Effects of mating delay and nutritional signals on resource recycling in a cyclically breeding cockroach

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Abstract

Ovarian apoptosis has been found to occur in the female cockroach \textit{Nauphoeta cinerea} in response to lack of mates. It has been proposed that ovarian apoptosis in continuously breeding insects is an adaptive mechanism for recouping resources in poor conditions (oosorption). However, \textit{N. cinerea} is a cyclically breeding insect and ovarian apoptosis may represent ageing and clearance of old unused oocytes. To test the hypothesis that oocyte resorption via apoptosis reflects the reclamation of resources, we delayed mating in combination with positive and negative nutritional signals. Females without access to food during sexual maturation invested less in reproduction and had elevated rates of ovarian apoptosis in the terminal oocyte. Starvation also induced apoptosis in non-vitellogenic oocytes of the vitellarium and germinarium, which would be used for future reproductive events. This is paradoxical as theory states that oosorption is an adaptive means of rerouting resources into investment in future reproduction, yet these oocytes do not represent a cache of resources and their loss could limit future reproduction.

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1. Introduction

The trade-off between gametes and soma is central to life-history evolution, yet the physiological mechanisms by which this trade-off is controlled remain poorly understood at all biological levels (Zera and Harshman, 2001; Harshman and Zera, 2006). One supposed mechanism in female insects is oosorption, the resorption of unfertilised oocytes, which is predicted to occur in response to environmental stress, such as lack of mates, food, host substrate, or parasitic attack (Bell and Bohm, 1975; Ohgushi, 1996; Kotaki, 2003; Wang and Horng, 2004). When conditions are not conducive to successful reproduction a developmental decision must be made by the female; should oocytes develop or die? If oocytes are resorbed, nutrients may be recouped and reinvested into somatic functions that increase lifespan (Bell and Bohm, 1975; Tatar and Carey, 1995; Kotaki, 2003; Wang and Horng, 2004).

Nutrient recycling potentially buys time in which conditions could improve, diverting resources into future reproductive opportunities (Roitberg, 1989; Rosenheim, 1999; Burger et al., 2004).

The hypothesis of nutrient recycling is supported by studies on continuously reproducing (synovigenic) insects that show sexual dimorphic survivorship under inadequate nutrition. In nutritionally poor conditions female longevity either increases (Chippindale et al., 1993; Ohgushi, 1996) or stays the same (Kaitala, 1991; Boggs and Ross, 1993), while male longevity decreases (Ohgushi, 1996). This effect on female lifespan can be explained by positive correlations between oocyte degradation and female longevity, suggesting that increased female lifespan is explained by the ability to recoup resources from eggs through oosorption (Credland et al., 1986; Ohgushi, 1996; Wang and Horng, 2004), while males do not have the additional resource store to exploit. However, given that this previous research has been limited to continuously reproducing insects, it is not clear that this mechanism will apply to insects with discrete reproductive cycles. In cyclically breeding organisms the
reproduction-survival trade-off is complicated by the need of the female to survive to the next reproductive cycle to realise future fitness or, in the case of ovovivipary and vivipary, survive through parturition of the embryos. Oocyte degradation in the cyclically breeding insect ovary may simply be the process of oocyte ageing and clearance, similar to follicular atresia seen in mammals (Tilly, 2001), required to maintain reproductive synchrony.

In the ovoviviparous cockroach *Nauphoeta cinerea*, females undergo ovarian apoptosis and lose fecundity when mating is delayed relative to sexual maturation (Moore and Moore, 2001; Moore and Sharma, 2005). There appear to be costs associated with maintaining mature oocytes (Moore et al., 2007) and consequently eggs that are not used from one reproductive cycle cannot be used in the next, and it appears that they may either undergo apoptosis (Moore and Sharma, 2005) or be dumped (Roth, 1964).

Here, we address the function of oocyte degradation via apoptosis in this species. Female fecundity is constrained by the number of 50 day reproductive cycles that are possible within a lifespan of approximately 250 days (Roth and Willis, 1954). Females that delay mating produce one fewer clutch than those that reproduce directly upon sexual maturity, and thus these females experience a direct loss of 20% of total reproductive output (Moore et al., 2007). However, females must also ensure adequate investment into somatic maintenance for survival, as although no nutrients are passed to developing embryos, females must survive through parturition in order to successfully produce a viable brood of offspring. Consequently, females must carefully balance resources between both gametes and soma.

This study aims to ascertain whether observed ovarian apoptosis (Moore and Sharma, 2005) is a mechanism for nutrient recycling or maintaining reproductive synchrony. We deprived *N. cinerea* females of mating stimuli in combination with adult nutritional manipulation. Mating deprivation past sexual maturation induces apoptosis (Moore and Sharma, 2005). We examined the influence of nutritional signals on ovarian physiological state in terms of oocyte size and levels of ovarian apoptosis over and above the influence of a delay in mating relative to sexual maturation. When we deprived females of food during sexual maturation females reduced overall investment in reproduction and increased rates of apoptosis throughout the ovary.

2. Materials and methods

2.1. Animal husbandry and experimental design

We removed late instar nymphs from the mass colony, which were then sexed and isolated in separate 32 × 24 × 10 cm plastic containers provided with ad libitum rat chow and water. Newly eclosed adults were removed from the nymph colonies daily and placed in individual 11 × 11 × 3 cm plastic boxes. All experimental animals were housed under standard rearing conditions of 28 °C with 12/12 h reversed light/dark cycle.

In order to examine the effect of nutritional signals on somatic growth and ovarian dynamics, we randomly allocated females to one of the two treatments. Females in the first treatment (n = 36) were provided with the standard laboratory diet (rat chow) and water ad libitum. Females in the second treatment (n = 36) were provided with ad libitum water but no food following adult eclosion. In both treatments females were held under these conditions for 18 days. At day 18 we dissected ovaries from all of the females of both treatments. At this age, females are past sexual maturity and have low fecundity and high levels of apoptosis in their ovaries (Moore and Sharma, 2005).

We weighed all females for the nutrition manipulation study at the end of the treatment period on day 18 post-adult emergence. We weighed a subset of females (n = 18 in both starved and fed treatment groups) on both the day of adult emergence, and on day 18 following treatment in order to determine the affect of treatment on change in body mass.

2.2. Analysis of nutrition and delayed mating on oocyte investment

*N. cinerea* has paired ovaries consisting of panoistic ovarioles connected to a lateral oviduct, as is typical for cockroaches (Chapman, 1998). The number of ovarioles is variable both between individuals and the different ovaries of an individual. Each ovariole consists of a distal germinarium containing oogonia and previtellogenic cells and the more proximal vitellarium, where the oocytes undergo vitellogenesis (Chapman, 1998; Moore and Sharma, 2005). The terminal (T) oocyte that would be the first to be ovulated is found in the vitellarium adjacent to the oviduct (Chapman, 1998). Progressing up the ovariole from the oviduct the vitellarium contains a linear array of primary oocytes, all of which have been clothed by follicle cells (Chapman, 1998), in various stages of maturation, from the penultimate oocyte (T-1) that would be ovulated in the second reproductive, to the next in line (T-2) that would be ovulated in the third reproductive cycle. The region of the ovariole most distal to the oviduct is the germarium, where the oocytes are produced (Chapman, 1998).

We removed one ovary, chosen at random from the pair, from each experimental female at 18 days post-eclosion, and mounted the ovary on a cavity slide in phosphate buffered saline (PBS; 10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.0). The number of ovarioles was counted using a Leica MZ12.5 dissecting stereomicroscope and a photograph was taken using a SPOT Insight camera (Diagnostic Instruments, Inc.). The height and radius of T, T-1 and T-2 oocytes from each individual were measured using Spot Basic version 4.1 (Diagnostic Instruments, Inc.). By treating the oocyte as a cylinder, we calculated oocyte

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volume as volume = \( \pi r^2 h \) where \( h \) is the height and \( r \) is the radius of the oocyte.

2.3. Analysis of nutrition and delayed mating on oocyte apoptosis

We removed one ovary from each experimental female and placed it in 1ml PBS. The ovaries were kept on ice until all ovaries had been dissected. Equal numbers of dissections from each treatment were done per assay period. We alternated dissections between treatments to randomise any effects on cell damage induced by time elapsed from removal from the body. When all dissections were complete, we stained ovaries using the Vybrant Apoptosis Assay kit #4 (Molecular Probes, Invitrogen, Eugene, OR, USA) as described by Moore and Sharma (2005). This contains two dyes, the dye YO-PRO-1 (green fluorescence) can enter apoptotic cells but is excluded from healthy cells, while propidium iodide (red fluorescence) cannot enter living or apoptotic cells due to its large molecular size and thus only stains cells which are either necrotic or in the late stages of apoptosis (Willingham, 1999; Moore and Sharma, 2005). Therefore differences in cell membrane permeability can be graded. Healthy oocytes are unstaught, apoptotic oocytes are green, oocytes in the late stages of apoptosis or that are necrotic are red (Moore and Sharma, 2005). The slides were examined using an Olympus BX51 epifluorescence microscope (Olympus UK Ltd., London, UK) for staining. We assayed for apoptosis oocytes by counting the number of ovarioles that displayed either green or red fluorescence in either the terminal oocyte or the primary oocytes of the vitellarium.

2.4. Statistical analysis

Statistics were done in SPSS v 14.0. Repeated measures ANOVA was used to analyse changes in mass (dependent variable) from eclosion to 18 days post-eclosion between the different diet treatments (independent variable). We used a MANCOVA with Type III sums of squares to test the effect of diet treatment (independent variable) on the volume of T1, T2 and T3 oocytes (dependent variables), using number of ovarioles in the ovary as the covariate as this was a potential confounding variable. We then used a MANCOVA with Type III sums of squares to test the effect of diet treatment (independent variable) on the presence of signs of apoptosis in both the mature and immature oocytes (dependent variables), again using the number of ovarioles as the covariate. We tested the influence of diet treatment on individual variables by using ANCOVAs and we used Levene’s test of homogeneity to look at differences in variance between the two diet treatments. We did an ANCOVA to look at the confounding effect of body mass on levels of ovarian apoptosis in both diet treatment groups; we then performed linear regressions to look at the different slopes of this relationship in the two groups.

3. Results

3.1. Changes in body mass

Mass was maintained in females that had sustained access to food (mean eclosion mass = 0.58 g, mean mass at day 18 = 0.58 g; repeated measures ANOVA, \( F_{1,34} = 0.001, p = 0.989 \)), whereas there was a significant decrease in the mass of individuals that received no food as adults (mean eclosion weight = 0.59 g, mean weight at day 18 = 0.51 g; repeated measures ANOVA, \( F_{1,34} = 18.3, p = 0.001 \)).

3.2. Investment in oocytes in the presence and absence of nutritional signals

A multiple analysis of covariance to compare the size of the T, T-1 and T-2 oocytes between females that were starved or fed demonstrated a significant overall effect of diet. We found no significant difference in the number of ovarioles per ovary in the two treatments (ANOVA, \( F_{1,70} = 0.6, p = 0.425, n = 36 \)), and ovariole number did not have any significant interactions with the dependent variables in this treatment (MANCOVA Wilk’s \( \lambda = 0.954 \), \( F_{1,33} = 1.64, p = 0.202 \)). Here we present the results of the individual ANCOVAs. Food had a significant effect on the volume of the T (Fig. 1A; ANCOVA, \( F_{1,38} = 12.39, p < 0.001 \)), T-1 (Fig. 1B; ANCOVA, \( F_{1,38} = 3.53, p < 0.001 \)) and T-2 oocytes (Fig. 1C; ANCOVA, \( F_{1,38} = 10.36, p = 0.003 \)). Oocytes of starved females were smaller across all three measured oocytes (Fig. 1). All females that were fed, deposited yolk in their terminal oocytes, whereas seven of the 36 starved females did not. There was significantly more variation in the terminal oocyte volume of starved females ovaries than that of the fed females (Levene’s test, \( F = 7.3, p = 0.009 \)). Females of greater body mass had larger terminal oocytes at 18 days post-adult eclosion in both the treatments with and without food (Terminal oocyte volume = \(-1.086 + 4.843 \times \text{mass} \), \( F_{1,69} = 22.2, p < 0.001 \)).

3.3. Oocyte apoptosis in the presence and absence of nutritional signals

We did a multiple analysis of covariance to compare presence of apoptosis in the terminal oocyte, and the T-1 and T-2 oocytes in females that were starved or fed, the overall analysis showed that starved females had significantly more apoptosis than fed females. Ovariole number did not have any significant interactions with the dependent variables in this treatment (MANCOVA Wilk’s \( \lambda = 0.938 \), \( F_{1,33} = 0.73, p = 0.542 \)). Here we present the results of the individual ANCOVAs. Females without access to food during sexual maturation had significantly more apoptosis in both the terminal oocytes (Fig. 2A; ANCOVA, \( F_{1,71} = 11.68, p = 0.001 \)) and the oocytes of the vitellariium (Fig. 2B; ANCOVA, \( F_{1,71} = 5.35, p = 0.024 \)) than females that had ad libitum access to food. During observations we noted that...
individual oocyte growth and degradation was varied and asynchronous with other oocytes within the same ovary.

Relationships of terminal oocyte apoptosis to female body mass were significantly different between the treatments (ANCOVA, $F_{3,68} = 9.06, p = 0.004$). In the treatment in which females were fed we found no significant relationship between apoptosis levels and female mass (terminal oocyte apoptosis $= 4.907 + 5.451*mass$, $F_{1,34} = 0.1, p = 0.746$). In the treatment in which females were denied food during sexual maturation we found a significant positive relationship between apoptosis levels and female mass, with heavier females having higher levels of apoptosis (terminal oocyte apoptosis $= -7.775 + 38.243*mass$, $F_{1,34} = 17.5, p < 0.001$).

4. Discussion

Our results clearly show that rates of ovarian apoptosis increase in response to starvation. Our work gives initial evidence that ovarian apoptosis in a cyclically reproducing insect is a mechanism for nutrient recycling and not just a mechanism to maintain reproductive synchrony.

4.1. Nutritional stress decreases reproductive investment and stimulates recuperation of invested resources

All fed females deposited yolk. Starved females did not consistently deposit yolk and had smaller oocytes at all stages throughout the ovariole. This reduction in reproductive investment is not simply due to lack of resources to produce ova, but due to the negative nutritional signals inhibiting juvenile hormone release, which is required for oocyte maturation (Roth and Stay, 1962; Roth, 1964; Schal et al., 1993, 1997; Engelmann and Mala, 2005). The effects...
of starvation on both female mass and yolk deposition we observed were concordant with other experiments on both *N. cinerea* (Roth and Stay, 1962) and other insects (Kotaki, 2003; Terashima and Bownes, 2004).

We found considerably more variation in oocyte size in starved females compared with fed females. The ovaries of starved females displayed a range of terminal oocyte development, from previtellogenic to fully mature stages, while all fed females had fully developed terminal oocytes. All females emerge into adults with undeveloped ovaries, so initial variation in fat body stores should lead to variation in the level of ovarian investment in females that receive no food as adults. There may also be individual differences in resource allocation between gametes and soma (de Jong and van Noordwijk, 1992). In both cases, which are not mutually exclusive, variation would be reduced in fed females as the nutrient pool available for investment is larger (Tatar and Carey, 1995).

Our finding that ovaries from starved females had significantly more apoptosis in their terminal oocytes than ovaries from fully fed females demonstrates that the observed apoptosis was influenced by the nutritional environment, and that females were capable of responding to nutritional environment during sexual maturation (Hatle et al., 2004). This suggests that females can recoup resources previously invested in reproduction back into the maintenance of the soma. This provides initial evidence that ovarian apoptosis is not simply a product of cellular ageing in a cyclically breeding insect, although ageing and oosorption may not be mutually exclusive (Reviewed in Tilly, 2001; Reviewed in McCall, 2004). We are confident that the mechanism of cell removal is apoptosis, rather than autophagy, or any other means of programmed cell death (Schwartz et al., 1993). Oocytes showed apoptosis-specific changes (Willingham, 1999; Moore and Sharma, 2005), including staining for activated caspase 3 (ELBB and PJM unpublished results). Apoptosis is a known mechanism for recouping resources (Schwartz et al., 1993; Tilly, 1996), although further studies using radiotracer assays would show where these resources were reallocated to, and in what form (Rivero et al., 2001).

We found that when larger females were starved they had a greater percentage of apoptotic oocytes than smaller females; this trend was not seen in fed females. Increased levels of apoptosis may be the result of the cost of maintaining a larger phenotype as standard metabolic rate scales approximately with mass (Coelho and Moore, 1989; Calder, 1996) and may put larger females at a reproductive disadvantage in situations of prolonged nutritional stress. Males are smaller than females in this species and there is no directional selection on male size (Schal and Bell, 1983). Given that larger females are not more fecund under optimal conditions (Moore et al., unpublished data), larger females resorb more in suboptimal conditions, and there are presumably developmental costs to growing large (Roff, 1981). Questions are raised as to what factors maintain the larger female phenotype in this species.

When mortality is high due to a poor quality environment current fitness is more secure than potential future fitness (Williams, 1957). We know starved female *N. cinerea* invest in ovarian development, albeit at a lower rate, and can reproduce successfully when mated (Roth, 1962; Roth and Stay, 1962). However, maintaining reproductive effort at the expense of soma is only the best strategy when nutrient stores are adequate to maintain survival through to parturition. When resources fall under this threshold, oosorption should be favoured. We found that readiness to adopt oosorption was variable in both treatments. Females that maintain oocytes have a fitness advantage if sperm and nutrient deprivation is only a short term problem. Oosorption is a better strategy if deprivation is long term, as rerouting resources into increased longevity gives more time to search for males and food (Roitberg, 1989; Ohgushi, 1996; Rosenheim, 1999; Wang and Horng, 2004; Moore et al., 2007). Dumping eggs without resorption wastes energy, and although we do not know the nutrient content of dumped eggs, it is surely better not to lose any resources. It may be that the window for reproductive plasticity is narrow (Hatle et al., 2004), and the decision of whether to resorb oocytes has to be made at an earlier stage of development, similar to the system in *Drosophila* (Drummond-Barbosa and Spradling, 2001; Smith et al., 2002; McCall, 2004). Once past this developmental point, they have gone past the point of no return and will either be fertilised or discarded.

As has been found in other insects (e.g. Søller et al., 1997; Terashima and Bownes, 2004, 2005), not all oocytes within the ovary are resorbed in *N. cinerea* females that delay mating. This gives further evidence that the observed apoptosis is selective oosorption and not simply a process of ageing and clearance. We might predict that stressed females resorb the minimal number of oocytes and channel available resources into production of fewer oocytes while maintaining somatic form by recouping some of the resources from reproduction. Selective apoptosis would allow balancing of resources for optimum fitness and survival, although the mechanism by which oocytes or ovarioles are saved while their neighbours die is not clear and remains a area requiring further research (Søller et al., 1997; Chapman, 1998; Tilly, 2001; Terashima and Bownes, 2004, 2005).
4.3. Consequences of oosorption on future reproduction

Delayed mating induces apoptosis in both the vitellogenic terminal and non-vitellogenic primary oocytes of the vitellarium (Moore and Sharma, 2005), and results in the loss of reproductive potential equivalent to one clutch (Moore et al., 2007). Here we found that levels of apoptosis in both categories of oocytes increased further when the females were nutritionally stressed. The destruction of non-vitellogenic oocytes which could potentially be used in future reproduction is paradoxical as oosorption is expected to be an investment in future reproduction (Bell and Bohm, 1975; Kotaki, 2003) and non-vitellogenic oocytes do not represent a large cache of invested resources. Induction of apoptosis in the non-vitellogenic primary oocytes may be ‘signal leakage’ from the removal of the terminal oocyte (Moore and Sharma, 2005) such that induction of apoptosis in the terminal, yolk-rich oocyte results in a loss of a functional ovariole. We only found apoptosis in the non-vitellogenic primary oocytes in the same ovarioles as had an apoptotic terminal oocyte. Apoptosis in non-vitellogenic primary oocytes may be the physiological insult behind this lifetime fecundity cost, resulting in a non-resource based reproduction-survival trade-off (Tatar and Carey, 1995; Harshman and Zera, 2006).

Despite the loss of fecundity, apoptosis in non-vitellogenic primary oocytes need not be maladaptive physiology. Here, apoptosis may be a mechanism to inhibit further egg production before conditions improve (Morita and Tilly, 1999; Kotaki, 2003; Terashima and Bownes, 2004). The substantial within population variation in ovariole number at sexual maturity further suggests that there are adaptive advantages of having different clutch sizes in different environments. Shutting down whole ovarioles via apoptosis may be a mechanism of pre-fertilisation brood reduction when the environment is too poor to realise potential fecundity. Resources invested in dead offspring at pre- or post-zygotic stages are not beneficial to the female and can decrease her overall fitness (Wang and Horng, 2004). Further work is required to understand how environmental induced changes in ovarian dynamics are related to reproductive strategy.

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