Journal of Stored Products Research 71 (2017) 64–71

Contents lists available at ScienceDirect

Journal of Stored Products Research

journal homepage: www.elsevier.com/locate/jspr

Effects of larval crowding and nutrient limitation on male phenotype, reproductive investment and strategy in *Ephestia kuehniella* Zeller (Insecta: Lepidoptera)

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ARTICLE INFO

Article history:
Received 19 April 2016
Received in revised form
24 August 2016
Accepted 26 January 2017

Keywords:
Male morphology
Dispersal
Sperm numbers
Mating frequency
Mediterranean flour moth

ABSTRACT

Food shortage experienced by juvenile insects affects adult morphology and life-history traits. Developmental plasticity and trade-off between ecological and sexual traits helps maximise individual fitness. *Ephestia kuehniella* were reared at different larval densities to investigate phenotypic shifts in adult males. Variation in ecological traits (sizes of forewing, head and thorax and adult longevity) and sexual traits (valva and aedeagus, sperm number, mating frequency) were compared. Males that emerged from highest density population (800) had lower body mass and small forewings, head and thorax, suggesting that they could not completely compensate for food shortage. The allometric relationship between body mass and forewing length also changed, and these males had relatively longer wings. This arrangement may enhance dispersal and assist in mate-searching at higher densities. Males from all larval densities achieved similar mating frequency but those from higher density produced fewer eupyrene sperm and had shorter adult lifespan. By mating more frequently and maintaining apyrene sperm production, males increase their reproductive success at sperm competition observed at higher densities. Food stress associated with high density populations did not affect valva and aedeagus size indicating that these traits may be insensitive to external environmental changes because they incur fitness costs to males.

1. Introduction

Organisms are constantly exposed to changing biotic and abiotic conditions that could reduce individual fitness (Koehn and Bayne, 1989; Rose, 1991). Changes in resource allocation pattern, trade-offs and plasticity in developmental and adult stages are expected to mitigate the detrimental effects of environmental variation on fitness (Monaghan, 2008). Although a critical component of evolutionary ecology, we still know little about the details of possible strategies (Boggs, 2009).

In many insects, population growth results in competition for resources, and larval crowding in species with discrete life-stages can restrict nutrient availability. The consequences of food shortage during juvenile stages may be experienced in subsequent developmental stages. This carry-over effect is most intense in species where adults do not feed and the nutrients necessary for the adult stage are obtained by larvae. Food-limitation during development lowers individual growth rate and survival (e.g. Bauerfeind and Fischer, 2005; Macia, 2009). Adults that emerge are typically small in size and have smaller body parts, lower reproductive success and shorter adult lifespan (e.g. Bhavanam et al., 2012; Gage, 1995; Hirschberger, 1999). Food shortage during juvenile stages has been observed to result in development of adults with large fat bodies (e.g. cockroach *Nauphoeta cinerea* - Barrett et al., 2009) or altered metabolic rates (e.g. butterfly *Bicyclus anynana* - Pijpe et al., 2008). Other species such as wing-dimorphic insects (e.g. aphids - Braendle et al., 2006; and crickets - Zera and Denno, 1997) and wing-monomorphic insects (e.g. lady bird beetles - Dmitriew et al., 2009; and speckled wood butterfly - Pellegroms et al., 2009) changes in wing morphology, body composition and behaviour occur that facilitate movement away from suboptimal conditions. However, a trade-off between resourcing flight and resourcing reproduction is documented in some female insects (Zera and Denno, 1997; Zera and Harshman, 2001).

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http://dx.doi.org/10.1016/j.jspr.2017.01.004

In general, primary sexual traits such as genitalia show a weaker response to environmental variation (e.g. Cayetano et al., 2011; House and Simmons, 2007; Simmons et al., 2009). Sperm production is however sensitive to larval rearing conditions in many insects (Indian mealmoth *Plodia interpunctella* - Gage and Cook, 1994; army worm *Pseudaletia separata* - He and Miyata, 1997; almond moth *Cadra cautella* - McNamara et al., 2010), and this may result in trade-off between the number of matings and the size of each ejaculate (e.g. Lewis et al., 2011) that affects lifetime mating success (the measure of male reproductive potential). Expression of secondary sexual traits such as weapons used in intrasexual competition (e.g. beetles - Johns et al., 2014) or ornaments for signalling females (e.g. damselflies - Outomuro et al., 2014; and flies - Rodriguero et al., 2002) is highly responsive to resource availability. Although nutrient limitation effects have been studied in several insects, few have dealt specifically with pests of stored products. Moreover, many studies focus on only a few life-history or morphological traits, largely ignoring the influence of environmental variation on multiple traits or a combination of life-history and morphological traits.

The Mediterranean flour moth, *Ephestia kuehniella* is a stored product pest that targets cereals and dead organic matter (Hill, 2002). The larval developmental period lasts for 20–30 days (Bhavanam et al., 2012), with all nutrients needed for adult reproduction being obtained during that stage as adults do not feed (Norris and Richards, 1932). Males and females sexually mature soon after emergence from pupa and engage in the first mating on the day of emergence (Calvert and Corbert, 1973). Males live to 14 days on average and mate up to 8 times in their lifetime. At each mating, males transfer a single spermatophore consisting of sperm and accessory gland products.

The varied reproductive strategies used by males to gain access to females provide an opportunity to test predictions on how resources between life-history and morphological traits are adjusted to maximise male reproductive success at high population density. We predicted that males that are resource-limited did not alter investment in genitalia, but would increase investment in ecological traits (wings, head, thorax and adult longevity) useful for dispersal and mate more often at the expense of gamete production.

2. Materials and methods

2.1. *Ephestia kuehniella*

Laboratory stock cultures were maintained in plastic containers (8 cm in diameter and 10 cm high). Larvae were reared on a diet of wholemeal wheat flour (43.5%), maize meal (43.5%), brewer's yeast (3%) and glycerine (10%) (Lima-Filho et al., 2001), at a density of 200 larvae to 50 g of food (Bhavanam et al., 2012) and kept at 25 ± 1 °C and 60 ± 10% r.h. on a 14L:10D photoperiod condition. Each container was provided with a crumpled paper towel (25 cm × 25 cm) for pupation.

2.2. Rearing densities

Eggs less than one day old were collected from stock culture females. A preliminary test showed that the hatching success of this culture was 96 ± 0.3%. The four experimental population densities: 100, 200, 400 and 800 were established by placing batches of 104, 208, 416 or 832 randomly selected eggs on 50 g of dietary medium in separate, clean containers, creating a range of food availability conditions. Mature pupae were collected and placed in individual 2 cm in diameter and 7.5 cm high glass vials. After emergence, adults were sexed be examination of genital structures and openings (Scoble, 1992) and used in the experiment.

2.3. Morphometric measurements

Thirty adult males from each density were randomly selected, anesthetized with CO_2, weighed individually using an electronic dual range balance (Mettler AE100, Mettler-Toledo Inc., Switzerland) with a precision of 0.1 mg and then frozen at −20 °C. After 24 h, forewing length (from the point of articulation to the distal end of the forewing), head and thorax width were measured for each individual (Fig. 1). They were then dissected so that valva length and aedeagus length (from the point of sclerotization to tip) could be measured. All morphological traits were measured using images captured by a USB 2.0 Video/Audio Grabber (Lindy Electronics Ltd, UK) with Universal Desktop Ruler software (AVPSoft, USA).

2.4. Male longevity, mating frequency and sperm numbers

Thirteen one day old virgin males were randomly selected from each of the 200, 400 & 800 population density cultures. Prior observations showed that individuals that developed at population densities of 100 and 200 larvae had similar levels of fitness (Bhavanam et al., 2012). Hence the population density of 100 larvae was excluded in this experiment. At the start of the scotophase, each male was provided with a one day old virgin female taken from the same experimental population density. If copulation occurred, the female was removed after mating termination, killed and dissected for spermatophore extraction. The numbers of infertile apyrene and fertile eupyrene sperm in the spermatophore was counted as described below. Male *E. kuehniella* require 24 h recovery time to produce a full spermatophore. Therefore, after 24 h each male was provided with another one day old virgin female and the same procedure was repeated for every 24 h until the male died. For each male, mating frequency and adult longevity were recorded.

2.4.1. Sperm count

To count the numbers of eupyrene and apyrene sperm transferred to a female during mating, females were frozen for 5 min at −20 °C immediately after mating. The female was dissected and the spermatophore extracted. The spermatophore was placed on a depression slide containing Barth saline and then ruptured with a fine needle to release sperm. During copulation, eupyrene sperm were transferred in bundles (Fig. 2) and each bundle consisted of 256 individual spermatozoa (Cook and Wedell, 1996). Therefore, to obtain the total numbers of eupyrene sperm transferred, the number of bundles was counted under ×40 magnification and then multiplied by 256. The sperm mass was then washed with Barth saline into a specimen tube, and the volume made up to 30 mL with distilled water. Six separate 10 μL subsamples were taken from the sample using a Gilson autopipette and each left to dry on separate microscope slide. The apyrene sperm (Fig. 2) in each dried subsample was counted using dark field phase-contrast microscopy at ×100 magnification. The average of the counts made from each 10 μL subsample was then multiplied by the dilution factor to obtain the total numbers of apyrene sperm transferred (Cook and Wedell, 1996).

2.5. Statistical analysis

Normality of the data was tested using Shapiro-Wilk and Kolmogorov-Smirnov tests using PROC UNIVARIATE and by visual comparison with qq probability plots. The effects of rearing population density on adult body mass, linear dimensions of

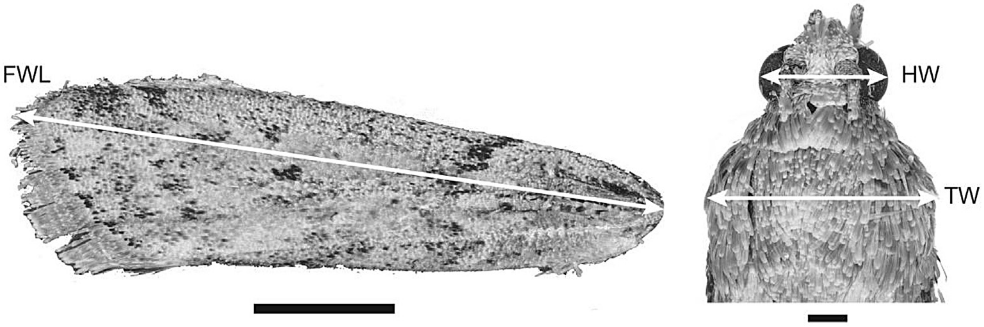

Fig. 1. Morphological measures made on male *Ephestia kuehniella*. FWL, HW and TW represent forewing length, head width and thorax width. Scale bars are 1 mm for FWL and 200 μm for HW and TW.

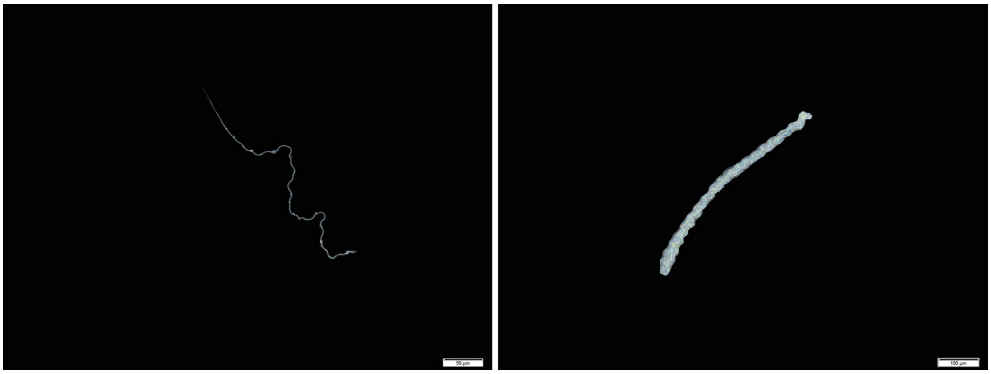

Fig. 2. Two types of sperm produced by male *Ephestia kuehniella*: eupyrene (right) and apyrene (left). Scale bars at 100 μm and 50 μm for eupyrene and apyrene sperm, respectively.

morphological traits, mating frequency, adult longevity and numbers of eupyrene and apyrene sperm were compared using ANOVA. The Tukey-Kramer HSD test was used as a multiple comparison procedure. The relationship between the size of various morphological traits and body mass was determined using regression analysis. The slopes of the regression lines were compared using ANCOVA. All statistical analyses were conducted using SAS version 9.3 and R version 2.5.1 (R Core Team, 2015) statistical software's. Graphs were plotted in R using the package cowplot (Wilke, 2015).

3. Results

3.1. Morphometric measurements

Adult males reared at population densities of 100 and 200 larvae were significantly heavier than those reared at population densities of 400 and 800 individuals (ANOVA: $F_{3,113} = 20.04$, $P < 0.0001$) (Table 1). There was a significant effect of population density on forewing length (ANOVA: $F_{3,113} = 3.03$, $P = 0.033$), head width (ANOVA: $F_{3,113} = 7.54$, $P = 0.0001$) and thorax width (ANOVA: $F_{3,113} = 28.48$, $P < 0.0001$) with the smallest sizes recorded at the highest (800) population density (Table 1). However, mean valva and aedeagus lengths did not differ significantly among males reared at different densities (ANOVA: valva: $F_{3,103} = 1.26$, $P = 0.291$; aedeagus: $F_{3,103} = 0.28$, $P = 0.838$) (Table 1).

With increasing body mass, forewing length increased significantly at population densities of 400 and 800, but body mass had no significant effect on forewing length at population densities of 100 and 200 (Table 2). ANCOVA further indicated that forewing length increased significantly faster at high population densities of 400 and 800 than at low population densities of 100 and 200 ($F_{3,109} = 2.72$, $P = 0.048$) (Fig. 3a). The size of the other morphological traits did not increase with body mass significantly ($P > 0.05$), except thorax width at the highest population density (800) and head width at population densities of 400 and 800 ($P < 0.05$) (Table 2). The slopes of linear regressions of these morphological traits over body mass did not differ significantly

Table 1
Mean (±SE) body mass (mg) and morphological trait sizes (mm) of *Ephestia kuehniella* males reared at four population densities. In each row, means followed by the same letter were not significantly different at $P = 0.05$.

Morphological trait	100	200	400	800
Body mass	15.21 ± 0.34 a	15.63 ± 0.34 a	13.89 ± 0.28 b	12.31 ± 0.37 c
Forewing length	8.76 ± 0.08 a	8.64 ± 0.08 a	8.52 ± 0.17 ab	8.30 ± 0.09 b
Head width	1.25 ± 0.01 a	1.25 ± 0.01 a	1.22 ± 0.01 a	1.19 ± 0.01 b
Thorax width	2.19 ± 0.03 a	2.20 ± 0.03 a	2.15 ± 0.03 b	2.13 ± 0.02 b
Valva length	1.34 ± 0.02 a	1.32 ± 0.01 a	1.33 ± 0.03 a	1.29 ± 0.02 a
Aedeagus length	1.55 ± 0.03 a	1.53 ± 0.03 a	1.56 ± 0.03 a	1.53 ± 0.02 a

Table 2
The relationship between log body mass (x, mg) and log morphological trait size (y, mm) in width and length of *Ephestia kuehniella* males that emerged from different population densities. Significant terms are indicated in bold. Regression lines were compared to determine their slope difference (Fig. 3).

Morphological trait	Density	Linear regression	R^2	*F*	*P*
Forewing length	100	y = 0.86 + 0.07 x	0.026	1,24 = 0.69	0.415
	200	y = 0.80 + 0.12 x	0.089	1,27 = 2.73	0.110
	400	y = 0.47 + 0.40 x	0.189	1,24 = 6.53	**0.016**
	800	y = 0.57 + 0.31 x	0.768	1,25 = 89.53	**<0.0001**
Head width	100	y = 0.02 + 0.06 x	0.024	1,24 = 0.64	0.432
	200	y = −0.04 + 0.11 x	0.019	1,27 = 2.99	0.095
	400	y = −0.19 + 0.24 x	0.241	1,24 = 8.88	**0.006**
	800	y = −0.12 + 0.18 x	0.318	1,25 = 12.60	**0.001**
Thorax width	100	y = 0.14 + 0.17 x	0.073	1,24 = 2.03	0.165
	200	y = 0.19 + 0.13 x	0.063	1,27 = 1.87	0.182
	400	y = 0.28 + 0.05 x	0.005	1,24 = 0.14	0.708
	800	y = −0.07 + 0.20 x	0.234	1,25 = 8.25	**0.008**
Valva length	100	y = −0.06 + 0.15 x	0.047	1,24 = 1.17	0.289
	200	y = 0.00 + 0.10 x	0.051	1,27 = 1.44	0.241
	400	y = −0.12 + 0.21 x	0.142	1,23 = 3.79	0.064
	800	y = −0.01 + 0.11 x	0.033	1,24 = 0.82	0.374
Aedeagus length	100	y = 0.26 + 0.00 x	0.000	1,24 = 0.00	0.997
	200	y = 0.18 + 0.01 x	0.000	1,27 = 0.00	0.971
	400	y = 0.01 + 0.15 x	0.070	1,24 = 1.74	0.201
	800	y = 0.16 + 0.02 x	0.002	1,25 = 0.06	0.815

between population densities (for thorax: $F_{3,109} = 0.41$, $P = 0.749$; head: $F_{3,109} = 1.10$, $P = 0.351$; valva: $F_{3,98} = 0.19$, $P = 0.905$; aedeagus: $F_{3,98} = 0.70$, $P = 0.553$) (Fig. 3b–e).

3.2. Male reproductive success

There was no significant difference in the average number of apyrene sperm produced by males reared at different densities ($F_{2,32} = 1.29$, $P = 0.29$) (Fig. 4a), but males reared at a population density of 200 produced more eupyrene sperm ($F_{2,32} = 3.33$, $P = 0.04$) (Fig. 4b) and lived for significantly longer periods than those at population densities of 400 & 800 ($F_{2,32} = 7.53$, $P = 0.002$) (Fig. 4c). Regardless of the number of eupyrene sperm produced and adult longevity, males taken from different densities mated a similar number of times during their lifetime ($F_{2,32} = 1.98$, $P = 0.16$) (Fig. 4d).

4. Discussion

We find that male *E. kuehniella* morphology and reproductive output is sensitive to larval population density and the developmental plasticity seen at that density maximises male reproductive success. Adult moths that emerged from the highest population density (800) were lighter in weight and had smaller forewings, heads and thoraxes than those reared at the low population densities (Table 1). These effects are most probably induced by a shortage of food at higher densities (Hooper et al., 2003). Moreover, competition between growing larvae for limited food and space (Hoffmann and Loeschcke, 2006) further reduced the time available for feeding (Tsuda and Yoshida, 1985). As a result, the resources needed for development of traits are disproportionately reduced. Nutrient availability also controls the duration and total amount of ecdysteriod hormone release and this influences the rate of cell proliferation and growth of the morphological trait. Starved larvae that have low levels of ecdysteriod titer develop smaller body parts (Nijhout and Grunert, 2010).

Male Lepidoptera produce both fertile eupyrene and infertile apyrene sperm. Eupyrene sperm production starts during early larval stage and is influenced by resource availability while apyrene sperm formation occurs in the last instar stage and hence responds to juvenile environment (Friedlander, 1997). Eupyrene sperm is more costly to produce (Silberglied et al., 1984). In the present study, it was evident that males from higher population densities (400 & 800) produced on average fewer eupyrene sperm than males reared at a lower population density (200) (Fig. 4b). This was consistent with observations of Indian mealmoth, *P. interpunctella* (Gage and Cook, 1994). In many insects, males transfer more sperm to females in each mating than is needed to fertilize available eggs (Arnqvist and Nilsson, 2000). As a result a decrease in eupyrene sperm number does not reduce female fertility in *E. kuehniella* and other insects (He and Miyata, 1997; Xu and Wang, 2009).

Population size did not affect the amount of apyrene sperm produced (Fig. 4a). In almond moth *C. cautella* (McNamara et al., 2010) and armyworm moth *P. separata* (He and Miyata, 1997) males that experienced crowding during juvenile stage transferred large numbers of apyrene sperm to females. The explanation for this appears to relate to the tendency of females to mate more frequently at higher densities (Gage, 1995) or when the population sex ratio is biased towards males (Gage, 1991). In these circumstances males experience a greater risk of sperm competition (Parker, 1982). When sperm from different males is of equal quality the paternity share of each male is proportional to his contribution to the total sperm present in the female reproductive tract. When mating order has a significant effect, large sperm numbers play a significant role. Apyrene sperm are less costly to produce and aid sperm competition by displacing the sperm of rival males and also assist in migration of eupyrene sperm to the fertilization site. Moreover, abundant apyrene sperm acts as filler and reduces female receptivity and so increases female remating interval (Silberglied et al., 1984). This might have favoured the production of large numbers of apyrene sperm in crowded *E. kuehniella* males indicating that selection might be strongly acting on apyrene but not eupyrene sperm production at higher population densities.

Sperm production incurs cost (Dewsbury, 1982), this might be especially pronounced in semelparous species as energy reserves are fixed. Under greater sperm competition risk, males transfer small ejaculates to females and under moderate or low sperm competition large ejaculates are allocated to females (Galvani and Johnstone, 1998). In the subsequent studies on white butterfly *Pieris rapae* (Wedell and Cook, 1999) and bush cricket species (Wedell, 1997) it is demonstrated that sperm number is strategically adjusted resulting in the trade-offs between the number of matings and ejaculate size. Male reproductive success increases with his mating rate (Bateman, 1948). Sperm replenishment takes

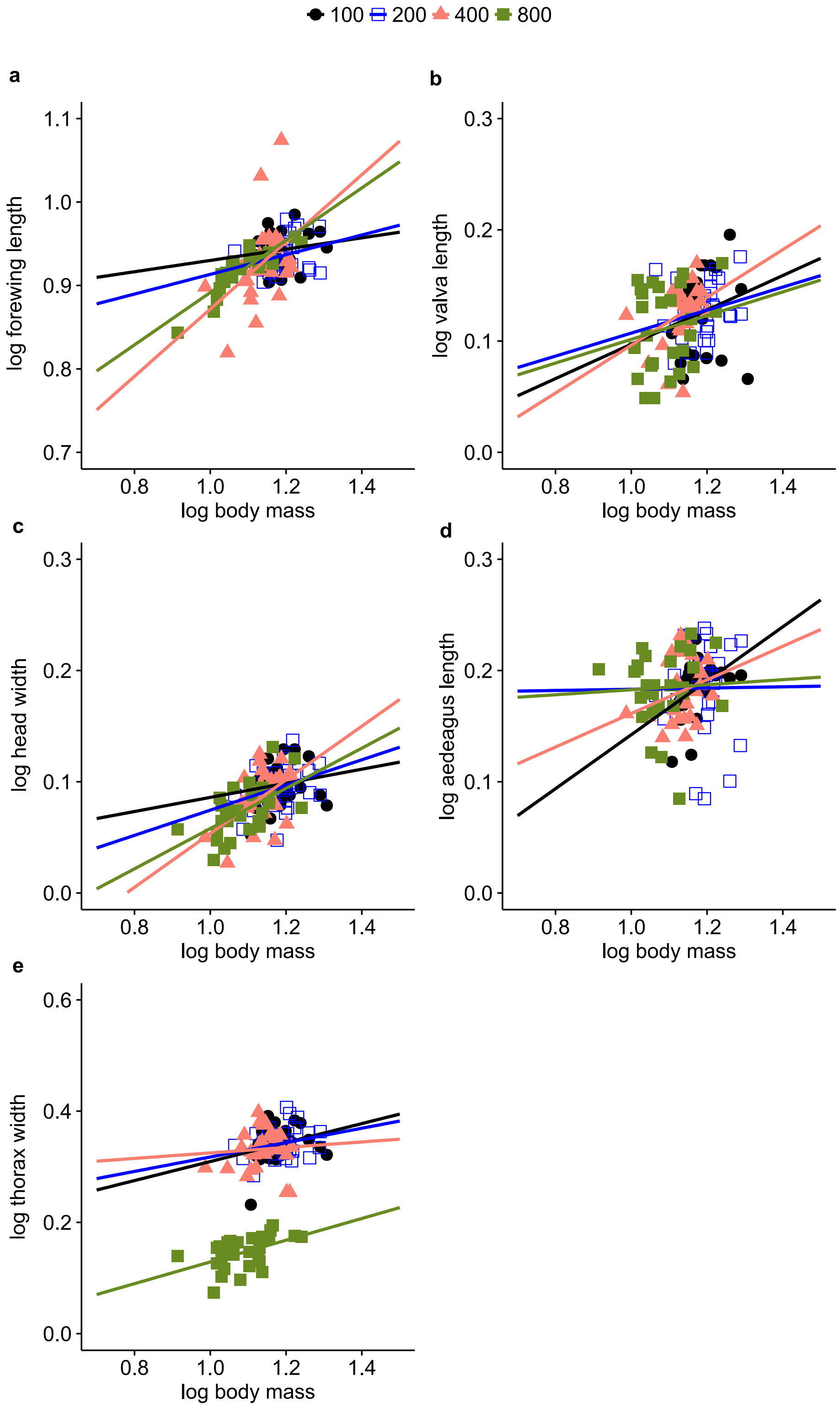

Fig. 3. The relationship between body mass and various morphological traits in male *Ephestia kuehniella* reared at different population densities. Slopes of regression lines obtained using Table 2 was compared using ANCOVA. The slope for forewing length was steeper at the higher population densities of 400 & 800 compared to the rest. For all the remaining traits the slopes were homogeneous. Adult body mass was measured in mg and the morphological traits in mm.

times (Dewsbury, 1982) and for *E. kuehniella* the recovery period is 24 h (Xu and Wang, 2009). This suggests that males from higher density populations have to invest more in survival period. Males investing more in survival can gain a net benefit as adults that live long have more chances of finding a mate. Here, we found that food shortage reduced adult longevity (Fig. 4c) but food-deprived males still mated as often as males developed at normal conditions (Fig. 4d). This indicates that selection pressure to increase relative

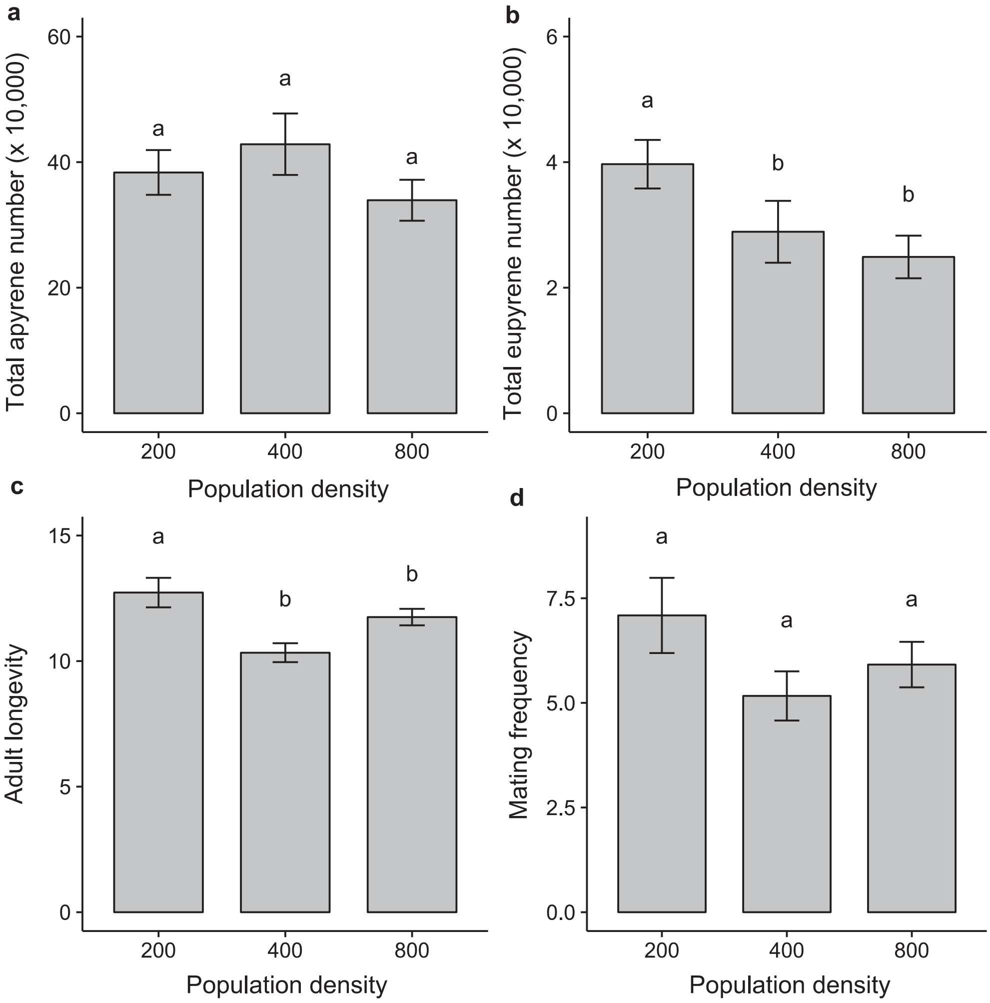

Fig. 4. (a) Total numbers of apyrene sperm and (b) eupyrene sperm, (c) adult longevity and (d) mating frequency of male *Ephestia kuehniella* emerged from three population densities. Error bars represent standard errors about the mean. For each parameter, bars with different letters are significantly different ($P < 0.05$).

investment in survival to increase their mating frequency and in turn increase their reproductive success.

The alteration of wing morphology may aid food-restricted males in mate-searching. The allometric relationship between forewing length and body mass and slopes differed among densities, with males at higher population densities (400 and 800) having larger wings relative to their body mass, as predicted; resource allocation shifted in response to food stress (Fig. 3a). In some butterflies, a similar trend has been observed; food-deprived adults have higher wing area (Pellegroms et al., 2009; Thomas et al., 1998). This when combined with decreased body mass due to nutrient limitation alters the wing loading. Likewise, *Drosophila* larvae in crowded conditions developed into adults with longer and more pointed wings (Bitner-Mathe and Klaczko, 1999). Alteration in wing morphology typically represents a difference in dispersal potential (Angelo and Slansky, 1984). Wing loading decreases with the increase in wing length (area)/body mass ratio (Dudley, 1999), which may affect flight efficiency and duration. Although flight is energetically expensive, lower wing loading reduces flight costs even for individuals in poor conditions. This indicates an adaptive strategy expressed in experimentally food stressed *E. kuehniella* males enhancing their ability to move away from low quality habitat.

At higher densities, male reproductive success may be more dependent on migration and mate-searching ability (e.g. Pomfret and Knell, 2006) if the population becomes male-biased due to skewed mortality (McLachlan and Neems, 1993), migration (Saastamoinen et al., 2010) or non- availability of mates during oviposition. It can be presumed that longer wings relative to the body mass developed by males that emerged from population densities of 400 & 800 increase the probability of mate finding due to greater agility. This would be a strategy for successful mate location that mitigates the disadvantages of small size when developed in dense populations. Such strategies have been reported from food-deprived populations of the butterfly *Pararge aegeria* (Berwaerts et al., 2002; Pellegroms et al., 2009) and Indian mealmoth *P. interpunctella* (Gage, 1995). Alternative reproductive tactics such as sneaky mating behaviour and greater agility developed by small males have evolutionary significance (Stockley et al., 1994). Whether male *E. kuehniella* emerged from high and low densities employ different mating tactics is not yet known.

We found that the lengths and allometries of the valva and aedeagus did not vary among treatments (Fig. 3b and d), which is consistent with the prediction that genitalia are insensitive to changes in environment and body mass (Tadler, 1999). In treehoppers *Enchenopa binotata* (Rodriguez and Al-Wathiqui, 2011), water striders *Aquarius remigis* (Bertin and Fairbairn, 2007) and the dung beetle *Onthophagus taurus* (Simmons et al., 2009) the weakest effects of environmental variation were on genital traits. This suggests that genital structures are selectively constrained and observations of several insect species confirm that natural and artificial genitalic variation has a strong influence on reproductive success (Koshio et al., 2007; Moreno-Garcia and Cordero, 2008; Stoffolano et al., 2000; Takami, 2003; Takami and Sota, 2007). Therefore, it is most likely that males with appropriate valva and adeagus length have an advantage and may be successful in intra and inter-sexual competition.

5. Conclusions

In *E. kuehniella* adult body mass, sizes of wing, head and thorax, but not lengths of valva and aedeagus of males were affected by larval growth conditions. Adults emerged from larvae reared at population densities of 400 & 800 had longer wings relative to their body mass that may facilitate dispersal. Eupyrene sperm production decreased with increased population density, however, the numbers of apyrene sperm and matings did not differ between the densities. There were no differences in the allometries of valva and aedeagus. Together these data suggests that males maintain their investment in traits that have a drastic effect on intromission success, however, gamete production and shifts in morphological trait expression occur in a manner that increases male reproductive success in a given environment.

Acknowledgements

Qiao Wang assisted with experimental design, Kay Sinclair helped with *E. kuehniella* colony establishment and Tracy Harris provided assistance with photographs. This research was partially supported by Massey University Doctoral Scholarship and funds from the Institute of Agriculture and Environment, Massey University.

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