Immune activation but not male quality affects female current reproductive investment in a dung beetle

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Changes in female reproductive investment can have large effects on offspring quality and thus maternal fitness. An immune activation is often expected to lead to a reduction in reproductive effort in order to release resources necessary for costly resistance to infection. Alternatively, an increase in investment in current reproduction may occur in order to maximize lifetime reproductive success: the so-called "terminal investment" effect. Additionally, females are expected to invest more in their reproductive effort when mating with higher quality males. Here, we investigate how an immune response and male quality affect the reproductive decisions made by female dung beetles, *Euoniticellus intermedius*. Female dung beetles invest a large amount of energy into each offspring, building large brood balls into which a single egg is laid. Maternal investment is therefore easily estimated from the number and size of the brood balls constructed. We found no affect of the mated male quality on female current reproductive investment. However, immune activation with lipopolysaccharide caused a significant reduction in the number of brood balls produced but did not affect brood ball size. An immune activation therefore caused resources to be diverted away from reproduction, as predicted by the "cost of immunity" hypothesis. Although life-history theory predicts 2 potentially opposing outcomes of an immune activation, a summary of studies investigating the trade-off between immune investment and current reproduction fails to provide any clear trends of what may predict the direction of this trade-off. *Key words:* dung beetle, *Euoniticellus intermedius*, female reproductive decisions, immune activation, life-history trade-offs, offspring quality, terminal investment. *[Behav Ecol 21:1367–1372 (2010)]*

The amount of resources that a female chooses to allocate to reproduction, and the manner by which those resources are distributed between offspring, is a critical determinant of her fitness. More offspring can obviously lead to higher fitness compared with producing fewer offspring, if they survive and reproduce. However, offspring growth and survival is often dependent on the amount and quality of resources allocated to each one. In many animals, offspring from large eggs generally grow faster, attain larger size, and have higher survivorship than those from small eggs (e.g., Rolff 1999; Schwanz 2008), meaning that females have to trade off offspring number against offspring quality. Females also require resources for somatic maintenance and diverting too much into reproduction can reduce fitness if a female becomes senescent or dies too early. Environmental and biological variation can result in the optimal reproductive strategy varying between females (Roff 1992). Two variables that are likely to affect reproductive decisions by females are exposure to pathogens and the quality of their mates.

Exposure to pathogens and parasites leads to activation of the host defenses. Because an immune response is costly, this is expected to result in reduced investment in other life-history traits, including reproductive effort (Sheldon and Verhulst 1996). There is support for this idea from both vertebrate and invertebrate studies: an immune activation caused a reduction in ovarian protein and egg production in the mosquito, *Anopheles gambiae* (Ahmed et al. 2002) and led to

a decrease in reproductive investment in terms of egg mass in female lizards, *Ctenophorus fordi* (Uller et al. 2006). Ultimately, however, allocation decisions between immunity and reproductive investment should favor individuals that maximize their lifetime reproductive success by trading off current and future reproduction (Magnhagen and Vestergaard 1991). When pathogens and parasites challenge the immune system, they are likely to alter an individual's probability of survival such that increased immune activity can be a cue to a reduced life expectancy (Bonneaud et al. 2004). Immune-challenged individuals should therefore invest more in current reproductive output because the chances of surviving to reproduce later in life are reduced. This "terminal investment" hypothesis predicts that instead of concentrating resources on an immune defense, individuals should allocate resources in favor of current reproduction, when life expectancy is reduced (Clutton-Brock 1984). Several recent studies have found that individuals increase, rather than decrease, their reproductive investment after an immune activation in a variety of species including crickets (Adamo 1999), mealworm beetles (Sadd et al. 2006), house sparrows (Bonneaud et al. 2004), and hamsters (Weil et al. 2006).

Females are also predicted to adjust their level of investment into offspring quality depending on the quality of the mated male (Sheldon 2000). Female banggai cardinal fish, *Pterapogon kauderni*, for example, produce heavier eggs when paired with larger preferred males (Kolm 2001). Increased investment in offspring quality when mating with a high-quality male is predicted to occur when reproduction is costly, there is a trade-off between current and future reproduction and mate quality affects offspring fitness (Sheldon 2000). Furthermore, allocation to offspring quality is expected to depend on the female's energetic state (Harris and Uller 2009). Females with low energy reserves are predicted to increase their investment

in offspring quality when mating with a high-quality male, whereas females with high energy reserves may increase investment with low-quality males (Harris and Uller 2009).

The effects of immune activation and male quality on female current reproductive investment are often investigated separately. However, given that immune investment is costly (Schmid-Hempel 2003), any resource limitations as a result of the immune activation might be expected to have strong implications for how male quality affects female investment in offspring quality. Overall, therefore, there are a number of theoretical questions regarding female reproductive investment, immune activation, and male quality that need to be addressed: first, do females increase (terminal investment) or decrease (the "cost of immunity" hypothesis) reproductive effort when given an immune challenge and second, does this interact with mate quality in the manner that theory would predict?

Here, we present an experimental test of the role of both of these factors, immune activation and mate quality, in determining current reproductive investment by females of the dung beetle *Euoniticellus intermedius*. Mature females build tunnels directly under a cow dung pat and construct large hollow brood balls from the dung at the end of these tunnels, in which a single egg is laid. The developing larva feeds on the dung in the brood ball and has no other food supply until it emerges as an adult. As a result, larval development and adult size are strongly dependent on the quantity of dung provided in the brood ball (Emlen 1994; Hunt and Simmons 1997; Head ML and Knell RJ, unpublished data). Brood ball weight therefore provides a reliable indicator of the offspring quality, and maternal investment can easily be estimated from the number and size of the brood balls constructed (Emlen 1994; Hunt and Simmons 1997). Males guard females by defending the entrance to the tunnel and fighting off rival males using the blunt curved horn on their head. Both male body size and horn size are known to be strong predictors of male quality in this species. Large males, and males with relatively long horns for their body size are more successful at guarding females (Pomfret and Knell 2006a), have stronger immune responses (Pomfret and Knell 2006b) and score higher for performance traits such as strength and endurance than do smaller males or those with relatively small horns (Lailvaux et al. 2005).

Euoniticellus intermedius therefore provides an interesting study species for examining how male quality and immune activation together affect female current reproductive investment. Unlike many insects, where females are able to produce hundreds of offspring that will forage independently, female dung beetles have to provide the resources for each developing offspring until it reaches adulthood. Female reproductive investment per offspring is therefore substantially greater than in most insect species. No previous studies have considered female reproduction following immune challenge in this or related species, but mate quality has been shown to have a considerable effect on female investment in offspring quality in the dung beetle *Onthophagus taurus*. Female *O. taurus* were found to manufacture more brood balls over their life time when mated with large males with long horns and provided substantially more resources for these offspring (Kotiaho et al. 2003).

In the experiments described here, we tested whether immune activation affected female current reproductive investment, in terms of number and size (quality) of brood balls produced. Because females were mated with males of varying quality, we were also able to test whether mate quality influenced female offspring investment. We elicited an immune response by injecting mature females with lipopolysaccharide (LPS), a component of bacterial cell walls that activates the humoral immune response in insects but is not itself a replicating pathogen. Because LPS does not generate its own metabolic products, unlike live pathogens, any changes in reproductive effort can be attributed to the host's immune activation alone (Kimbrell and Beutler 2001). In light of current theory, we make the following predictions: 1) females will decrease current reproductive investment, in both number and size of brood balls produced, if resources are diverted away from reproduction after an immune activation; 2) alternatively, immune-activated females will increase their current reproductive investment if they terminally invest in reproductive output; and 3) females that experienced an immune activation, and are therefore in a less energetic state than unaffected females, should increase offspring investment when paired with a high-quality male.

We also provide a synopsis of published studies to date that have investigated the effects of immune activation on current reproductive investment in order to assess whether there are any obvious patterns in the relationship between life history and an individual's decision to increase or decrease reproductive output after an immune activation.

MATERIALS AND METHODS

Stock beetles were originated from wild-caught animals (~700 individuals) collected in New South Wales, Australia in November 2007. Beetles were reared at 28 °C with a 12:12 light:dark cycle. Breeding pairs were kept in 1000-ml pots with 600 ml damp sand and 150 ml defrosted cow dung. After 5 days, brood balls were removed and kept in damp sand until eclosion. Newly emerged females were placed in individual containers with sufficient cow dung until sexually mature (between 14 and 20 days after eclosion).

Females were weighed and measured for body size and then randomly assigned to 1 of 3 treatment groups; 1) unwounded control: chilled for 1 min ($N = 43$); 2) procedural control: chilled for 1 min before being injected with 1 µl of phosphate-buffered saline (PBS) (pH 6.8) between the abdominal sternites with a 5 µl Hamilton syringe ($N = 37$); and 3) LPS injection: similarly injected with 1 µl of a 0.1% solution of LPS (derived from *Serratia marcescens*; Sigma L6136-25MG) dissolved in PBS (1 mg/ml, $N = 34$). This is a low dose of LPS comparable with that used in other studies on insects of similar size (e.g., *Allonemobius socius*, Fedorka and Mousseau 2007). Hemolymph loss during injecting was minimal and unlikely to have had a significant effect on reproduction.

Females were returned to individual containers with fresh (homogenized) cow dung. After 24 h, a mature male was placed in the container with the female, and they were left for 24 h to allow them to mate. The male was then removed and photographed using a Nikon Coolpix 950 camera mounted on a dissecting microscope. Male horn and pronotum length were measured using Image J v.1.40 (Rasband 1997–2009). Females were individually placed in 1000-ml pots with 500 ml damp sand and 100 ml of fresh (homogenized) cow dung for 3 days. Each female's brood balls were then collected from the sand and dried to a constant weight at 60 °C, to eliminate any variation caused by soil and dung moisture. Any uncompleted brood balls were discarded. Brood balls were weighed on a Sartorius Balance (Model: BP 221S). The experiment was carried out in 3 temporal blocks. There was no difference in the size of females in each treatment group ($F_{2,111} = 0.202$, $P = 0.82$) or size of the males introduced to females in each treatment group ($F_{2,111} = 0.846$, $P = 0.43$).

A second experiment was performed to determine if the probability of mating was affected by the treatments. Twenty females were allocated to each of the 3 treatment groups

described above until the point where they were separated from the male. Females were then euthanized using ethyl acetate and the spermatheca was checked for the presence of sperm.

Statistical analysis

All statistical analysis was performed on R 2.10 (R Development Core Team 2009). The number of brood balls produced was analyzed by fitting a generalized linear model with Poisson errors and a log link. Whether a female reproduced at all was analyzed by coding females as 0 (no brood balls) or 1 (at least one brood ball) and fitting a generalized linear model with binomial errors and a logit link, and for the females that did reproduce the mean brood ball weight per female was log-transformed to correct heteroscedasticity and analyzed with a general linear model. The initial set of predictor variables for each model was treatment, female body length, female weight, male body length, male horn length, and block; plus the 2-way interactions between treatment and the other predictor variables; plus the 3-way interaction between male horn length, female weight, and treatment. Model reduction was carried out via sequential removal of nonsignificant terms to produce a minimal adequate model (Crawley 2002; Zuur et al. 2009). None of the interaction terms used in the analysis were retained in any of the minimal adequate models and these will not be discussed in detail. When a significant effect of treatment was found, the effects of the different factor levels were tested by fitting models with either LPS and the procedural control or the unwounded control and the procedural control combined into single factor levels, and the goodness of fit of these models compared with the that of the original model with all 3 factor levels. A significant reduction in fit when levels are combined indicates that the factor levels in question have a significantly different effect (Crawley 2002; Zuur et al. 2009).

RESULTS

Brood ball number

The number of brood balls produced was positively correlated with female weight, with heavier females producing more brood balls over the 3 days (likelihood ratio = 12.94, df = 1, $P < 0.001$). Neither male body length nor horn length had any effect on the number of brood balls produced (main effects: male body length, likelihood ratio = 0.176, df = 1, $P = 0.68$; male horn length, likelihood ratio = 0.036, df = 1, $P = 0.85$). There was a significant effect of block because females in block 2 produced more brood balls than those in blocks 1 and 3 (likelihood ratio = 8.11, df = 2, $P = 0.02$), but note that there was no significant interaction between block and treatment (likelihood ratio = 4.50, df = 4, $P = 0.34$), indicating that the treatment effects were independent of the block effect.

There was a significant effect of treatment on the number of brood balls manufactured by females (likelihood ratio = 51.58, df = 2, $P < 0.001$; Figure 1). Although females given the procedural injection produced significantly fewer brood balls than unwounded control females, the LPS injection caused the largest decline in the number of brood balls produced (unwounded control: 4.3 ± 2.1 standard deviation [SD], $N = 43$; procedural control: 2.4 ± 2.0 SD, $N = 37$; LPS treatment: 1.7 ± 1.9 SD, $N = 34$). A model fitted to the data with the LPS and procedural control treatments combined into a single factor level gave a significantly worse fit than a model with the 2 treatments separated (likelihood ratio = −5.506, df = 1, $P = 0.03$), indicating that the effect of LPS was significantly greater than that of the procedural control.
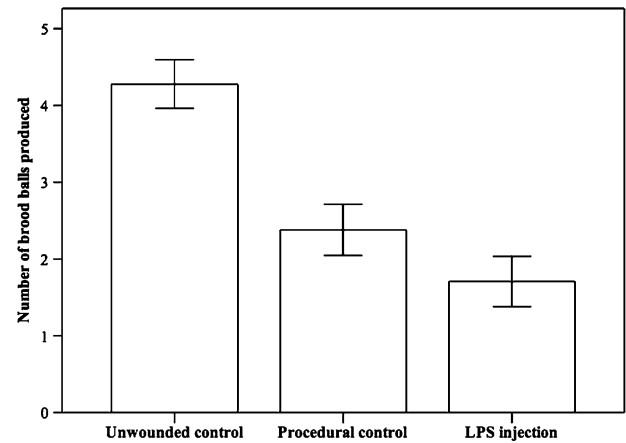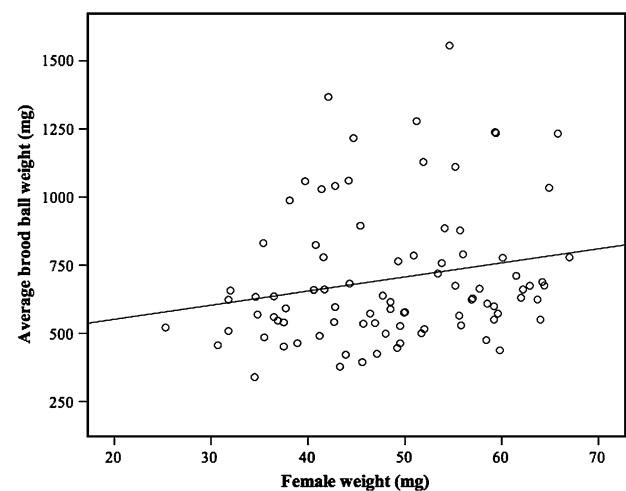

Figure 1
The number of brood balls (mean ± standard error) produced by unwounded control females ($N = 43$), females that received a procedural control injection ($N = 37$), and females that received an injection with 0.1% LPS solution ($N = 34$).

The minimal adequate model for whether females reproduced or not had 2 significant main effects: female body length (likelihood ratio = 4.53, df = 1, $P = 0.03$) and treatment (likelihood ratio = 11.72, df = 2, $P = 0.003$). Longer females were more likely to reproduce (standardized coefficient = 1.28, standard error = 0.615) no matter what the treatment, but females given either LPS or the procedural injections were less likely to produce brood balls than unwounded controls. Fitting a model with the LPS and procedural control treatments combined into a single treatment gave a fit that was not significantly worse than the model with all 3 treatments (likelihood ratio = 3.0896, df = 1, $P = 0.08$). Again, neither body nor horn length of the male was related to whether a female produced brood balls or not (main effects: male body length likelihood ratio = 0.005, df = 1, $P = 0.94$; horn length likelihood ratio = 0.165, df = 1, $P = 0.69$).

Brood ball weight

Brood ball weight was positively correlated with female weight ($F_{2,82} = 27.99$, $P < 0.001$, Figure 2). Male horn and body length had no effect on brood ball weight (main effects: male

Figure 2
Relationship between female weight (mg) and the average weight of each brood ball (mg). $N = 88$.

body length, $F_{1,80} = 0.0384$, $P = 0.85$; male horn size, $F_{1,80} = 0.0278$, $P = 0.87$). Once again, there was a significant effect of block because females in block 2 manufactured heavier brood balls than those in blocks 1 and 3 ($F_{2,82} = 32.66$, $P < 0.001$). Neither the procedural control nor the LPS injections had an effect on the average weight of brood balls produced ($F_{2,82} = 0.996$, $P = 0.37$: unwounded control: 0.670 ± 0.224 g SD, $N = 39$; procedural control: 0.732 ± 0.272 g SD, $N = 29$; LPS treatment: 0.700 ± 0.251 g SD, $N = 20$).

Female mating probability

The probability of a female mating at least once was unaffected by treatment, with the majority of females from the second experiment having stored sperm in their spermathecae after 24 h with a male ($\chi_2^2 = 2.88$, $P = 0.24$; females with stored sperm: unwounded control: 18/20, procedural control: 20/20, LPS injection: 19/20).

DISCUSSION

Male quality had no effect on female offspring investment, both in terms of brood ball number and quality. This is a surprising result as maternal investment has been shown to vary with male quality in another species of dung beetle (Kotiaho et al. 2003). Furthermore, a recent model by Harris and Uller (2009) shows that intraindividual variation in female allocations decisions is likely to depend on energetic state, with females in a less energetic state being more likely to increase offspring investment when paired with a high-quality male. Given that immune activation is costly, the LPS treatment would be expected to amplify any increase in offspring investment with increasing male quality. Only female quality, however, significantly predicted reproductive investment, with larger females producing both more and larger brood balls. Although differential maternal investment as a result of male quality appears to be widespread (Sheldon 2000; Harris and Uller 2009), female quality appears to have much larger effects on offspring quality than does male quality in E. intermedius.

Females that received either a procedural control injection or an LPS injection were less likely to reproduce compared with unwounded females, suggesting that females from both treatments experienced an immune activation from the procedure. This pattern is unlikely to be related to changes in mating probability with treatment because the probability of a female E. intermedius having mated at least once was unaffected by treatment. When the total number of brood balls produced was analyzed, we found that LPS-injected females made significantly fewer brood balls than either procedural controls or unwounded control females, suggesting that the LPS produced a stronger immune response that wounding alone. An immune challenge therefore resulted in a decrease in female current reproductive investment, a finding that supports the cost of immunity hypothesis.

Although the number of brood balls produced declined with an immune challenge, the average weight of each brood ball manufactured was unaffected by treatment. This suggests that female investment in offspring quality was not compromised after an immune activation. The size of each offspring is an important fitness component because the amount of resources allocated per offspring can strongly affect offspring fitness and thus maternal fitness (Roff 1992). The females that do reproduce after an immune challenge therefore appear to maintain offspring quality at the expense of offspring number.

Although the LPS injections caused the greatest decline in the production of brood balls, females receiving the procedural injections also manufactured fewer brood balls than unwounded females. Altincicek et al. (2008) similarly found that a procedural control had a significant effect on the number of viviparous offspring produced compared with unwounded controls in the pea aphid, Acyrthosiphon pisum. In vitro studies have found that the prophenoloxidase cascade, a crucial element of insect innate immune systems, has a mechanistic role in hemolymph coagulation (Nagai and Kawabata 2000; Bidla et al. 2005), and 2 recent in vivo studies have shown that wounding also leads to other components of the immune system being upregulated (Haine et al. 2007; Wigby et al. 2008), although LPS injection is known to lead to a stronger and longer upregulation (e.g., Jacot et al. 2005). Wounding itself may therefore have caused a large enough immune response to affect current female reproductive effort. Studies investigating the effect of an immune investment on other life-history traits commonly use assays that involve procedural injections as the only control group (see Table 1). Our procedural control gave distinctly different results from our unwounded control and has allowed us to separate out the effect of the wounding alone from the effect of the immune activation by LPS on female reproductive investment. The use of only a procedural control is therefore likely to obscure any subtle effects that may occur and limit the interpretation of studies investigating the effects of immune response on life-history traits (Wigby et al. 2008).

Published studies to date that have investigated the effects of immune activation on current reproductive investment show a clear disparity of results (Table 1), with some studies reporting increases and some reporting decreases in current reproductive output when the immune system is stimulated and very few reporting no response. One explanation for this disparity in response is that the optimal strategy for an individual who has had an immune activation may vary according to the biology of the species, as well as individual condition and age. The contrast between crickets, where immune activation has been found to increase short-term oviposition in Acheta domesticus (Adamo 1999, but see Shoemaker and Adamo 2007) and E. intermedius is likely to reflect differences in the cost of increasing short-term reproduction in the 2 species. Crickets are able to store mature eggs in the lateral oviduct, enabling females to increase their oviposition rate at little cost by laying already mature eggs immediately after an immune activation, and once an egg is matured there is no female investment beyond oviposition. Female E. intermedius, in contrast, have only a single oviduct and do not appear to store more than 1 mature egg at a time (Knell RJ, unpublished data). Furthermore, egg laying in E. intermedius not only requires resources for egg production itself but energy is required for manufacturing the brood ball. Females collect and drag down portions of dung that will provision each larva for its entire developmental period. Each brood ball takes several hours to construct and they weigh on average 26 times the weight of the female (Reaney LT, unpublished data). The decrease in the number of brood balls produced by female in E. intermedius after an immune insult is therefore not surprising.

In the absence of a proper theoretical study of the effects of immune activation on reproductive effort, it is difficult to ascertain how other biological and life-history traits should affect an individual's decision to increase or decrease reproductive output. However, some obvious candidates are the relative cost of an immune response, the virulence of the parasites that a species or population is commonly exposed to, reproductive life span, and whether reproduction is iteroparous or semelparous. It is difficult to see any trends in our summary of the current research, however (Table 1). Regarding life span, both the shorted-lived pea aphid (Altincicek et al. 2008) and the long-lived eider duck (Hanssen 2006), for example, show an increase in their investment in

Table 1

Summary of studies that have investigated current reproductive output after an immune challenge

Study species	Sex	Immune challenge used	Reproductive output measured	Response	Unwounded control	Reference
Pea aphid (*Acyrthosiphon pisum*)	F	Bacteria (heat inactivated)	Offspring number	Increase	Yes	Altincicek et al. (2008)
Cricket (*Gyrllus campestris*)	M	LPS	Daily calling rate	Decrease	No	Jacot et al. (2004)
Cricket (*Cyphoderris strepitans*)	M	LPS	Daily calling rate	Decrease	Yes	Leman et al. (2009)
Cricket (*Acheta domesticus*)	F	LPS	Egg-laying rate	Increase	Yes	Adamo (1999)
		Bacteria	Egg-laying rate	Increase	Yes	
		Parasitoid	Egg-laying rate	No effect	Yes	
Cricket (*Gryllus texensis*)	F	Bacteria	Egg-laying rate	Increase (on moist soil)	No	Shoemaker et al. (2006)
			Egg-laying rate	Increase (at high doses)	No	
Cricket (*Gryllus texensis*)	F	LPS	Egg-laying rate	No effect	Yes	Shoemaker and Adamo (2007)
			Egg weight	Decrease (at high doses)	Yes	
Mosquito (*Anopheles gambiae*)	F	LPS	Egg production	Decrease	Yes	Ahmed et al. (2002)
Mealworm beetle (*Tenebrio molitor*)	M	Parasite	Pheromone production	Decrease	N/A	Worden et al. (2000)
Mealworm beetle (*Tenebrio molitor*)	M	Nylon implant	Pheromone production	No effect	Yes	Vainikka et al. (2007)
		LPS	Pheromone production	No effect	No	
		Micro-latex beads	Pheromone production	No effect	No	
Mealworm beetle (*Tenebrio molitor*)	M	Nylon implant	Pheromone production	Increase	Yes	Sadd et al. (2006)
Dung beetle (*Euoniticellus intermedius*)	F	LPS	Egg investment	Decrease	Yes	This study
Snails (*Biomhalria glabrata*)	F	Parasite	Egg-laying rate	Increase	N/A	Minchella and Loverde (1981)
Lizard (*Ctenophorus fordi*)	F	LPS	Clutch size	No effect	No	Uller et al. (2006)
			Egg mass	Decrease	No	
Blue tits (*Parus caeruleus*)	F	Vaccine	Parental effort (feeding rate)	Decrease	No	Råberg et al. (2000)
Pied flycatchers (*Ficedula hypoleuca*)	F	Vaccine	Reproductive output	Decrease	No	Ilmonen et al. (2000)
House sparrow (*Passer domesticus*)	F	Vaccine	Clutch replacement	Increase	No	Bonneaud et al. (2004)
Eider duck (*Somateria mollissima*)	F	Red blood cells	Brood care	Increase	No	Hanssen (2006)
Blue-footed booby (*Sula nebouxii*)	M	LPS	Reproductive success	Decrease (mature males)	No	Velando et al. (2006)
			Reproductive success	Increase (old males)	No	
Hamster (*Phodopus sungorus*)	M	LPS	Testes investment	Increase	No	Weil et al. (2006)

We report papers that deal with changes in only current reproductive investment for mature males and females. Using the Web of Science (http://isiknowledge.com), we searched for papers using the following key words: "immune activation," "reproductive investment," "cost of immunity," and "terminal investment." We also located papers from cited references. We report the type of immune challenge used, the reproductive output measured, and whether the immune challenge caused an increase or decrease in reproductive output. Species given in this order: hemimetabolous insects, holometabolous insects, molluscs, reptiles, birds, and mammals.

reproduction after an immune activation. Furthermore, some results are ambiguous with effects only apparent under certain conditions (e.g., Shoemaker et al. 2006) and there are inconsistent results within the same study species (e.g., Sadd et al. 2006; Vainikka et al. 2007). There is also a strong bias toward specific study animals, with nearly half of the studies on invertebrates being performed on crickets and mealworm beetles. Finally, few studies report no changes in reproductive effort after an immune activation (Shoemaker and Adamo 2007; Vainikka et al. 2007), suggesting a "file drawer effect," whereby null results are not published. Clearly, more research is needed, both theoretically and empirically, if we wish to understand how reproductive decisions are made in the presence of parasites and pathogens.

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