Larval developmental temperature and ambient temperature affect copulation duration in a seed beetle

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Abstract

The effects of temperature on cellular, systemic and whole-organism processes can be short-term, acting within seconds or minutes of a temperature change, or long-term, acting across ontogenetic stages to affect an organism's morphology, physiology and behavioural phenotype. Here we examine the effect of larval development temperature on adult copulatory behaviour in the bruchid beetle, *Callosobruchus maculatus*. As predicted by temperature's kinetic effects, copulation duration was longest at the lowest ambient temperature. However, where ambient temperature was fixed and developmental temperature experimentally varied, males reared at the highest temperature were least likely to engage in copulation, whilst those reared at the lowest temperature copulated for longer. Previous research has shown males reared at cooler temperatures inseminate fewer sperm. Thus, in this species longer copulations are associated with reduced sperm transfer. We argue that knowledge of preceding ontogenetic conditions will help to elucidate the causes of variation in copulatory behaviour.

Keywords

mating behaviour, *Callosobruchus maculatus*, ectotherms, developmental plasticity, sperm transfer, thermal heterogeneity.

1. Introduction

The rate at which biochemical reactions take place is constrained by temperature, which in due course affects the rates of cellular, systemic and whole-organism processes, including the behavioural phenotype (Gillooly

et al., 2001; Angilletta, 2009; Abram et al., 2017). In ectothermic animals, the rates at which behaviours are performed increase with increasing temperature up to a thermal optimum, thereafter decreasing to a point at which the behaviour can no longer be performed (Huey & Stephenson, 1979; Abram et al., 2017). Temperature's kinetic effects can also be seen in the ontogenetic development of an organism. Changes in temperature during development can alter the pattern of resource allocation to different functions resulting in long-term changes in the adult phenotype (Deeming & Ferguson, 1991; Deeming, 2004; Angilletta, 2009; Abram et al., 2017). Such developmental plasticity can have wide-ranging consequences for adult behaviour including locomotion, foraging, learning and reproduction (Deeming, 2004; Abram et al., 2017; Matsubara et al., 2017).

One behaviour that has rarely been studied in relation to ontogenetic environment is copulation duration. This is surprising because most ectothermic animals are exposed to a wide range of temperatures during development (Kvist et al., 2013; Penttilä et al., 2013) and since the pioneering work of Parker (1970a, b) copulation duration is generally perceived as a high fitness trait (Kelly & Jennions, 2011). Intraspecific variation in the duration of copula is substantial and has been associated with variation in the biotic environment experienced by adults, including male and female size, the operational sex ratio, risk of sperm competition, and male and female mating status, i.e., whether they are virgin or not (Simmons, 2001). It has also been associated with variation in ambient temperature (Wang et al., 2016; Delisle et al., 2016), exposure to heat shock (Zhang et al., 2016), and photoperiod (Wang et al., 2013).

Biological variation in the developmental environment can also affect the ontogeny, and ultimately the function of primary reproductive traits. For example, in the moth *Plodia interpunctella* (Gage, 1995) and the dung fly *Scatophaga stercoraria* (Stockley & Seal, 2001) an increase in larval density resulted in greater resource allocation to testes and ejaculatory traits. In strains of the bruchid beetle *Callosobruchus chinensis*, characterised by high female polyandry, increased larval density resulted in males transferring a greater number of sperm during longer copulations (Yamane & Miyatake, 2005). Variation in the abiotic environment during ontogeny can also affect reproductive trait development. It has been known for many years that extreme developmental temperatures can induce male sterility in Drosophilid fruit flies (David et al., 2005; Rohmer et al., 2004), while in the dung fly

S. stercoraria, the guppy *Poecilia reticulata*, the land snail *Arinata arbustorum*, and the bruchid beetle *C. maculatus* (Blanckenhorn & Hellriegel, 2002; Breckles & Neff, 2013; Minoretti et al., 2013; Vasudeva et al., 2014) developmental temperature is known to affect spermatogenesis. These developmentally derived plastic changes in traits associated with the production of functional ejaculates are likely to be matched by corresponding alterations to copulatory behaviour. However, this has rarely been tested beyond whether copulation is more or less likely (Delisle et al., 2016; Westerman & Monterio, 2016; Taylor et al., 2017) and to our knowledge only a handful of studies have reported the effects of developmental temperature on the duration of copulation. In the butterfly *Bicyclus anynana*, dry season males (associated with cool developmental temperatures) produce resource-rich ejaculates (Prudic et al., 2011) and copulate for longer than warm-reared (wet season) males (Westerman & Monterio, 2016) and in *C. maculatus* pre-imaginal exposure to heat shock resulted in a 25% increase in the duration of copulation (van Lieshout et al. 2014). However, in the predatory mite *Neoseiulus californicus*, developmental temperature had no effect on subsequent mating behaviour (Nguyen & Amano, 2010).

To address this shortfall, we report the results of a study into the effects of developmental temperature on subsequent copulatory behaviour in *Callosobruchus maculatus* (Coleoptera: Bruchinae). In *C. maculatus* copulation follows two phases (Eady, 1994): the start-to-kick phase is characterised by a quiescent period following the genital union of the male and female, and the kick-to-end phase is typified by female's vigorously kicking at the mounted male with her hind legs until genital union is terminated. Ejaculate transfer appears to occur during the quiescent phase before female kicking begins (Dougherty & Simmons, 2017). Vasudeva et al. (2014) previously reported that males from larvae reared at temperatures above and below their optimal thermal range (Stillwell et al., 2007) inseminated fewer sperm and it is known that in this species, sperm-limited males remain in copula for longer (Eady & Brown, 2017). Therefore, we predict that males experiencing either high or low developmental temperatures will remain in copula for longer during the 1st phase of copulation (when ejaculate transfer takes place) and in total, in comparison to males reared at intermediate temperatures that are closer to their developmental optimum.

2. Material and methods

2.1. Study population

The *Callosobruchus maculatus* beetles used in this study were derived from a large, outbred population (approximately 5000 adults) cultured for 24 generations on moth beans (*Vigna aconitifoli*) in an insectary maintained at 27°C, 32% relative humidity and a 16 hours light and 8 hours dark photoperiod (Vasudeva et al., 2014). The parental stock population originated from Niamey, Niger, and had been kept on black-eyed beans (*Vigna ungliculata*) for more than 100 generations at the University of Lincoln. Moth beans were used instead of larger black-eyed beans because only one adult tends to emerge from each seed.

2.2. Ambient temperature and copulation

The effect of ambient temperature on copulation duration was investigated by initially rearing (from eggs) larvae in the insectary at 27°C. Approximately 1000 adults (estimated by mass) were housed with 200 g of moth beans (about 7500 beans) for one hour at 27°C. After one hour, the adults were sieved from the beans and the remaining egg-laden beans incubated at 27°C. Just prior to adult eclosion the beans were transferred into the individual cells of 25 cell replidishes (Sterilin™, www.fishersci.co.uk) and sealed with glass lids, from which emergent beetles could be collected and adult virginity ensured. Adults (0–2 h old) were randomly transferred into separate incubators (Lucky Reptile Herp Nursery II, The Reptile Shop, Morecambe, UK) maintained at 17, 25, 27 or 33°C for 24–48 h before being paired in clear Perspex (50 mm diameter) Petri dishes. These temperatures were chosen as previously we have shown this temperature range to affect spermatogenesis within this species (Vasudeva et al., 2014) and because they are within the natural range of temperatures experienced by *C. maculatus* in field conditions (Germain et al., 1987). The likelihood of engaging in copulation within 30 min of observation and, if successful, the duration of copulatory phases were recorded through the window of the incubator. Copulation duration was recorded as the time (in seconds) from the onset of genital coupling to the onset of female kicking behaviour (Phase I) and from the onset of female kicking behaviour to genital uncoupling (Phase II). The sample sizes were; 17°C ($N = 17$), 25°C ($N = 14$), 27°C ($N = 15$) and 33°C ($N = 15$).

2.3. Larval rearing temperature: development and copulation

To determine the effect of temperature on egg-to-adult development, eggs and subsequent larvae were raised in incubators set at 17, 25, 27 and 33°C. Initially egg-laden seeds were collected from the stock 27°C population as described above and transferred to the appropriate incubator on the day they were laid. In order to compare the growth rates of larvae, a sample of egg-laden beans from each temperature regime were cracked open at intervals (dependent on development time) and the larvae removed from the seeds and their length determined under a stereo-microscope, before being euthanised. Larvae reared at 25, 27 and 33°C were sampled every two days following the appearance of the first larval instar, whilst those reared at 17°C were sampled at intervals of between 2–8 days (reflecting the large difference in development time, see results), with an average of 8 larvae being measured at each time period.

To obtain virgin adults for the copulation assays, egg-laden seeds were plated out into the individual cells of 25-cell replidishes just prior to adult eclosion. Post emergence, the adults were sexed and maintained at the same temperature as larval development until 24–48 h old. Virgin males reared at different environmental temperatures (17, 25, 27 and 33°C) were then moved to the insectary (maintained at 27°C) for 1 h to reach thermal equilibrium before being paired to virgin females (24–48 h from eclosion) derived from the 27°C stock population, in 50 mm Petri dishes. The likelihood of engaging in copulation within 30 min of observation was recorded and, if successful, the duration of the copulatory phases was recorded as described above. The sample sizes for this experiment were 17°C ($N = 20$), 25°C ($N = 22$), 27°C ($N = 21$) and 33°C ($N = 21$).

2.4. Statistical analysis

Likelihood of engaging in copulation was analysed using a binary logistic regression. The durations of the copulatory phases were Log_{10}-transformed prior to being analysed via a one-way analysis of variance (ANOVA) on SPSS version 20 (IBM) and statistically significant ($p < 0.05$) differences between groups were established using post-hoc Tukey test. Significant differences between the different ambient and larval temperatures are indicated with an asterisk (*) across both the experiments. Graphs were created in R (R Core Team, 2015) using ggplot2 (Wickham & Chang, 2015).

3. Results

3.1. Effects of ambient temperature on copulation

For beetles initially raised at 27°C, the ambient temperature at which mating took place affected the likelihood of copulation and the duration of copulation. Copulation was less likely to occur at the highest ambient temperature with all males copulating at 25 and 27°C, but only 88 and 75% of males achieving copulation at 17 and 33°C, respectively ($\chi_2 = 8.95$, df = 3, $p = 0.03$). The duration of the start-to-kick phase was longest when copulation occurred at 17°C, being approximately two times longer than the durations of this phase recorded at 25, 27 and 33°C, which were statistically equivalent (ANOVA: $F_{3,60} = 27.37$, $p < 0.0001$, effect size = 0.59, Figure 1). By contrast, the kick-to-end phase was unaffected by ambient temperature (ANOVA: $F_{3,60} = 1.61$, $p = 0.198$, effect size = 0.08, Figure 1). However, since the duration of the first phase of copula equates to 60–70% of the total duration of copulation, it was not surprising that total copula-

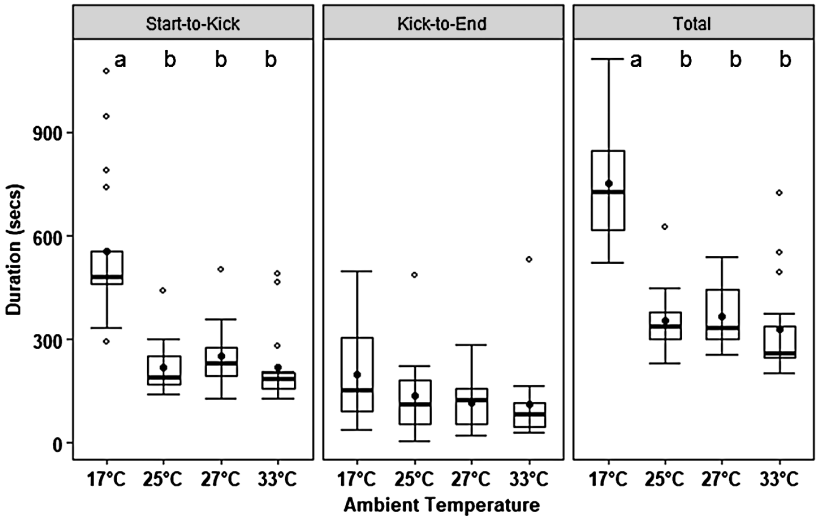

Figure 1. Box-whisker plots of untransformed durations (s) of the start-to-kick phase, kick-to end phase and total copulation in relation to ambient temperature. Thick horizontal lines indicate the median, bold dots the mean, boxes the 1st and 3rd quartiles and whiskers the upper (maximum) and lower (minimum) values that are not outliers. Outliers are shown as small open circles. Box plots with different superscripts indicate significant differences ($p < 0.05$) between groups as determined by post-hoc Tukey tests on the transformed data.

tion duration was longest for those beetles that mated at 17°C (ANOVA: $F_{3,60} = 35.69$, $p < 0.0001$, effect size = 0.65, Figure 1).

3.2. Effects of larval rearing temperature on copulation

Larval growth followed the usual growth trajectory of holometabolous insects: a period of exponential growth up to a critical size after which growth slows but continues up to a peak size (denoted by arrows in Figure 2) after which feeding and growth cease as metamorphosis proceeds (Nijhout et al., 2006). Egg-to-adult development at 17°C took on average 87 days whilst at 33°C it was just 22 days. The thermal environment at which egg-to-adult development took place affected the likelihood of males engaging in copulation with virgin stock (27°C) females with 100% of males developed at 27°C engaging in copulation but 90, 83 and 67% males copulating from the 17, 25 and 33°C treatments, respectively ($\chi_2 = 12.35$, df = 3, $p = 0.006$). Despite copulation being observed at the same ambient temperature, the start-to-kick phase was longest for those beetles reared at 17°C (ANOVA: $F_{3,83} = 6.44$, $p = 0.0005$, effect size = 0.19, Figure 3). The kick-to-end phase was unaffected by larval rearing environment (ANOVA: $F_{3,83} = 0.27$, $p = 0.84$, effect size = 0.01, Figure 3). However, when the two phases were combined

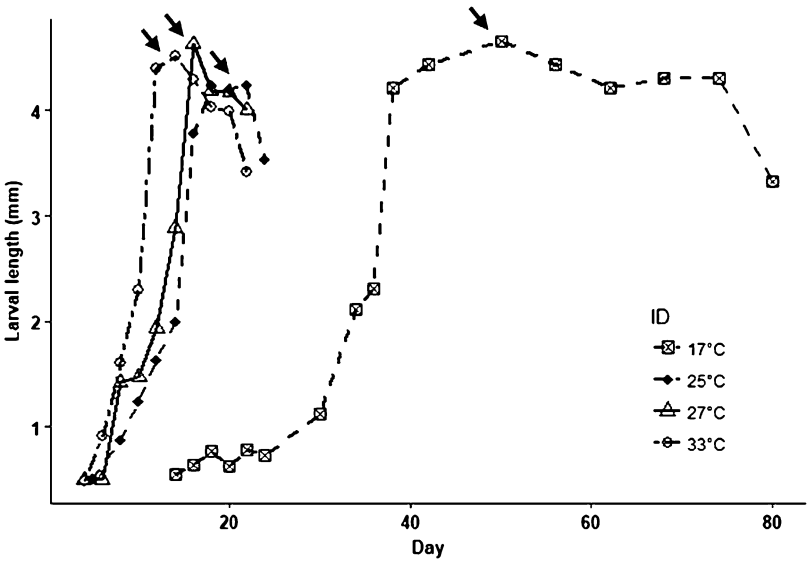

Figure 2. Total larval length (mm) as a function of the time elapsed since egg laying in relation to developmental temperature. Arrows indicate peak size (see text for details).

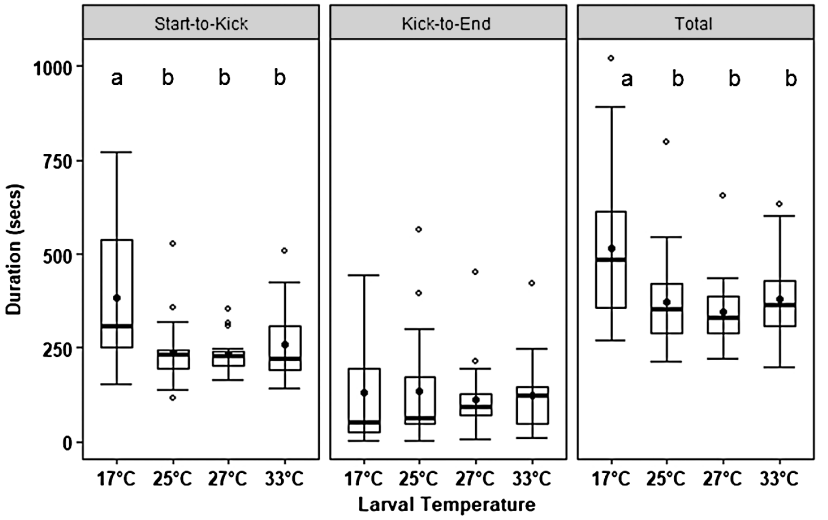

Figure 3. Box-whisker plots of untransformed durations (s) of the start-to-kick phase, kick-to end phase and total copulation in relation to development temperature. Thick horizontal lines indicate the median, bold dots the mean, boxes the 1st and 3rd quartiles and whiskers the upper (maximum) and lower (minimum) values that are not outliers. Outliers are shown as small open circles. Box plots with different superscripts indicate significant differences ($p < 0.05$) between groups as determined by post-hoc Tukey tests on the transformed data.

the total duration of copulation was greatest in those males that were reared at the lowest developmental temperature (ANOVA: $F_{3,83} = 5.19$, $p = 0.002$, effect size $= 0.16$, Figure 3).

4. Discussion

Egg-to-adult development was longest at the lowest developmental temperature, and development time accelerated as rearing temperature progressively increased. Both ambient and developmental temperature affected the likelihood of males engaging in copulation and the duration of copulation. Males were least likely to engage in copulation at the highest ambient temperature and the highest developmental temperature. Similar effects of temperature on mating likelihood have been reported in the hemlock looper *Lambdina fiscellaria* (Delisle et al., 2016) and the polyphenic butterfly *B. anynana* (Westerman & Monterio, 2016), whilst in *Drosophila pseudoobscura* and *Drosophila melanogaster* rearing temperature had no effect on the likelihood of female re-mating (Taylor et al., 2017; Pavković-Lučić & Kekić, 2013). Va-

sudeva et al. (2014) reported that male *C. maculatus* reared at 33°C had the smallest relative and absolute testes size in comparison to males reared at cooler temperatures. Thus, thermal stress associated with high developmental temperature appears to impact negatively on both reproductive physiology and behaviour in this species.

Given the kinetic effects of temperature on ectothermic animals it was not surprising that adult beetles reared within the optimum temperature range of 25–30°C (Stillwell et al., 2007) but subsequently paired with females at 17°C had the longest copulation duration. However, when adults mated under a constant 27°C but experienced different thermal regimes during egg-to-adult development, copulation duration was longest for those beetles reared at the coolest temperature (17°C). This increase in the duration of copulation could be explained as entirely due to an increase in the first phase of copulation as rearing temperature had no effect on the duration of the kick-to-end phase of copulation. In *C. maculatus*, sequentially mating males to females results in a dramatic decline in ejaculate size (Fox et al., 1995; Savalli & Fox, 1999), the number of sperm transferred (Eady, 1995), and an increase in the duration of the first phase of copulation (Eady & Brown, 2017), which is when ejaculate transfer takes place (Dougherty & Simmons, 2017). In essence, ejaculate-limited males take longer to elicit the kicking behaviour of females, which is possibly triggered by stretch receptors in the wall of the bursa copulatrix, where the ejaculate is received (Eady, 1994; Dougherty & Simmons, 2017). Our finding that the first phase of copulation was longest when developmental temperature was lowest is consistent with this mechanism because Vasudeva et al. (2014) found males reared at 17°C transferred significantly fewer sperm at copulation than males reared at higher temperatures (see also van Lieshout et al., 2014). With regard to the kick-to-end phase of copulation, Eady & Brown (2017) found no effect of sequential mating, and hence sperm limitation, on the duration of this phase of copulation. This suggests that the duration of the kicking phase of copula is unrelated to sperm transfer (see also Dougherty & Simmons, 2017). The non-significant effect of larval rearing temperature on the duration of the second phase of copulation reported here would support this suggestion.

In the present study, copulation duration of adults reared at 27°C increased with decreasing ambient temperature (i.e., 17°C). Again, this appears to be entirely due to an increase in the first phase of copulation. The inverse relationship between temperature and the duration of phase I of copulation may

reflect the fact that this is when the ejaculate transfer takes place (Dougherty & Simmons, 2017). It is reasonable to assume that the rate at which sperm and seminal fluid are transferred is likely to become progressively slower with decreasing ambient temperature and, in line with earlier arguments, is likely to result in a longer latency to female kicking. The transfer of seminal fluid at low temperatures could be constrained by physiology because metabolism slows at lower temperature, and/or fluid viscosity because fluids become more viscous at lower temperatures (Purchase et al., 2010). Why ambient temperature had no effect on the duration of phase II of copulation is surprising given the effect of temperature on metabolic rate and thus activity (Nijhout, 2003; Kingsolver et al., 2004; Angilletta, 2009). One possible explanation for this could be that the termination of copulation is under the influence of both male and female attributes (Eady & Brown, 2017). Thus, at low temperatures females kick with less intensity/frequency but the male's ability to maintain genital contact is also reduced, producing an overall neutral effect of ambient temperature on the duration of phase II of copulation. An increase in phase I and no effect on phase II of copulation, resulted in an extended total copulation duration at lower ambient and rearing temperatures. A number of studies have shown copulation duration to increase with decreasing ambient temperature (Gering, 1953; Rovner, 1971; Cook, 1994; Horton et al., 2002) including the bruchid beetle *C. chinensis* in which longer copulations at lower temperatures were associated with greater sperm transfer (Katsuki & Miyatake, 2009).

The studies discussed above suggest the relationship between temperature, copulation duration and sperm transfer is not straightforward. For example, in *D. subobscura* male fertility is a product of population origin, larval temperature, adult temperature and their interaction (Porcelli et al., 2017). In addition, medium-term kinetic effects (reversible acclimation; Abram et al., 2017) can also affect reproductive performance; Jørgensen et al. (2006) found 'hardening' (the pre-treatment exposure to heat stress) reduced the subsequent effects of heat stress on male fertility and success in obtaining copulations in *Drosophila buzzatii*. Such interrelationships are further complicated by the fact that copulation is essentially an interaction between the behavioural, morphological and physiological traits of males and females (Eberhard, 1996; Eady & Brown, 2017). Here we have focused on the effect of male developmental temperature on copulatory behavior, primarily based on the observation that developmental temperature affects resource

allocation to ejaculatory traits (Vasudeva et al., 2014). However, developmental temperature can also affect the ontogeny of female reproductive traits (Berger et al., 2011), thus it is likely that the developmental environment experienced by males and females will impact on the duration of copulation (although see Westerman & Monterio, 2016). Therefore, we propose that future studies elucidate the relationship between male and female developmental temperature on copulatory behaviour. This is important because in nature the developmental environment can fluctuate dramatically. For example, Penttilä et al. (2013) report that dung pat temperatures can range from 10 to 35°C over a matter of days, thus exposing dung fly (*S. stercoraria*) larvae to a wide range of temperatures during their development. Recent studies suggest this level of variation could impact on the reproductive anatomy and physiology of subsequent ontogenetic stages (Blanckenhorn, 2000; Blanckenhorn & Hellriegel, 2002; Blanckenhorn & Henseler, 2005) and ultimately their reproductive behaviour.

Acknowledgements

We thank Maya Krishnan Kumar for help with 'R' and 'ggplot' and University of Lincoln for the financial support to RV to conduct the doctoral research.

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