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Nutrient limitation results in juvenile hormone-mediated resorption of previtellogenic ovarian follicles in mosquitoes

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Abstract

Juvenile hormone (JH) is a central hormonal regulator of previtellogenic development in female *Aedes aegypti* mosquitoes. JH levels are low at eclosion and increase during the first day after adult emergence. This initial rise in JH is essential for female reproductive maturation. After previtellogenic maturation is complete, the mosquito enters a 'state-of-arrest' during which JH synthesis continues at a slower pace and further ovary development is repressed until a blood meal is taken. By examining the relationships between juvenile hormone, follicular resorption and nutrition in *A. aegypti*, we were able to define a critical role of JH during the previtellogenic resting stage. The rate of follicular resorption in resting stage mosquitoes is dependent on nutritional quality. Feeding water alone caused the rate of follicular resorption to reach over 20% by day 7 after emergence. Conversely, feeding a 20% sucrose solution caused resorption to remain below 5% during the entire experimental period. Mosquitoes fed 3% sucrose show rates of resorption intermediate between water and 20% sucrose and only reached 10% by day 7 after emergence. Follicular resorption is related to JH levels. Ligated abdomens separated from a source of JH (the corpora allata) showed an increase in resorption comparable to similarly aged starved mosquitoes (16%). Resorption in ligated abdomens was reduced to 6% by application of methoprene. The application of methoprene was also sufficient to prevent resorption in intact mosquitoes starved for 48 hours (14% starved vs. 4% starved with methoprene). Additionally, active caspases were localized to resorbing follicles indicating that an apoptotic cell-death mechanism is responsible for follicular resorption during the previtellogenic resting stage. Taken together, these results indicate that JH mediates reproductive trade-offs in resting stage mosquitoes in response to nutrition.

Keywords

Juvenile Hormone; Mosquito; Oosorption; Sugar feeding; Reproduction; Apoptosis

1. INTRODUCTION

The appropriate allocation of nutrients between competing demands such as reproduction, growth, maturation or flight is a critical component of an insect's life-history strategy (Boggs, 1992; Stevens et al., 2000; Wheeler 1996). Insects must not only allocate nutrients properly within each developmental stage, but must also consider the effects of immediate resource allocations on future reproduction and overall fitness (Boggs, 1981; Pianka and

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Parker, 1975; Rivero et al., 2001). Any over-allocation of resources to one specific activity will have adverse effects on other physiological activities. These life-history and resource allocation trade-offs can be best uncovered with examples of competition between nutrient intensive processes such as flight and reproduction (flight-oogenesis syndrome) (Oliveira et al., 2006), sexual advertisement and immunity (immuno-competence handicap hypothesis) (Rantala et al., 2003), nutrition and adult phenotype (horned beetle polyphenism) (Moczek, 1998; Moczek and Nijhout, 2004) or with examples where nutrient limitation forces developmental, reproductive or other nutrient allocation decisions to be made (Nijhout and Wheeler, 1982; Noriega, 2004; Wheeler 1996).

Reproductive trade-offs in adult insects occur primarily through the process of oosorption. By resorbing excess reproductive tissues, insects can alter previous reproductive decisions by redirecting resources away from reproduction in favor of competing physiological activities (Boggs and Ross, 1993; Ohgushi, 1996; Osawa, 2005). The redirection of reproductive nutrients to support alternative processes is supported by studies that explored the differences in female/male survivorship under starvation conditions which have demonstrated that the resources contained within the ovary can be a valuable nutrient store for females (and one unavailable to males) when dealing with environmental constraints (Ohgushi, 1996; Osawa, 2005). Other work has suggested that the nutritional resources recovered from the ovary can be used to improve survival, extend search time for resources or hosts, and maximize overall fitness (Boggs and Ross, 1993; Ohgushi, 1996; Osawa, 2005; Richard and Casas, 2009; Rosenheim et al., 2000). Like many resource allocation decisions, the balance between nutrition, reproduction, and survival is likely to be mediated hormonally (Flatt et al., 2005; Trumbo and Robinson, 2004)

Juvenile hormone (JH) has been suggested as the main hormonal factor that controls follicular resorption in some insects, a role that conveniently meshes with JH's broader participation in mediating resource allocation trade-offs (Reviewed in Bell and Bohm, 1975). In many insects, yolk protein production is dependent on JH synthesis and overall nutritional status. Nutritional limitation often leads to cessation of JH synthesis (Caroci et al., 2004), a reduction of yolk protein synthesis and increased oosorption (Barrett et al., 2008; Bell and Bohm, 1975). The developmental pauses, unique pattern of hormonal secretions and discrete feeding habits of anautogenic mosquitoes have made a regulatory scheme based on nutrition production and JH synthesis more difficult to define. Thus, oosorption in mosquitoes has only been well-described after a blood meal (Carwardine and Hurd 1997; Clements and Boocock, 1984; Uchida et al., 2004); the highest rates of oosorption are seen during this time when up to 27% of follicles will become resorbed despite advanced vitellogenic development (Clements and Boocock, 1984). Follicular resorption after a blood meal can be greatly increased by *Plasmodium* infection in *Anopheline spp*. mosquitoes (Ahmed and Hurd, 2006; Carwardine and Hurd 1997; Hopwood et al., 2001).

Studies exploring the relationship between sugar feeding and reproduction in mosquitoes have clearly demonstrated that sugar feeding improves fecundity in some circumstances but have failed to provide a mechanistic or hormonal reason for the observed alterations in reproductive allocations. (Nayar and Sauerman, 1971; Briegel, 1990; Naksathit et al., 1999; Foster, 1995). In adult *A. aegypti*, JH's most clearly defined role is as the hormone responsible for regulating previtellogenic reproductive development. A role for JH in coordinating nutrition and reproduction by regulating the resorption of follicles before a blood meal has not been described in any mosquito.

Descriptions of apoptosis and oosorption have rarely been described in mosquitoes. Reports by Hopwood et al. (2001), Ahmed and Hurd (2006), and Uchida et al. (2004) have all

attempted to clarify the progression of resorption following a blood meal. The description by Uchida et al. (2004) identifies active caspases solely in the follicular epithelium. Hopwood et al. (2001) and Ahmed and Hurd (2006) have described markers of apoptosis occurring in patches of the follicular epithelium as preceding resorption of the follicle in *Plasmodium*-infected mosquitoes. From these observations, a regulatory role for the follicular epithelium in resorption has been surmised. It is not clear if resorption, in sugar fed *A. aegypti*, also occurs through apoptotic mechanisms and if follicular resorption is mediated by the follicular epithelium.

In this study, we specifically investigated the relationships between nutrition, juvenile hormone and reproduction in *A. aegypti* during the previtellogenic resting stage by exploring three main questions: 1) Does nutrition during the resting stage affect follicular resorption and overall fecundity? 2) Does JH during the previtellogenic resting stage prevent follicular resorption? And finally, 3) which cell-death mechanisms are responsible for follicular resorption in a previtellogenic mosquito? Each of these interrelated factors is an important component of the fitness and life-history strategy of *A. aegypti* which also makes them important for understanding the relationship between this disease vector and its environment. The conspicuous and observable nature of the insect ovary in conjunction with its close dependence on hormonal and nutritional status makes oosorption an ideal experimental mechanism for exploring resource and reproductive trade-offs. By answering these questions it is clear that JH synthesis during the resting stage participates in the regulation of fecundity by preventing apoptosis and resorption in previtellogenic follicles. Furthermore, the resorption of follicles is dependent, at least in part, on the quality of nutrition during the resting stage.

2. METHODS

2.1 Insects

A colony of *Aedes aegypti* of the Rockefeller strain was maintained at 28°C with 80% relative humidity under a 16 hour day-8 hour night regime. Males and females emerging during the same 15 hour overnight period were collected and transferred to a screened container. Mated adults were offered a cotton pad soaked in 3% sucrose solution.

2.2 Observations of oosorption in previtellogenic follicles

Adult mosquitoes were fed 3% sucrose from eclosion until 2 days after emergence to allow normal previtellogenic follicular maturation. After the second day post- emergence, mosquitoes were transferred to one of three nutritional regimes: 1) 20% sucrose 2) water only (starved) or 3) continued on 3% sucrose. Ten female mosquitoes were collected each day for 5 days for each nutritional regime beginning on day 3, post-emergence. Mosquitoes within each feeding regime were collected for at least three independent replicates. All mosquitoes were anesthetized by chilling for 5–10 minutes at 4°C prior to dissection. Ovaries from these mosquitoes were removed, rinsed in *Aedes* physiological saline (APS) ($MgCl_2$ 0.6mM; KCl 4.0mM $NaHCO_3$ 1.8mM; NaCl 150.0 mM; HEPES 25.0 mM; $CaCl_2$ 1.7mM) and stained with 0.5% neutral red solution in acetate buffer at pH 5.2 (Sigma Aldrich, St. Louis, MO) for 10 seconds to visualize resorbing follicles. Neutral red stains the lysosomes associated with resorbing follicles and can clearly indicate follicle status (Winckler, 1974; Bell and Bohm, 1975; Clements and Boocock, 1984; Hopwood et al., 2001). The ovaries were rinsed a second time in APS and placed under a coverslip. Photographs were taken of the previtellogenic ovaries using a DM 5500 B Leica fluorescence microscope, a Leica DFC 310 FX mounted camera and Leica LAS imaging software. Ovaries were later scored using Leica LAS imaging software for total follicle count and also for the presence of any resorbing follicles.

2.3 Abdominal ligations

Fifteen female mosquitoes were randomly selected from a mixed-gender population fed 3% sucrose until 3 days old. After being cold anesthetized for 5–10 minutes a fine Kevlar thread (Stren Powerbraid, Wilmington, DE) was firmly tied around the mosquito anterior to abdominal segment I and posterior to the halters. The abdomen was removed and the anterior end sealed with wax (Boekel tackiwax, Philadelphia, PA). The loose end of the tied Kevlar thread was hung from a strip of parafilm (Pechiney Plastic Packaging; Chicago, Illinois) and placed inside of a humid glass chamber (~700cm^3)(Supplementary Fig. 1). The hanging abdomens were treated with either 500 ng of the juvenile hormone analogue Methoprene (Zoecon Co., Palo Alto, CA) in 0.5 μl acetone or 0.5 μl of acetone alone. After 48 hours, viable abdomens were dissected, and the ovaries rinsed with APS. The ovaries were stained with neutral red for 10 seconds, rinsed again and placed under a coverslip. Four independent replicates were conducted. Photographs were taken of the previtellogenic ovaries and scored as previously described.

2.4 Hormonal manipulations of intact mosquitoes

At 3 days post-emergence, randomly selected mosquitoes from a mixed gender population were cold anesthetized and topically treated with either 500 ng of methoprene dissolved in 0.5 μl acetone or with 0.5 μl acetone alone. Insects were then transferred to a water-only diet for 2 days before being dissected and assayed with neutral red, photographed and scored as previously described. Two independent replicates were conducted.

2.5 Active caspase in situ assay

Active caspases were detected in the ovaries of previtellogenic mosquitoes using a sulforhodamine multi-caspase activity kit (AK-115, Enzo life sciences, Plymouth Meeting, PA). In this kit, sulforhodamine labeled valylalanylaspartic acid fluoromethyl ketone (SR-VAD-FMK) enters the cell and acts as a specific inhibitor of apoptosis by covalently binding to the reactive cysteine residue indicative of an active caspase. The sulforhodamine label allows detection and localization of active caspases by fluorescence microscopy. Ovaries were removed from 4 day old previtellogenic mosquitoes that were fed 3% sucrose, rinsed in APS, and incubated in the 1/3X reaction medium (10 μl of 30× SR-VAD-FMK in 290 μl of PBS) for 1.5 hours at 26°C. The labeled ovaries were washed first for 6 hours at 26°C and then overnight in 1X wash solution at 4°C, rinsed with 1X PBS and placed under a coverslip. Photographs were taken of the previtellogenic ovaries as described. Visible light and florescence images taken with a Leica Texas red filter set were merged.

3. RESULTS

3.1 Nutrition affects previtellogenic follicular resorption

The nutritional factors that affect follicular resorption were explored by rearing adult mosquitoes under three nutritional regimes: 1) water alone 2) 3% sucrose in water or 3) 20% sucrose in water. Follicles were scored as resorbing by possessing one of the two following indicators: 1) Condensed chromatin (pyknotic nuclei) in nurse cells in conjunction with intense staining of cytoplasmic regions of nurse cells or oocyte with neutral red (early resorbing) (Supplementary Fig. 2) or 2) Intense staining of follicular epithelial cells with neutral red combined with absence of identifiable interior cells types (late resorbing) (Fig. 1A). Follicles not conforming to either of the two criteria were not counted as resorbing. The rate of resorption increases daily in mosquitoes fed 3% sucrose reaching a rate of nearly 10% on day 7 (Fig. 1B). The increase in the number of resorbing follicles was inversely proportional to the decrease in JH synthesis (Fig. 1B).

Resorbing follicles at day 3 already comprised between 3% and 7% of the total follicles in mosquitoes fed either 3% sucrose, 20% sucrose or water alone, indicating that the process of follicular resorption begins prior to day 3 and may be concurrent with previtellogenic development (Figs. 1 and 2). An even steeper increase in resorption is seen in mosquitoes fed water only. By day 7 over 20% of the follicles are resorbing in starved mosquitoes. The increase in resorption seen in mosquitoes fed water is significantly greater than mosquitoes fed 3% and 20% sucrose on every day examined (unpaired *t*-test; $P<0.0001$) (Fig. 1 and 2). In conjunction with the overall upward trend in starved mosquitoes, this result indicates that an increase in the rate of resorption begins quickly under starvation conditions.

The rate of follicular resorption in mosquitoes fed 20 % sucrose declines slowly until day 5. On days 6 and 7 the rate of follicular resorption begins increasing again but always remains below the rate of resorption for either 3% sucrose fed or water only mosquitoes (Figs. 1 and 2). By day 7 only 4% of follicles are resorbing; a rate that is only half of the rate found in mosquitoes fed 3% sucrose. In fact, the rate of resorption seen in mosquitoes fed 3% sucrose is significantly greater than those of mosquitoes fed 20% sucrose in each of the days examined (unpaired *t*-test; $P<0.0001$).

3.2 Methoprene prevents follicular resorption in ligated abdomens

To establish that juvenile hormone can prevent follicular resorption, abdomens from 3 day old mosquitoes were ligated, treated with 500 ng of methoprene or acetone and incubated for 48 hours. Abdominal ligation is a well-established experimental technique to test the effect of JH deprivation on abdominal tissues (Noriega et al., 1997). By isolating the ovaries from the corpora allata, their endogenous source of JH was removed and the rate of follicular resorption increased to over 16% (Fig. 3A), a level only seen in intact but starved mosquitoes (Fig. 2). Topical application of 500 ng of methoprene was sufficient to prevent the increase in follicular resorption seen in ligated abdomens and limit the rate of follicular resorption to 6% (Fig. 3A), a level comparable to 5 day old intact mosquitoes (Fig. 2).

3.3 Methoprene prevents follicular resorption in starved mosquitoes

To investigate the connection between nutritional status, hormonal levels and follicular resorption in intact animals, we treated mosquitoes with either 500 ng of methoprene or acetone alone. These mosquitoes were subsequently starved (fed water only) for two days and assayed as previously described. By applying hormone to starved mosquitoes, we were able to prevent follicular resorption and alter the normal resource allocation response to starvation. Mosquitoes treated with acetone alone and starved for 48 hours showed levels of follicular resorption (14 %) (Fig. 3B) comparable to similarly aged (5 days old) starved mosquitoes (Fig. 2). Mosquitoes treated with methoprene showed only a 3.4% rate of resorption (Fig. 3B). This was comparable to similarly aged mosquitoes fed 20% sucrose (Fig. 2).

3.4 Active caspase in-situ assay

An active caspase *in situ* assay was conducted on ovaries from 4 day-old mosquitoes to explore the cell-death mechanisms of follicular resorption in *A. aegypti*. Follicles undergoing resorption contained active caspases as indicated by the sequestration and concentration of a sulforhodamine-labeled caspase inhibitor probe (SR-VAD-FMK) (Fig. 4A). Those follicles that were not undergoing resorption in the same ovary did not sequester the probe (Fig. 4A).

In follicles that still contained identifiable nurse cells and oocytes, the intracellular spaces between cell types generally contained the most intense caspase activity (Fig. 4B). As interior cellular boundaries became less well-defined, caspase localization became more

diffuse throughout the interior of the follicle (Fig. 4C). Follicles that no longer contained identifiable internal cell types had active caspases localized throughout the follicle but most intensely in the remains of the follicular epithelium (Fig. 4D). The pattern of caspase localization in resorbing follicles matches closely the pattern of neutral red staining i.e. resorption appears to begin first within the follicle followed by the follicular epithelium. The follicular epithelium in the final stages of resorption had lost integrity as a covering and also contained the most intense caspase signal. At this late stage, the interior of these follicles contained no remaining cellular structures and did not contain active caspases (not shown).

4. DISCUSSION

4.1 Nutrition, JH synthesis, and follicular resorption are linked

In *A. aegypti*, the nutritional regulation of JH synthesis has been described as a mechanism to control reproductive maturation (Noriega, 2004). Immediately following emergence, a peak of JH synthesis (nearly 35 fmol/CA pair/hour) directs the maturation of the ovary and other tissues to support an adult phenotype capable of blood-feeding and reproduction (Caroci et al., 2004; Li et al., 2003; Noriega, 2004; Raikhel and Lea, 1984; Raikhel and Lea, 1991). After 60 hours, maximum previtellogenic development is reached and the mosquito enters a developmental resting stage that feeds exclusively on sugar or nectar until a host is found (Clements, 1992; Hagedorn et al., 1977). From day 3 until day 7 post-emergence, JH synthesis declines from 15 fmol/pair CA/hour to 5 fmol/pair CA/ hour (Li et al., 2003) while follicular resorption increases from 3% of total follicles per female to nearly 10% per female during the same period in mosquitoes fed 3% sucrose (Fig. 1). Results from hormonal manipulations in isolated abdomens confirmed that the increase in follicle resorption during the resting stage is probably the result of falling JH titers. This hypothesis was further validated since methoprene was able to prevent follicular resorption in intact but starved mosquitoes. Taken together, these results provide strong evidence that follicular resorption is under the control of juvenile hormone and ultimately, nutrition during the resting stage.

Reproduction and nutrition are closely connected through JH titers. In many insects, starvation or other nutritional limitation leads to reduced JH synthesis, which in turn, leads to resorption, arrest or other reproductive trade-offs (Schal et al., 1993; Tobe and Chapman, 1979; Trumbo and Robinson, 2004; Yin et al., 1999). The connection of reproduction with nutrition through JH synthesis is also true in *A. aegypti*, albeit this relationship has only been previously demonstrated immediately following emergence. Hormonal and nutritional manipulations have shown that females emerging with low teneral reserves will fail to develop ovaries properly (Caroci et al., 2004; Hernandez-Martinez et al., 2007; Feinsod and Spielman, 1980). A clear function for JH during the resting stage in previtellogenic mosquitoes has, to our knowledge, been previously overlooked (Hagedorn et al., 1977; Lea, 1963; Raikhel and Lea, 1984; Raikhel and Lea, 1991).

The experiments described here indicate that JH synthesis during the resting stage is an integral component of the allocation and reproductive decisions made as part of the life-history strategy of *A. aegypti*. These results are generally in agreement with data obtained by Soller et al., (1999) that showed methoprene application reduces the apoptosis of nurse cells in the follicles of *Drosophila melanogaster* and others who have shown the hormone-dependent nature of follicular resorption (Bell and Bohm, 1975). The hormonal basis for follicular resorption may not depend on JH alone. Work with *D. melanogaster* showed starvation-induced apoptosis of follicular cells to be caused by an increased ecdysteroid titer and methoprene prevented this apoptosis by reducing ecdysteroid titers; a result which appears contradictory to observations made in mosquitoes (Terashima et al., 2005). In *A. aegypti*, ecdysteroid titers are very low prior to a blood meal and are not reduced further by methoprene application at that time (Borovsky et al., 1986). However, many differences

exist in the way that JH and ecdysteroid signaling pathways interact, making comparisons between species difficult (Reviewed in Spindler et al., 2009).

Work that explored the interaction between methoprene, ecdysteriods and gene expression changes during midgut remodeling and metamorphosis in *A. aegypti* has shown that methoprene blocks apoptosis in midgut cells by modulating the expression of ultraspiracle A (Usp A) and ecdysone receptor B (Ecr B) as well as genes important to apoptosis (Wu et al., 2006). Later work showed that transcription of AeDronc (an initiator caspase and key component of apoptosis) as well as overall caspase activity were both increased by ecdysone treatment in larval mosquitoes (Cooper et al., 2007). In the adult mosquito ovary, ecdysone treatment caused a nearly 3-fold increase in AeDronc transcription (Cooper et al., 2007). It is not yet clear if ecdysone increases apoptosis and follicular resorption in previtellogenic ovaries of *A. aegypti* but we cannot reject the idea that the balance of the two hormones may be an important factor in determining the fate of the follicles (Terashima and Bownes, 2004).

4.2 Follicular resorption as a resource allocation decision

Previous studies on the effects of sugar feeding on reproduction in *A. aegypti* have shown that sugar can improve fecundity under some circumstances, although the mechanism for an improvement (or reduction) in fecundity was not elucidated in previous studies (Reviewed in Foster, 1995). Our results demonstrate that sugar-feeding can prevent follicular resorption during the previtellogenic resting stage. By manipulating the feeding regime in resting stage mosquitoes, we were able to show that the rate of follicular resorption increased as the concentration of the sugar meal decreased.

A high sucrose meal was sufficient to prevent a reduction in fecundity as there were significantly more follicles remaining at the end of 7 days in those fed the higher concentration food (148.5 follicles vs. 139.8 follicles; $P<.05$; $n=60$; $df=58$; t-test). These results clearly demonstrate that reproductive trade-offs are made specifically in response to carbohydrate feeding. More specifically these results demonstrate that sugar feeding can prevent follicular resorption and enhance fecundity.

While we did not explore how the nutrients from resorbed follicles were metabolically allocated, other studies exploring starvation may help explain how these nutrients are reused. Sugar feeding in mosquitoes primarily supports flight activity and lipid synthesis. The majority of lipids (teneral and newly synthesized) are stored in the fat body and are mostly used to provision eggs after the first blood meal (Briegel, 1990; Briegel et al., 2002; Foster, 1995; Zeigler and Ibrahim, 2001; Zhou et al., 2004). The synthesis of lipids from sugar feeding has been reported many times and generally occurs whenever excess carbohydrates are available (Briegel, 1990; Briegel et al., 2001; Foster, 1995; Nayar and Van Handel, 1971). Briegel (1990) found that starvation leads to drastic reductions in whole body lipid and carbohydrate content and much smaller reductions in protein content. Since lipids are primarily stored in the fat body and very little are stored or synthesized in the ovary of previtellogenic mosquitoes (Troy et al., 1975; Zeigler and Van Antwerpen, 2006; Zeigler and Ibrahim, 2001), it does not seem likely that during starvation ovaries are resorbed exclusively for their lipid content. This likelihood is further supported by observations demonstrating a limited role for lipids in supporting flight activities (Briegel et al., 2002). If lipids cannot be used for anything but egg provisioning and sugar feeding only supports flight and/or lipid synthesis, then it is hard to see the immediate linkage between sugar feeding and reproduction. Briegel et al., (2001) found anything above a 0.5% sucrose solution adequate to extend survivorship and stimulate reserve lipid synthesis in nutrient-deficient mosquitoes. When we fed mosquitoes 3% sucrose, resorption increased on each day examined until reaching nearly 10% on day 7 (Fig. 1). This indicates that 3% sucrose

(or even 20% sucrose), while potentially adequate to stimulate synthesis of lipids is not adequate to stop resorption and further suggests that resorption in *A. aegypti* is not occurring because of a shortage of lipids and/or carbohydrates. It is also possible that follicular resorption occurs to limit or reduce the expenditure of energy required to maintain a large tissue mass such as the ovary.

Another possible reason for follicular resorption in previtellogenic mosquitoes may be due to a nitrogen/protein limitation. Support for a protein-limitation scenario can be found in a report by Judson and DeLumen (1976), who described that treatment of mosquitoes with synthetic analogues of JH (Cecropia hormone and methoprene) causes increases in ovarian protein content and an increase in overall ovarian size as compared to controls. They did not report the specific nature of the protein increase nor did they explore any effect of juvenoids or juvenile hormone analogues on resorption. An increase in oocyte and follicle size after methoprene application has been subsequently confirmed in our lab during explorations of this topic (unpublished results). Conversely, starvation until death was found to reduce whole body protein levels by 14%. This reduction in protein content may reflect the catabolism of proteins to support survival and may be occurring through the resorption of ovarian tissues (Briegel, 1990). Therefore, it is possible that ovarian follicles are resorbed exclusively for their protein content and this allocation is mediated by juvenile hormone.

Further support for this possibility comes from a basic examination of the life-history strategy of *A. aegypti*. The majority of proteins/nitrogen must be carried over from larval feeding as sugar feeding mosquitoes have limited access to nitrogenous compounds. Qualitative nutritional deficiencies (especially protein) have been reported before as a cause of resorption (Bell and Bohm, 1975). Although the synthesis of some non-essential amino acids does occur from sugars, it is not clear how sugar feeding would dictate protein and reproductive allocations (Zhou et al., 2004).

4.3 Follicular resorption as a life-history strategy

The resorption of follicles represents a reversal of nutrients away from reproduction and towards alternative activities and reflects the need to balance present and future reproduction to maximize fitness (Boggs and Ross, 1993; Ohgushi, 1996; Rosenheim et al., 2000). Studies exploring reproductive trade-offs and life-history strategies may offer some clues to explain the reasons for the follicular resorption we observed in previtellogenic mosquitoes. In the nectivorous butterfly, *Speyeria mormonia*, nutritional limitation causes reductions in fecundity via follicular resorption but does not cause reductions to life span, suggesting survival needs get “first claim” on any resources (Boggs and Ross, 1993). Studies exploring oosorption and survival in the lady beetle, *Epilachna niponica*, found that females were able to tolerate periods of starvation better than males due to their ability to resorb follicles and may realize significant future benefits to fitness by resorbing eggs in the present (Ohgushi, 1996). Theoretical models using the wasp, *Aphytis aonidiae*, demonstrated that the resorption of follicles only improved fitness if it increased life-span and more specifically, increased host-seeking time (Rosenheim et al., 2000). The translation of reproduction into flight (i.e. host-seeking time) is supported by observations in the blood-sucking bug, *Rhodnius prolixus*. Fecundity was significantly reduced in bugs subjected to daily exhaustive flight due to increased lipid oxidation (Oliveira, et al., 2006). However, in Aedine mosquitoes, lipids are generally not used to support flight (Briegel et al., 2002; Nayar and Handel, 1971). Despite this, flight and host-seeking are metabolically costly in the mosquito and the resorption of follicles to support these costs might make sense from a life-history strategy viewpoint even if the biochemical nature of this trade-off is not immediately obvious (Foster, 1995).

Another explanation for the pattern of follicular resorption we observed in *A. aegypti* may be better illustrated by work that examined the adaptive and nutritional value of resorption in the parasitoid wasp, *Eupelmus vuilleti*. In this species, the resorption of a single egg is only able to cover less than 10% of daily energy needs (Casas et al., 2005). In *E. vuilleti* as well as the mosquito, a simple relationship between nutrition and resorption is not clearly evident as resorbed eggs do not seem to be able to provide the kinds of benefits to survival that one would expect (Richard and Casas, 2009). Richard and Casas (2009) explain resorption as a buffer against stochasticity in the environment whereby a low value resource (ovarian follicles) can confer a fitness advantage primarily because of the controllability of this resource. While many of the observations stated here have not been specifically described in mosquitoes, they do demonstrate that additional layers of decision-making are likely to exist which need to be considered when explaining resorption in mosquitoes.

4.4 Progression of follicular resorption in previtellogenic mosquitoes

The progression of resorption in previtellogenic follicles supports the assertion by Uchida et al. (2004) that the follicular epithelium participates in the regulation of resorption in mosquitoes. Additional observations about the progression of resorption and localization of caspases in mosquito follicles were possible in this study due to the lack of vitellogenin synthesis and visibility of interior cells in sugar-fed mosquitoes In all follicles examined that contained identifiable structures (i.e. nurse cells and oocytes) active caspases were localized to the interior portion of the follicle, especially in the interior spaces between epithelial and nurse cells as well as the spaces between nurse cells and oocyte (Fig. 4; panel B). In many cases, caspases could be visualized within the remains of the oocyte (Fig. 4; panel C). From these observations, it seems that apoptosis (as indicated by active caspase localization) occurs first in the interior cells (nurse cells and oocyte) of the follicle, followed later by the epithelial cells. These observations represent a new detail to the understanding of the process of resorption as previous descriptions were unable to describe events occurring within the follicle possibly due to the obfuscation of interior structures by dense yolk proteins in follicles after a blood-meal (Uchida et al., 2004).

Previous reports have described apoptosis in patches of follicular epithelium cells as preceding resorption of the entire ovarian follicle in *Anopheline spp.* mosquitoes infected with *Plasmodium* or artificial immune-elicitors (Ahmed and Hurd, 2006; Hopwood et al., 2001). These observations conflict somewhat with observations made here as well as with previous reports of resorption in *Culex pipiens pallens* by Uchida et al., (2004). It is possible that the progression of apoptosis and resorption seen in infected *Anopheles spp.* is a unique feature of *Plasmodium spp.* infection, is a unique feature of immune response, or is a species-specific response. Also, each of the previous reports explored only vitellogenic follicles. It is possible that vitellogenic resorption progresses differently than previtellogenic resorption. More than likely, the discrepancies reported here reflect a more complicated relationship between various cells of the follicle than was previously supposed.

4.5 Conclusions

By combining abdominal ligations, hormone application and nutritional manipulations we were able to determine that JH likely mediates reproductive trade-offs in *A. aegypti* during the resting stage by preventing apoptosis and resorption of reproductive tissues in a nutrition-dependent manner. However, some key questions remain: 1) How are the resources from resorbed follicles being used? 2) Why does sugar feeding that is adequate for lipid synthesis and metabolism not prevent resorption? And finally 3) How does JH prevent follicular resorption during the resting stage?

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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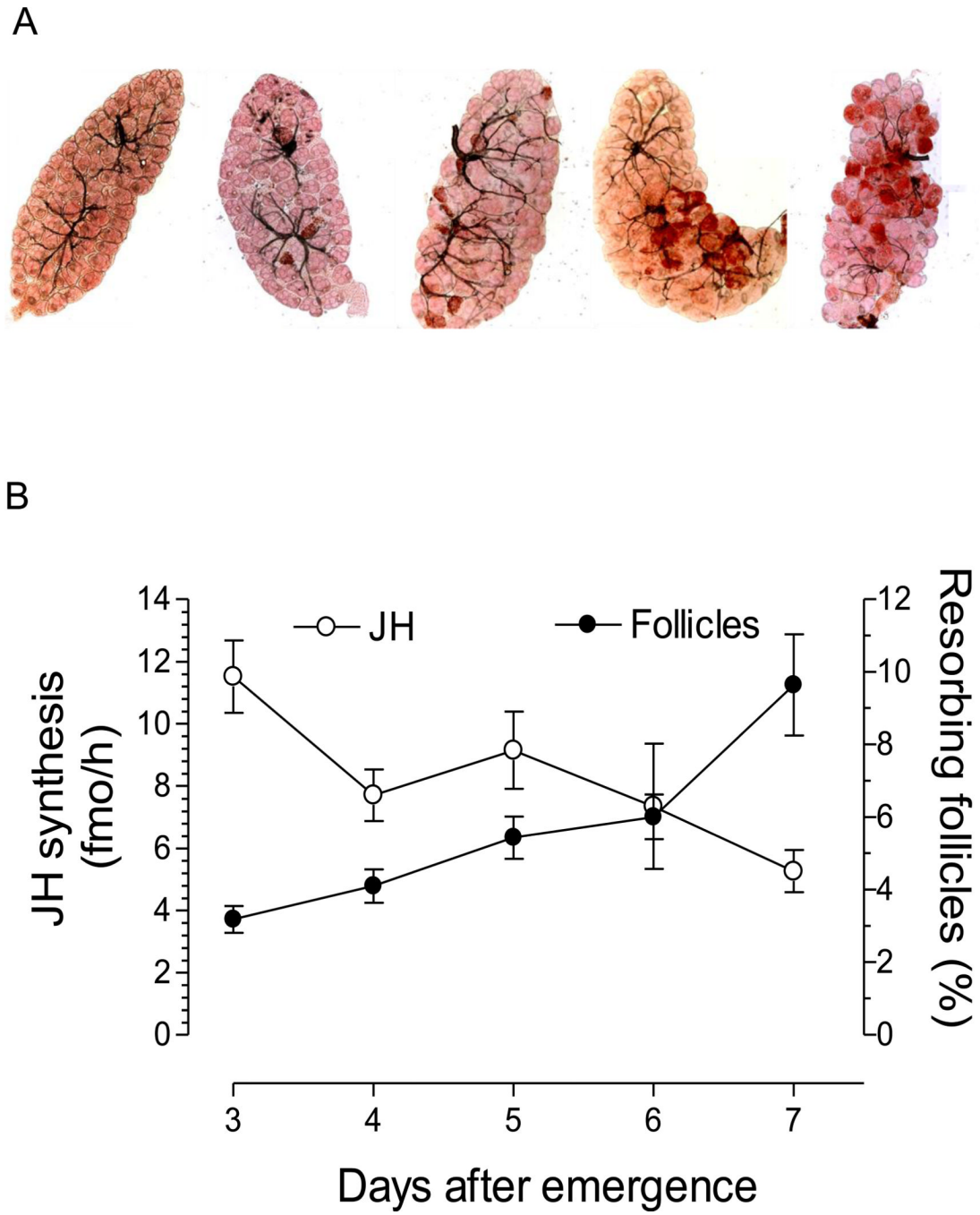

Figure 1. Changes in the rate of previtellogenic follicular resorption

A: Representative images of the progression of resorption in ovaries from day 3 until day 7 after emergence. Ovaries are stained with neutral red. **B**: Average percent of resorbing follicles per day in female mosquitoes fed 3% sucrose (filled circles) (Mean ± SEM of five independent replicates of groups of at least 10 ovaries). JH biosynthesis values (empty circles) are from Li et al., 2003, and are expressed as femtomole of JH III per hour.

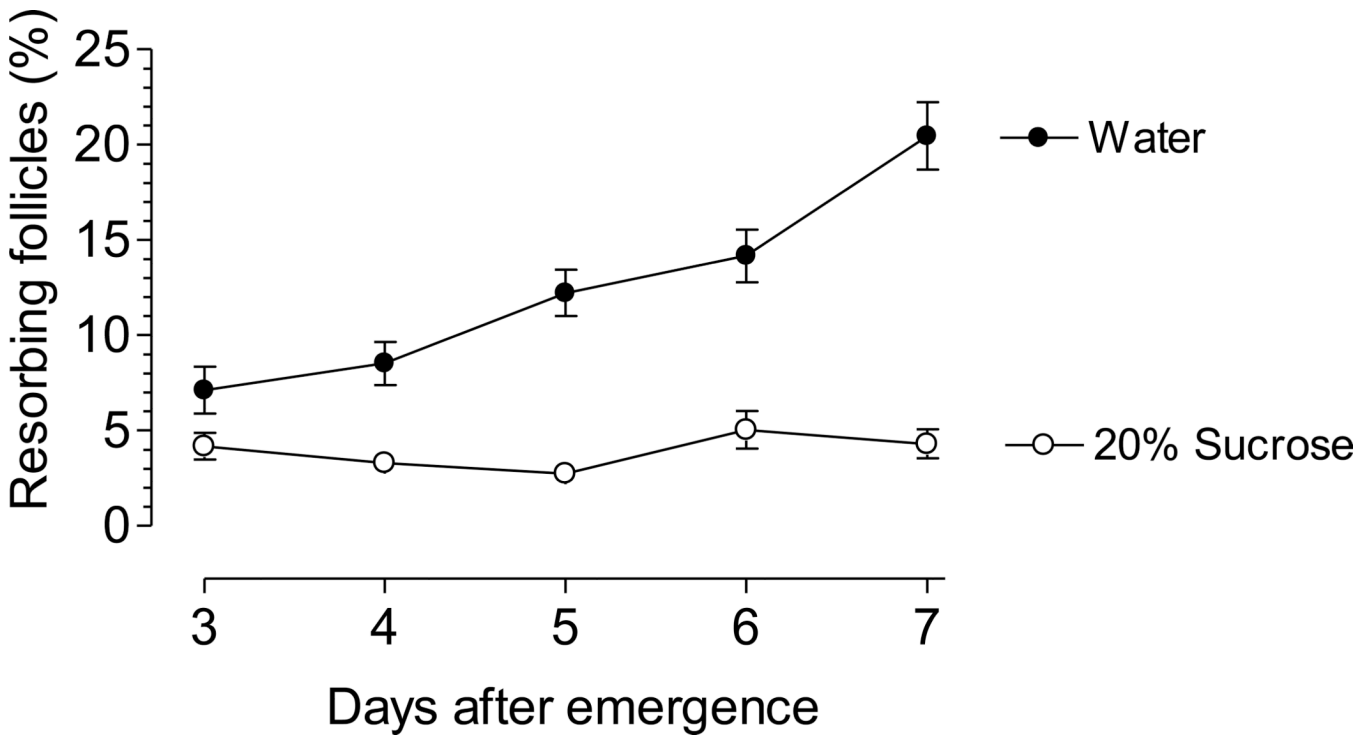

Figure 2. Effect of nutrition on the rate of previtellogenic follicular resorption

Average percent of resorbing follicles from day 3 to day 7 after emergence in females fed with either 20% sucrose solution (empty circles) or water alone (filled circles). (Mean ± SEM of three independent replicates of groups of 10 ovaries), The increase in resorption seen in mosquitoes fed water is significantly greater than mosquitoes fed 20% sucrose on every day examined (unpaired *t*-test; $P<0.0001$).

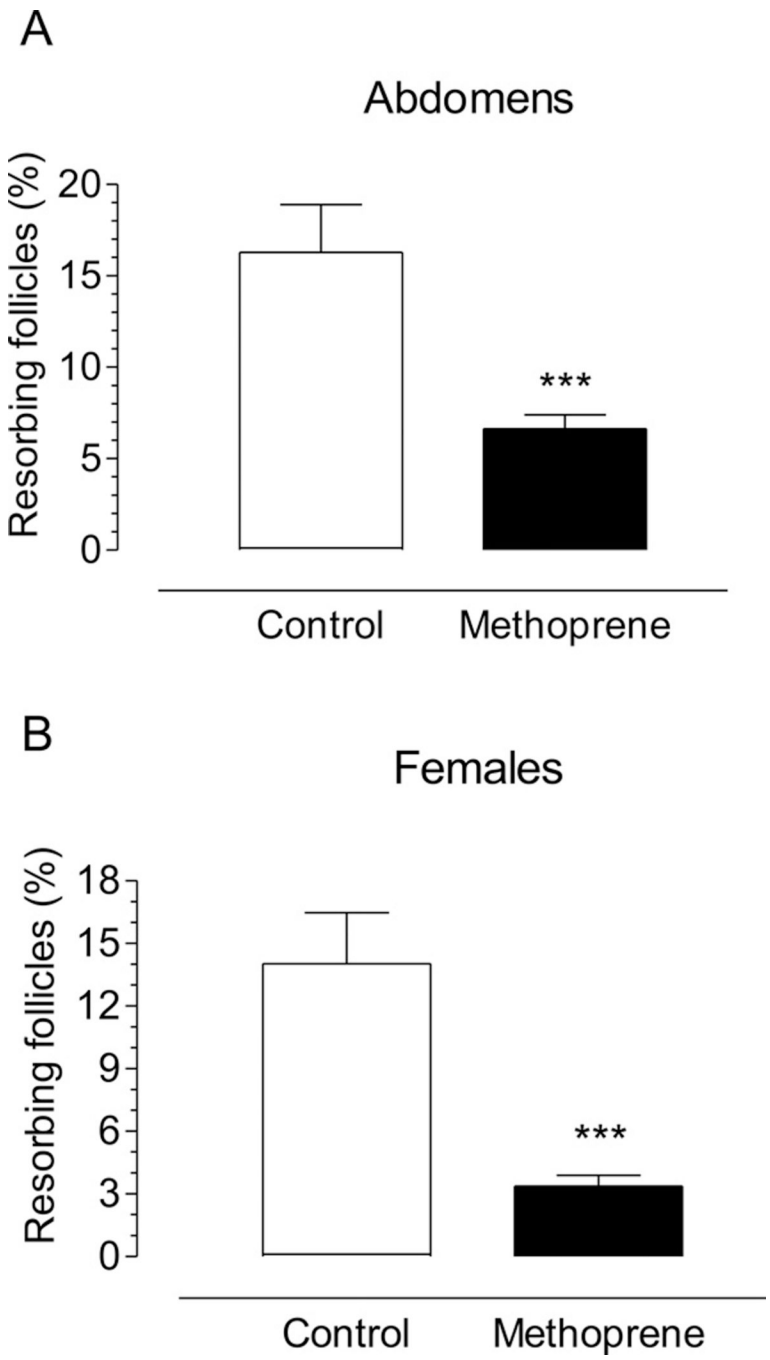

Figure 3. Methoprene prevents follicular resorption in ligated abdomens and in starved mosquitoes

A) Abdomens were ligated 3 days after emergence and treated with either acetone as a control (empty bar) or 500 ng of methoprene dissolved in acetone (filled bar). Follicular resorption was evaluated after 48 hours. Bars represent the means ± SEM of four independent replicates of groups of at least 10 ovaries (unpaired *t*-test; ***$P \leq 0.001$).

B) Mosquitoes were fed 3% sucrose until being treated with either acetone as a control (empty bar) or 500 ng of methoprene dissolved in acetone (filled bar). Follicular resorption was evaluated after 48 hours. Bars represent the means ± SEM of two independent replicates of groups of 30 ovaries (unpaired *t*-test; ***$P \leq 0.001$).

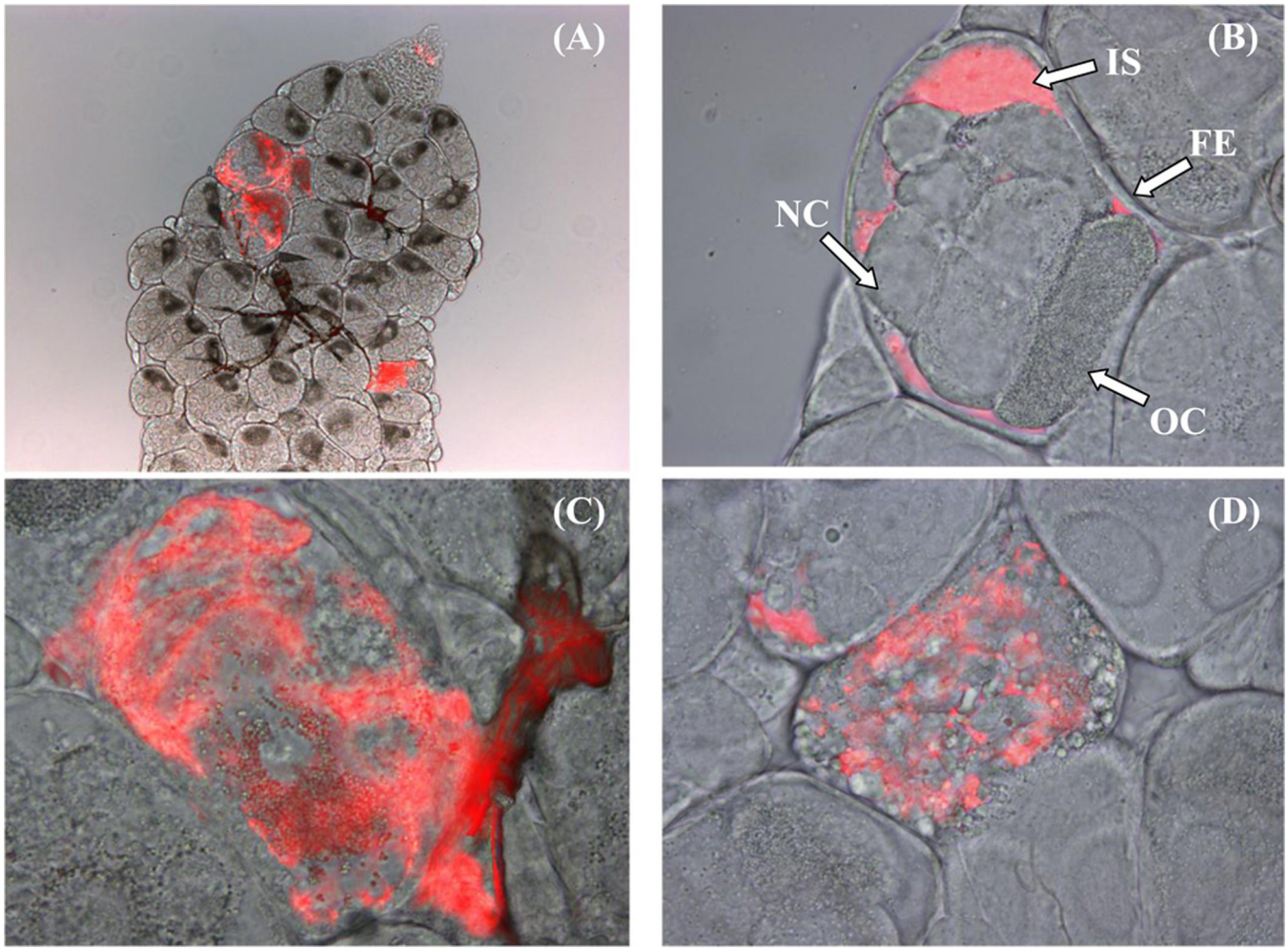

Figure 4. Active caspase localization in previtellogenic follicles

Active caspases were visualized by merging a light micrograph with a fluorescent micrograph. (A) Resorbing follicles can be identified by the presence of pyknotic nuclei and accumulation of a sulforhodamine-labeled caspase inhibitor probe (SR-VAD-FMK). Normally developing follicles fail to accumulate significant amounts of SR-VAD-FMK and show uncondensed nurse cell nuclei. (B) Early resorbing follicles with definable interior structures had active caspases localized solely to intercellular spaces and not within follicle cells, nurse cells or the oocyte. (C) As resorption progresses, interior cell types become degraded and caspases can be visualized throughout the interior of the follicle. (D) In late resorbing follicles all interior structures have been degraded and the remaining cells of the follicular epithelium contain active caspases. FE, Follicular epithelium; NC, Nurse cell; OC, Oocyte; IS, Intercellular space.