Paternal investment directly affects female reproductive effort in an insect

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Female reproductive effort can be influenced by the quality of her mate. In some species, females increase their reproductive effort by differentially allocating resources after mating with high-quality males. Examination of female reproductive effort in relation to male quality has implications for estimating the evolvability of traits and for sexual-selection models. Accurate quantification of reproductive investment is not possible in many species. Butterflies are an exception, as most nectar-feeding species emerge with almost intact reproductive resources, and in some species males provide nutrients at mating that enhance female fecundity. By manipulating male donations and using radioactive isotopes, we quantified the effect of variation in nutrient provisioning on female reproductive effort in two butterfly species. In the green-veined white butterfly, *Pieris napi*, females increased their reproductive effort after receiving large male donations. By contrast, in the speckled wood, *Pararge aegeria*, where males do not provide nutrients, female reproductive effort was independent of male ejaculate. Increased reproductive effort in *Pieris napi* resulted from the production of more eggs, rather than from investing more resources per egg. In this species donating ability is heritable; hence females laying more eggs after mating with high-donating males benefit both through higher fecundity and through the production of high-donating sons.

Keywords: paternal investment; maternal effort; differential allocation; *Pieris napi*; *Pararge aegeria*; sexual selection

1. INTRODUCTION

Females generally make a larger investment in reproduction than males (Bateman 1948). Reproductive effort is central to life-history theory (Roff 1992), as the amount of resources invested by females in current reproductive events will affect their future reproduction and survival (Williams 1966; Trivers 1972). Recently, it has been realized that a female's reproductive effort may be influenced by the quality of her partner (Burley 1986; Eberhard 1996). By differentially allocating resources to reproduction, females can increase their reproductive effort after mating with attractive males (Burley 1986, 1988). This can be achieved by increasing investment in offspring production, biasing resources towards the more attractive sex (i.e. sons) or varying the sex ratio to more sons (see Sheldon (2000) for a review). The increased cost to females of higher reproductive investment would be expected to be balanced by higher future reproductive fitness of their offspring, mainly through higher reproductive success (Weatherhead & Robertson 1979).

There has recently been a vigorous debate regarding the importance of female reproductive effort in relation to male attractiveness (Cunningham & Russell 2000, 2001; Sheldon 2000, 2001; Colegrave 2001; Gil & Graves 2001; Petrie *et al.* 2001). Understanding how females vary their reproductive investment in relation to the quality of their partners has several important implications. First, it is a prerequisite for demonstrating female post-copulatory choice (Thornhill 1983; Eberhard 1996), through increased investment in reproduction, increased egg-laying rate or biasing sperm use in favour of high-quality males (e.g. Thornhill 1983; Pizzari & Birkhead 2000; Saino *et al.* 2002). Second, female variation in reproductive investment has obvious impacts on models of sexual selection (Wolf *et al.* 1997; Qvarnström & Price 2001): it has been argued that differential allocation in relation to male attractiveness will reinforce the response to sexual selection (Sheldon 2000; Qvarnström & Price 2001). Finally, differential allocation can directly affect the expression of condition-dependent traits and also has ramifications for estimating the heritability of traits (Møller & Thornhill 1998; Mousseau & Fox 1998). Without considering the presence of maternal or paternal effects, simple models of heritabilities may lead to an under- or over-estimation of the potential for traits to evolve (McAdam *et al.* 2002). If females vary investment in offspring in relation to male genetic quality, it has been argued that similarities between half-sibs cannot always be attributed to paternal or maternal genetic effects (Gil *et al.* 1999; Cunningham & Russell 2000; but see Sheldon 2000, 2001).

Examination of differential allocation requires that the amount of resources invested by the female in offspring production can be reliably estimated. The total amount of resources invested in offspring needs to be determined, as does the amount of resources invested per offspring. For many bird and mammal species this is not feasible, unless variation in resources invested per egg is revealed by egg weight variation (e.g. Cunningham & Russell 2000). However, this does not always accurately reflect resource investment (see Petrie *et al.* 2001). As proxys for differential allocation, many studies have examined variations in clutch size and parental feeding effort. This has revealed that females of several bird species behave as predicted by

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theory (Burley 1986, 1988): they lay more eggs and work harder at feeding their young after mating with attractive males (e.g. Burley 1988; Yamamoto *et al.* 1989; de Lope & Møller 1993; Petrie & Williams 1993; Forkman & Corr 1996). However, not all species (and not all birds) show variation in egg weight that is directly related to variation in the degree of female investment (Karlsson & Wiklund 1985; Wedell 1996), and many species do not provide parental care, making it difficult to quantify differential allocation by females.

In several insects males provide nutrients to the female at mating that are converted into more eggs, resulting in higher lifetime fecundity (reviewed in Vahed 1998). Many butterfly species emerge as adults with the majority of the nitrogenous resources they will require for reproductive investment derived from reserves allocated during larval feeding. They have limited possibilities to replenish these nitrogenous compounds through adult feeding. This is because nectar contains mainly carbohydrates and only traces of the amino acids needed for the production of additional eggs (Karlsson & Wickman 1990; Boggs 1997). However, these energy reserves provide carbon skeletons for egg production in some species (Boggs & Ross 1993), and can prolong adult life, enabling sufficient time for the conversion of larval-derived nutrients to reproduction (Boggs 1997; Karlsson 1998). In addition, in species in which males provide nutrients females can obtain more resources by essentially foraging for matings. These features make it possible to quantify accurately the investment in reproduction of limiting resources by females, either resources derived from larval feeding or those received from male donations. It has been shown, using radioactive isotopes, that females of some butterflies use male-derived nutrients for egg production (Boggs & Gilbert 1979; Wiklund *et al.* 1993; also Vahed 1998) and also differentially allocate their resources in relation to males' nutrient donations (Wedell 1996). This makes butterflies ideally suited for examining the effect of variation in male quality (i.e. nutrient provisioning) on female reproductive effort.

We examine the effect of variation in male nutrient provisioning on female reproductive effort in two butterfly species. In one, the green-veined white butterfly (*Pieris napi*), males provide nutrients to the female at mating (Wiklund *et al.* 1993, 1998); in the other, the speckled wood (*Pararge aegeria*), males do not give donations (Svärd 1985). We used radioactive isotopes: one for tracing the fate of resources invested in reproduction by the female from larval resources, and another for tracing resources provided by the male. This allows quantification of: (i) the amount of resources provided by the male and (ii) the effect this has on female reproductive effort, both in terms of the number of offspring produced and the amount of resources invested per offspring. This provides information not only on whether females increase their reproductive effort after mating with high-donating males as predicted by theory, but also on whether females produce more offspring and/or increase investment in each offspring. If females vary their reproductive effort in relation to male nutrient provisioning, we expect to see differential allocation in *Pieris napi*, but not in *Pararge aegeria* where males do not provide resources.

2. MATERIAL AND METHODS

(a) *Butterfly rearing and incorporation of isotopes*

Female butterflies of the two species were captured around the campus of Stockholm University, Sweden, and allowed to lay eggs. When the eggs hatched, larvae were reared on their host plant until their last instar. *Pieris napi* were fed garlic mustard leaves (*Alliaria petiolata*), and *Pararge aegeria* were fed *Poa annua* grass. When larvae reached their final instar they were fed with either 5 µl of U-^{14}C protein hydrolysate (specific activity of 1.85 Mbq ml^{-1}, concentration of 50 µCi ml^{-1} in 2% ethanol; Amersham) or 5 µl of ^{3}H amino acid mixture (specific activity of 37.0 Mbq ml^{-1}, concentration of 1.0 mCi ml^{-1} in 2% ethanol; Amersham). There is no difference in the proportions of the various amino acids in the ^{14}C and ^{3}H mixtures. Individuals were kept without food the night before receiving the radioactive mixture. Each label was placed on either a piece of *A. petiolata* leaf or a piece of *Poa annua* grass stem, and the ethanol was allowed to evaporate. Each individual larva received a piece of host plant labelled with 5 µl isotope. If the larva had not consumed the entire piece of *A. petiolata* leaf or grass straw within 3 h, they were discarded. Males and females of the two species were randomly allocated to receive one of the labels, with roughly equal numbers of ^{14}C and ^{3}H individuals for each sex.

On the day of adult eclosion all individuals were weighed and placed in flight cages according to species, sex and label, and provided with a 10% sucrose solution added to flowers. On the day following eclosion individual females were randomly paired with a male labelled with the opposite isotope to their own. This ensured that all females were of the same age. Cages were checked every 15 min and pairs were confined to a 0.5 l cup from detection of the mating until the mating had ended. Following mating each female was placed in an individual 1.5 l plastic cup and provided with either *A. petiolata* leaves (*Pieris napi*) or *Poa annua* blades (*Pararge aegeria*) for egg laying and a piece of cotton wool soaked in 10% sugar solution twice a day. Host plants were checked daily for eggs, which were removed. After 7 days of egg laying females were placed in the freezer. We chose 7 days of egg laying as female *Pieris napi* mated to a virgin male remate, on average, after 5.5–7 days (Kaitala & Wiklund 1995; Cook & Wedell 1999), and will mate, on average, 2.03 times in their lifetime (Wiklund & Forsberg 1991). By contrast, Swedish female *Pararge aegeria* are monogamous (Svärd & Wiklund 1989).

To generate males providing varying amounts of nutrients to the female we used the fact that once-mated *Pieris napi* males provide a significantly smaller spermatophore (the sperm packet including nutrients) on their second mating than do virgin males (Kaitala & Wiklund 1995). A similar decline in spermatophore size with mating has been shown in several other butterfly species (reviewed in Vahed 1998). After their first mating, males were allowed to recuperate for 1 day before mating with a 1-day-old virgin female. Both females had been fed on the opposite label to the male. These females were allowed to lay eggs for 7 days as before. This procedure ensured that some female *Pieris napi* received substantially smaller donations from mated males. Following the second mating males were killed by putting them in a freezer, and their abdomens were dissected from their thoraces with a pair of micro-scissors and dried at 60 °C for 48 h. Females were also dissected after 7 days of egg laying and their body parts were dried. Similarly, female *Pararge aegeria* were also mated to non-virgin males. In total 22 *Pieris napi* and 16 *Pararge aegeria* matings were achieved this way.

(b) *Isotope quantification*

The dried abdomens and thoraces of males and females, and the eggs each female laid during the 7 days after mating were placed in separate scintillation vials with 1–2 ml of tissue solubilizer (Soluene 350, Packard). Abdomens, thoraxes and eggs were incubated at 35 °C and stirred daily until the tissue had completely dissolved. Abdomens, thoraxes and eggs were tested for radioactivity by adding 5 ml of scintillation fluid (HiSafe, Fermenta) and radioactivity was counted with a 1217 RackBeta LKB Wallac scintillation counter. Each sample was counted for 3 min, and quenching was by internal standard. Counting efficiency was *ca.* 85% for ^{14}C and 30% for ^{3}H. Since labels varied in both concentration (^{3}H containing 20 times the concentration of ^{14}C) and counting efficiency, total counts (DPM = disintegrations per minute) were adjusted accordingly to allow direct comparisons.

The total amount of label (DPM) for males was calculated as the total label present in the male's abdomen and thorax, plus the total amount of label originating from the male that was incorporated into eggs or present in the female's abdomen after her death. Similarly, for females, the total amount of label was calculated as the total label present in her abdomen and thorax plus the total amount of female label found in her eggs. When examining differences in reproductive investment between males and females of the two species, proportion invested in reproduction was calculated as the total amount of label minus the amount of label incorporated into the thorax. Hence, the entire label found in abdomen, eggs and spermatophore represents an individual's total investment in reproduction. This represents both realized (DPM in eggs laid over the 7 days for females and in spermatophores for males) and non-realized reproductive investment (DPM left in the abdomen). Since the larvae may have varied in both metabolic rate and the duration between consumption of the label and pupation, the amounts of label used in reproduction from each adult were converted into a proportion of the total amount found in eggs, spermatophores and abdomens.

Male spermatophore donation was calculated as the proportion of male label incorporated into eggs or found in females' abdomens. Male and female investments in eggs were calculated for each female as (total DPM in eggs from either the male or the female/number of eggs) divided by (total DPM in eggs from both sexes/number of eggs). This provides a measure of mean male or female investment per egg. There is no effect of the isotope (either ^{14}C or ^{3}H) on the total amount of DPM incorporated by females (*Pieris napi*: $F_{1,20} = 2.902$, $p > 0.11$; *Pararge aegeria*: $F_{1,14} = 0.840$, $p > 0.37$) or males (*Pieris napi*: $F_{1,11} = 0.148$, $p > 0.71$; *Pararge aegeria*: $F_{1,9} = 0.371$, $p > 0.56$) in either of the two species. Similarly, there is no effect of isotope on either the number of eggs laid (*Pieris napi*: $F_{1,20} = 0.592$, $p > 0.45$; *Pararge aegeria*: $F_{1,14} = 0.107$, $p > 0.75$) or the amount incorporated in the spermatophore by males (*Pieris napi*: $F_{1,20} = 0.400$, $p > 0.53$; *Pararge aegeria*: $F_{1,14} = 0.930$, $p > 0.35$). All statistics were calculated using STATISTICA 99 Edition, all proportions were square-root–arcsine-transformed and results are presented as means ± s.e.

3. RESULTS

In summary, *Pararge aegeria* females invest more resources (71%) than males (32%) in reproduction ($F_{1,24} = 44.700$, $p = 0.0001$), whereas in *Pieris napi* males and females allocate similar proportions of their resources
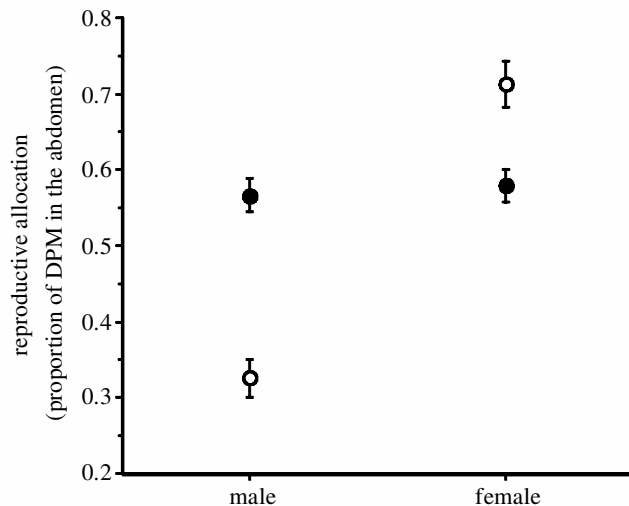

Figure 1. Relative investment of resources in the abdomens of male and female *Pieris napi* (filled circles) and *Pararge aegeria* (open circles) butterflies. DPM, disintegrations per minute. Means ± s.e.

to reproduction (females 58% versus males 57%, $F_{1,32} = 0.151$, $p > 0.7$; figure 1). This is not surprising, as male *Pieris napi* provide nutrients in the spermatophore to the female at mating (Wiklund *et al.* 1993, 1998), whereas this is not the case for *Pararge aegeria*. However, male *Pieris napi* provide fewer resources in the spermatophore on their second mating than on their first mating (3790 ± 2080 versus 7250 ± 2800 DPM, $F_{1,20} = 10.3$, $p = 0.0044$, corresponding to 10% versus 21% of the total amount of resources, $F_{1,20} = 17.6$, $p = 0.0005$), indicating that our manipulation was successful. By contrast, no effect of male mating history on spermatophore quality was found in *Pararge aegeria* ($F_{1,14} = 0.182$, $p > 0.68$).

Therefore, do females vary their reproductive effort in relation to male spermatophore quality? As predicted, female *Pieris napi* receiving more resources from the male increase their own investment in egg production (figure 2*a*). By contrast, in *Pararge aegeria*, female reproductive effort is independent of male spermatophore investment (figure 2*b*), which is not surprising, as males do not provide nutrients in this species. This is mirrored in the lack of detection of any male-derived isotopes in *Pararge aegeria* eggs laid by females.

The increased investment by *Pieris napi* females after receiving larger donations from the male is expressed by the production of more eggs (figure 3). However, *Pieris napi* females do not appear to increase their investment in each individual egg in relation to the amount of resources provided by the male. There is no relationship between the average amount invested per egg by the female (mean female-derived DPM/egg) and the investment received from the male (mean male-derived DPM/egg; $r = -0.036$, $p > 0.87$, $n = 22$). This suggests that there are no obvious maternal effects in terms of resource provisioning per offspring in relation to male investment in *Pieris napi*.

4. DISCUSSION

As predicted by the differential-allocation hypothesis, female *Pieris napi* increase their reproductive effort after

Figure 3. Number of eggs laid by female *Pieris napi* in relation to the amount of nutrients (DPM, disintegrations per minute) received from males ($r = 0.73$, $p = 0.0001$, $n = 22$).
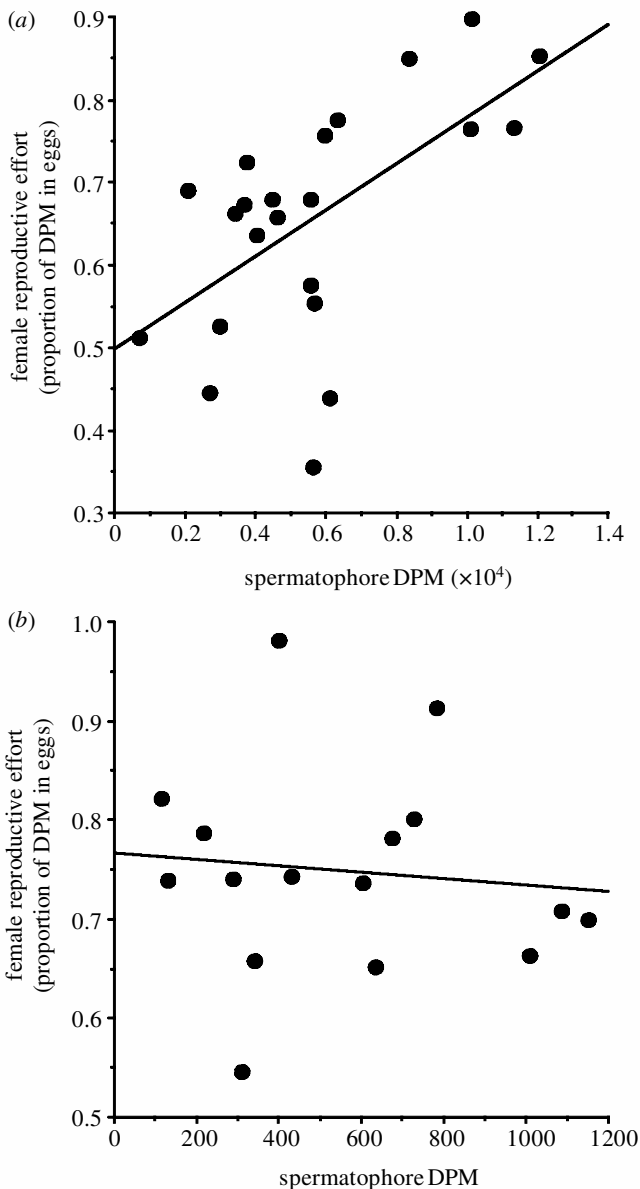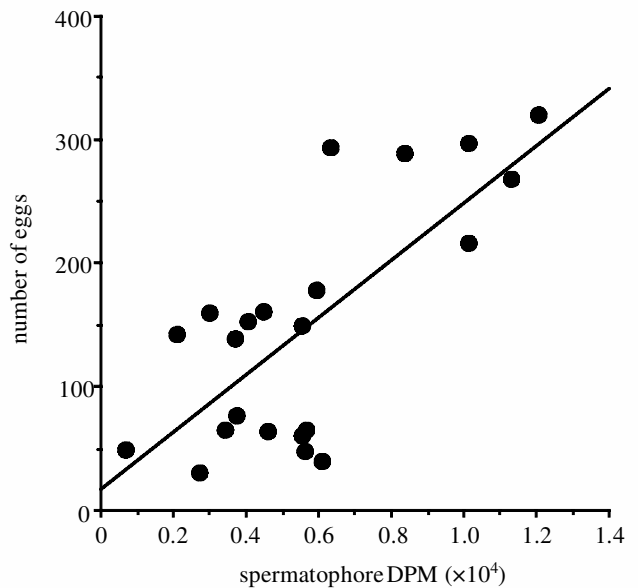

Figure 2. Female reproductive effort in relation to the amount of nutrients (DPM, disintegrations per minute) received from the male in (*a*) *Pieris napi* ($r = 0.60$, $p = 0.004$, $n = 22$) and (*b*) *Pararge aegeria* ($r = -0.101$, $p > 0.7$, $n = 16$).

mating with males providing high-quality donations. After receiving large donations they produce more eggs. This is not simply a function of females converting male nutrients into eggs. The difference in the amount of resources present in a spermatophore between a virgin and a mated male is equivalent to the production of approximately 21 eggs (Karlsson 1998), whereas the smallest difference in the number of eggs laid by females mated to virgin and mated males is almost 100 eggs. This implies that females are increasing their egg laying after receiving larger donations. However, females do not vary the amount invested per offspring in relation to male investment. The amount of resources invested per egg is independent of the resources received from the male. By contrast, female *Pararge aegeria* do not vary their reproductive investment in relation to the male's spermatophore investment. This is as predicted, since males do not provide resources to the female at mating in this species.

In *Pararge aegeria*, females make a larger investment in reproduction (measured as DPM in the abdomen) than males. In *Pieris napi*, however, investment in reproduction is of a similar magnitude for males and females, *ca.* 60% of the total amount of resources. This assumes that DPM (^{14}C or ^{3}H) accurately reflects resource investment. We believe this to be the case. For example, in *Pararge aegeria* heavier females allocate a higher proportion of their total carbon to the abdomen, resulting in higher lifetime fecundity (Karlsson & Wickman 1990), and, in *Pieris napi*, the amount of carbon is found to covary with the amount of nitrogen invested in spermatophores (Karlsson 1998). Nitrogen is frequently a limiting resource for reproduction in many insects including butterflies (Boggs 1981, 1997; Karlsson 1995). Our finding is in agreement with previous work showing that female *Pieris napi* eclose with 60% of their total nitrogen allocated to their reproductive reserves (Karlsson 1998). We found that males invest *ca.* 20% of their total resources in their first spermatophore (measured as DPM), which is a substantial donation to females. This donation represents *ca.* 10% of the female's total body reserves of nitrogen, and is equivalent to the amount of nitrogen in approximately 70 eggs (Karlsson 1998). This is corroborated by results showing that a spermatophore provided by a virgin male represents the production of an additional 50–80 eggs to the female (Karlsson 1998; Wiklund *et al.* 1998).

Male-derived nitrogen is an important supplement to the pool of nitrogen that females derive from the larval diet. Both affect egg production in *Pieris napi* (Karlsson 1998). Similarly, a recent study on a moth found that essential amino acids used in egg production originate entirely from the larval diet (O'Brien *et al.* 2002). As females age, the carbon skeleton used for the production of non-essential amino acids is, increasingly, derived from adult nectar sources. However, although dietary sugars (e.g. carbon) are converted into non-essential amino acids,

all essential amino acids derive from larval feeding, thus setting the upper limit on the effect that the adult diet may have on reproduction (O'Brien *et al.* 2002). In this study, egg production was measured in females of the same age that were given free access to sugar solution as adults, ensuring that any effect of variation in female age on reproductive investment did not influence our results.

Unlike *Pieris napi*, *Pararge aegeria* is a territorial species. Differences in mate-locating tactics have been shown to affect flight design, including investment in flight muscles (Wickman 1992). However, while it is possible that this may partly explain why male *Pararge aegeria* invest fewer resources in reproduction than *Pieris napi*, we think that the main reason for this difference is a variation in nutrient provisioning. This is corroborated by the finding that *Pieris napi* have relatively larger abdomens than *Pararge aegeria* (Karlsson 1995) with higher nutritional quality (Karlsson 1996) rather than proportionally smaller flight muscles (Wickman 1992), showing that they invest more resources overall in reproduction.

It has been argued that increased female reproductive output after mating to high-quality males does not necessarily imply differential allocation, but could instead be the result of male manipulation (Colegrave 2001; Cunningham & Russell 2001; Gil & Graves 2001). In many insects, males transfer substances in the ejaculate that stimulate female oviposition rate (Chen 1984; Cordero 1995; Gillott 2003). Males can vary in how efficient they are at elevating female egg-laying rate (Service & Vossbrink 1996: Chapman 2001). The extent to which male *Pieris napi* can stimulate female egg laying is not known. There is no genetic difference between males in the egg-laying rate they confer on females immediately after first mating, despite documented effects of male genotype on female longevity and lifetime fecundity (N. Wedell, unpublished data). In this study the same male was mated to two different females, one receiving a large donation, the other substantially less. The possibility that mated males also transfer smaller amounts of oviposition stimulants in the ejaculate cannot be ruled out; nutrient provisioning may covary with the level of oviposition stimulants in the spermatophore. However, female egg-laying rate has a genetic basis in *Pieris napi*. The degree of polyandry is genetically determined (Wedell *et al.* 2002), and singly mated 'polyandrous' females lay eggs at a higher rate than 'monogamous' females (Wiklund *et al.* 1993). The rapid shift in reproductive effort by females in relation to the variation in nutrient provisioning may be adaptive regardless of whether it is caused by male manipulation or increased female resource allocation.

At the current time, the adaptive significance to females of varying their reproductive effort, either in relation to male attractiveness or to the quality of males' donations, is unknown. Theory predicts that females that differentially allocate their resources in relation to male quality should have higher fitness than females that do not (Sheldon 2000). Several studies have demonstrated a direct benefit to females of receiving male donations (Vahed 1998), but the long-term fitness effects on females of varying their reproductive investment are largely unknown. Male nutrient donation is heritable in *Pieris napi* (N. Wedell, unpublished data); therefore females mating with high-donating males will produce sons with high-quality donations, and

hence may choose to increase their egg production after mating with these males. It is not known whether *Pieris napi* females are able to vary the sex ratio of their offspring and produce more sons after mating with high-donating males, but it seems unlikely. *Pieris napi* is a protandrous species with early-emerging males having a higher probability of mating with virgin females (Wiklund & Fagerström 1977). However, protandry is caused by a higher male growth rate rather than by females laying more sons at the beginning of the reproductive season (Wiklund *et al.* 1991). Finally, there appears to be no cost to females of increasing their reproductive effort, as females mating with virgin males and receiving large donations both produce more eggs and live longer than females receiving smaller donations from mated males (Karlsson 1998; Wiklund *et al.* 1998).

The results of this study have implications for estimating heritability and for models of sexual selection. Demonstrating a lack of maternal effects in terms of resources invested per offspring in relation to male quality has two obvious implications. First, the similarities between offspring and parents are not confounded by variation in female investment. This suggests that it should be possible to detect males' genetic contributions across dams to their offspring in this species using a half-sib design without the very large sample sizes that are required when maternal influences are strong. Second, maternal (and paternal) effects in terms of increased resource provisioning to individual offspring can influence the expression of condition-dependent traits and therefore offspring attractiveness (Qvarnström & Price 2001). Hence, confirming an increased reproductive effort in terms of the production of more young rather than an increased investment per offspring reduces the likelihood of this potentially confounding effect. In most species, excluding the non-genetic influence on the expression of traits is difficult unless the maternal and paternal contributions to offspring can be precisely quantified, as is the case in *Pieris napi*.

The differential-allocation hypothesis states that females should gain indirect benefits by the increased production of attractive offspring (Burley 1986); hence demonstrating differential allocation may indicate the presence of genetic effects. This prediction is corroborated in birds, where differential allocation is predominantly found in species where females receive genetic benefits from mate choice (Møller & Thornhill 1998). It is likely that female *Pieris napi* also gain genetic benefits by producing more eggs after mating with high-donating males, as spermatophore size is heritable in this species (N. Wedell, unpublished data). Additionally, females receive direct benefits from males in terms of nutrients that enhance both fecundity and longevity, which may also offset some of the cost to females of increased reproductive effort (cf. Wedell 1996). However, it is unlikely that females can preferentially choose to mate with high-donating males, as they appear to be unable to discriminate between males on the basis of their mating status in both the field and the laboratory (Kaitala & Wiklund 1995). This may have promoted the evolution of female differential allocation of resources post-mating, promoting higher egg production after receiving large donations, as the donating ability will be passed on to sons.

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