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Changes in the Fat Body during the Post-Embryonic Development of the Predator *Toxorhynchites theobaldi* (Dyar & Knab) (Diptera: Culicidae)

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Abstract

Several studies have focused on understanding the biochemistry and morphology of the fat body of the hematophagous mosquito *Aedes aegypti* (L.) (Diptera: Culicidae). In contrast, few studies, if any, have focused on morphological characters of the fat body in other mosquitoes, especially non-hematophagous taxa such as the culicid *Toxorhynchites*. Larvae of *Toxorhynchites* prey upon the larvae of other mosquito species and are used in vector mosquito control. We investigated aspects of the fat body trophocytes, including the morphometric analyses of the lipid droplets, protein granules and nuclei, during *Toxorhynchites theobaldi* (Dyar & Knab) post-embryonic development. Following the body weight increase from larval stage L2 to L4, the size of lipid droplets within the trophocytes also increase, and are likely the result of lipogenesis. Lipid droplets decrease in size during L4 to the female pupal stage and increase once again during the period from newly-emerged to mature adult females. Protein granules are observed for the first time in female pupae, and their appearance might be related to protein storage during metamorphosis. The size of the nucleus of trophocytes also increases during larval development, followed by a decrease during metamorphosis and an additional increase as adult female ages. In conclusion, the morphology of the fat body of *T. theobaldi* changes according to the developmental stage. Our study provides for the first time important insights into *T. theobaldi* fat body development and contributes to understand this species biology.

Introduction

The fat body plays an important role in energy storage and utilization providing nutrients for insect growth, flight and reproduction (reviewed in Roma *et al* 2010). The trophocytes are the major cell type of the fat body that lines the abdominal cavity and is located underneath the insect epidermis (Cruz-Landim 1975, Martins & Pimenta 2008). The trophocyte cytoplasm is filled with lipid droplets, proteins granules and glycogen (Martins *et al* 2011). While lipid droplets are widely found in trophocytes during developmental stages of all insects, protein granules generally appear during metamorphosis. In general, protein granules are consumed during energetically cost activities, such as migration and reproduction (Locke & Collins 1965, Zara *et al* 2003, Zara & Caetano 2004).

Holometabolous insects obtain their adult biomass during larval growth. Thus, food consumption is intense and the fat body enlarges during larval development. However, holometabolous insects do not feed during metamorphosis and simply utilize the nutrients stored

during their larval growth. During metamorphosis, the fat body is rebuilt through cellular turnover to the extent that when the adult insect emerges, the fat body has been reshaped or completely replaced (Larsen 1976, Grzelak & Kumaran 1986, Wang & Haunerland 1991, 1992).

The non-hematophagous mosquitoes *Toxorhynchites* spp. (Culicidae) are predators of several aquatic invertebrates (reviewed in Focks 2007). The predatory behavior in these species occurs throughout their larval development (reviewed in Steffan & Evenhuis 1981), while adults feed solely on plants (Trpis 1972, Trimble & Smith 1978, Chowanadisai *et al* 1984). Larvae of *Toxorhynchites* are found widely distributed in natural and artificial containers and are potential natural agents for the control of other Culicidae, such as *Aedes albopictus* (Skuse) and *Aedes aegypti* (L.) (Tikasingh & Eustace 1992, Kesavaraju & Juliano 2004, Kesavaraju *et al* 2007, Albeny *et al* 2010) and other Diptera (Lounibos & Frank 1987). In spite of *Toxorhynchites* relevance for biocontrol of mosquito vectors, little is known in terms of their physiology and internal morphology, including the lack of information regarding changes in fat body cells used for nutrient storage during post-embryonic development. In contrast, several studies to date were focused on understanding the fat body of the hematophagous mosquitoes, mainly *A. aegypti* (Wigglesworth 1942, Raikhel & Lea 1983, Martins & Pimenta 2008, Martins *et al* 2011) and *Culex quinquefasciatus* (Say) (Alves *et al* 2010, Cardoso *et al* 2010, Martins *et al* 2011, in press).

In the present work, we investigated morphological changes in the fat body of *T. theobaldi* during its post-embryonic development. The results obtained indicate significant differences for trophocytes, especially related to lipid droplet size increase during the larval development and during aging of the adult mosquito. The presence of protein granules and changes in the size of the nucleus are also discussed regarding their possible role in *T. theobaldi* fat body physiology. Our study provides insights into *T. theobaldi* fat body development, contributing to understand aspects of the physiology this tissue in the studied species. Potentially, how *T. theobaldi* trophocytes respond to biotic (e.g, mosquito development and age) or abiotic (i.e., environmental cues) factors can have an impact on its ability to function as a biocontrol agent. Identifying such responses is a prerequisite for the efficient use of such species in biocontrol programs against other mosquito larvae.

Material and Methods

Mosquitoes

Larvae of *T. theobaldi* were collected from artificial containers in the field and maintained in dechlorinated tap water under laboratory conditions. Seven days after adult emergence, insects were allowed to mate. All developmental stages were obtained from the F1 generation. Seven groups, with six individuals/group, of F1 mosquitoes were used in the studies described here: larval stages one (L1) through four (L4) (fed on *A. aegypti* larvae *ad libitum*), two-day old female pupae, newly-emerged and four-to-six day-old fertilized females. These adult females were fed on 50% honey solution *ad libitum*. All individuals were maintained using a 12/12 photoperiod at 28 ± 3°C and 80% humidity.

Body mass

Larvae and pupae were gently dried in bath tissue and adults were anesthetized with CO_2 and immediately weighted using a precision scale. The body mass (mg) was recorded for six individuals from each of the samples described above.

Dissection and fixation

Specimens were dissected under a stereoscope using 0.1 M phosphate buffered saline (PBS) at pH 7.2. The abdomen was separated from the thorax, cut at the last abdominal segment and stored in 4% formaldehyde fixative in PBS until use.

Morphometry

Samples were rinsed in PBS, dehydrated in a graded ethanol series (30-100%) and embedded in historesin (Leica). Four-μm thin sections were stained with hematoxylin and eosin. These histological sections were observed and photographed with an Olympus BX60 light microscope coupled with an Olympus Q-color3 digital camera. Lipid droplets were measured individually in all seven groups described above. Protein granules were measured from pupae, while nuclei were measured from L2 to four-to-six day-old adult females. For all measurements taken, each cell component was randomly selected and measured for a total area per group of individuals equivalent to 800 μm^2 in histological sections. Also, for each of the three cell components, their respective areas were determined considering their boundaries with the software Image ProPlus™.

Statistics

Body weight, lipid droplet and nuclei of different *T. theobaldi* developmental stages were compared using analysis of variance (ANOVA). All analyses were carried out using generalized linear models (GLM) (Crawley 2002). Analyses were performed using the free software R (R Development Core Team 2006), followed by residual analyses to verify error distribution. The confidence interval was set at 5%.

Results and Discussion

The fat body of *T. theobaldi* is located underneath the epidermis and is formed basically by a mass of trophocytes. Visual inspection using light microscopy reveals that the trophocytes that comprise the fat body contain large lipid droplets and protein granules. Also, nuclei of trophocytes are easily recognized in almost all developmental stages. Well developed nucleolus similar to previously described for other dipterans (Sohal 1973, Tobe & Davey 1974, Stoppie *et al* 1981, Martins *et al* 2011) are also present (Figs 1A-D).

A significant increase in body mass was detected during development of *T. theobaldi* ($F_{1;34}$ = 84.83, P < 0.0001) (Fig 2A), with a 52-fold body mass increase from L1 to L4 (Fig 2A). During metamorphosis, there is a reduction of nearly 0.87-fold in the body mass of L4 larvae to female pupae and another decrease of approximately 3.5-fold from pupae to newly-emerged females (Fig 2A). However, adult females have a 2-fold increase in their body mass on their fourth to sixth day of adulthood (Fig 2A).

Trophocytes have a cytoplasm rich in lipid droplets in all samples analyzed (Fig 1B, C). As with body mass, the area of the lipid droplets of trophocyte varies according to the developmental stages ($F_{1;342}$= 39.89, P < 0.0001). However, differences are not statistically significant from L1 (0.36 ± 0.039 μm^2) to L2 larvae (0.35 ± 0.050 μm^2). On the other hand, a 1.4-fold increase in size is detected from L3 (3.16 ± 0.22 μm^2) to L4 (4.54 ± 0.40 μm^2), but once females pupate, a ~0.9-fold decrease in the area of the lipid droplets occurs. After metamorphosis, the size of the lipid droplets decreased by ~0.8-fold in the newly-emerged females in comparison to pupae, increasing once again in four-to-six day-old females (~1.2-fold) (Fig 2B).

Based on the body mass and on the morphometric analysis of the trophocyte lipid droplets, it can be inferred that the lipid storage increases during larval development. It is interesting to note that in spite of a body mass increase of ~9.5-fold from L1 to L2, the lipid droplets do not significantly increase in size. This suggests that body weight gain is not solely associated to the increase in lipid droplet size, and may be due to an increase in other structures or organelles as well as increase in the number of lipid droplets per cell. However, as the fat body cells are so tightly clustered, accurate assessment of cell limits and of the number of droplets per cell is difficult. The increase in size of the lipid droplets continues until the L4 stage, when these cells reach their maximum size (Fig 2B). The increase in the lipid droplet size may be correlated with the overall weight gain by the larvae. For instance, from L1 and L2 to L4, the lipid droplet increases ~13-fold, while body mass increases ~52-fold.

There is a marked increase in the size of lipid droplets from L2 to L3 (Fig 2B), suggesting that the *T. theobaldi* lipid storage is possibly enhanced during this development interval than any other point during larval development. Thus, food deprivation during the L2-L3 transition may be more critical for larval development than in other stages. Future studies shall clarify the importance of feeding and lipid storage during these early larval stages.

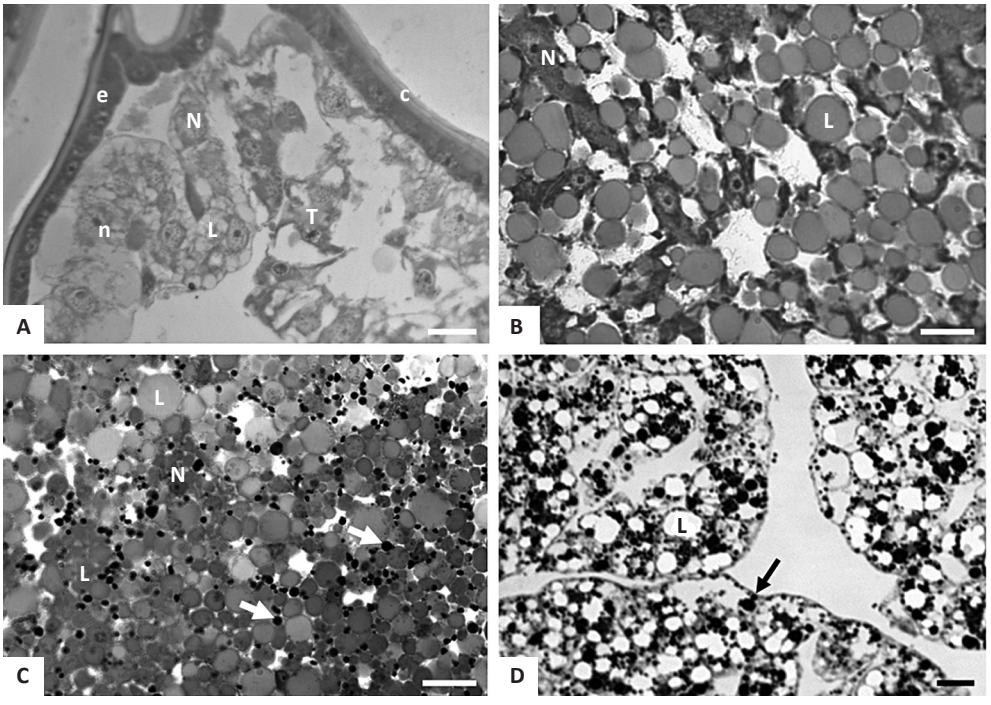

Fig 1 Histological sections of the *Toxorhynchites theobaldi* fat bodies stained with hematoxylin and eosin. A) L2 fat body. Trophocytes (T) form a cell mass located underneath the epidermis (e) and the cuticle (c). These cells have a prominent nucleus (N) with a developed nucleolus (n); B) L4 fat body. Cells are filled with large lipid droplets (L) and also have a prominent nucleus (N); C) Fat body of two-day-old female pupa. Protein granules (arrows) are strongly stained and noticeable between the lipid droplets (L); D) Four-to-six day-old female fat body. Lipid droplets (L) are weakly stained in contrast with the protein granules (arrow). Bars = 10 μm.

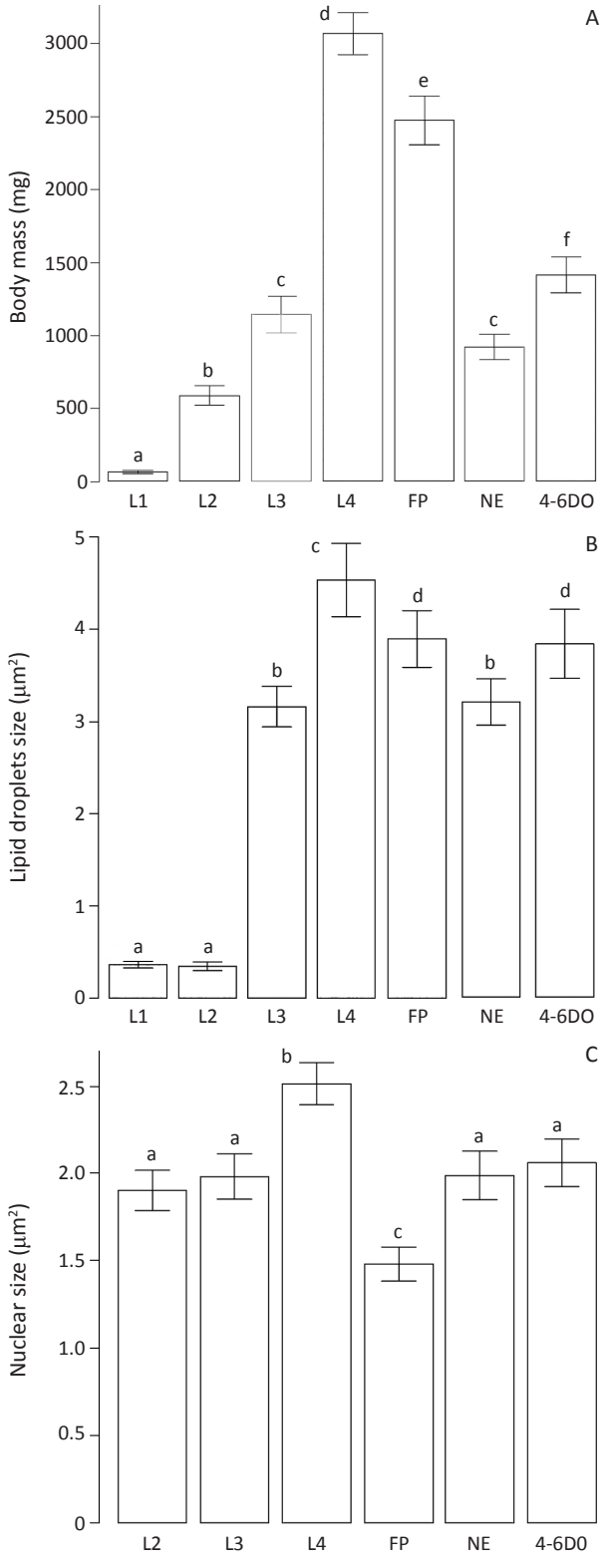

Fig 2 Body mass, trophocyte lipid droplet and nuclear sizes at different development stages of *Toxorhynchites theobaldi*. Vertical bars denote standard error. Letters (a-f) indicate the significant differences of: A) body mass ($F_{1;34}$= 84.83, P < 0.0001); B) lipid droplet size ($F_{1;342}$= 39.89, P < 0.0001); and C) nuclear size ($F_{5;294}$ = 7.14, P < 0.0001). L1-L4 - larvae; FP - two-day-old female pupae; NE - newly-emerged and 4-6DO - four-to-six day-old adult females.

During insect larval development, there is a need for nutrient storage for building the adult body; therefore, it is expected that the lipid reserves increase mainly prior to metamorphosis. Even though *Toxorhynchites* is predatory during their entire larval stage, fourth-instars are the most robust and efficient predators (reviewed in Steffan & Evenhuis 1981). Prey consumption is thought to be higher during the fourth-instar larvae to assure nutrient storage prior to metamorphosis (Trpis 1972, Trimble & Smith 1978, Chowanadisai *et al* 1984). With this in mind, such high predation may explain why the body and the lipid droplets reach their maximum size in L4 (Fig 2B). Otherwise, poorly fed larvae delay adult sexual development and have reduced egg production (Amalraj *et al* 2005).

Considering that the female pupae and the newly-emerged females do not feed, decrease in body mass and lipid storage due to storage nutrient utilization during metamorphosis is expected. Additionally, the decrease in body mass is higher in comparison to lipid droplet size, and it can be partially explained by lipid utilization during metamorphosis. However, as the body mass and the lipid droplets size increase in the four-to-six day old females, it suggests that individuals that feed on sugars undergo lipogenesis during adulthood, as reported for *A. aegypti* adult females (Ziegler & Ibrahin 2001) and other insects (reviewed in Canavoso *et al* 2001).

Protein granules become clearly visible in the cytoplasm of trophocytes from female pupae and remained in adult females of *T. theobaldi* (Figs 1C, D). The protein granule size ranged from 2.92 ± 0.43 µm² in female pupae to 2.54 ± 0.16 µm² and 3.45 ± 0.34 µm² in newly-emerged and in four-to-six day old females, respectively. Nevertheless, the protein granule size are similar in both pupae and adult females ($F_{1;150}$ = 2.69, P = 0.074). The storage of protein granules in the fat body after insect larval development is thought to come mainly from the accumulation of tyrosine-rich proteins, such as vitellogenin and arylphorin (Haunerland *et al* 1990). These proteins are consumed during aging of adult females and are a key source of nutrients that are mobilized during egg development in the anautogenous yellow fever mosquito *A. aegypti* (Raikhel & Lea 1983) and other hematophagous (Tobe & Davey 1974) and non-hematophagous dipterans (Thomsen & Thomsen 1974, Stoppie *et al* 1981).

Besides lipid droplets, the trophocyte nuclear size also varies between some *T. theobaldi* developmental stages ($F_{1;294}$ = 7.14, P < 0.0001). At the beginning of its larval development, no significant difference in nuclear sizes is detected between L2 and L3. However, nuclear size increases ~1.3-fold from L3 to L4, and decreases 1.7-fold during metamorphosis. Conversely, after emergence, nuclei size increases ~1.3-fold, and it increases ~1.04-fold in 4-6 day-old females in

comparison to the newly-emerged ones. In spite of this, the nuclei of trophocytes in adults still have the same size as in the L2-L3 (Fig 2C).

Ploidy levels and DNA synthesis are known to be common to the fat body of insects as reported to several species (Wigglesworth 1967, Butterworth & Rash 1986, Butterworth *et al* 1988, Dittmann *et al* 1989) and are generally association with the increase in the nuclei size of trophocytes in insect fat bodies. The association of nuclei development in fat body cells as the result of DNA synthesis in larvae and adult insects also seems to be the case here for the nuclei of trophocytes in *T. theobaldi* larval and adult stages. However, the reduction in size of the trophocyte nuclei detected in pupae is still unclear.

In conclusion, we identified for the first time the morphological changes during the post-embryonic development of *T. theobaldi*, and we showed that the fat body in this species undergoes significant changes at the macro (overall structural changes) and cellular levels. *Toxorhynchites* is an important natural enemy of mosquito vectors and is a widely studied model to control mosquito larvae. The better understanding of *Toxorhynchites* biology can improve mass rearing, leading to the development of homogenous populations with synchronous metamorphosis, eclosion and uniform adult size that can then be applied towards mosquito vector control.

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