Paternal effects correlate with female reproductive stimulation in the polyandrous ladybird *Cheilomenes sexmaculata*

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Abstract

Components of male seminal fluids are known to stimulate fecundity and fertility in females of numerous insect species and paternal effects on offspring phenotype are also known, but no studies have yet demonstrated links between male effects on female reproduction and those on progeny phenotype. In separate laboratory experiments employing 10-day-old virgin females of *Cheilomenes sexmaculata* (F.), we varied male age and mating history to manipulate levels of male allomones and found that the magnitude of paternal effects on progeny phenotype was correlated with stimulation of female reproduction. Older virgin males remained in copula longer than younger ones, induced higher levels of female fecundity, and sired progeny that developed faster to yield heavier adults. When male age was held constant (13 days), egg fertility declined as a function of previous male copulations, progeny developmental times increased, and the adult weight of daughters declined. These results suggest that male epigenetic effects on progeny phenotype act in concert with female reproductive stimulation; both categories of effects increased as a consequence of male celibacy (factor accumulation), and diminished as a function of previous matings (factor depletion). Male factors that influence female reproduction are implicated in sexual conflict and parental effects may extend this conflict to offspring phenotype. Whereas mothers control the timing of oviposition events and can use maternal effects to tailor progeny phenotypes to prevailing or anticipated conditions, fathers cannot. Since females remate and dilute paternity in polyandrous systems, paternal fitness will be increased by linking paternal effects to female fecundity stimulation, so that more benefits accrue to the male's own progeny.

Introduction

Maternal effects, the epigenetic modification of offspring development by mothers, can have dramatic consequences for progeny phenotype and life history (Mousseau & Dingle, 1991; Mousseau *et al.*, 2009). Transgenerational signals of maternal origin have received considerable research attention, especially in insects, since the publication of

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Mousseau & Fox (1998). Until recently, less attention was directed toward the potential significance of paternal effects (but see Hunt & Simmons, 2000; Bonduriansky & Head, 2007; Adler & Bonduriansky, 2013). Insect seminal fluids have been a focus of research attention largely because of their many effects on female reproductive functions, ranging from inhibition of receptivity on the one hand, to stimulation of fecundity and fertility on the other (Chen *et al.*, 1988; Gillott, 2003; Avila *et al.*, 2011). Seminal fluids also transmit the paternal epigenome, whether via genomically imprinted sperm, or transcribed factors such as allohormones, as exemplified by the sex peptide of *Drosophila melanogaster* (Gioti *et al.*, 2012). However, the role of insect seminal fluids in mediating transgenerational signals of paternal origin is not yet fully understood.

If the influence of paternal effects on offspring is underestimated, their potential roles in sexual conflict remain largely unexplored (but see Qvarnstrom & Price, 2001). Intersexual conflict arises when the reproductive interests of male and female are not congruent (Eberhard, 1996, 1997). When both parents exert parental effects, the potential exists for sexual conflict over offspring phenotype development (e.g., Miller *et al.*, 2006; Simmons & Garcia-Gonzalez, 2007). As noted by Brown *et al.* (1997), the degree of control exerted by each sex over events in the mating sequence can have a strong influence on evolution of the mating system and on the level of sexual conflict. Female insects clearly hold the balance of power in this regard, having effectively decoupled the process of fertilization from insemination through evolution of sperm storage in the spermatheca, selecting in turn for male abilities to coerce aspects of female reproduction and so regain some control of the process (Eberhard, 1996).

In the case of aphidophagous coccinellids, which are highly promiscuous, unidentified male factor(s) appear to stimulate egg production by females. This has been demonstrated using repeated matings to boost male factor levels in females, first in *Adalia bipunctata* (Semyanov, 1970) and subsequently in a number of other species (Majerus, 1994; Omkar & Mishra, 2005; Omkar & Pervez, 2005), including *Cheilomenes sexmaculata* (F.) (Bind, 2007). In *Propylea dissecta* and *C. sexmaculata*, the optimum number of matings required to maximize female fitness in the laboratory was estimated to be about 13 (Omkar *et al.*, 2006*a*, *b*). But repeated matings can be costly for males; a study in two *Coccinella* spp. revealed that serial copulations progressively diminished male ability to stimulate female reproduction, suggesting male factor(s) are subject to depletion (Michaud *et al.*, 2013). The same study found negative consequences of previous paternal matings for progeny fitness (lower survival, slower development, smaller adult size), which suggested possible linkage between female reproductive stimulation and paternal effects. The age of beetles was held constant in most of the studies cited above, but age at first mating can also have a significant effect on female reproductive performance. For example, Omkar *et al.* (2010) showed that fecundity and egg fertility in *Coelophora saucia* (Mulsant) was maximized when females and males were about 20 and 30 days old at first mating, respectively. This increase in female reproductive performance with male age up to 30 days could be inferred to reflect male factor accumulation during the period of mate deprivation.

Given that coccinellid males have no direct control over egg fertilization by females, the timing of oviposition, or the placement of eggs, we hypothesized that paternal effects on progeny will not be contingent on any specific environmental conditions, but rather should be expressed in a fixed pattern, i.e., they will be 'selection-based', rather than 'detection-based' (*sensu* Shea *et al.*, 2011). Since female polyandry results in a progressive dilution of the first male's paternity, males in such systems would benefit from coupling paternal effects with female fecundity stimulation, thus ensuring that their epigenetic investment contributes primarily to the condition of their own progeny. In the present study, we varied both male age at first mating and male mating history in *C. sexmaculata* in order to effect the accumulation and depletion of male factor, respectively, and observed the consequences for both female reproductive performance and offspring development. We hypothesized that prolonged male celibacy would lead to accumulation of male factor, so that female reproductive performance (as measured by time to oviposition, fecundity and fertility) and offspring quality (as measured by survival, developmental rate and body mass) would both improve with male age at first mating. The second hypothesis was that matings will deplete male factor, so that male influences on both female reproduction and offspring condition will diminish as a function of the number of previous male copulations for males of a given age.

Materials and methods

Insect colony

A colony of *C. sexmaculata* was established from about 100 adult beetles collected from a pistachio orchard (GPS coordinates 30°23′39.07″N and 55°55′39.30″E) infested with pistachio psyllid, *Agonoscena pistaciae* Burckhardt and Lauterer at Vali-e-Asr University of Rafsanjan, Rafsanjan, Iran in September 2012. Prior to use in experiments, the beetles were reared for two generations on frozen eggs of *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) in a climate-controlled growth chamber set to $26 \pm 1°C$, $60 \pm 5\%$ RH and 16L: 8D photoperiod. Adults were held in a ventilated plastic box ($20.0 \times 30.0 \times 10.0$ cm) and fed initially with psyllid-infested leaves of pistachio. Eggs of *E. kuehniella* were obtained from a colony maintained in the insectary of the University of Rafsanjan and were never stored for more than 1 month prior to use. Mated females were isolated in plastic Petri dishes (6.0 cm diameter × 1.5 cm height) and paired with a male from the stock colony for 2–3 h every other day during the course of oviposition to maintain fertility. Eggs were collected every 12 h and females were fed *E. kuehniella* eggs daily. Upon hatching, first instar larvae were transferred to plastic Petri dishes (6.0 cm diameter), one per dish, and fed eggs of *E. kuehniella* daily until they emerged as adults. Callow adults were transferred to plastic boxes (as above), about 25 pairs per box, where they were fed eggs of *E. kuehniella* daily.

Experiments

All experiments were conducted in growth chambers under constant physical conditions ($27.5 \pm 1°C$, $55 \pm 10\%$ RH and 16L: 8D photoperiod). Forty mated female *C. sexmaculata* from the stock colony were isolated in plastic Petri dishes (6.0 cm) and provisioned with *E. kuehniella* eggs ad libitum, refreshed twice daily. Petri dishes were observed twice daily and changed following each oviposition. Egg clusters were kept in an incubator until eclosion under the same physical conditions as larvae. First instar larvae were isolated in Petri dishes and fed every 12 h until pupae formed. Adult beetles were weighed on an electronic balance (accuracy = 0.1 mg)

Table 1. Copulation, reproduction and progeny development data (means ± SE) for *C. sexmaculata* pairs in which 10-day-old virgin females were mated once with a virgin male either 5, 15 or 30 days old. Means bearing the same letter were not significantly different within columns (Bonferroni, α = 0.05).

Male age (day)	n	Time to copula (min)	Duration of copula (min)	Fecundity (no. eggs)	Fertility (% hatching)	n (progeny)	Progeny development time (days)
Five	20	4.18 ± 0.70a	130.7 ± 9.9b	159.8 ± 25.5b	83.2 ± 2.0b	85	11.26 ± 0.10a
Fifteen	20	2.85 ± 0.65ab	170.6 ± 12.6a	241.9 ± 40.2ab	80.9 ± 5.5ab	60	11.25 ± 0.10a
Thirty	16	2.28 ± 0.49b	183.0 ± 9.6a	326.0 ± 39.8a	83.3 ± 6.2a	65	10.58 ± 0.05b
F		2.30	6.17	5.79	0.09		17.93
df		2,53	2,53	2,47	2,47		2,207
P		0.11	0.004	0.006	0.915		<0.001

within 12 h of emergence and sexed. Males and females were distinguished by the shape of the terminal abdominal segment, which is more rounded in females and more pointed in males when viewed ventrally. The average fresh mass of female parents was 9.53 ± 0.26 mg and of male parents, 6.72 ± 0.17 mg (n = 70 in each case).

For the male age experiment, all beetles were isolated in Petri dishes and fed daily until they reached the required age. Virgin males aged 5, 15 and 30 days (n = 20 per treatment) were each paired once with a 10-day-old virgin female in a Petri dish. Time to copula was tallied as the time elapsed between introduction of male and female into the dish until onset of copula. Duration of copula was tallied as the time between onset of copula and male dismount. After a copulation, females were isolated in Petri dishes with *E. kuehniella* eggs and observed twice daily for 20 days. Egg retention time (ERT) for females post-copula was tallied as the median time between observations during the period when the eggs were laid. Fecundity (no. of eggs laid) and egg fertility (percentage of eggs hatching) were recorded for each female, along with the developmental time (egg incubation + larva + pupa) and adult fresh weight of five of her offspring. Fresh weight and sex were recorded for all offspring within 24 h of emergence.

For the mating history experiment, females from the stock colony were used to create males with different mating histories. Once-mated males were permitted a single copulation on their 11th day of adult life, five-times-mated males, five copulations on days 7–11; and ten-times-mated males daily copulations from days 2 to 11 inclusive. All copulations were visually confirmed. No copulations were permitted on the 12th day and all males were 13 days old when paired with a single focal, 10-day-old, virgin female. The same physical conditions were employed as in the previous experiment and all the same biological parameters were measured.

Data analysis

Data were checked for normality using the Kolmogorov–Smirnov test (K–S test) and all were found to be normally distributed. Percentages (e.g., egg fertility) were arcsine square root-transformed prior to analysis. Data on time between introduction to start of mating, duration of copulation, fecundity, percentage egg hatch and developmental time for immature stages were subjected to one-way ANOVA followed by a Bonferroni test to separate means (α = 0.05; SPSS, 2006), with either male age or mating history as the independent factor. A two-way ANOVA was used to test effects of male age/mating history and offspring sex on offspring weight. Percent survival and offspring sex ratio were analyzed by Chi-square (χ^2).
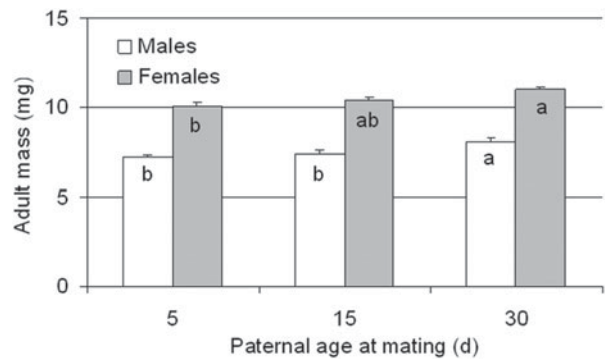

Fig. 1. Mean ± SE adult fresh mass of *Cheilomenes sexmaculata* progeny sired by virgin males either 5, 15 or 30 days old with 10-day-old virgin females. Columns bearing the same letter were not significantly different from others of the same sex (Bonferroni, α = 0.05).

Results

Effects of male age at first mating

There were significant effects of male age on duration of copula and female fecundity, but not on the time to initiate copula, or egg fertility (table 1). Older males tended to remain longer in copula and stimulated female fecundity more than younger males. One female in treatments one and three and four females in treatment two failed to oviposit and were excluded from analyses of fecundity and fertility. There were significant effects of male age on the total developmental time of progeny from egg to adult; faster development was obtained by the offspring of the oldest fathers, although the magnitude of the difference between five and 30-day-old fathers was <1 day over the entire period of development. Mean offspring survival was 93.3 ± 2.8, 81.2 ± 5.0 and 94.3 ± 3.3% for 5-, 15- and 30-day-old sires, respectively, the difference between 15- and 30-day-old sires being the only significant difference (χ^2 = 4.84, P = 0.028). The sex ratio of offspring did not vary among treatments (χ^2 = 0.829, df = 2, P = 0.661) and was not significantly different from 50% (χ^2 = 0.5, P = 0.480). A two-way ANOVA of paternal age and offspring sex on offspring fresh mass was significant overall (F_{5202} = 83.08, P < 0.001) and revealed significant main effects of age (F_{2202} = 12.32, P < 0.001) and sex (F_{1202} = 376.84, P < 0.001) without significant interaction (F_{2202} = 0.01, P = 0.987). Females were heavier than males and offspring of older fathers tended to be heavier than those of younger fathers, regardless of whether they were male or female (fig. 1).

Table 2. Copulation, reproduction and progeny development data (means ± SE) for *C. sexmaculata* pairs in which 10-day-old virgin females were mated once with a 13-day-old male that was either virgin or mated once, five or ten times previously. Means bearing the same letter were not significantly different within columns (Bonferroni, α = 0.05).

Male mating history	n	Time to copula (min)	Duration of copula (min)	Fecundity (no. eggs)	Fertility (% hatching)	n (progeny)	Progeny development time (day)
Virgin	16	1.13 ± 0.26b	151.4 ± 13.1a	260.7 ± 54.7a	86.0 ± 1.6a	62	10.63 ± 0.08c
Mated × 1	13	2.19 ± 0.36ab	118.1 ± 14.9a	180.2 ± 34.6a	74.6 ± 3.7ab	55	10.95 ± 0.07ab
Mated × 5	14	1.36 ± 0.29b	113.9 ± 9.9a	142.2 ± 40.0a	70.5 ± 5.0b	44	11.16 ± 0.11a
Mated × 10	15	3.40 ± 0.55a	117.9 ± 7.0a	150.0 ± 33.5a	70.1 ± 4.1b	48	10.79 ± 0.06bc
F		7.51	2.46	1.71	4.49		7.01
df		3,54	3,54	3,51	3,51		3,205
P		<0.001	0.095	0.270	0.870		<0.001

Effects of male mating history

There were significant effects of male mating history on the time required to initiate copula and egg fertility, but not on duration of copula or fecundity (table 2). One female failed to oviposit in each of the first three treatments and was excluded from analysis of fecundity and fertility. Time to copula tended to increase with number of previous paternal matings, suggesting a reduction in male mating proclivity with increased numbers of copulations.

Differences in female fecundity did not reach significance at $P = 0.05$ because of high within-group variation among females. However, there was a clear downward trend in fecundity with number of previous male matings, and a negative quadratic regression line could be fitted to the four data points with $r^2 > 0.85$. Egg fertility declined progressively and significantly and there were also significant effects of male mating history on the total developmental time of progeny. Although the progeny of 5× mated males required the longest time to complete development, those of 10× mated males developed as quickly as virgin males. Mean survival was 86.7 ± 4.6, 88.3 ± 3.8, 75.0 ± 6.0 and 83.6 ± 3.6% for offspring of virgin, one, five and ten times mated males, respectively ($\chi^2 = 9.49$, df = 3, $P = 0.023$) and revealed no clear trend. The sex ratio of offspring did not vary among treatments ($\chi^2 = 3.76$, df = 3, $P = 0.289$) and was not significantly different from 50% ($\chi^2 = 2.48$, df = 1, $P = 0.116$). The two-way ANOVA of paternal mating history and offspring sex on offspring fresh mass was significant overall ($F_{7,202} = 34.72$, $P < 0.001$) and revealed significant main effects of mating history ($F_{3201} = 5.15$, $P = 0.035$) and sex ($F_{1201} = 380.51$, $P < 0.001$), without a significant interaction ($F_{3201} = 1.51$, $P = 0.213$). The mass of daughters decreased with increased number of paternal matings but the mass of sons did not (fig. 2).

Discussion

The first experiment revealed that increasing paternal age at first mating up to 30 days of adulthood is associated with increased female reproductive performance, as measured by 20-day fecundity (table 1). Previously, Omkar *et al*. (2006*a*, *b*) found that female fecundity in *C. sexmaculata* increased with male age up to 60 days, provided males were prevented from mating during this period. Also similar to the observations of Omkar *et al*. (2006*a*, *b*), older *C. sexmaculata* males initiated copula more quickly and exhibited longer copulations, which are thought to correlate with the transfer of larger volumes of seminal fluid (Obata, 1987). A very similar pattern was observed for *C. saucia* Mulsant (Coleoptera: Coccinellidae); in a study that spanned a greater range of male ages, fecundity
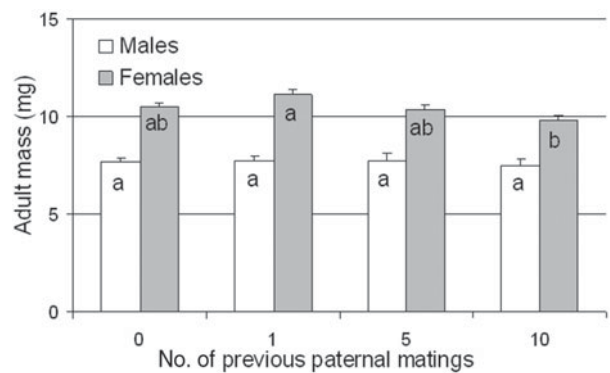

Fig. 2. Mean ± SE adult fresh mass of *Cheilomenes sexmaculata* progeny sired by 13-day-old males of different mating histories with 10-day-old virgin females. Columns bearing the same letter were not significantly different from others of the same sex (Bonferroni, α = 0.05).

stimulation peaked at 30 days and declined thereafter (Omkar *et al*., 2010). Likewise, Srivastava & Omkar (2004) found fertility in *Coccinella septempunctata* L. to be dependent on paternal age, peaking for males 20–30 days old. Although an increase in sperm number with age could contribute to some of these effects, male allohormonal factors would seem to be implicated, as egg maturation should not depend on the number of sperm available.

Male age effects on female reproduction were mirrored by paternal effects on offspring fitness, confirming our primary hypothesis; 30-day-old males sired progeny that developed faster (table 1) and yielded heavier adults (fig. 1) than did 15-day-old males, with better survival to the adult stage. This appears consistent with previous observations by Omkar & Singh (2009) who found that younger adults of *C. sexmaculata* produced faster developing offspring than did older pairs, although the ages of both parents were varied in that study. We infer that either the same male factors stimulating female fecundity in *C. sexmaculata* are responsible for the paternal effects on offspring development, or independent factors are responsible, which are accumulated/depleted in tandem. For example, the male sex peptide of *D. melanogaster* elicits a cascade of genetic expression in mated females (Gioti *et al*., 2012) and even demonstrates cross-species activity in other insect orders (Hanin *et al*., 2012), although any possible effects on progeny phenotype have not been explored. Once again, simple differences in sperm numbers received during mating are unlikely to affect progeny development post-fertilization,

so changes in other components of seminal fluids would seem to be implicated.

The results of the mating history experiment, in which treatments effectively imposed different levels of male factor depletion prior to a focal mating, partly confirmed the second hypothesis. Female fertility declined with increasing numbers of previous paternal matings, although changes in fecundity were not significant due to high variation within treatments (table 2). As numbers of previous paternal matings increased, progeny developed more slowly to yield lighter female adults, although the mass of male progeny appeared unaffected (fig. 2). Previous work has shown that matings are costly for coccinellid males and diminish their ability to invest in subsequent copulations, both behaviorally and physiologically (Michaud *et al.*, 2013; Omkar *et al.*, 2013). We infer that reserves of male allomonal factor(s) accumulate slowly through adult life, prior to onset of senescence, until they are depleted by mating events.

Female coccinellids control the timing of oviposition and the placement of eggs, and can alter progeny phenotypes to suit predictable dynamics of resource availability, even when this requires producing higher condition progeny later in the reproductive cycle, rather than early (e.g., Vargas *et al.*, 2012*a*, *b*). Such tactics are beyond the ability of males that have little, if any, control over sperm utilization by females. In polyandrous systems, paternal effects will be most profitably coordinated with female fecundity stimulation because paternity is diluted over time by female remating (Wedell *et al.*, 2006). Thus, male *C. sexmaculata* coerce the early and rapid production of high-quality offspring by females, to the extent they are able.

Fecundity stimulation would seem a rather inefficient male strategy for maximizing paternity, but may represent an alternative strategy for males when females are able to resist male inhibition of female receptivity post-copula. Clearly, females can only benefit from paternal effects that improve offspring condition, and probably rely upon them to realize their own fitness potential, but matings beyond some optimum number will reduce female fitness (i.e., Omkar *et al.*, 2006*a*). Sexual conflict may arise over female reproductive rate, simply because males will strongly discount residual (future) female reproductive effort and favor a higher rate of oviposition than that which may be optimal for female lifetime fitness. Indeed, laboratory studies which have demonstrated reductions in female longevity as a cost of repeated matings (i.e., Mishra & Omkar, 2006) may well underestimate them, as longevity is greatly extended by artifacts of confinement (reduced energy expenditure, protection from predation, ad libitum food, etc.). However, most studies of coccinellids that have followed lifetime reproductive success reveal no indication of a fecundity/longevity tradeoff; rather, the most fecund individuals seem to be the longest lived (e.g., Michaud & Qureshi, 2006; Omkar *et al.*, 2010). It is also possible that male fecundity stimulation exacts a genetic cost for females; a sub-maximal oviposition rate might enable them to mate with a larger number of males and thus produce more genetically diverse progeny, or counter the infertility risks associated with mating with related males (Tregenza & Wedell, 2002). It is our hope that the present work will provide impetus for exploration of paternal effects in other insect systems where male factors have been shown to stimulate female reproductive performance, especially in those for which the chemical factors responsible have been identified.

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