

Sexual activity diminishes male virility in two *Coccinella* species: consequences for female fertility and progeny development

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Abstract

Male contributions, both chemical and behavioural, can influence female sperm usage and reproductive success. To determine whether such male factors are subject to depletion in *Coccinella septempunctata* and *Coccinella transversalis*, we tested the effects of male mating history on male virility, as estimated by measures of mating behaviour, female reproductive success and progeny fitness, with parental age held constant. Overt measures of male mating effort (wriggling duration, number of shaking bouts and total copula duration) all diminished from virgin to 5×mated males and were mirrored by concurrent declines in female fecundity and fertility (measured over 20 days). Paternal effects were also observed which diminished as a function of mating history, suggesting that transgenerational signals of male origin are also subject to depletion. Progeny of virgin fathers had higher rates of survival (*C. transversalis*) and faster development (both species) than progeny of 5×mated fathers. Seminal fluid proteins are known to have allohormonal properties and can stimulate female fecundity and fertility in a number of insects, making them strong candidates for depletion as a function of mating activity. However, it is also possible that sperm limitation and/or reduced tactile stimulation of females by multiple-mated males may have contributed to some of the observed effects.

Keywords: developmental time, fecundity, fertility, paternal effects, seminal fluid proteins

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Introduction

The evolution of the spermatheca in female insects effectively uncoupled fertilization from insemination and gave rise to novel forms of intersexual conflict (Eberhard, 1996). Once females achieved postcopulatory control over sperm utilization, mating *per se* was no longer a guarantee of paternity for males. Sperm competition and cryptic female choice became important selective forces shaping the

evolution of secondary male strategies to influence sperm usage patterns in females (Parker & Simmons, 1989; Eberhard, 1997; Simmons & Siva-Jothy, 1998; Fisher *et al.*, 2006). Consequently, males of polyandrous insect species evolved a diversity of tactics to foil male competitors (e.g., sperm removal), reduce female receptivity, prevent females from remating (e.g., mate guarding, mating plugs) and stimulate female oviposition and hence rapid sperm utilization (reviewed by Eberhard, 1996; Gillott, 2003; Wedell, 2005). A variety of compounds generally referred to as 'seminal fluid proteins' (SFPs) are produced in insect male accessory glands (Chen, 1984) and can have a wide range of physiological effects on females, ranging from fecundity and fertility stimulation to reduction of female receptivity (Avila *et al.*, 2011). When sperm competition is intense, or when male

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ejaculates contain nutrients or expensive-to-produce SFPs, the costs of ejaculate production can be much higher than once assumed by the 'sperm is cheap' hypothesis of sexual differentiation (Bonduriansky, 2001; Lupold *et al.*, 2010; Perry & Rowe, 2010; Avila *et al.*, 2011). Furthermore, it has been argued theoretically that if fecundity stimulation by males is sufficiently strong, it will actually attenuate sperm competition among males because those mating with promiscuous females 'cooperatively' fertilize more eggs than those who inseminate monogamous females (Alonzo & Pizzari, 2010).

Aphidophagous coccinellids are known to obtain fitness benefits from multiple matings (Quinones Pando *et al.*, 2001; Omkar & Srivastava, 2002; Omkar & James, 2005; Omkar & Pervez, 2005; Bind, 2007; Omkar *et al.*, 2010a), particularly when females mate with multiple males (Majerus, 1994; Omkar & Mishra, 2005a; Srivastava & Omkar, 2005; but see Haddrill *et al.*, 2007). Although many species produce a spermatophore in the bursa copulatrix of the female (e.g., Obata, 1987; Omkar & Mishra, 2005b), others such as *Cheilomenes sexmaculata* (F.) and *Coelophora saucia* (Mulsant) engage in direct sperm transfer (Omkar *et al.*, 2006) and there is variation in this regard even within coccinellid sub-families (e.g., Fisher, 1959; Katakura *et al.*, 1994; Kaufmann, 1996). Among spermatophore-forming species, females may or may not eject and consume the spermatophore following termination of copula (e.g., Obata, 1987; Perry & Rowe, 2010), raising the possibility of a male nutritional contribution, or 'nuptial gift'. However, Eberhard (1997) argues that allohormonal substances in male seminal products tend to have more important effects on female reproduction than nutritional contributions and a study by Perry & Rowe (2008) suggests that spermatophore consumption by coccinellid females yields little, if any, nutritional benefit. Wilson *et al.* (1999) attributed elevated fecundity resulting from multiple matings in two bruchid species to the action of 'oviposition stimulants' delivered by males, rather than any nutritional donation and similar female benefits from multiple matings have been demonstrated for *Coccinella septempunctata* (Bista & Omkar, 2012).

If ejaculates are costly to produce, the virility of coccinellid males should decline as a function of repeated matings, and that could result in diminished fecundity and/or fertility in their mates. Furthermore, if male seminal products mediate paternal effects, there may be a reduction in offspring quality as a function of paternal mating history. The present study used two congeneric ladybird species, *C. septempunctata* (L.) and *Coccinella transversalis* F., to test whether increased male mating activity would affect either the quantity or quality of progeny while controlling for effects of both male and female age. Both species co-occur widely throughout Asia where *C. septempunctata* contributes to control of aphids on many herbaceous crops (Hodek, 1996; Hodek & Michaud, 2008), with *C. transversalis* more specific to legumes and mustards (Hodek, 1996; Yadav *et al.*, 2011). However, females of *C. septempunctata* eject and consume spermatophores, whereas females of *C. transversalis* eject, but do not consume them, providing an opportunity to control any nutritional effects of spermatophore consumption.

On the basis of the foregoing, we propose two hypotheses with respect to the influence of male virility on female fertility and progeny fitness. We hypothesize that female fecundity and fertility should decline with increasing history of sexual activity in her mate, on the assumption that female reproductive stimulation will represent a physiological expense for

males. Second, we hypothesize that some measures of progeny fitness should diminish with increasing history of paternal sexual activity, assuming that there are paternal effects on offspring quality.

Materials and methods

Insects

Adults of *C. septempunctata* and *C. transversalis* ($n \sim 50$ of each species) were collected from agricultural fields around Lucknow, Uttar Pradesh, India, brought to the laboratory and used to establish stock colonies in climate-controlled growth chambers. Physical conditions were set at $27 \pm 1^\circ\text{C}$, $65 \pm 5\%$ RH and 14L:10D photoperiod. Adult beetles were sexed, paired in plastic Petri dishes (9.0×2.0 cm) and fed cowpea aphids, *Aphis craccivora* Koch, reared in a glasshouse on cowpea plants, *Vigna unguiculata* L. All egg clusters were collected daily and isolated in Petri dishes (as above) until hatching. Upon eclosion, larvae were transferred to plastic beakers (14.5×10.5 cm), 10 per beaker and provisioned with an *ad libitum* supply of *A. craccivora* on cowpea shoots that were replenished daily until the larvae pupated. Adults for use in experiments were isolated individually in Petri dishes upon emergence and fed cowpea aphids *ad libitum*, refreshed daily.

Experimental protocol

Behavioural observations were made on a laboratory bench at $25 \pm 2^\circ\text{C}$ under fluorescent lights; otherwise all experimental insects were held under the same physical conditions as the stock colony. In order to determine the effect of mating history on male mating behaviour, maternal reproduction and progeny development, four types of mate crosses ($n=10$ each) were conducted with both *C. septempunctata* and *C. transversalis*:

- (A) 10-day-old virgin female × 10-day-old virgin male.
- (B) 10-day-old virgin female × 10-day-old male mated once (on day 9).
- (C) 10-day-old virgin female × 10-day-old male mated twice (on days 8 and 9).
- (D) 10-day-old virgin female × 10-day-old *C. transversalis* male mated five times (on days 5–9); 12-day-old *C. septempunctata* mated five times (on days 7–11).

In a normal mating sequence, males of *C. septempunctata* initiate a series of vertical thrusting movements with the abdomen (strokes) shortly after genital contact is established with the female. These are followed by wriggling movements (a gentle forward and backward movement of the abdomen with a simultaneous side-to-side component) and then by a series of shaking bouts (rapid lateral oscillations of the abdomen separated by resting intervals). Strokes and wriggling movements are observed only during the initial stages of mating. The mating behaviour of *C. transversalis* is similar in all respects, but lacks the period of vertical strokes.

All matings were subject to continuous direct observation and the following data were recorded: The initial latent period [=time from genital contact to first stroke (*C. septempunctata*), or to first wriggling movement (*C. transversalis*)]; number of strokes (C7); number of wriggling movements; total duration of wriggling

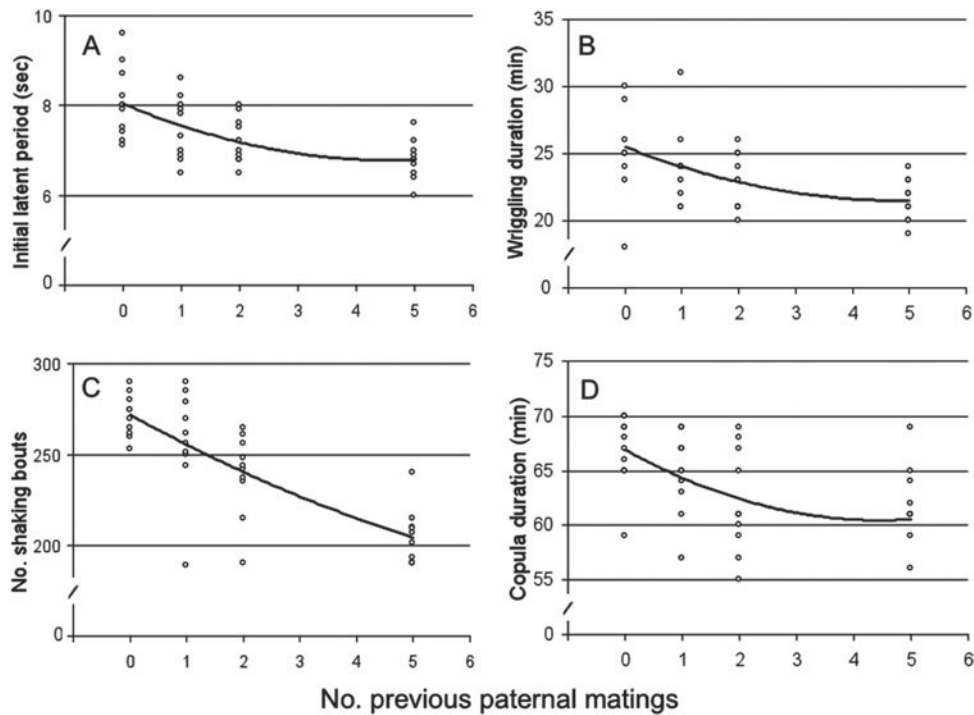


Fig. 1. Quadratic regression of various components of male mating behaviour for 10 day-old *C. septempunctata* males of different mating histories (5 × mated males were 12-day-old) mated with virgin 10-day-old females. See text for behaviour definitions.

movements; number of shaking bouts; total duration of shaking bouts; duration of resting intervals between bouts; terminal latent period (period from last shaking bout until pair separation); and total mating duration (period from initial genital contact until pair separation).

At the end of each mating, the female was isolated in a Petri dish (as above) with an *ad libitum* supply of *A. craccivora* replenished daily, and her eggs were collected daily for the next 20 days. All the eggs were incubated until eclosion and rates of hatching were recorded. Ten larvae were selected from each experimental female over the first two days of her reproduction and reared to follow progeny development. Neonates were transferred to plastic beakers (14.5 × 10.5 cm, 10 per beaker) covered with muslin secured with a rubber band and provisioned daily with an *ad libitum* supply of cowpea aphids on plant shoots. Developmental time (from egg to adult emergence) and stage-specific mortality data were recorded for each replicate.

Statistical analysis

All data were subjected to regression analysis and trend lines were fit using non-linear regression; in all cases, quadratic equations provided lines of best fit.

Results

C. septempunctata

There was a significant effect of male mating history on length of the initial latent period ($F_{1,37}=10.79$, $P<0.001$, $R^2=0.368$); more matings resulted in shorter initial latent

periods (fig. 1a). Male mating history did not affect the number of strokes prior to wriggling ($F_{1,37}=1.56$, $P=0.224$, means ± SE = 101.6 ± 5.03). However, more matings decreased the duration of wriggling movements ($F_{1,37}=6.22$, $P=0.005$, $R^2=0.252$, fig. 1b) and the number of shaking bouts ($F_{1,37}=30.06$, $P<0.0001$, $R^2=0.619$, fig. 1c). There was no effect of male mating history on either the time spent in resting intervals between shaking bouts (means ± SE = 22.0 ± 0.9, 21.4 ± 1.0, 21.4 ± 1.2 and 18.4 ± 2.2 min for 0 ×, 1 ×, 2 × and 5 × mated males, respectively; $F_{1,37}=2.82$, $P=0.072$), or in the duration of the terminal latent period (means ± SE = 22.9 ± 0.6, 22.8 ± 0.8, 22.5 ± 0.7 and 22.1 ± 0.6 s for 0 ×, 1 ×, 2 × and 5 × mated males, respectively; $F_{1,37}=0.32$, $P=0.729$), but more previous male matings diminished the duration of copula overall ($F_{1,37}=5.71$, $P=0.007$, $R^2=0.236$, fig. 1d). Pre-oviposition periods (means ± SE) averaged 4.0 ± 0.2, 3.7 ± 0.4, 3.8 ± 0.2 and 4.5 ± 0.3 days for females mated to males with 0, 1, 2 and 5 previous matings, respectively ($F=1.29$; $df=3,36$; $P=0.176$). The number of eggs laid by females over a 20-day period declined as the mating history of their males increased ($F_{1,37}=4.97$, $P=0.012$, $R^2=0.212$, fig. 2a) and so did their fertility ($F_{1,37}=146.19$, $P<0.0001$, $R^2=0.888$, fig. 2b).

Immature survival (from eclosion to adult emergence) appeared to decline as a function of paternal mating history, but the regression was not significant ($F=1.92$; $df=3,39$; $P=0.144$; fig. 2c). However, more paternal matings significantly increased total developmental time of progeny ($F_{1,37}=20.02$, $P<0.0001$, $R^2=0.520$, fig. 2d), due to increases in the duration of the incubation period ($F_{1,37}=8.05$, $P=0.001$), the first instar ($F_{1,37}=6.06$, $P=0.005$), and the fourth instar ($F_{1,37}=4.57$, $P=0.017$).

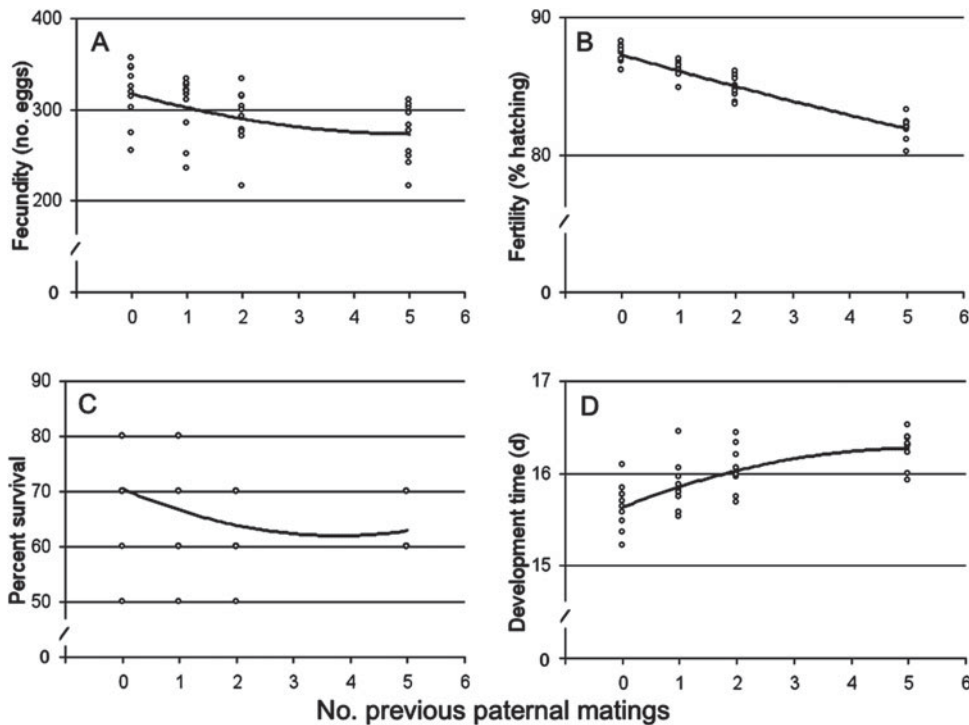


Fig. 2. Quadratic regression of reproductive data and development of progeny produced during the first five days of oviposition by 10-day-old *C. septempunctata* females mated as virgins with 10-day-old males of different mating histories (5× mated males were 12-day-old).

C. transversalis

There was a significant effect of male mating history on length of the initial latent period ($F_{1,37}=39.10$, $P<0.0001$, $R^2=0.679$); more matings resulted in shorter initial latent periods (fig. 3a). More matings decreased the duration of wriggling movements ($F_{1,37}=4.06$, $P=0.025$, $R^2=0.18$, fig. 3b) and the number of shaking bouts ($F_{1,37}=20.62$, $P<0.0001$, $R^2=0.527$, fig. 3c) and resulted in longer periods spent in resting intervals (means±SE=12.0±0.1, 12.2±0.2, 12.4±0.1 and 12.5±0.1 min for 0×, 1×, 2× and 5× mated males, respectively; $F_{1,37}=5.34$, $P=0.009$, $R^2=0.224$). The total duration of copula decreased as the number of previous male matings increased ($F_{1,37}=21.26$, $P<0.0001$, $R^2=0.535$ fig. 3d), although there was no effect of mating history on the terminal latent period (means±SE=8.1±0.3, 8.6±0.3, 8.4±0.2 and 7.8±0.3 min for 0×, 1×, 2× and 5× mated males, respectively; $F_{1,37}=1.97$, $P=0.154$). There was a tendency for preoviposition period (means±SE) to lengthen as the number of previous male matings increased from 0 to 5 (1.9±0.3, 2.4±0.3, 2.9±0.3 and 3.1±0.5), but means did not differ significantly ($F_{3,39}=2.31$, $P=0.093$). As in *C. septempunctata*, the number of eggs laid by females in a 20-day period declined as the mating experience of their males increased ($F_{1,37}=15.81$, $P<0.0001$, $R^2=0.461$, fig. 4a) and so did their fertility ($F_{1,37}=54.57$, $P<0.0001$, $R^2=0.747$, fig. 4b).

Immature survival (1st instar to adult) diminished as the number of paternal matings increased ($F_{3,39}=4.60$, $P=0.008$, fig. 4c). More paternal matings also increased total developmental time of progeny ($F_{1,37}=15.76$, $P<0.0001$, $R^2=0.460$, fig. 4d), due largely to increases in the duration of the incubation period ($F_{1,37}=12.12$, $P<0.001$), and the fourth instar ($F_{1,37}=6.42$, $P=0.004$).

Discussion

The fecundity and fertility of females of both species were affected by paternal mating history, as hypothesized, indicating that male sexual activity diminishes male virility as reflected in female reproductive success. Notably, between-treatment variation in number of shaking bouts, duration of wriggling behaviour and overall duration of copula mirrored variation in female fecundity and fertility, suggest that these male behaviours correlate with male mating effort and differences in female reproductive performance. In both species, the number of shaking bouts was diminished by approximately 30% in 5× mated males compared with virgins, although the corresponding reduction in female fecundity was proportionally greater in *C. transversalis*, the more fecund species.

Insect seminal fluids are very complex and may contain a large number of substances (Reinhardt *et al.*, 2009). If males use seminal fluids to stimulate female reproductive activity, these products may become depleted in males over the course of multiple matings. Alternatively, depletion of sperm itself might contribute to the observed results, if sperm have a direct stimulatory effect on female reproductive processes. Heifetz *et al.* (2001) used mutant males of *Drosophila melanogaster* deficient in male accessory gland proteins and/or sperm to demonstrate that both factors exert complimentary effects on the onset and rate of female oogenesis. In subsequent work, Heifetz & Wolfner (2004) demonstrated different neurological responses of the female reproductive tract to sperm, accessory gland proteins and either mechanical mating stimulation or some other unknown seminal fluid component. Since the duration of copula and numbers of shaking bouts in these

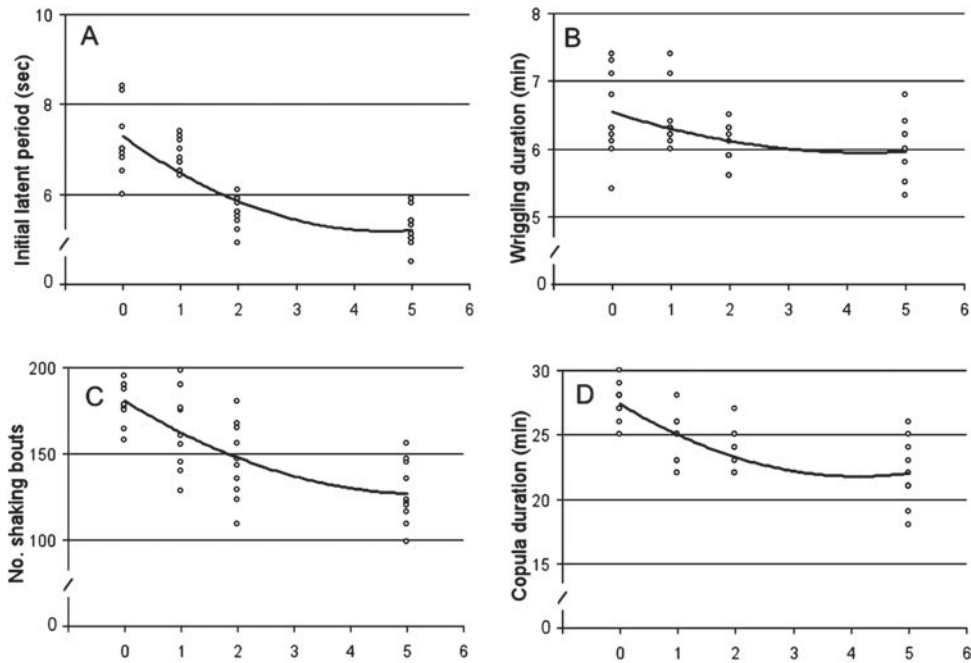


Fig. 3. Quadratic regression of various components of male mating behaviour for 10-day-old *C. transversalis* males of different mating histories mated with virgin 10-day-old females. See text for behaviour definitions.

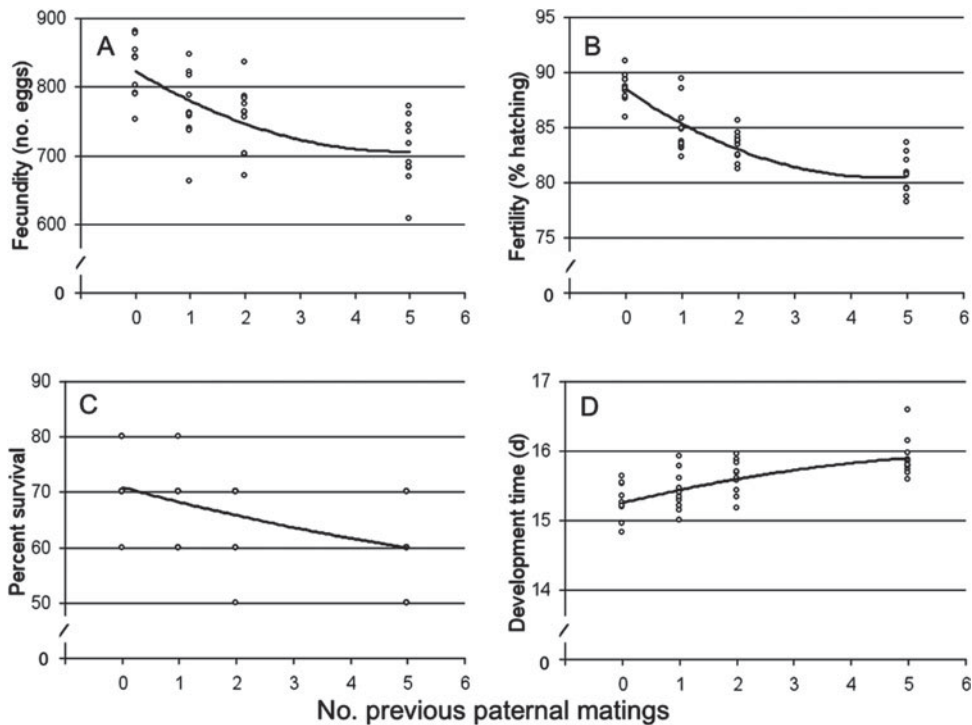


Fig. 4. Quadratic regression of reproductive data and development of progeny produced during the first five days of oviposition by 10-day-old *C. transversalis* females mated as virgins with 10-day-old males of different mating histories.

Coccinella spp. also diminished as a function of previous matings, tactile stimulation of the female would also have been reduced and, if such stimulation has neurosecretory

consequences, this may have contributed also to the reduced reproductive success of females mated to non-virgin males.

The similarity of responses in both species suggest the observed effects on female reproduction are not related to spermatophore consumption, as *C. transversalis* females do not consume them, even though those of *C. septempunctata* do. Previously, Omkar *et al.* (2006) examined mating duration in *C. sexmaculata* and *C. saucia* and found that preoviposition periods became shorter and fecundities increased as females were permitted to mate for longer periods, suggesting a progressive transfer of seminal fluids to the female during copula. In the latter species, female fecundity was also shown to decline as a function of male age at mating (Omkar *et al.*, 2010b), suggesting that male virility declines with age. Perry & Rowe (2010) showed that low-condition *Adalia bipunctata* L. males produced ejaculates with higher relative sperm content, suggesting they reduced non-sperm components of seminal fluid more than sperm under conditions of resource limitation. If non-sperm components of seminal fluids are more costly to produce than the sperm itself, depletion of these materials may limit coccinellid male fertility more than the rate of spermatogenesis. Such is the case for the bed bug, *Cimex lectularius* L. (Reinhardt *et al.*, 2011) despite a very different fertilization mechanism (traumatic insemination).

Although it would make sense for coccinellid females to lay only eggs that have sperm available to fertilize them, they do not have perfect control over the fertilization process. For example, a short (10s) period of mating in *C. sexmaculata* and *C. saucia* is not enough to permit sperm transfer in these species and yet it still stimulates some oviposition of infertile eggs by females (Omkar *et al.*, 2006). The observed changes in egg fertility as a function of increased male mating history in these experiments may reflect either qualitative (sperm viability) or quantitative (sperm number) effects, or some combination thereof. Repeated matings may also deplete males of factors associated with sperm activation, storage, or nourishment (Poiani, 2006). Yaginuma *et al.* (1996) demonstrated that structural proteins of the *Tenebrio molitor* L. spermatophore and associated sugars may be implicated in both the activation of sperm in the bursa copulatrix of female, and their subsequent nourishment in the spermatheca. Other studies have identified sex peptides of male origin in Diptera and Lepidoptera that trigger neuroendocrine responses in females (Chen *et al.*, 1988; Hanin *et al.*, 2012; LaFlamme *et al.*, 2012). Work on both *Danaus plexippus* (L.) (Herman, 1993) and *Tribolium castaneum* (Herbst) (Parthasarathy *et al.*, 2009) has implicated juvenile hormone as an important factor mediating the expression of accessory gland proteins and their resulting effects on female fertility. The present results indicate that *Coccinella* spp. males are able to affect female fertility and fecundity, presumably for their own benefit, but this capacity is significantly diminished as a function of mating activity.

Male mating history also had effects on offspring fitness, although the magnitude of the effects were not great, indicating paternal effects on offspring survival and development that are likewise diminished as a function of male sexual activity. The trend towards a reduction in progeny survival by about 10% after five paternal matings was significant only in *C. transversalis*, due to high within-treatment variation in *C. septempunctata* (figs 2c and 4c). However, there was a significant increase in the developmental time of offspring in both species with increased paternal matings (figs 2d and 4d), suggesting that seminal fluids contain allohormonal factors that either serve to accelerate offspring development directly,

or trigger maternal responses that have this effect. Although the difference in developmental time across treatments was slightly less than one day in both species, even small delays in development can have negative consequences for aphidophagous coccinellids. Fast progeny development is especially important to parental fitness in these species because developmental time is negatively correlated with body size and larvae must exploit ephemeral prey, usually aphids, to achieve a critical pupation weight within a narrow time window (Kindlmann & Dixon, 1993).

Most studies of transgenerational phenotypic plasticity in insects have examined maternal effects, the epigenetic mechanisms whereby females can affect phenotype development in their progeny. Although the existence of maternal effects has been recognized for nearly 80 years (Dobzhansky, 1935), it is only since the publication of Mousseau & Fox (1998) that they have become the focus of significant research attention, especially in insects (Fox & Mousseau, 1998; Mousseau *et al.*, 2009). However, paternal effects have received less attention, despite the fact that male insects often influence female reproductive physiology (reviewed in Ridley, 1988; Alexander *et al.*, 1997), and hence potentially the development of their progeny. For example, female condition had no effect on progeny body size in the neriid fly *Telostylinus angusticollis* (Enderlein), but the offspring of high-condition males were larger than those of low-condition males (Bonduriansky & Head, 2007). The herbivorous coccinellid *Epilachna paenulata* Germar synthesizes defensive alkaloids which both parents contribute to the eggs (Camarano *et al.*, 2009). Previous studies on *Coleomegilla maculata* (DeGeer) and *Hippodamia convergens* Guerin-Meneville have demonstrated effects of maternal age on progeny development and examined how maternal body size can act as a constraint on these signals (Vargas *et al.*, 2012a, b, c). However, since body size was manipulated simultaneously in both males and females in these studies, it seems likely that paternal effects contributed at least partly to some results, such as the rapidly declining fertility observed in pairs of small *H. convergens* beetles (Vargas *et al.*, 2012b).

The multiple effects of male factors on female fecundity, fertility and progeny fitness may select abilities in coccinellid females to distinguish male mating status and discriminate, either overtly or cryptically, in favour of virgin males. Female coccinellids often exert mate preference based on genetic or phenotypic criteria (O'Donald & Majerus, 1984; Wang *et al.*, 2009; Wang *et al.*, 2013) and when subclasses of males are disfavoured, this can theoretically lead to differential patterns of male investment in sperm versus seminal fluids (Cameron *et al.*, 2007). Similarly, if matings exact a cost in terms of male virility, we might expect selection of males to discriminate female mating status and adjust their mating effort accordingly. Since some sperm mixing occurs within multiply-mated coccinellid females (de Jong *et al.*, 1998; Haddrill *et al.*, 2008) males may invest more reproductive effort in virgins than in previously mated females. Future experiments could test for male age-mating status interactions and effects of male condition on paternal effects. Furthermore, body size has a range of reproductive consequences in coccinellids (e.g., Vargas *et al.*, 2012a, b, c) and its possible effects on male virility have not yet been explored. Subsequent experiments could test for effects of body size on male virility and for abilities of both sexes to detect the mating status of sexual partners and alter reproductive investment accordingly.

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