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ORIGINAL PAPER

Experimental evolution reveals that population density does not affect moth signalling behaviour and antennal morphology

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Abstract Population density can play a vital role in determining investment in reproductive behaviours and morphologies of invertebrates. Males reared in high-density environments, where competition is high but difficulties in locating mates are low, may invest more in reproductive structures associated with sperm competition such as testes, at the expense of those traits associated with mate location, such as antennae. In species where females advertise for mates, such as most moths, a high-density environment may also lead to a reduction in pheromonal signalling (calling) length and frequency as a result of high mate abundance. While such responses have been shown at the phenotypically plastic level in moths, heritable evolutionary adaptations have seldom been tested, and studies of how population density influences pheromone signalling strategies are scarce. Here we use behavioural assays and scanning electron microscopic measurements to test whether larval population density influences, at the genetic level, the ability of males to locate females and male investment into antennal morphology, in addition to its effect on the frequency and duration of female calling. We used two replicated populations of the Indian meal moth *Plodia interpunctella* that had experimentally evolved under high or low population densities for 35 generations. We found no significant divergence in antennal morphology or mate acquisition behaviours between the two density populations. These findings suggest that although population density has the ability to create plastic changes in both morphological and behavioural traits, this factor alone is unlikely to be causing evolutionary change in male and female signalling in this species.

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Introduction

For sexually reproducing animals the process of signalling for, and responding to mates can be costly (Andersson 1994), as a consequence, animals must balance investment in the structures and behaviours associated with signalling and receiving messages (Harari et al. 2011). Although finding a mate is paramount, the effort expended searching for a mate is often notably different between the sexes (Ambrogi et al. 2008; Barnes 1982). For moths, it has often been assumed that because the amount of pheromones produced by a female is diminutive, that this behaviour has no cost to females (Greenfield 1981); however potential costs of signalling include the energetic costs of pheromone synthesis and production, and the potential for parasites and predators to detect the same cues (Dicke and Sabelis 1992; Engqvist et al. 2014). Additionally, although females who release pheromones ("call") more frequently and for longer durations of time may be more successful in attracting a mate, particularly in low-density environments, excessive advertisement may attract less sensitive and potentially less reproductively viable males, especially in high-density environments (Umbers et al. 2015). Consequently, it is predicted that female moths should adjust their calling strategy in relation to the population density of the environment (Umbers et al. 2015).

Male moths have more obvious mobility-related mate acquisition costs, including metabolically expensive flying, an increased risk of predation and potential sexual competition upon arrival (Greenfield 1981; Lewis et al. 2013). Many moth species use scramble competition, where male–male competition is high and being the first to reach a female may significantly increase the chances of successful fertilisation (Emlen and Oring 1977; Rutowksi 1982). This may be particularly relevant in species with first male sperm precedence or where males protect their reproductive investment via mate guarding (Atwell and Wagner 2014; Kokko and Rankin 2006). Therefore, heightened response and mate-location traits should be selected for within populations where fittest could simply mean fastest (Lewis et al. 2011).

Chemical signals are almost exclusively received on the antennae, a highly diverse structure across many invertebrate species (Callahan 1975; Schneider 1964; Shields and Hildebrand 2001). The extraordinary diversity of antennae can be seen across many moth species, with some having simple filiform antennae while others have feathered 'bipectinate' and 'quadripectinate' antennae (Mankin and Mayer 1984; Symonds et al. 2012). Antennae are commonly more elaborate in males than females, giving rise to theories that associate antennal size and structure with pheromone output and overall sensitivity (Bau et al. 2005; Greenfield 1981; Steinbrecht 1996). Furthermore, in males, larger antennae typically contain a greater number of olfactory receptors (Chapman 1982), which suggests that these structures are strongly linked to the ability of males to detect female pheromones (Chapman 1982; Svensson 1996).

There is remarkably limited evidence so far to conclude how or why diversity in antennal morphology arises; however, several studies have suggested that population density may play an important role in determining investment in male morphology

associated with reproduction (Gage 1995; Holwell et al. 2007; McNamara et al. 2010; Symonds et al. 2012). Individuals reared in high-density environments, where competition is high but difficulty finding a mate is low, may invest more in reproductive structures such as testes, at the expense of those traits associated with locating mates such as antennae (Holwell et al. 2007).

Although studies have found that density has the ability to create plastic responses in moth reproductive traits such as testes size and number of sperm (Gage 1995; McNamara et al. 2010), it is yet to be investigated whether these changes are heritable and derive from adaptive evolution. In this study, we investigate the role density may play in the evolution of moth signalling behaviours and receiving structures. Here, we examine evolutionary changes in female calling behaviours and male antennal morphology and mate-locating ability of *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), in relation to population density. This species is an ideal model system as we already know that density affects a number of male phenotypic reproductive and morphological traits, including plastic investment in mate-locating structures (Gage 1995). This species is a stored product pest that naturally exists at high population densities, which allows for a clear distinction between high and low density treatments (Brady and Smithwick 1968). Additionally, the female pheromone blend has been characterised and is commercially available.

We examine male and female evolutionary responses using replicated experimental evolution populations that have been evolving for 35 generations under high or low population density. In light of the limited experimental evidence on the mechanisms driving evolutionary change in male and female morphological and behavioral traits, we set out to investigate if low and high rearing densities can affect the evolution of behavioral and morphological traits using scanning electron microscopy and behavioural assays.

Materials and methods

Laboratory culturing

A stock population of *P. interpunctella* was obtained from two separate wild populations (approximately 150 individuals) in Perth, Australia in 2011. All individuals were maintained in the laboratory at 25 ± 2 °C on a 12:12 h L:D (light–dark) photoperiod and reared on a diet of wheat bran, yeast and glycerol (in a ratio of 10:1:1).

Experimental population lines

From the stock population of *P. interpunctella*, six experimental evolution populations were established at the University of Western Australia. Larvae were reared from the first instar under two treatments: high and low density. The treatments are identical to those used in earlier work on density effects on reproductive traits in this species (Gage 1995). There were three independent replicate lines for each treatment, making six replicate lines in total. Within each replicate there were a total of 400 larvae. All larvae were raised in 7 × 7 × 5 cm plastic containers, which were sealed with a tightly fitting lid. Larvae in both treatments had access to the same amount of food (350 mg per capita). The high-density treatment consisted of 20 containers, each containing 20 larvae and the low-density treatment consisted of 80 containers, each containing 5 larvae.

As adults emerged they were removed and isolated into 5 ml vials. Adults were pooled from across all the containers until sufficient numbers (80 males and females) were acquired for each replicate population. Males and females were haphazardly selected to contribute to the next generation in order to limit the likelihood of container effects and inbreeding. High-density and low-density individuals were mated in different conditions: high-density mating containers consisted of a single 2 l glass container comprising of 40 males and 40 females; low-density individuals were mated in two 2 l containers with 20 males and 20 females in each. We did this to limit differences in the effective population size between the treatments, while allowing the adult mating density to approximate the larval rearing environment. Each mating container was covered with mesh and inverted over paper, allowing eggs to collect and hatch. First-instar larvae were collected from the paper and were used to establish the next generation.

Laboratory culturing for moths used in behavioural and morphological assays

The individuals used in this study represent the offspring of the 35th generation of experimental evolution lines described above. Here, 30 adult males and females were collected from each of the three replicate lines for each treatment. The 60 males and females from each replicate lines were then placed in a single 2 l container to mate. The collected eggs were then sent to Deakin University between March and May 2015, where they hatched. All individuals were reared in a 'common garden' environment—where density was consistent (see below) across all lines. Doing so ensured that any differences in morphology and behaviour between lines were the result of evolutionary, not plastic responses to population density.

Once the eggs had hatched, for each high and low-density population, we transferred 12 larvae with 4.2 g (350 mg per capita) of food into 7 × 7 × 5 cm containers that were sealed with a tightly fitting lid. Upon adult emergence, we sexed individuals and isolated them in 100 ml glass vials to prevent mating. The weight of each moth at emergence was recorded to four decimal places using a Sartorius Extend ED1245 balance. We chose individuals arbitrarily as they emerged from the containers, but recorded the container identity for use in subsequent analysis.

Behavioural assays

Male mate attraction

To examine the effect of ancestral rearing density on the ability of males to locate females, we recorded the time taken for male moths to locate an artificial pheromone lure. At one end, inside a netted butterfly enclosure (dimensions 50 × 50 × 150 cm), an artificial (HOVEX) lure in a gauze-covered container (diameter 8 cm, depth 2 cm) was secured. The lure, weighing 0.38 g has as an active constituent 4.9 g/kg (Z, E)-9,12-tetradecadien-1-yl-acetate, and is commonly used as an attractant for pantry moths (Burks and Kuenen 2012). Each lure remains active for 3 months, however we replaced lures every 4 weeks.

For each assay, an individual male was introduced into the enclosure at the opposite end from the lure. We then recorded the time it took for each male to locate the lure. Males were determined to have successfully located the lure when they landed on the outside of the lure container. We set a maximum timeframe of 45 min for each male to locate the

lure. Any male failing to locate the pheromone source in this period was recorded as a 'non-successful response'. We conducted a total of 192 trials, with each male being used only once. After completing behavioural assays, males were analysed for antennal morphology (see below).

Female mate attraction

To examine the effect of ancestral rearing density on the investment of females into mate attraction, we observed the frequency and duration of female calling behaviour. Calling behaviours begin when a female moth starts to release attractant pheromones; this behaviour can be seen when a female parts her wings and raises her abdomen, exposing her ovipositor (McNamara et al. 2010). This behaviour is concomitant with pheromone production and release (Brady and Smithwick 1968). Under red light, to mimic the scotophase, we recorded both the number of times females raised their abdomen and extruded their ovipositor and the total duration for which their abdomens were raised over a 3-h period on the first day after adult eclosion (Zavodska et al. 2012). This process was repeated 5 days after eclosion to assess whether any differences in female calling efforts appeared with age (Umbers et al. 2015); however no such differences in behaviour were found, consequently our analysis used solely the observations from the first round of assays. A total of 197 females were observed within 24 h of eclosion.

Male antennal morphology

To assess difference in male investment into antennal morphology, we took measurements of males including antennal length, antennal sensilla density and average antennal sensilla length (described below). These measures were assessed through images taken using a Phillips XL20 scanning electron microscope (SEM) and a JEOL NeoScope JCM-5000 SEM. Using a scalpel and forceps, we excised heads with antennae attached from each male and prepared the specimens for imaging and morphological measurements. A scale bar was embedded into each image and measurements were conducted using the image analysis software package ImageJ^{TM} (Schneider et al. 2012). For each male, three measurements were taken from a single antenna. First, we calculated antennal length by measuring from the base to the tip of the full antenna (Fig. 1a). Second, we calculated the density of sensilla at the base of the antennae by measuring an area located on antennal segments one to three (whichever was most visible) and counting the number of antennal hairs within this area then dividing the number of sensilla observed by the area measured. The specific antennal segment used in sensilla measurements had no bearing on the calculation of sensilla length (linear model: $t_4 = 0.23$, $P = 0.83$) or sensilla density ($t_4 = 0.20$, $P = 0.85$). All areas measured were above a set minimum of $1500\mu m^2$ and only hairs that were visible to the base were counted (Fig. 1b). Finally, we measured the length of five sensilla where the entire hair could be seen within the aforementioned area and calculated the average sensilla length for each individual (Fig. 1c).

Statistical analysis

All analyses were conducted using R (R 3.2.2, R Core Team, 2015) and the package *lme4* (Bates et al. 2015). Generalised linear mixed-effects models (GLMMs) and mixed effects

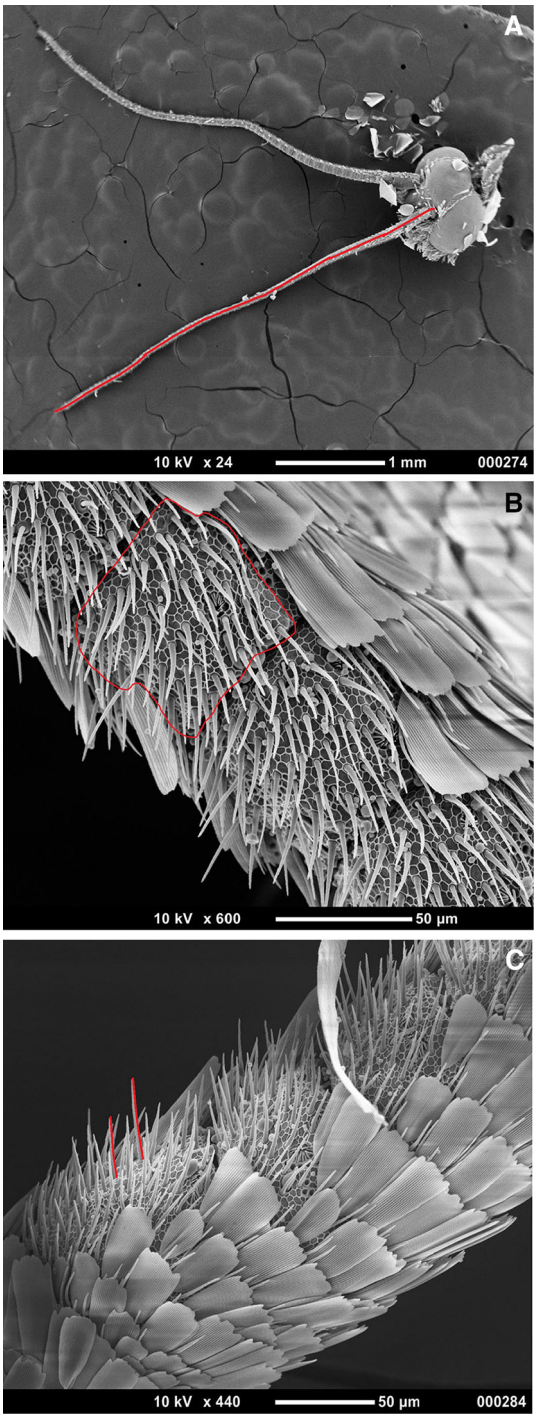

Fig. 1 SEM pictures of **a** moth head, with the *red line* representing the measure of total antennal length (×24 magnification); **b** antenna, with the area inside the *red line* representing the area used to calculate density of sensilla (×600 magnification); **c** antenna, with the *red line* representing the line traced along the sensilla to calculate average sensilla length (×440 magnification)

binomial logistic regression models were used to analyse variation in moth behavioural and morphological traits in relation to rearing density. For all analyses, container nested within evolutionary replicate line was included as a random effect to control for potential pseudo-replication in using individuals from the same rearing container, and for using multiple experimental evolution replicate lines. Because body size could influence behavioural and morphological traits, and itself be influenced by population density, body weight at eclosion was included as a covariate in all analyses.

To analyse male success rate of locating the pheromone lure we used a mixed-effect binomial logistic regression model with density treatment as a predictor variable and the categorical successful/non-successful response as the response variable. To investigate the effect population density had on male morphology and on the time taken to reach the pheromone lure, we created separate GLMMs with antennal length, sensilla density, and time to locate lure as the response variables, and density treatment as a predictor variable.

For female data, we constructed a logistic model, with proportion of time during the observation period spent calling as the response variable, and ancestral density treatment as predictor. As the response variable is a proportion of time and is restricted to values between 0 and 1, we used a mixed effects logistic regression model with a binomial error and logit-link function (fitted by maximum likelihood). This views each minute of observation as a 'presence' (i.e. abdomen raised/calling) or 'absence' (i.e. abdomen not raised/not calling) behaviour within a fixed number of Bernoulli trials (total minutes of observation). We also used GLMMs to analyse variation in the frequency of calling during the observation period in relation to ancestral rearing density.

Results

Male behaviour

Of the 192 males that were tested, 84 males successfully located the pheromone lure (48/123 from low density, 36/69 from high density).

There was a weak tendency for individuals from a high-density background to be *more* successful than low-density derived individuals at locating the pheromone lure (rate of successful location of lure; low density: 39.02 %, high density: 53.62 %, Table 1a), but no significant effect of population density on the time taken for males to locate the lure (Table 1b; Fig. 2a).

Female behaviour

Of the 197 females that were tested, 87 females called for the entire testing period (57 from low density, 30 from high density), while 17 failed to call at all (5 from low density, 12 from high density).

Of the females that commenced calling, we found no significant effect of density on calling duration (Table 1c; Fig. 2b) or on frequency of calling during the observation period (Table 1d; Fig. 2c). Additionally, selection density treatment had no effect on the weight of females (low-density $= 0.25 \pm 11.80$ mg, high-density $= 0.33 \pm 10.91$ mg; Estimate ($\pm$SE) $= 0.00 \pm 0.00$, $t_4 = 1.19$, $P = 0.30$).

Table 1 Summary of models exploring male and female behavioural responses to population density, controlling for body mass as a fixed effect

	Estimate	SE	Statistic	*P*
(a) Male success at locating lure (mixed effect logistic regression model)				
Density	−0.66	0.38	z = −1.72	0.08
Body mass	154.01	98.43	z = 1.56	0.12
(b) Male time taken to locate lure (GLMM)				
Density	2.27	8.57	t = 0.26	0.80
Body mass	−4100.29	2957.69	t = −1.39	0.17
(c) Proportion of time females called (mixed effect logistic regression model)				
Density	−0.13	0.53	z = −0.24	0.81
Body mass	−55.46	4.72	z = −11.7	<0.001
(d) Female frequency of calling (GLMM)				
Density	−0.13	0.36	t = −0.36	0.74
Body mass	38.51	26.59	t = 1.45	0.15

Model type is indicated in brackets

Male morphology

We assessed 47 low-density and 47 high-density derived males for antennal morphology. We found no significant effect of density treatment on antennal length (Table 2a; Fig. 2d), sensilla density (Table 2b; Fig. 2e), average sensilla length (Table 2c; Fig. 2f) or weight (low-density = 0.15 ± 7.63 mg, high-density = 0.21 ± 7.70 mg; Estimate (±SE) = 0.001; $t_4 = -0.002$, $P = 0.99$).

Discussion

We sought to examine whether population density could be a mechanism for evolutionary change in pheromone receiver structure morphology and mating behaviours. Previous studies have demonstrated environmentally plastic effects of density on these traits (Atwell and Wagner 2014; Gage 1995; Holwell et al. 2007; McNamara et al. 2010; Sheehan and Tibbetts 2011). Contrary to prediction, we found no significant evolutionary consequences of variation in population density on antennal morphology or mate attraction behaviours in *P. interpunctella*. These findings suggest that although population density has the ability to create both plastic morphological and behavioural changes, this factor alone is unlikely to be causing evolutionary change in these traits.

Although we did not find population divergence in signalling morphology and female pheromone-producing behaviour it appears that this is not due to an insufficient time-frame for selection, nor a lack of genetic variation in the original stock population. A study on the same experimental evolution populations of *P. interpunctella* (using common garden populations of an earlier generation) found evolutionary divergence in both male reproductive and immunological investment (K.B.M unpublished data). The only pattern that emerged from the data was that, unexpectedly, males from high-density backgrounds

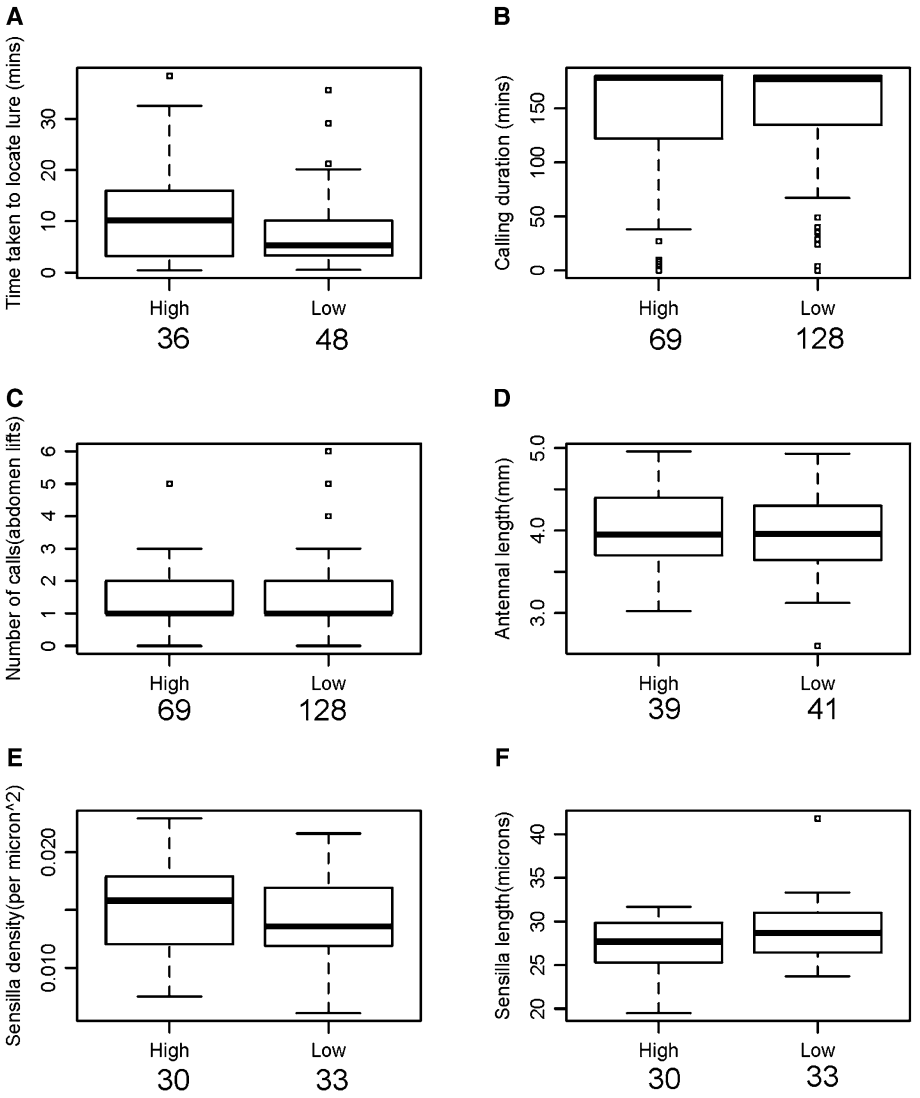

Fig. 2 Box plots showing the effect of larval rearing density (high vs. low) on **a** time taken for male moths to locate a pheromone lure, **b** calling duration of female moths, **c** calling frequency of female moths, **c**, **d** antennal length of male moths, **e** sensilla density of male moths, **f** sensilla length of male moths. Sample sizes are given beneath the *x-axes*

tended to be more successful at locating an artificial pheromone lure than low-density derived individuals.

Previous theoretical and empirical studies have explored the plastic, within-generational effects of population density on receiver structures, and reproductive behaviour (Kokko and Rankin 2006; Stelinski et al. 2014). Although we found no evolutionary consequences of population density on antennal structures in *P. interpunctella*, mechanisms that allow such plastic changes in antennal investment are therefore likely to exist in other species. Additionally, these findings are restricted to the measurements performed, which cover

Table 2 Generalised linear mixed effect models exploring male antennal morphology in response to population density, controlling for body mass as a fixed effect

	Estimate	SE	Statistic	*P*
(a) Male antennal length				
Density	−0.15	0.13	t = −1.17	0.31
Body mass	85.91	25.09	t = 3.42	0.001
(b) Male sensilla density				
Density	−0.00	0.00	t = −0.69	0.53
Body mass	−0.79	0.25	t = −3.19	0.003
(c) Male sensilla length				
Density	1.55	1.18	t = 1.31	0.26
Body mass	386.28	232.51	t = 1.66	0.11

only a subset of the parameters of the antennal structure and the female signaling behavior necessary to fully assess how selection may operate on this system under high and low density conditions.

Similarly, population density showed no significant effect on female signalling behaviour. It may be that signalling effort is determined plastically by using cues to determine appropriate calling effort (Kasumovic and Brooks 2011; Simmons 2015). In insects, the primary modality of communication is chemical (Wyatt 2010). If chemical cues are able to convey information on the sex ratios within a population, females may be able to adjust their calling effort according to both population density and the number of competing females (Brady and Smithwick 1968; Kasumovic et al. 2009; Kokko and Rankin 2006; Umbers et al. 2015). There are substantial difficulties in quantifying the metabolic costs of pheromone production, and due to the small amounts of pheromone produced, there is very little experimental evidence to date that overcomes the perception that the female cost of pheromone production/emission is low (Cardé and Baker 1984; Greenfield 1981).

Several recent studies suggest that there are, indeed, costs associated with calling in moths (Harari et al. 2011, 2015; Stelinski et al. 2014; Umbers et al. 2015). The costs, despite being potentially small, influence the life history of females, which may in turn affect male life histories due to their reliance on female signalling to successfully find a mate and reproduce (Johansson and Jones 2007; Kokko and Rankin 2006). Although females of some species may be able to adjust signalling effort according to population density (Stelinski et al. 2014), our findings suggest that female *P. interpunctella* does not evolve in response to population density.

Although ancestral density had no significant effect on any of the traits measured, it remains possible that there was divergence in other yet unquantified traits. For example, under differing population densities, maturation, mortality rate, testes size, eye size, warning colouration patterns and patterns of ovipositional activity and female fitness have all shown environmental plastic responses (Atwell and Wagner 2014: female mate choice plasticity is affected by the interaction between male density and female age in a field cricket; Bonduriansky 2007: diet condition influences body size of *Telostylinus angusticollis*; Brent 2010: larval density influences maturation time and mortality rates in the western tarnished plant bug, *Lygus hesperus*; Gage 1995: population density influences testes, body size and sperm numbers in *P. interpunctella*; McNamara et al. 2010: Larval population size influences sperm traits, somatic characters and reproductive behaviours in the almond moth, *Cadra cautella*). That these traits are affected plastically by population density suggest some capacity for evolutionary change should those densities be

maintained through time (He and Miyata 1997; Peters and Barbosa 1977). In particular, one aspect that was not assessed in this study was female pheromone concentration and composition. While there is remarkable consistency in pheromone output by adult *P. interpunctella* females across a range of ages, suggesting that pheromone concentration does not vary over time (Brady and Smithwick 1968), little is known about how the relative proportion of pheromone components varies across individuals. In a phylogenetic comparative analysis of moths, Symonds et al. (2012) found that species with larger elaborate antennae had lower male population densities, and lower molecular weights of female sex pheromones. An analysis of potential divergence in pheromone composition between the two density treatments in this study could provide insight into these complex processes.

Relatively little is known about how environmental variables such as density, influence the nature of biological trade-offs in invertebrates, particularly in regard to communication systems. This study builds on the limited work investigating the variation and evolution of signalling and receiving structures. Explaining the variation that occurs in reproductive investment is fundamental to understanding the life history and mating strategies of many invertebrates. Although density does not appear to be a key mechanism for the evolution of communication structures and calling behaviour, these insights highlight the need for further investigation into other potential factors that may shape the evolution of the morphological and behavioural facets of signalling.

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