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Exposure to juvenile males during development suppresses female capacity for parthenogenesis in a stick insect

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The rarity of facultative asexuality in animals is an evolutionary puzzle. It has been hypothesized that male factors that influence female performance could be key to this paradox because parthenogens that fail to obtain fitness-increasing stimulation from males at certain life stages may reproduce poorly via parthenogenesis. However, given that certain male factors can reduce female fitness, an alternative hypothesis is that exposure to male factors could exert a sexually antagonistic suppressive effect on females' capacity for parthenogenesis. To test the contrasting predictions of these two hypotheses, we used the spiny leaf stick insect, *Extatosoma tiaratum*, a species capable of both sexual and asexual reproduction, to investigate developmental age-dependent effects of nonmating exposure to males on female behaviour and parthenogenetic performance, with exposure to other females as a control. We found that females reared with immature males were more likely than controls to show resistance-like behaviours as juveniles. Moreover, as adults, females reared with immature males produced asexual eggs with greatly depressed hatching success, resulting in a two-fold reduction in asexual performance compared to controls. By contrast, nonmating exposure to adult males at maturity had little effect on female behaviour or performance. However, females maintained exclusively with other females had slightly reduced fecundity, perhaps due to deprivation of fecundity-increasing male stimulation. Our results suggest that interactions with juvenile males can suppress the development of females' parthenogenetic capacity, an effect that appears to be sexually antagonistic. Such effects could help to explain the rarity of facultative asexuality in animal systems.

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Sexual reproduction is associated with both benefits and costs, but it remains unclear why obligate sex is so widespread in animals (Bell, 1982; Maynard Smith, 1978; Williams, 1975). Theory suggests that facultative strategies that incorporate both sexual and asexual reproduction are superior to obligate sex because they provide benefits of each reproductive mode with fewer associated costs (Burke & Bonduriansky, 2017; D'Souza & Michiels, 2010; Hurst & Peck, 1996). However, facultative strategies, including facultative parthenogenesis, where reproduction is sexual when eggs are fertilized and asexual otherwise, are rare in animals (Bell, 1982). Why this is so remains unclear.

One way that facultative parthenogenesis could fail to spread is if females perform poorly when reproducing asexually. This could occur if asexually reproducing females miss out on important fitness-increasing stimulation from males (Neiman, 2004, 2006; West-Eberhard, 2003). Indeed, in many obligately sexual species, copulatory stimulation is necessary for optimal coordination of female reproductive processes. (Arnqvist & Nilsson, 2000; Carroll, Erskine, & Lundell, 1985; Dufy-Barbe, Franchimont, & Faure, 1973; Wildt, Seager, & Chakraborty, 1980). Since sexual reproduction is ancestral in animals (Simon, Delmotte, Rispe, & Crease, 2003), asexual descendants could inherit their sexual ancestors' dependence on male stimuli (Neiman, 2004). Thus, the widely observed phenomenon of lower asexual fecundity compared to sexual fecundity (reviewed by (Lamb & Willey, 1979; Levitis, Zimmerman, & Pringle, 2017)) could arise because parthenogens cannot usually obtain copulatory stimulation without mating (Neiman, 2004). In facultatively parthenogenetic animals, where only virgin females reproduce asexually, crucial male stimuli linked to sperm delivery (such as physical contact or seminal proteins) might be unobtainable without mating, thereby constraining asexual performance. However, facultative parthenogens could feasibly bypass this constraint by responding instead to nonmating factors that are known from sexual systems to increase fecundity, such as

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pheromones (Carter, Getz, Gavish, Mc Dermott, & Arnold, 1980; Gelez & Fabre-Nys, 2004), scents (Hurst, 2009), cuticular hydrocarbons (Ali & Tallamy, 2010) and microbes (Hoffmann & Harshman, 1985).

Regardless of the source of factors, stimuli originating from males are not always beneficial to females. Seminal factors can be sexually antagonistic, promoting high reproductive performance in males at the expense of females (Andersson, Borg-Karlson, & Wiklund, 2000; Chapman, Liddle, Kalb, Wolfner, & Partridge, 1995). Even noncopulatory stimuli, such as male pheromones, can mediate sexual conflicts over reproduction (Moore, Gowaty, Wallin, & Moore, 2001). Such effects are unlikely to be limited to sexual lineages though, as male traits that function to coerce females into mating or to elevate female fecundity after mating can be strongly favoured in facultative systems as well (Burke & Bonduriansky, 2017). Thus, costs imposed by male stimuli, particularly nonmating stimuli, could offer an alternative explanation for the relatively low performance and rarity of parthenogenesis in animals. To our knowledge, this hypothesis has not been explicitly proposed or tested before, and, thus, the relative importance of stimulatory versus antagonistic male effects on parthenogenesis remains unclear.

Another important factor in understanding effects of nonmating stimuli is female sensitivity to stimuli, which may be age dependent. Some types of stimuli, such as pheromonal stimulation, can occur prior to copulation and could therefore affect females at early developmental stages. This occurs in the desert locust, *Schistocerca gregaria*, whereby exposure to adult male pheromones hastens the development of immature females (Loher, 1961). Similarly, in the house mouse, *Mus musculus*, juvenile females exposed to the urine scents of adult males reach maturity faster than nonexposed females (Colby & Vandenbergh, 1974), and sex hormones released in utero by male mouse embryos induce phenotypic effects on the development of adjacent sisters (vom Saal & Bronson, 1978). More recent examples in the cockroach *Nauphoeta cinerea* (Moore, Gowaty, & Moore, 2003) and the nematode *Caenorhabditis elegans* (Aprison & Ruvinsky, 2016) suggest that male-induced effects on development can be costly to female lifetime fecundity and ageing, respectively. For females of facultatively parthenogenetic taxa, exposure to males at immature stages could influence investment in developmental pathways for parthenogenesis versus sex, with potential flow-on effects for future performance and fitness. Females may benefit by producing eggs parthenogenetically in early life, but males will lose fertilization opportunities if this happens. Males may therefore be selected to antagonistically induce females to produce fewer parthenogenetic eggs so that more eggs can be available for fertilization instead. However, less investment in parthenogenesis following early exposure to males could, alternatively, indicate strategic allocation of resources by females to the mode of reproduction most likely to occur in adulthood. One way to disentangle these contrasting interpretations is through an assessment of female behaviour during male exposure, with resistance-like behaviours likely to suggest antagonistic suppression of parthenogenesis.

Here, we ask how nonmating exposure to males at different ontogenetic stages affects the asexual reproductive performance of females in the facultatively parthenogenetic spiny leaf stick insect, *Extatosoma tiaratum*, a sexually dimorphic phasmatid native to the tropical rainforests of Queensland, Australia (Brock & Hasenpusch, 2009). Females of this species appear to be sensitive to the presence of male stimuli at both juvenile and adult stages, with one study finding a negative relationship between asexual egg output and nonmating male exposure (Schneider & Elgar, 2010), and another showing that leaves previously inhabited by adult males repel prereproductive adult females (Burke, Crean, & Bonduriansky, 2015). However, it is unclear at what ontogenetic stages females are most sensitive to male stimuli, whether pheromones or physical contact are most important, or how exposure to males affects overall reproductive success of females that reproduce parthenogenetically. Mature females of this species also engage in a range of resistance-related behaviours, including leg kicking, abdomen curling and excretion of repugnatorial scents, which may assist in repelling unwanted mating attempts (Burke et al., 2015). Whether juvenile females show similar behaviours in the presence of males is unknown.

To investigate effects of male stimuli on asexual reproduction, we exposed females to males (or other females as a control) during juvenile and adult stages (without the possibility of mating) in a fully crossed design, and asked whether parthenogenetic performance depended on the timing of male exposure (juvenile versus adult) and type of adult male stimulation (pheromones without mating versus pheromones and physical contact without mating). We expected that if stimuli were sexually antagonistic, they would elicit resistance-related behavioural responses in females, with the strongest resistance occurring at ages when the cost to asexual performance was greatest. Conversely, we expected neutral or beneficial stimuli to elicit no resistance and to induce either equivalent or higher asexual performance compared to controls.

METHODS

Animal Maintenance

To obtain focal females and pairing partners, sexually produced eggs were taken from laboratory stocks and hatched in damp cocopeat at room temperature (21–27 °C) under ambient light conditions. First-instar nymphs were housed in a 90-litre plastic tub until they could be sexed at the second or third instar. Females at this age were distinguished from males by dorsal spikes on the abdomen. Upon sexing, females were allocated to either the male juvenile exposure treatment or the juvenile control (female-only exposure). Because of the long development time and intensive husbandry requirements of this species, juvenile exposure did not take place in separate individual enclosures. Instead, focal females were reared together with multiple female or male nymphs in 90-litre tubs. Each level of the juvenile treatment ('male-exposed' versus all-female 'control') consisted of three tubs of 35 focal juvenile females housed with either 35 juvenile males or 35 other juvenile females, all at similar stages of instar development. Tubs containing males were separated from all-female tubs at opposite sides of the laboratory (approximately 5 m apart) to reduce potential between-tub pheromonal effects. Although juvenile stick insects tend to experience higher average densities than adults in the wild (Willig, Presley, & Bloch, 2011), the density at which juveniles were maintained in our experiment may have been higher than what is typical for wild populations. This is unlikely to bias our results though, as the same density was used for the juvenile treatment as for the control. Juvenile exposure lasted for the duration of focal female development (i.e. from sexing to final ecdysis). Insects at all developmental stages were kept in the laboratory under ambient light and temperature conditions and fed *Agonis flexuosa* leaves which were changed each week and sprayed with water every other day for insects to drink.

Effect of Male Stimuli on Juvenile Behaviours

Approximately 4 months after the establishment of juvenile tubs, we conducted a behavioural assay on juvenile females to test for treatment effects on the expression of resistance-related behaviours. We did this by tapping the tip of female abdomens with a

pencil for 10 s at a rate of approximately 4 taps/s. This procedure was designed to mimic the physical stimulus that females receive from males during copulation attempts whereby males repeatedly try to grasp onto the ventral lamella of the female abdomen with their vomer, or clasping organ (N.W. Burke, personal observation). Since *E. tiaratum* is most active at night (which made direct observations of male–female interactions very difficult), we used these behavioural assays as a proxy for female behaviour in the enclosures. We were unable to designate focal versus density-control females at the juvenile stage because individuals could not be tracked through instar moults while housed in groups. Consequently, focal females were designated only after final moult. Thus, the control for the juvenile assay consisted of all focal and density-control females pooled. We recorded the incidence of swaying (rocking), raising forelegs, walking away, abdomen curling, raising hindlegs, kicking, playing dead (thanatosis) and excreting of repugnatorial scents as binary presence–absence tallies. Of these behaviours, swaying and raising forelegs were considered 'neutral' as they are thought to play a role in motion camouflage (Bian, Elgar, & Peters, 2016; N.W. Burke, personal observation), whereas the remaining behaviours were considered 'defensive' or 'resistance-like' since adult females are known to deploy them during sexual interactions (Burke et al., 2015). A nonlinear principal components analysis (NLPCA) was performed on the tallies of these behaviours, with each treated as a nominal variable in the analysis. NLPCA reduces the dimensionality of multivariate categorical data (including presence/absence data) into a set of noncorrelated dimensions, allowing for nonlinear relationships between reduced and unreduced variables (Linting, Meulman, Groenen, & van der Koojj, 2007). We performed this analysis using the homals R package (de Leeuw & Mair, 2009). Given that dimension 1 explained the most variation in behavioural responses (23%; dimension 2 explained 13%), with more defensive behaviours having higher loadings on this dimension (see loadings plot in Fig. A1a), we used these scores as a composite metric for female resistance. (Note that the relatively low percentage of variance explained by dimension 1 here is due to low intraindividual correlations between behaviours.) We analysed composite resistance in a linear mixed-effects model (LMM) fitted with Gaussian error structures and identity link functions. Juvenile treatment was fitted as a categorical effect, female developmental age (i.e. instar number) was a fixed continuous covariate, and tub identity was included as a random effect.

Effect of Male Stimuli on Adult Behaviours

At final ecdysis into the adult stage, focal females were taken from juvenile tubs and placed in individual cylindrical enclosures (20 cm diameter x 40 cm high). To maintain equal sex ratios in juvenile tubs, a male or nonfocal female was removed at the same time as focal females to control for potential density effects. Males that moulted to adulthood were removed from juvenile enclosures and replaced with immature males from the stock population. Density-control females were marked with a felt-tip pen at final ecdysis to distinguish them from focal females in later pairings.

One week after final ecdysis and prior to the onset of oviposition, adult focal females were paired with a mature male (or an ovipositing female as a control) in a cylindrical plastic enclosure (20 cm diameter x 40 cm high). These females were exposed to one of two types of stimulus: physical and pheromonal stimulus or pheromonal stimulus only. In the pheromonal stimulus treatment, females were separated from partners by a flyscreen barrier that prevented physical contact but enabled pheromonal interaction. In the physical and pheromonal stimulus treatment, focal females and partners could freely interact both physically and pheromonally in nonpartitioned enclosures. However, male partners had a skirt of greaseproof paper taped around the end of the abdomen to prevent copulation but allow excretion. Female partners were also fitted with a paper skirt as a control. Adult pairings lasted for 15 days. Partners were replaced by a different partner every 5th day to avoid potential habituation effects. Adult exposure was shorter than juvenile exposure, reflecting the much longer prereproductive developmental period that occurs in stick insects at the juvenile stage, and the greater opportunity for social effects during this period.

To assess adult female responses to male exposure, we repeated the behavioural assay as described above for juveniles immediately following the conclusion of adult pairings. We obtained a composite score of resistance-like behaviours for each female by taking dimension 1 scores from an NLPCA of adult behaviours. Again, this dimension was chosen as it captured the most variation in behavioural responses (dimension 1: 23% of variance explained; dimension 2: 13% of variance explained), with defensive behaviours having higher loadings on dimension 1 (see loadings plot in Fig. A1b). We analysed these scores in an LMM fitted with Gaussian error structures and identity link functions. Juvenile exposure, adult exposure and type of adult exposure were the interacting fixed effects. The day that females moulted to adult instar was included as a fixed continuous covariate to control for seasonality effects associated with the staggered entry of females into treatment groups. The sequential order in which females moulted to the adult instar within each juvenile tub was also included as a covariate to account for the potential effect of decreasing juvenile density in tubs over time. We also included female body length (mouth to ovipositor, in mm) as a covariate to account for body size effects. Juvenile tub identity was included in the model as a random effect.

Effect of Male Stimuli on Asexual Performance

Following adult exposure, focal females were left to oviposit parthenogenetically for 3 weeks, and then humanely killed by freezing. To test for male effects on egg output, we analysed egg counts using a GLMM with a Poisson error structure and a log link function. The interacting fixed effects, covariates and random effects in this model were the same as those used in the adult behaviour model, except an additional observation level random effect was included to account for overdispersion.

A subsample of 20 eggs was collected from each female and hatched in damp coco-peat under ambient light and temperature conditions. Two females that produced fewer than 20 eggs were removed from subsequent analyses. Hatchlings were counted three times per week and killed by freezing soon after emergence. Approximately 4 months after the last emergence, unhatched eggs were dissected to assess the presence of dead embryos, which were identified by their desiccated state and black coloration. We did not observe any live embryos in dissected eggs. However, a small number of unhatched eggs (<10%) may have been in a state of extended pre-embryotic diapause (Bedford, 1978) as they possessed liquid yolk with no obvious embryo. We did not attempt to quantify variation in the numbers of such eggs because we could not reliably determine whether eggs were alive or dead when no embryo was present.

To test for male effects on hatching rate and embryo development, we used GLMMs with binomial error structures and logit link functions. The response variables in these analyses were number of hatched eggs and number of unhatched eggs containing embryos, respectively, each treated as presence–absence binomial proportions. The structure of these models was the same as the adult behaviour models described above. Observation level random

effects were also included to account for overdispersion in each model.

We produced a composite estimate of asexual reproductive performance for each female calculated as the product of hatching rate and total egg output. We analysed this score in an LMM with a Gaussian error structure and identity link function. The structure of this model was the same as that of the adult behaviour model. We used the same LMM structure to analyse the onset of parthenogenetic oviposition (i.e. the time elapsed from adult ecdysis to first oviposition).

We used likelihood ratio tests (LRTs) to determine the significance of each model effect. The significance of covariates was assessed by removing each one independently from the full model. The significance of fixed effects and interactions was assessed by removing each one from a reduced model that had nonsignificant higher-level interactions removed. All LMMs and GLMMs were fitted as random intercept models by maximum likelihood using the lmer and glmer functions in the lme4 R package (Bates, Sarkar, Bates, & Matrix, 2007). Bonferroni post hoc tests were used to determine differences between factor levels involved in interaction effects. The unit of replication was the focal female. Sample sizes for each treatment combination are provided in the Appendix (Tables A1 and A2). The experimental period was from September 2015 to August 2017.

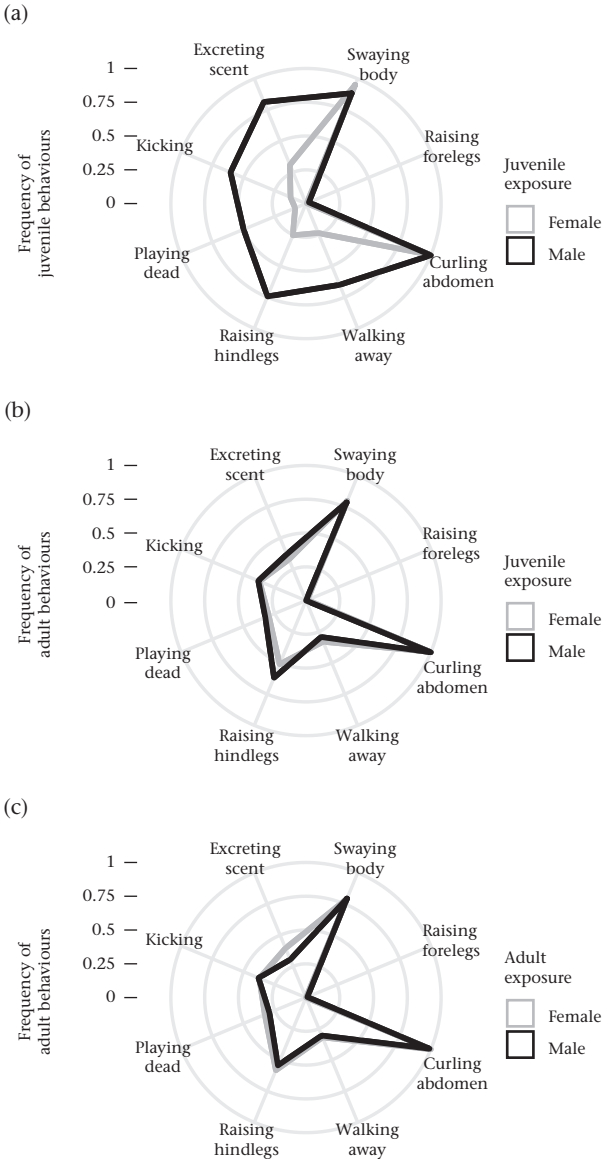

Figure 1. Radar plots showing the frequency of behaviours shown by females (a) as juveniles and (b, c) as adults. Adult behaviours are plotted as a function of (b) the juvenile exposure treatment and (c) the adult exposure treatment. 'Swaying body' and 'raising forelegs' are interpreted as nondefensive behaviours; all other behaviours are indicative of resistance/defence.

RESULTS

Juvenile and Adult Behaviours

We found striking differences in the behaviours of immature females between mixed- and same-sex cohorts (Fig. 1a). Immature females reared with juvenile males had a higher composite score for resistance-like behaviours (walking away, raising hindlegs in a defensive pose, kicking legs, playing dead, secreting repugnatorial scents) than did females reared without males (coefficient = 0.09; $\chi^2 = 22.83$, $P < 0.001$). Higher scores were also associated with older instars (coefficient = 0.003; $\chi^2 = 8.79$, $P = 0.003$). When each behaviour was analysed separately (see Table A3), behaviours associated with motion camouflage (i.e. raising forelegs and swaying) were the only ones that did not differ between exposure treatments, suggesting that juvenile males made juvenile females stressed or defensive rather than simply more active.

Exposure to males had little effect on the behaviour of adult females (Fig. 1b and c). Composite scores for resistance-like behaviours in adult females were unaffected by juvenile or adult exposure to males, the type of adult exposure, or any two- or three-way interaction ($-0.01 \leq$ coefficients ≤ 0.01; LRTs: $0.004 \leq \chi^2 \leq 0.88$, $0.35 \leq P \leq 0.95$). All covariates were nonsignificant ($0.0005 \leq$ coefficients ≤ 0.0008; $0.06 \leq \chi^2 \leq 0.31$, $0.58 \leq P \leq 0.81$), except for day of final moult (coefficient = -0.01; $\chi^2 = 9.51$, $P = 0.002$), such that earlier-moulted females had higher composite resistance scores.

Separate analyses of each juvenile and adult behaviour are shown in the Appendix (Tables A3 and A4).

Asexual Performance

Oviposition latency was not affected by juvenile or adult exposure to males, the type of adult exposure, or any interaction of these treatments ($-2.07 \leq$ coefficients ≤ 0.40; LRTs: $0.03 \leq \chi^2 \leq 1.01$, $0.32 \leq P \leq 0.87$; Fig. 2a). However, larger body size (coefficient = -1.27; $\chi^2 = 9.33$, $P = 0.002$) and later moulting date (coefficient = -1.25; $\chi^2 = 5.09$, $P = 0.02$) were associated with significantly reduced latencies.

We observed a significant juvenile exposure*adult exposure interaction effect on egg output (coefficient = -0.28; $\chi^2 = 7.73$, $P = 0.005$; Fig. 2b). Females that had never been exposed to males produced fewer eggs than females exposed to juvenile females and adult males ($Z = -3.77$, $P = 0.001$). All other pairwise comparisons were nonsignificant ($-2.17 \leq Z \leq 1.65$, $0.18 \leq P \leq 1.00$). All other interaction effects in the model were also nonsignificant ($-0.22 \leq$ coefficients ≤ 0.16; LRTs: $0.0002 \leq \chi^2 \leq 3.77$, $0.052 \leq P \leq 0.99$). Type of adult exposure had no effect on egg output (coefficient = 0.18; $\chi^2 = 3.72$, $P = 0.054$). However, body size (coefficient = 0.08; $\chi^2 = 11.31$, $P < 0.001$) and day of final moult (coefficient = -0.06; $\chi^2 = 5.84$, $P = 0.02$) were positively and negatively associated with egg output, respectively.

Juvenile exposure to males resulted in a 49.4% reduction in mean hatching rate of asexual eggs (coefficient = -1.69; $\chi^2 = 7.31$, $P = 0.007$; Fig. 2c). Adult exposure (coefficient = -0.40; $\chi^2 = 0.29$, $P = 0.59$) and type of adult exposure (coefficient = -0.91; $\chi^2 = 2.75$,

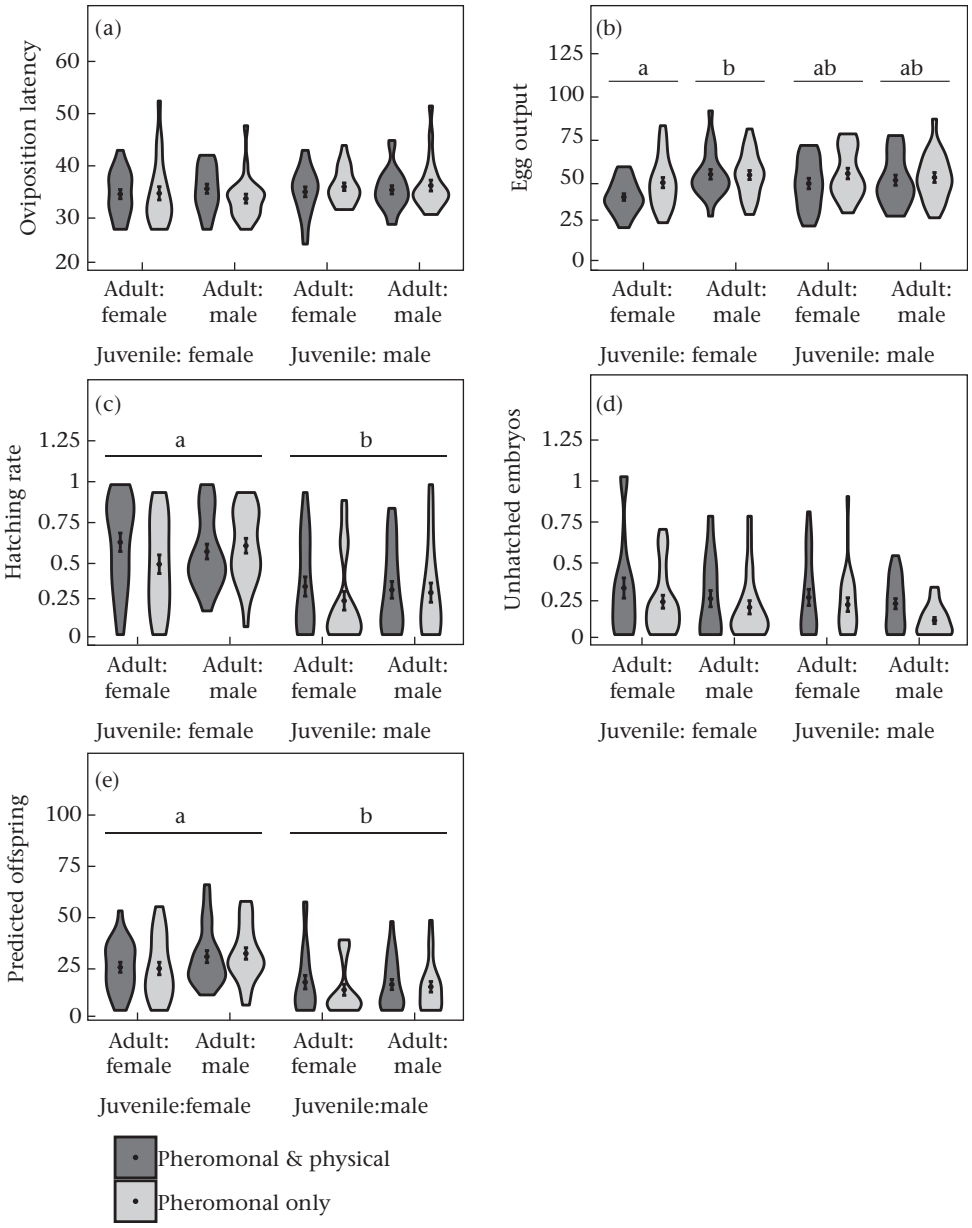

Figure 2. Violin plots with means ± SEs for measures of asexual performance across all treatment combinations. (a) Oviposition latency (days), (b) number of eggs produced, (c) hatching rate, (d) proportion of unhatched eggs and (e) predicted number of offspring. Labels along the x-axis indicate the levels of juvenile exposure (bottom line) and adult exposure (top line). Dissimilar letters above violin plots in (c) and (e) indicate significant differences between treatments according to likelihood ratio tests, and in (b) according to Bonferroni post hoc tests.

$P = 0.10$) had no effect on hatching rate. There were no significant covariate or interaction effects (-0.66 ≤ coefs ≤ 1.08; LRTs: 0.0002 ≤ χ^2 ≤ 2.76, 0.10 ≤ P ≤ 0.99).

The proportion of unhatched eggs containing (dead) embryos (16.9%) was unaffected by juvenile exposure, adult exposure, the type of adult exposure, or any interactions or covariates (-0.44 ≤ coefficients ≤ 0.01; LRTs: 0.18 ≤ χ^2 ≤ 3.16, 0.08 ≤ P ≤ 0.67; Fig. 2d). This suggests that differences in hatching rate were not driven by suspended embryo development (i.e. diapause) in the juvenile male exposure group, but by higher embryo viability in the juvenile female exposure control group.

Overall, we found that females exposed to males during juvenile development experienced a 49.6% reduction in asexual reproductive performance, quantified as the estimated total number of nymphal offspring (coefficient = -1.23; χ^2 = 5.60, P = 0.02; Fig. 2e). No other treatments, interactions or covariates affected females' net asexual performance (-0.49 ≤ coefs ≤ 0.70; LRTs: 0.03 ≤ χ^2 ≤ 2.87, 0.10 ≤ P ≤ 0.76).

Summary statistics for all behaviours and performance measures are provided in the Appendix (Tables A1 and A2).

DISCUSSION

Our findings are broadly consistent with the hypothesis that obligate sex is maintained in animals because coercive males prevent the optimal expression of asexual traits in females. We found striking negative effects of nonmating juvenile male (but not adult male) stimuli on the hatching rate of asexually produced eggs and estimated offspring counts. However, asexual egg output was lowest in females that never encountered male stimuli, suggesting

that exposure to males may provide at least some stimulatory benefit, although this benefit was not sufficient to compensate for the negative effect of males on egg hatching rate. Male-exposed juvenile females also showed the highest frequency of resistance-like behaviours. Taken together, these results suggest that males may be selected to reduce female investment in parthenogenesis, and that females may be selected to behaviourally avoid this manipulation to optimize asexual performance.

Juvenile females from mixed-sex cohorts showed a high frequency of resistance-like behaviours, including walking away, raising hindlegs, kicking, playing dead and excreting repugnatorial scents, which were remarkably similar to the resistance-related behaviours previously reported for adult females of this species (Burke et al., 2015), suggesting that resistance to male exposure might similarly occur in juveniles. However, juvenile behavioural responses did not persist into adulthood, and adult male stimuli had little influence on adult female behaviour. Besides these direct effects on juvenile behaviour, we observed strong flow-on effects of developmental exposure to males into adulthood. Females reared with males as juveniles produced asexual eggs with a much lower hatching rate than eggs of control females, which translated into a near 50% reduction in the estimated total number of asexually produced nymphs. By contrast, adult exposure to males had no effect on hatching rate or estimated offspring counts. These results provide evidence that parthenogenetic reproduction in *E. tiaratum* is heavily influenced by the social environment at the juvenile stage. Although females in our experiment experienced higher densities in juvenile stages than in adulthood, which is typical of stick insects in the wild (Willig et al., 2011), density is unlikely to have confounded our behavioural results as density levels were identical between the juvenile treatment and juvenile control groups. The longer period of exposure in the juvenile than the adult treatment may also have contributed to the stronger juvenile effect. Indeed, long developmental periods during early ontogeny are characteristic of many hemimetabolous insects, and could therefore provide a wider window of opportunity for environmental effects than later ontogenetic stages (West-Eberhard, 2003). Our results suggest that aspects of female reproductive physiology that make parthenogenetic reproduction possible in this species begin to develop before adult ecdysis and are subject to environmental effects. This capacity for developmental plasticity underpins the reduction in asexual reproduction that we observed in females that developed with males. There are two possible interpretations of this result.

One interpretation posits adaptive developmental plasticity in females: if females benefit by reproducing sexually when males are available, selection may have favoured females that adaptively reduce their own investment in asexual reproduction when encountering males during juvenile development. Adaptive plasticity may explain why females exposed to juvenile males during development adjusted their developmental trajectory and reduced investment in parthenogenetic reproduction. The presence of males during development may have induced females to optimize their reproductive system for sex in expectation of future mating. Our finding of greatly reduced hatching success following exposure to juvenile males could therefore be interpreted as evidence of a mismatch between expected and actual mating environments, such that eggs primed for fertilization are less likely to hatch when left unfertilized. However, it is difficult to reconcile this adaptive plasticity interpretation with the resistance-like behaviours that juvenile males elicited in juvenile females.

An alternative interpretation posits sexual conflict over mating: if females benefit by reproducing parthenogenetically, selection may have favoured sexually antagonistic male traits that enable juvenile males to suppress the development of females' capacity for parthenogenetic reproduction. Sexual conflict may be the more plausible interpretation of both the behavioural and reproductive data. Not only did males elicit defensive, resistance-like behaviours in juvenile females, but these behaviours were most frequent during the period of development when males had the greatest negative effect on parthenogenetic performance (i.e. during the juvenile stage), a pattern predicted by sexual conflict theory (Arnqvist & Rowe, 2005). These behaviours could function to repel juvenile males and facilitate avoidance of costly male stimuli, but closer observation of these defensive behaviours in juveniles is required to verify this.

Our results highlight the potential for female development to be an arena of sexual conflict. In organisms with plastic reproductive strategies, developmental trajectories can be optimized for either sex or parthenogenesis. For example, in cyclically asexual organisms, such as aphids and water fleas, development of sexual versus asexual eggs is tightly controlled by environmental cues, ensuring that investment in each mode of reproduction is optimally timed (Gerber, Kokko, Ebert, & Booksmythe, 2018). In facultatively parthenogenetic organisms, such as stick insects and mayflies, the mode of reproduction is determined by whether females mate, or not, providing greater scope for social conditions to influence reproductive investment. Thus, in facultative parthenogens, sexual conflict over female development can arise as a consequence of the sexes having different interests in whether females should develop eggs ready for parthenogenesis or for fertilization. If these interests do not align, males will be selected to coerce females to develop towards the male optimum, leading to counter-selection on females to resist male manipulation. Our findings are broadly consistent with this explanation. Indeed, our results suggest that sexual conflict over female development could even manifest at the juvenile stage.

Developmental coercion is likely to increase male fitness in *E. tiaratum* because females are incapable of flight and probably remain on the same tree or branch throughout their life. Resident males may therefore encounter the same individual females across juvenile and adult stages. Thus, males that suppress asexual egg development will increase their own fitness because the females they encounter in adulthood will have reduced capacity for parthenogenetic reproduction and be less reluctant to mate. There is also the potential that if exposure is initiated later in development, some offspring could be produced parthenogenetically even after mating. Although unknown in this species, postmating parthenogenesis has been observed in other insects (Arbuthnott, Crespi, & Schwander, 2015; Chang, Ting, Chang, Fang, & Chang, 2014). It is currently unclear what stimuli might be involved in developmental coercion in *E. tiaratum*, but analogous effects are known in other species. For example, sexually antagonistic pheromones produced by *C. elegans* males promote accelerated development but faster ageing of hermaphrodites/females (Aprison & Ruvinsky, 2016). Male *N. cinerea* use sexually antagonistic pheromones to lengthen the duration of development of their mates' offspring so that females remain gravid for longer and lose future opportunities to remate with other males (Moore et al., 2003). These examples demonstrate the potential for males to use nonmating stimuli to manipulate the development of conspecific females for their own reproductive benefit, and, in conjunction with our own findings, suggest that sexual conflict over female development could be more widespread than currently thought.

It is important to note that females in facultatively parthenogenetic systems will only be selected to avoid diverting their reproductive development to a sexual pathway if parthenogenetic reproduction is more advantageous than sex. Although we did not assess the relative costs and benefits of sex versus parthenogenesis

in this study, previous work on *E. tiaratum* suggests that asexual reproduction can be superior to sex in at least some circumstances. Burke et al. (2015) showed that asexually reproducing females that mate and switch to sex die faster and produce fewer eggs than females that reproduce either asexually or sexually their whole lives. A subsequent study showed that females of parthenogenetic origin that hatch from unfertilized eggs might maximize their reproductive output by reproducing parthenogenetically (Burke & Bonduriansky, 2018). Thus, a solely asexual strategy may be optimal for this species in some contexts. However, what may be optimal at one point in time may be suboptimal at another, even within a single lifetime (Fricke, Green, Mills, & Chapman, 2013). This suggests the potential for suppressive effects of male exposure to operate in age-dependent ways, which remain to be tested.

One of the surprising findings from our study is that despite the strong juvenile exposure effect on hatching rate and estimated offspring counts, females that never encountered males had the lowest fecundity. A similar result has been reported for an asexual freshwater snail where competition for limited resources between females, but not between females and males, results in lower egg output (Neiman, 2006). Competition resulting in resource limitation is an unlikely explanation for our result because females were supplied with food (leaves) ad libitum at all developmental stages, and females separated from partners by a barrier produced as many eggs as females that accessed the same food as partners. It is possible that lower fecundity was induced by inhibitory chemicals produced by surrounding female competitors, a phenomenon well documented in social insects (Keller & Nonacs, 1993), fish (Gerlach, 2006) and rodents (Koyama, 2004). If this were the case, we would have expected exposure to adult females to have resulted in lower fecundity regardless of juvenile exposure, but adult exposure by itself had no effect on fecundity. A more probable explanation is that stimuli from adult and juvenile males increased egg production. Such stimulatory effects are common in internally fertilizing animals (Aron, 1979; Gillott, 2003) and can either benefit or supress female fitness (Bonduriansky, 2014; Bonduriansky, Wheeler, & Rowe, 2005; Perry & Rowe, 2008; Wigby & Chapman, 2005). Given that the modest increase in fecundity we observed in our study was entirely cancelled out by the much larger negative effect of juvenile male exposure on subsequent hatching rate, the net effect of nonmating exposure on asexual reproduction appears to be negative.

We also found that male effects on female reproduction were not mediated by type of adult exposure (pheromonal contact without mating versus pheromonal and physical contact without mating). Previous work has shown that females and males of this species both respond to scents of the opposite sex (Burke et al., 2015; Schneider & Elgar, 2010). Our results extend this work by showing that stimulation effects are probably most potent at the juvenile stage. However, it remains to be tested whether the type of stimulation mediating these effects in juveniles is chemical or physical.

In summary, we found that stimulation from subadult males of the spiny leaf stick insect, *E. tiaratum*, induced defensive, resistance-like behaviours in juvenile females, and greatly reduced the hatching rate of the asexually produced eggs that these females subsequently laid, resulting in a nearly two-fold reduction in asexual reproductive output. Our results suggest the possibility of sexual conflict over females' developmental trajectory mediated by behavioural interactions among juveniles. Our study demonstrates the potential for male stimuli to influence asexual fecundity and, in particular, the potential for males to suppress females' capacity for asexual reproduction. Our results thus support the idea that sexual conflict could maintain obligate sex in animals by inhibiting parthenogenetic reproduction.

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Appendix

We examined the effect of juvenile male exposure on the behavioural responses of juvenile females in a separate set of analyses that treated each behaviour as a separate response variable. Each behavioural tally was treated as a binomial presence–absence proportion in a separate GLMM fitted with binomial error structures and logit link functions. Each model included juvenile treatment as the fixed effect, female developmental age (i.e. instar number) as a continuous covariate and tub identity as the random effect. The same model structure was used to analyse adult behaviours, except juvenile exposure, adult exposure and adult exposure type were the interacting fixed effects, order of ecdysis, day of final moult and body length were included as covariates, and juvenile tub identity was the random effect. Results of these analyses are provided in Tables A3 and A4.

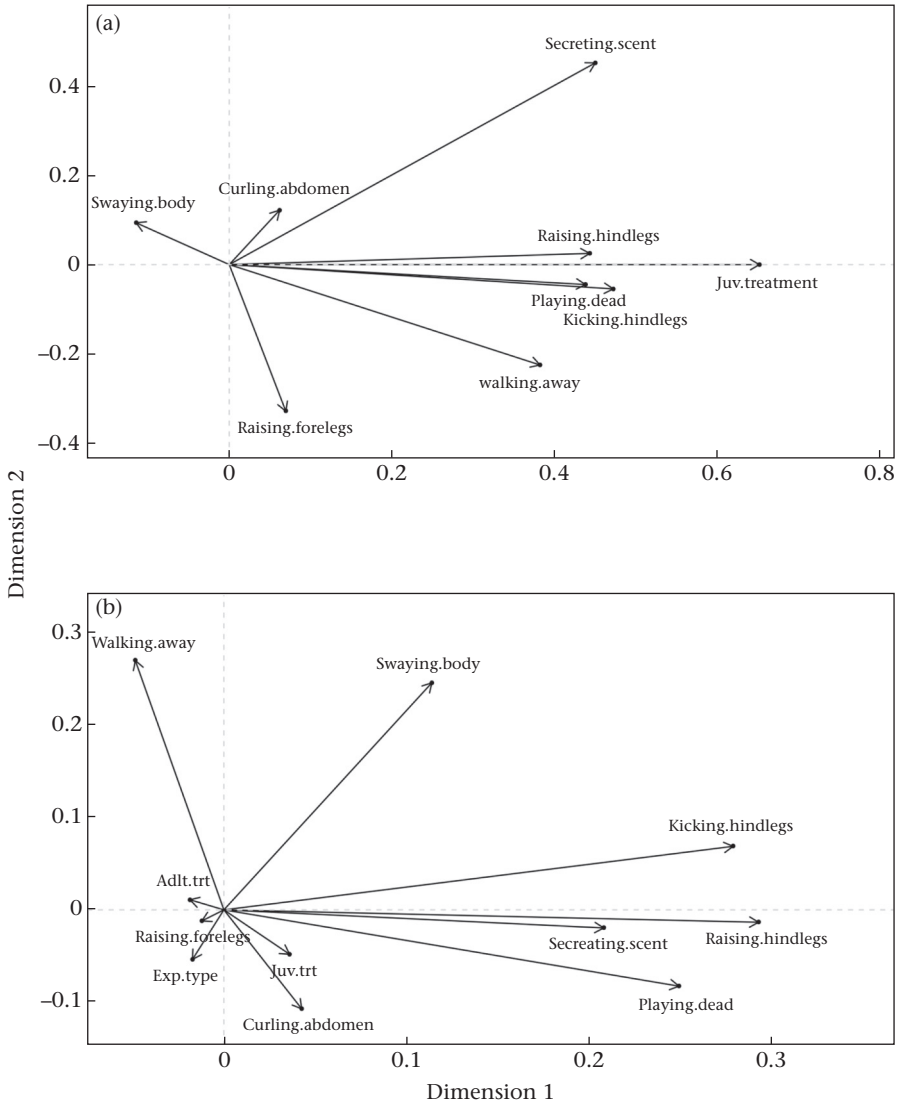

Figure A1. Loadings plots showing the nonlinear PCA loadings for dimensions 1 and 2 for female behaviours during (a) the juvenile and (b) the adult stages. Dimension 1 is interpreted as the 'resistance axis' because neutral behaviours such as body swaying and foreleg raising load closer to the origin, while more stressful responses related to resistance, such as kicking, excreting repugnatorial scent and playing dead, load further along the x-axis. Loadings for treatments ('juv.treatment' in (a), and 'juv.trt', 'adlt.trt' and 'exp.type' in (b)) are also shown.

Table A1
Summary statistics for juvenile behavioural responses

Response	Juvenile exposure to females	Juvenile exposure to males
Walking away	0.24 (0.43) *161*	0.65 (0.48) *86*
Curling abdomen	0.99 (0.11) *161*	1 (0) *86*
Raising hindlegs	0.25 (0.44) *161*	0.74 (0.44) *86*
Kicking	0.12 (0.33) *161*	0.60 (0.49) *86*
Playing dead	0.09 (0.28) *161*	0.50 (0.50) *86*
Excreting scents	0.31 (0.46) *161*	0.81 (0.39) *86*
Swaying body	0.95 (0.22) *161*	0.88 (0.32) *86*
Raising forelegs	0.01 (0.08) *161*	0.02 (0.15) *86*

Values are means, SDs (in parentheses) and sample sizes (in italics).

Table A2
Summary statistics for adult behavioural and reproductive responses

Response	Juvenile exposure to females				Juvenile exposure to males			
	Adult exposure to females		Adult exposure to males		Adult exposure to females		Adult exposure to males	
	PP	P	PP	P	PP	P	PP	P
Walking away	0.42 (0.50) *24*	0.33 (0.48) *24*	0.17 (0.39) *23*	0.39 (0.50) *23*	0.32 (0.48) *22*	0.19 (0.40) *21*	0.25 (0.44) *24*	0.41 (0.50) *22*
Raising hindlegs	0.54 (0.51) *24*	0.50 (0.51) *24*	0.48 (0.51) *23*	0.52 (0.51) *23*	0.59 (0.50) *22*	0.71 (0.46) *21*	0.71 (0.46) *24*	0.45 (0.51) *22*
Kicking	0.38 (0.49) *24*	0.33 (0.49) *24*	0.35 (0.49) *23*	0.43 (0.51) *23*	0.27 (0.46) *22*	0.52 (0.51) *21*	0.38 (0.50) *24*	0.36 (0.49) *22*
Curling abdomen	1 (0) *24*	1 (0) *24*	1 (0) *23*	0.96 (0.21) *23*	1 (0) *22*	1 (0) *21*	1 (0) *24*	1 (0) *22*
Playing dead	0.21 (0.41) *24*	0.33 (0.49) *24*	0.35 (0.49) *23*	0.30 (0.47) *23*	0.36 (0.49) *22*	0.43 (0.51) *21*	0.25 (0.44) *24*	0.27 (0.46) *22*
Excreting scents	0.33 (0.48) *24*	0.38 (0.49) *24*	0.30 (0.47) *23*	0.30 (0.47) *23*	0.45 (0.51) *22*	0.43 (0.51) *21*	0.33 (0.48) *24*	0.27 (0.46) *22*
Swaying body	0.71 (0.46) *24*	0.88 (0.34) *24*	0.83 (0.39) *23*	0.78 (0.42) *23*	0.77 (0.43) *22*	0.81 (0.40) *21*	0.83 (0.38) *24*	0.73 (0.46) *22*
Raising forelegs	0 (0) *24*	0 (0) *24*	04 (0.21) *23*	0 (0) *23*	0 (0) *22*	0 (0) *21*	0 (0) *24*	0 (0) *22*
Oviposition latency	33.13 (4.57) *24*	33.29 (6.59) *24*	34.22 (4.48) *23*	32.22 (4.38) *23*	33.59 (4.71) *22*	34.62 (3.56) *21*	34.00 (4.14) *24*	34.86 (5.19) *22*
Egg output	37.58 (10.05) *24*	46.38 (15.30) *24*	51.43 (14.15) *23*	51.00 (13.26) *23*	45.82 (15.00) *22*	51.90 (14.79) *21*	47.83 (15.30) *24*	49.50 (14.19) *22*
Offspring emergence	0.58 (0.29) *24*	0.45 (0.29) *24*	0.53 (0.23) *23*	0.56 (0.23) *23*	0.30 (0.29) *22*	0.21 (0.27) *21*	0.28 (0.26) *24*	0.27 (0.29) *22*
Unhatched embryos	0.29 (0.31) *24*	0.21 (0.20) *24*	0.23 (0.25) *23*	0.17 (0.20) *23*	0.24 (0.24) *22*	0.19 (0.20) *21*	0.20 (0.16) *24*	0.09 (0.09) *22*
Predicted total offspring	21.81 (2.57) *24*	21.18 (3.17) *24*	27.21 (3.06) *23*	28.80 (2.94) *23*	14.27 (3.43) *22*	10.39 (2.81) *21*	13.09 (2.59) *24*	11.92 (2.69) *22*

Values are means, SDs (in parentheses) and sample sizes (in italics). PP = pheromonal and physical exposure; P = pheromonal exposure only.

Table A3
GLMM analyses of female behaviours during juvenile development

Model effect	Walking away		Raising hindlegs		Kicking		Playing dead		Excreting scent		Swaying body		Raising forelegs	
	GLMM	LRT	GLMM	LRT	GLMM	LRT	GLMM	LRT	GLMM	LRT	GLMM	LRT	GLMM	LRT
Juvenile exposure	1.81	9.45	2.40	10.16	2.82	10.16	2.86	10.09	2.78	6.33	-0.93	3.43	1.36	0.80
(♂)	(0.35)	***0.002***	(0.49)	***0.001***	(0.57)	***0.001***	(0.59)	***0.001***	(0.87)	***0.01***	(0.50)	*0.06*	(1.43)	*0.37*
Instar number	-0.02	0.01	0.43	7.63	0.69	14.83	0.88	19.68	0.32	3.64	-0.06	0.05	0.15	0.06
	(0.15)	*0.90*	(0.16)	***0.006***	(0.19)	***<0.001***	(0.21)	***<0.001***	(0.17)	*0.06*	(0.25)	*0.82*	(0.64)	*0.81*

GLMM coefficients are reported above SEs (in parentheses). Chi-square statistics (all $df = 1$) from likelihood ratio tests (LRTs) are reported above *P* values (in italics). Bold *P* values indicate significant effects according to LRTs. Analysis of 'curling abdomen' could not be performed due to lack of variation in tallies of this behaviour.

Table A4
GLMM analyses of female behaviours during adulthood

Model effect	Walking away		Raising hindlegs		Kicking		Playing dead		Excreting scent		Swaying body	
	GLMM	LRT	GLMM	LRT	GLMM	LRT	GLMM	LRT	GLMM	LRT	GLMM	LRT
Juvenile exposure (♂)	-0.70 (0.72)	0.00	0.64 (0.71)	0.26	0.36 (0.68)	1.18	-0.03 (0.67)	0.42	0.02 (0.63)	0.02	-0.61 (0.84)	0.17
		1.00		*0.61*		*0.28*		*0.52*		*0.88*		*0.68*
Adult exposure (♂)	0.27 (0.61)	–	0.09 (0.64)	0.37	0.56 (0.66)	0.04	-0.10 (0.66)	0.22	-0.31 (0.63)	1.65	-0.64 (0.80)	<0.001
		–		*0.54*		*0.84*		*0.64*		*0.20*		*0.98*
Type of adult exposure (P)	0.30 (0.61)	–	0.20 (0.64)	0.51	0.21 (0.67)	0.88	-0.74 (0.70)	0.38	-0.21 (0.62)	0.03	-1.06 (0.77)	0.06
		–		*0.48*		*0.35*		*0.54*		*0.86*		*0.80*
Juv(♂)*Adult(♂)	0.82 (0.94)	1.64	-1.35 (0.97)	0.14	-1.23 (0.95)	0.19	-0.57 (0.95)	2.12	-0.35 (0.91)	0.38	0.20 (1.09)	0.004
		0.20		*0.71*		*0.66*		*0.15*		*0.54*		*0.95*
Juv(♂)*Type(P)	0.35 (0.95)	0.31	-0.67 (0.98)	0.45	-1.31 (0.97)	0.27	0.58 (0.97)	0.05	0.40 (0.88)	0.31	0.87 (1.08)	0.69
		0.58		*0.50*		*0.60*		*0.82*		*0.58*		*0.40*
Adult(♂)*Type(P)	-1.45 (0.93)	4.60	-0.37 (0.91)	0.83	-0.70 (0.94)	0.10	0.89 (0.96)	0.49	0.19 (0.89)	0.058	1.28 (1.08)	1.97
		0.03		*0.36*		*0.75*		*0.48*		*0.81*		*0.16*
Juv(♂)*Adult(♂)*Type(P)	0.06 (1.34)	0.002	2.20 (1.37)	2.62	1.88 (1.35)	1.97	-0.84 (1.37)	0.38	-0.08 (1.27)	0.004	-0.47 (1.50)	0.10
		0.96		*0.11*		*0.16*		*0.54*		*0.95*		*0.76*
Order of ecdysis	-0.06 (0.23)	0.06	-0.04 (0.23)	0.03	0.23 (0.24)	0.85	0.19 (0.24)	0.65	0.02 (0.22)	0.01	0.17 (0.26)	0.43
		0.81		*0.87*		*0.36*		*0.42*		*0.92*		*0.51*
Body length	-0.13 (0.20)	0.42	0.15 (0.20)	0.53	0.02 (0.20)	0.01	-0.05 (0.21)	0.05	-0.02 (0.19)	0.01	-0.02 (0.23)	0.01
		0.51		*0.47*		*0.92*		*0.82*		*0.92*		*0.93*
Day of final moult	0.19 (0.26)	0.51	-0.91 (0.27)	9.80	-1.19 (0.32)	8.59	-1.00 (0.32)	11.07	0.40 (0.27)	2.25	-0.29 (0.30)	0.96
		0.48		***0.002***		***0.003***		***<0.001***		*0.13*		*0.33*

GLMM coefficients are reported above SDs (in parentheses). Chi-square statistics ($df = 1$) from likelihood ratio tests (LRTs) are reported above *P* values (in italics). LRTs of lower-level effects involved in higher level interactions are not reported but denoted with a dash. Bold *P* values indicate significant effects according to LRTs. Analysis of 'raising forelegs' could not be performed due to lack of variation in tallies of this behaviour. P = pheromonal exposure only.