and during horn growth. We found that phenoloxidase 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 two 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.

Keywords: condition-dependence, dimorphism, immunocompetence, phenoloxidase, sexual selection, trade-offs
Introduction

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 (Ahtiainen et al., 2005; Jacot et al., 2004; Pomfret and Knell, 2006; Rantala and Kortet, 2003; Rantala et al., 2000; Simmons et al., 2005; Siva-Jothy, 2000; but see Jacot et al., 2005; Kurtz and Sauer, 1999 for correlations with larval immune function). These positive correlations suggest that only high quality individuals, i.e. 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.

Scarabeid 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) (Emlen and Nijhout, 1999; Hunt and Simmons, 1997). 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, whilst 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 whilst 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 (Cerenius and Soderhall,
2004; Hagen et al., 1994; Hung and Boucias, 1996; Ourth and Renis, 1993; Rantala
and Roff, 2007; Rowley et al., 1990; Washburn et al., 1996; Wilson et al., 2001). 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 (Rantala et al., 2003; Rolff and Siva-Jothy, 2002). 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 asked the following questions:

1. Do patterns of investment in PO activity differ between the sexes, or between
   larvae that will develop into minor males and major males?
2. Does PO activity increase throughout the final instar, as JH levels decrease in
   preparation for the molt?
3. Is variation in immunity reflective of nutrient availability, i.e. can investment
   in immunity be predicted by condition?

**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 one week and then females were established in individual breeding
chambers: 30-cm long, 9-cm diameter sections of PVC piping, three-quarters filled
with moist sand topped with 250 ml of fresh cow dung. *O. taurus* females dig tunnels
directly under a dung pat, and lay a single egg inside a ball of dung, known as a brood
Chambers were left at 25°C for one week before being sieved and brood balls collected. Brood balls were buried en masse in moist sand in 6 liter containers.

**Hemolymph collection and larval staging**

Larval development occurs entirely within the brood ball; *O. taurus* go through three larval instars before pupation with the majority of the larval stage being spent in the third instar (egg, first and second instar ~ one week, third instar ~ two weeks, (Emlen and Nijhout, 1999). The third instar can be further subdivided into five morphologically and behaviorally distinct stages: stages 3I to 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, 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 brood boxes were used for the experiment, each of which contained approximately 200 brood balls. Each day a sub-sample of brood balls from each box were randomly chosen. Over a period of 2 weeks, from approximately one week after laying, brood balls were opened, and third instar larvae were staged using the criteria described above (for further detail 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 larvae was also weighed to 4 decimal places prior to haemolymph sampling. Hemolymph samples were immediately frozen.
at -80°C for later analysis. PO and protein were both measured in each haemolymph 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. Haemolymph was sampled from 500 larvae in total, 373 of which emerged as adults, comprising 180 females and 193 males. However, in some cases there was insufficient haemolymph for the PO and protein measurements and so 158 females and 175 males were used for the final analyses.

**Phenoloxidase assay**

Hemolymph PO was measured using a modified version of the method described in Cotter & Wilson (2002). In brief, 4 μl of hemolymph were 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) for 10 minutes at 25°C. PO activity was expressed as the slope of the line over 10 minutes 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/PBS 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 switchpoint function is used to determine the point at which the scaling relationship changes. Male morph was determined using the two switchpoint functions described in Kotiaho and Tomkins (2001).

1. \( Y = \alpha + \beta_1 X + \beta_2 (X - X_D)D + \beta_3 D + \varepsilon \)
2. \( X = \alpha + \beta_1 Y + \beta_2 (Y - Y_D)D + \beta_3 D + \varepsilon \)

Where \( Y \) is horn length, \( X \) is pronotum width and \( Y_D \) and \( X_D \) are the proposed switchpoints. \( D = 0 \) 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 (K. Wilson, unpublished code).

Importantly, this statistically determined switch point coincides with a change in
reproductive behaviour adopted by alternative male phenotypes (Hunt & Simmons 2000).

Statistical analyses

The determination of male morph was carried out in S Plus 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 REML models in Genstat 8, which are more robust with regards to unbalanced designs than ANOVA 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 ($R^2 = 0.838, \beta_3 = 0.751, P < 0.001$) the horn length switch point was 0.30 mm ($R^2 = 0.830, \beta_3 = 0.114, P = 0.045$). These are very similar to the switch point values reported for a laboratory colony of O. taurus by Kotiaho and Tomkins (2001) (body size of 5.14 mm and horn length of 0.31 mm).
**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 following the gut purge at the beginning of the fourth stage. It was at this time that differences between the morphs became apparent (Fig. 2). The predicted curves for the females and minor males were quite similar with weight peaking at stage 3 then dropping to stage 5 (Fig. 2). However, in the major males the maximum weight was reached at stage 4 then dropped to stage 5.

From stage 3 onwards, there were significant differences between the morphs. At stage 3, females were just significantly heavier than minor males ($t_{18} = 2.12, P = 0.048$), for major males the difference was marginally non-significant ($t_{23} = 1.81, P = 0.08$) and major males and females were not significantly different from each other ($t_{33} = 0.26, P = 0.79$). At stage 4 major males and females were significantly heavier than minor males (female vs. minor male, $t_{30} = 2.14, P = 0.04$; major male vs. minor male, $t_{16} = 2.77, P = 0.01$), though not significantly different from each other ($t_{30} = 2.43, P = 0.02$). 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 ($t_{12} = -1.62, P = 0.13$) or minor males ($t_6 = 1.58, P = 0.14$).
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^2 ($F_{2,326} = 5.46, P = 0.005$) and stage^3 ($F_{2,326} = 9.14, P < 0.001$). This again resulted in separate curves being fitted for each of the morphs. The predicted curves for the males were quite similar (Fig. 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 ($t_{24} = 2.07, P = 0.04$) and minor males ($t_{61} = 2.03, P = 0.046$), whereas females and minor males were not significantly different from each other ($t_{107} = 0.04, P = 0.97$). At stage 5, major males had significantly higher protein levels than either minor males ($t_{33} = 4.67, P < 0.001$) or females ($t_{43} = 9.63, P < 0.001$), and minor males had significantly higher protein levels than females ($t_{40} = 5.00, P < 0.001$).

Hemolymph phenoloxidase activity

Fig. 4 shows that PO activity increases slowly during the first four stages of the third instar but then increases sharply at the fifth stage. A cubic linear regression model was fitted to the phenoloxidase data with morph, and third instar stage as main effects. All interactions were non-significant 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 (SE) Vmax values for the untransformed PO data were as follows, Major males
Vmax = 22.59 (0.91), females Vmax = 21.21(0.65) and minor males Vmax = 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 either male morph (minor males, $t_{327} = 1.76$, $P = 0.079$; major males, $t_{327} = -1.00$, $P = 0.319$) although it was closer to major males. There was a significant effect of stage ($F_{1,327} = 3.96$, $P = 0.047$), $stage^2$ ($F_{1,327} = 5.49$, $P = 0.019$) and $stage^3$ ($F_{1,327} = 9.02$, $P = 0.003$) on hemolymph PO levels, with PO activity levels per ml 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 is driven by differences in condition, the analysis for PO activity was repeated including hemolymph protein level as a covariate (Cotter et al., 2004). Haemolymph 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 of 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 RSS = 230.17, df = 295, final model RSS = 261.79, df = 326, $F = 1.31$, $P = 0.13$).

Next, the same linear regression model was fitted to the subset of phenoloxidase data with weight included as a covariate. PO is also correlated with body weight ($r = 0.17$, $t_{251} = 3.04$, $P = 0.003$), however, when included in the model weight was marginally non-significant ($F_{1,134} = 3.74$, $P = 0.055$). 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 non-significant ($F_{1,286} = 0.40, P = 0.53$).

However, if it was retained in the model the main effect of morph was still significant ($F_{1,286} = 3.82, P = 0.023$). 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 (Kurtz and Sauer, 1999; Pomfret and Knell, 2006; Sheldon and Verhulst, 1996).

For the sexually dimorphic dung beetle *Onthophagus taurus*, male morph is determined late in the final instar upon the attainment of a critical size, which is dependent upon the availability of resources to the developing larva (Emlen and Nijhout, 1999). The investment in energetically-costly horn production does not occur until after this point (Emlen, 2000). If the trade-off between immunity and sexually selected traits occurs in this species, and is driven by resource availability, we should
see a different pattern of investment in immunity in males as they divert resources to
horn growth. Moreover, we might only expect males to differ after the point at which
resource availability determines which morph they will develop into. In this study, we
examined changes in the activity of the immune system enzyme phenoloxidase (PO),
during the final larval instar of *O. taurus*, incorporating the time at which male larvae
divert resources to horn growth. We found significant differences in PO activity
between larvae that were destined to become major males, minor males and females
before the time when horn growth occurs, and more importantly, before the point at
which male morph is determined. PO activity was highest in major males,
intermediate in females and lowest in minor males throughout the final instar, despite
minor and major males being indistinguishable during the first three stages of the
instar. In addition, we also found that levels of PO activity changed markedly
throughout the final larval instar in all three groups (females, minor males and major
males), despite the risk of parasitism presumably remaining constant. PO levels were
found to increase throughout the final instar, increasing sharply in all three morphs
from stages four to five.

Whilst it is possible that PO activity could increase with larvae size due to an
increased availability of resources to divert to immunity, changes in PO did not follow
the changes in larval weight. Larval weight increased rapidly during the first three
stages of the final instar at which time PO activity remained relatively constant. It was
only when larval weight peaked and then began to fall that PO activity increased
rapidly. This increase in PO activity prior to the pupal molt may be adaptive as PO is
involved in the melanization of the cuticle. Alternately, higher PO levels might be
beneficial in reducing the risk of septicemia during metamorphosis, as gut bacteria
may present a potential risk to the pupa, especially in dung beetles whose environment is rich in bacteria.

The two estimates of condition, larval weight and hemolymph protein levels, varied considerably throughout the final instar, and differed between the sexes and the morphs. Larval weight increased rapidly during the first three stages of the instar, with minor males only being moderately lighter at stage three. However, from stage four all three groups lost weight in preparation for the molt, and minor males reached a final weight that was significantly lower than major males. For minor males it is the inability to maintain a threshold weight during this critical period that determines their hornless status (Emlen and Nijhout, 2001), which is primarily determined by the availability of the food resource.

Protein levels also varied markedly throughout the final instar, increasing with weight in all three groups initially. However, whereas in females protein levels followed the change in weight, decreasing through stages four and five, in males, protein levels increased at this time. Moreover, the increase in major males was significantly higher than that shown in minor males. Therefore, using weight or protein as a surrogate for condition would predict that minor males are in poorer condition than major males in stages four and five only.

The differences in protein levels between the sexes suggest that something other than condition is driving the changes at this time. Proteins are usually removed from the hemolymph and stored in the fat body prior to pupation (Chapman, 1998). One possibility is that females start this process slightly earlier than males, or that females are sequestering proteins to be used in ovarian growth and egg production. The synthesis and storage of proteins is regulated by both juvenile hormone and ecdysteroids (Chapman, 1998), therefore the sex differences may be due to
differences in hormone profiles at this time. Despite this, not all hemolymph proteins
decline in females, since hemolymph PO levels increased during stages four and five
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 three groups. Neither larval
weight nor hemolymph protein levels were significant predictors of variation in PO
activity. An alternative hypothesis is that the patterns are driven by other
physiological differences between the morphs, such as different hormone profiles
during larval development.

Juvenile hormone (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 three onwards (Emlen and Nijhout, 1999). PO levels
follow these changes in hormone titers, increasing as JH levels are predicted to drop
from stage three. It is possible, therefore, that PO activity is reduced by the presence
of JH in the hemolymph (Rantala et al., 2003; Rolff and Siva-Jothy, 2002), 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 two morphs show different levels of susceptibility to
methoprene, a JH analogue, when it is applied topically during the final instar (Emlen
and Nijhout, 1999; Emlen and Nijhout, 2001; Moczek and Nijhout, 2002).
Application after gut purge can induce small, normally hornless males to develop horns (Emlen and Nijhout, 1999; Moczek and Nijhout, 2002) suggesting that JH titer at this time is linked to horn growth. Application earlier in the final instar, at the time when larvae are assessing body size in order to determine which morph they will develop into, 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 et al., 2005; Emlen and Nijhout, 2001). 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.

References


Figure legends

Fig. 1. Switchpoint values used to predict male morph

Pronotum width is plotted against horn length in males. The sigmoidal relationship is associated with a bimodal frequency distribution of horn lengths in the population. The switchpoints were calculated for both horn length and body size as described in Kotiaho and Tomkins (2001). The body size switch point was 5.16 mm ($R^2 = 0.838$, $\beta_3 = 0.751$, $P < 0.001$) the horn length switch point was 0.3005 mm ($R^2 = 0.830$, $\beta_3 = 0.114$, $P = 0.045$)

Fig. 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 $\pm$ 1 SE. Minimal model: $weight \sim morph + stage + stage^2$. 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.

Fig. 3. The relationship between hemolymph protein levels and stage

Cubic curves were fitted for each morph, the bars represent the group means $\pm$ 1 SE. Minimal model: $protein \sim morph + stage + morph:stage^2 + morph:stage^3$. Fitted values are plotted for each morph and stage and the predicted curve for each morph is shown.
Fig. 4. The relationship between hemolymph PO activity and stage

Cubic curves were fitted for each morph, the bars represent the group means ± 1 SE.

Minimal model: $PO\ activity \sim morph + stage + stage^2 + stage^3$. Fitted values are plotted for each morph and stage and the predicted curve for each morph is shown.
Figures

Figure 1.

![Graph showing pronotum width and horn length in mm with switchpoints for major and minor categories.](image-url)
Figure 2

![Graph showing mean weight in mg +/- SE for different instar stages for Female, Minor male, and Major male.](graph.png)

**Third instar stage**

- 50
- 70
- 90
- 110
- 130
- 150

**Mean weight in mg +/- SE**

- Female
- Minor male
- Major male
Figure 3

The graph shows the mean log protein levels in mg +/- SE for the third instar stage. The x-axis represents the third instar stage, and the y-axis represents the mean log protein levels in mg +/- SE. The graph includes data for Female, Minor male, and Major male. The graph indicates a trend where protein levels increase with the stage, with Major males showing the highest levels.
Figure 4

Third instar stage

Mean log PO activity +/- SE

Female
Minor male
Major male