J E B	12431	WILEY	Dispatch: 21.5.14	CE: Gowthaman S.
Journal Code	Manuscript No.		No. of pages: 8	PE: Amudhapriya

doi: 10.1111/jeb.12431

Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in *Callosobruchus maculatus*

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Keywords:

ontogeny;
phenotypic plasticity;
primary reproductive traits;
reaction norm;
sexual selection.

Abstract

The outcome of post-copulatory sexual selection is determined by a complex set of interactions between the primary reproductive traits of two or more males and their interactions with the reproductive traits of the female. Recently, a number of studies have shown the primary reproductive traits of both males and females express phenotypic plasticity in response to the thermal environment experienced during ontogeny. However, how plasticity in these traits affects the dynamics of sperm competition remains largely unknown. Here, we demonstrate plasticity in testes size, sperm size and sperm number in response to developmental temperature in the bruchid beetle *Callosobruchus maculatus*. Males reared at the highest temperature eclosed at the smallest body size and had the smallest absolute and relative testes size. Males reared at both the high- and low-temperature extremes produced both fewer and smaller sperm than males reared at intermediate temperatures. In the absence of sperm competition, developmental temperature had no effect on male fertility. However, under conditions of sperm competition, males reared at either temperature extreme were less competitive in terms of sperm offence (P_2), whereas those reared at the lowest temperature were less competitive in terms of sperm defence (P_1). This suggests the developmental pathways that regulate the phenotypic expression of these ejaculatory traits are subject to both natural and sexual selection: natural selection in the pre-ejaculatory environment and sexual selection in the post-ejaculatory environment. In nature, thermal heterogeneity during development is commonplace. Therefore, we suggest the interplay between ecology and development represents an important, yet hitherto underestimated component of male fitness via post-copulatory sexual selection.

Introduction

Since the pioneering work of Parker in the 1970s (Parker, 1970) and the application of DNA fingerprinting in the 1980s (Burke, 1989), it has become apparent that female monogamy is the exception rather than the rule (Smith, 1984; Eberhard, 1996; Birkhead & Møller, 1998; Simmons, 2001). The post-copulatory sexual selection generated by female promiscuity has provided a valuable framework for the evolutionary interpretation of often bizarre behavioural, morphological and physiological traits that are directly involved in reproduction, such as the giant sperm of *Drosophila bifurca*, that measure about 20 times longer than the fly itself (Pitnick *et al.*, 1995).

At its simplest level, post-copulatory sexual selection requires a female to mate with two males within a single reproductive event. However, predicting the outcome of this three-player interaction has proved difficult because it depends on characteristics of the female (Wilson *et al.*, 1997; Miller & Pitnick, 2002; Simmons & Kotiato, 2007), the 1st male to mate, the 2nd 4 male to mate, the interaction between the ejaculates of the two males and male–female interactions that can be

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manifest at the level of molecules (Swanson & Vacquier, 2002) through to populations (Kelly & Jennions, 2011). This picture is further complicated by the fact that sperm have to function in two environments: the pre-ejaculatory environment of the male reproductive organs and the post-ejaculatory environment. Thus, heterogeneity in one or both environments is likely to influence the structure and function of ejaculates and the subsequent dynamics of post-copulatory sexual selection (Morrow *et al.*, 2008).

Environmental sex determination (Valenzuela & Lance, 2004) is testament to the profound influence of the ontogenetic environment on the development of primary reproductive traits and it is perhaps surprising that relatively few studies have examined the effect of developmental environment on sperm structure and function (Sharpe, 2010). Those that have point to sperm morphology being remarkably canalized; variation in nutrition and/or density (socio-sexual environment) frequently affects sperm number (Kelly & Jennions, 2011) but rarely affects sperm size (Gage & Cook, 1994; Hellriegel & Blanckenhorn, 2002; Amitin & Pitnick, 2006; Gay *et al.*, 2009; although see Crean & Marshall, 2008). An exception to this appears to be environmental temperature; in the dung fly *Scathophaga stercoraria*, males that experienced intermediate or high temperatures during development produced the largest sperm (Blanckenhorn & Hellriegel, 2002), whereas in the land snail, *Arianta arbustorum* (Minoretti *et al.*, 2013), and the guppy, *Poecilia reticulata* (Breckels & Neff, 2013), sperm length was greatest when developmental temperature was reduced.

Thermal plasticity in female reproductive architecture has also been reported in the dung fly (Berger *et al.*, 2011; Schäfer *et al.*, 2013); the frequency at which females expressed four as opposed to three spermathecae increased with developmental temperature (Berger *et al.*, 2011). Such plasticity is likely to be particularly relevant to the dynamics of post-copulatory sexual selection because females tend to set the rules by which sperm competition is played out (Eberhard, 1996). For example, in *Drosophila melanogaster*, artificial selection for seminal receptacle length drives the co-evolution of sperm length via sperm–female interactions during sperm competition; males with long sperm outcompeted males with short sperm, but only when the competition occurred in females with long seminal receptacle lengths (Miller & Pitnick, 2002; see also Simmons & Kotiato, 2007). Correlated evolution of this type is also apparent in numerous comparative studies (Snook, 2005; Rugman-Jones & Eady, 2008).

Plasticity in male and female primary reproductive traits may represent an important component of their evolution. It has been argued that plasticity promotes the accumulation of cryptic genetic variation 'hidden' in developmental and metabolic pathways that is subsequently released and exposed to selection during environmental change, resulting in rapid and divergent evolution (Lande, 2009; Pfennig *et al.*, 2010; Espinosa-Soto *et al.*, 2011; Gomez-Mestre & Jovani, 2013). However, despite renewed interest in the role of phenotypic plasticity in the process of evolution and the demonstration of plasticity in several ejaculatory traits (Gage, 1991; Blanckenhorn & Hellriegel, 2002), few studies have examined how this plasticity affects the dynamics of post-copulatory sexual selection. Here, we expose the bruchid beetle, *Callosobruchus maculatus*, to a range of temperatures during larval development and report their effects on ejaculatory traits and the outcome of sperm competition.

Materials and methods

Study organism

Callosobruchus maculatus is a cosmopolitan pest of legumes. Following copulation, females oviposit on the surface of suitable host seeds. Upon hatching, the larvae burrow through the base of the egg and directly enter the seed cotyledon where they pass through four larval instars before pupating, with overall egg-to-adult development taking ~21 day at 27 °C (Devereau *et al.*, 2008). Females tend to mate with more than one male under laboratory conditions (Eady *et al.*, 2000). Males transfer large ejaculates at copulation (Fox *et al.*, 1995), which probably accounts for the last-male sperm precedence observed in this species (Eady, 1991, 1995).

The wild-type beetles used in this study were originally derived from Niamey, Niger, and have been in culture for ~16 years, or about 200 generations at 27 °C, ~35% RH and a 16L:8D photoperiod. After ~10 years, a mutant black-line culture was established from the original wild-type population. Black-line males and females are entirely black and breed true when mated. By contrast, black-line females mated to wild-type males produce dark-tan offspring that have distinctive tan forelegs and tan tips to their antennae.

Sperm number and size

Approximately 1000 wild-type adults (estimated by mass and derived from the stock culture) were housed with 200 g (~7500 beans) of moth beans (*Vigna aconitifolia*) for one hour at 27 °C, during which females laid eggs on the beans. Approximately 1500 egg-laden beans were placed into four triple vent 110-mm Petri dishes (Fisherbrand, www.fisher.co.uk), before being transferred to one of four separate incubators (Lucky Reptile Herp Nursery II, The reptile shop, UK) set at 17, 25, 27 or 33 °C. These temperatures reflect the cosmopolitan nature of the species distribution and the temperature fluctuations likely to be experienced in bean stores (Appleby & Credland, 2007).

Prior to adult emergence, the beans were plated out into the individual cells of 25 cell repli-dishes (one bean per cell) and sealed with a glass lid. As adults emerged from the seeds, they were isolated within the cells ensuring virginity. Moth beans only have sufficient resources to support one developing larvae, thus only one adult per bean generally eclosed. The adult beetles were kept in their respective environments until they were 24–48 h old. A subset of these beetles were euthanized and stored in a freezer (−5 °C) for later dissection. Another subset of male beetles were permitted a single copulation with a virgin female from the wild-type stock culture, raised at 27 °C. Immediately following the termination of copulation, pairs were frozen for subsequent dissection to determine the number of sperm transferred at copulation.

Sperm number was determined for 17–20 males per treatment across two replicates. The spermatophore was dissected from the female bursa copulatrix (Eady, 1994), before being transferred to 100 μL of insect saline mixed with liquid biological detergent (ratio 10 : 1) to free the sperm from the seminal fluid. The solution was mixed with an entomological pin for 60 s after which 20 μL was transferred to a haemocytometer and the number of sperm heads determined. Three measures per sample were taken (intraclass correlation coefficient $r_I = 0.83$, $F_{39,80} = 16.1$, $P < 0.0001$).

Testes size and sperm length were determined following the methods of Gay *et al.* (2009). Briefly, testes were dissected free under insect saline, and digital images captured under an Olympus SZH dissecting microscope prior to measuring their cross-sectional area using IMAGE J (Abramoff *et al.*, 2004). The testes were then ruptured under saline to release sperm. Ten randomly selected sperm were viewed using a Nikon Eclipse E600 microscope, and sperm length measured using the segmented hand tool of IMAGE J. Measures of testes size and sperm length have previously been shown to be highly repeatable in this species (Rugman-Jones & Eady, 2008). Male elytra length was measured as an estimate of male size (Wilson & Hill, 1989).

Sperm competition

Male fertilization success was determined using the genetic marker technique (Eady, 1991). Essentially, virgin black-line females (raised at 27 °C) were mated to virgin black-line males (raised at 27 °C) followed by virgin wild-type treatment males (and *vice versa*) such that the P_2 and P_1 of the wild-type treatment males could be determined. The sperm competition experiment ran over an 8-week period because of the large difference in development times of the wild-type treatment males reared at 17, 27 or 33 °C (development time ~80, 25 and 21 day, respectively). In order to stagger the emergence of black-line adults, batches of fresh moth beans were housed with the black-line culture every 24 h. To determine the effect of developmental temperature on P_2, virgin black-line females were permitted a single copulation with a virgin black-line male. Following copulation, females were housed in individual Petri dishes containing 30 moth beans for 24 h prior to being mated to a virgin wild-type male derived from either the 17, 27 or 33 °C treatment ($n = 20$ per treatment). After the second copulation, the female was transferred to an individual Petri dish containing 30 fresh moth beans on which to oviposit until her natural death. The egg-laden beans were incubated at 27 °C in the insectary until the imagos emerged. For estimates of P_1, the above protocol was repeated except that black-line females were mated first to a wild-type treatment male before being re-mated to a black-line stock male. Mean P_2 and P_1 values were calculated from the ratio of black to dark-tan offspring (Eady, 1991).

Statistical methods

Testes size, sperm number and sperm length were analysed using a two-way ANOVA with treatment (temperature) as a fixed factor and replicate as a random factor. All interaction terms were nonsignificant ($P > 0.05$) and thus removed from the models. Data on offspring number were analysed using a log-linear model, whereas P_1 and P_2 data were analysed using logistic regression with a binomial error distribution and a logit link function. In both analyses, division of the residual scaled deviation by the residual degrees of freedom indicated overdispersion, thus statistical significance was evaluated using *F* tests (Crawley, 2002).

Results

Males reared at the highest temperature had the smallest absolute testes size (Fig. 1): two-way ANOVA developmental temperature ($F_{3,70} = 9.38$, $P < 0.0001$) and replicate ($F_{1,70} = 2.28$, $P = 0.14$). However, developmental temperature ($F_{3,70} = 12.54$, $P < 0.0001$) but not replicate ($F_{1,70} = 0.45$, $P = 0.5$) also affected body size, such that males reared at the lowest temperature were the largest adults (*post hoc* $P < 0.05$: mean elytra length (±SE) 17 °C = 2.13 ± 0.03, 25 °C = 1.84 ± 0.03, 27 °C = 1.94 ± 0.03 and 33 °C = 1.81 ± 0.05). Subsequently, ANCOVA revealed a significant effect of elytra length ($F_{1,70} = 6.89$, $P = 0.011$) and developmental temperature ($F_{3,70} = 7.46$, $P < 0.0001$) on testes size, with *post hoc* analyses showing the 33 °C males to have the smallest relative testes ($P < 0.05$).

The number of sperm transferred at copulation varied in relation to developmental temperature, with males inseminating fewest sperm when incubated at 17 °C (Fig. 2a). A two-way ANOVA revealed a significant effect of treatment ($F_{3,100} = 7.91$, $P < 0.0001$) but not replicate ($F_{1,100} = 1.73$, $P = 0.19$). Sperm size also varied with respect to developmental temperature but not

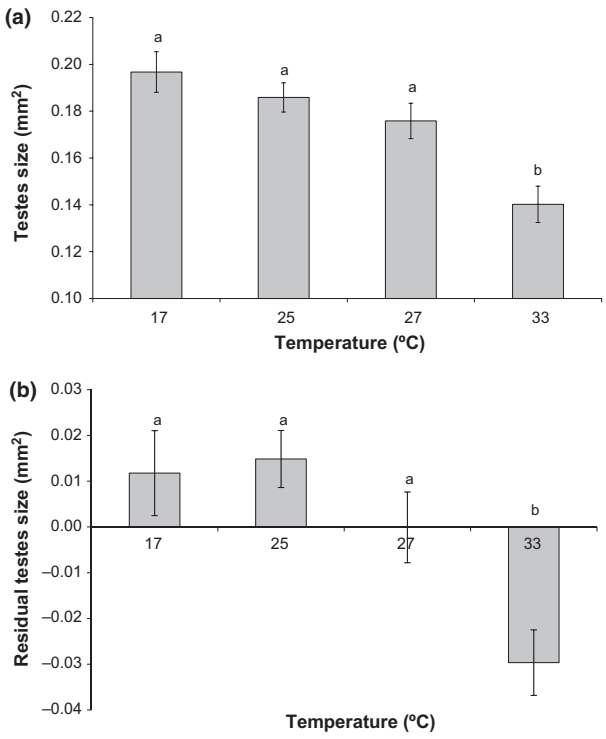

Fig. 1 Mean (±SE) absolute (a) and relative (b) testes area (mm^2) in relation to developmental temperature. Treatments with different superscripts represent significant differences ($P < 0.05$) following *post hoc* tests.

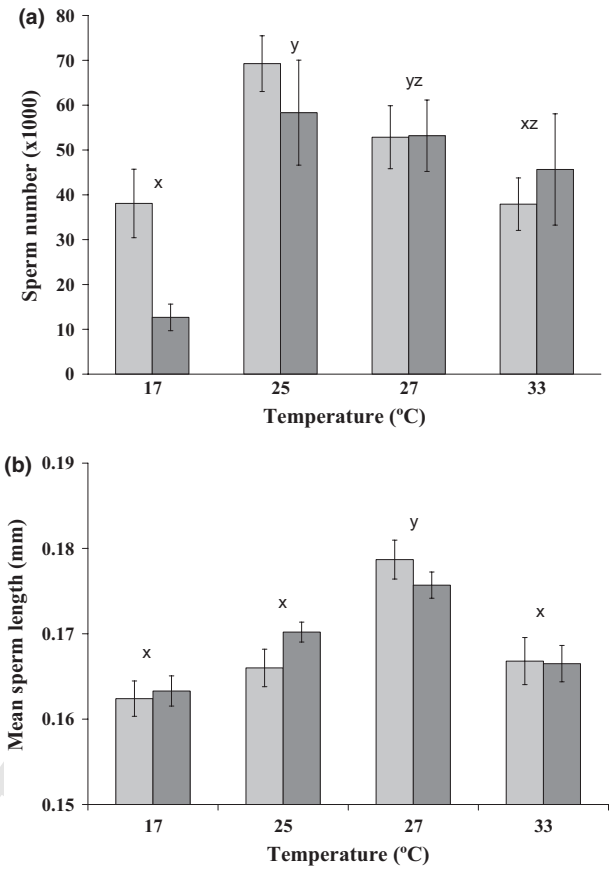

Fig. 2 Mean (±SE) number of sperm transferred (a) and sperm length (b) in relation to developmental temperature. Differently shaded bars represent replicate #1 and replicate #2, respectively. Treatments with different superscripts represent significant differences ($P < 0.05$) following *post hoc* Tukey tests.

replicate; two-way ANOVA treatment ($F_{3,74} = 17.53$, $P < 0.0001$) and replicate ($F_{1,74} = 0.11$, $P = 0.74$). Males reared at either temperature extreme produced small sperm relative to those reared at 27 °C (Fig. 2b).

Males reared at 17 °C were less likely to copulate with the black-line females (41% failed to copulate) in comparison with those reared at 27 and 33 °C (11% and 15%, respectively; $\chi^2 = 12.7$, d.f. = 2, $P < 0.01$). However, if copulation was observed, the mean (±SE) number of offspring produced by the black-line females in the 24 h following a single copulation with 17, 27 or 33 °C wild-type males was 22.5 (±2.7), 25.8 (±2.6) and 26.1 (±1.3), respectively (GLM log-linear regression: Δ deviance = 6.85, $F_{2,62} = 0.91$, $P = 0.4$). This suggests that developmental temperature had no effect on male fertility in the absence of sperm competition.

However, under conditions of sperm competition, males reared at 17 and 33 °C performed poorly in comparison with those reared at 27 °C with regard to sperm offence (P_2) (GLM binomial logistic regression: Δ deviance = 290, $F_{2,69} = 23.2$, $P < 0.0001$; *post hoc* comparison of model fit following systematic collapse of factor levels revealed all three levels to be significantly different from each other at $P < 0.05$). The sperm of males reared at 17 °C also performed poorly in terms of sperm defence (P_1) (Δ deviance = 109, $F_{2,62} = 7.3$, $P = 0.0014$; *post hoc* analysis revealed 17 °C males to have lower P_1 than both 27 and 33 °C males, the P_1 of which were statistically equivalent, Fig. 3). When reared at 27 °C, the P_2 of treatment males = 0.89, similar to that reported previously in this species (Eady, 1991, 1994).

Discussion

Adult size followed the temperature-size rule (Bergmann, 1847; Atkinson, 1994), such that males reared at the highest temperature eclosed at the smallest body size. Males reared at the highest temperature also had smaller testes (both absolute and relative), suggesting high-temperature stress is associated with a reduced investment in testes. Thermal stress also affected ejaculatory traits. Males reared at the highest and lowest temperatures produced fewer and shorter sperm than those reared at intermediate temperatures. This could represent a developmental trade-off, such that under

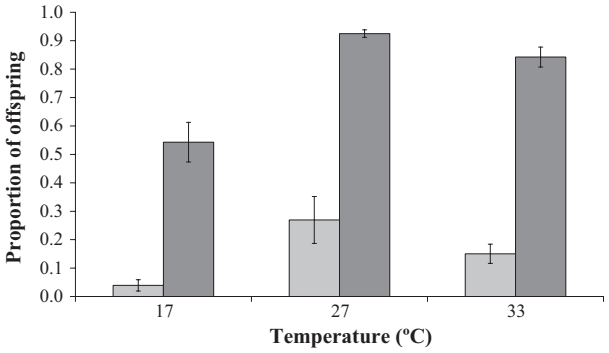

Fig. 3 The mean (±SE) proportion of offspring fathered by the wild-type treatment males that experienced 17, 27 or 33 °C during development when in competition against the sperm of males reared at 27 °C. Light grey bars represent P_1, the proportion of offspring fathered by the 1st male to mate, and dark grey bars P_2, the proportion of offspring fathered by the 2nd male to mate.

thermal stress, resources get diverted from reproductive development and homoeostasis to somatic growth and development. In terms of causal mechanisms, there is evidence in *Drosophila* that the functionality of genes important in the regulation of gonad morphogenesis is temperature sensitive (Schütt & Nöthiger, 2000). Additionally, the lipid contents of cell membranes are known to exhibit thermal plasticity (Van Dooremalen *et al.*, 2011), which could affect cell membrane permeability and thus circulating titres of hormones that are important to developmental regulation.

In the absence of sperm competition, the sperm of males exposed to the different temperature regimes were functionally equivalent (i.e. fertility was unaffected by developmental temperature). By contrast, under conditions of sperm competition, males derived from the low- and high-temperature treatments were less competitive in terms of sperm offence and sperm defence when competing with stock males raised at the intermediate temperature, indicating selection on sperm function to be stronger under polyandry (Kvarnemo & Simmons, 2013). Thus, the developmental pathways that regulate the phenotypic expression of ejaculatory phenotypes are subject to both natural selection (in the pre-ejaculatory environment) and sexual selection (in the post-ejaculatory environment).

Plasticity in primary reproductive traits in response to developmental temperature is likely to be commonplace in nature because ectothermic animals are likely to experience thermally variable environments during ontogeny both within and between generations. For example, in the dung fly, a classic model of sperm competition (Parker, 1970; Parker & Simmons, 1994) development takes place within dung pats that can experience temperature fluctuations of > 25 °C within just a few days (Penttilä *et al.*, 2013). Similar levels of thermal heterogeneity are experienced by larvae of the Glanville fritillary, *Melitaea cinxia* (Kvist *et al.*, 2013). In *C. maculatus*, a stored product pest of beans and pulses, the localized temperature of infested, in contrast to uninfested seed stores, can be up to 8 °C higher (Appleby & Credland, 2007). This scale of thermal heterogeneity could maintain intraspecific variation in sperm length despite strong stabilizing selection on this trait (Calhim *et al.*, 2007; Immler *et al.*, 2008; Fitzpatrick & Baer, 2011; although see Sharma *et al.*, 2013) and could help explain some of the residual variation in male fertilization success that is inherent in sperm competition studies (Simmons, 2001).

Genotype × environment interactions during ontogeny and spermatogenesis (i.e. the pre-ejaculatory environment; Sharpe, 2010) and during male–female and sperm–sperm interactions (the post-ejaculatory environment) are likely to preserve genetic variation in sperm size (see also Morrow *et al.*, 2008). In the dung fly, genotype × environment interactions affect the expression of female sperm storage organ morphology (Berger *et al.*, 2011), adding a further level of complexity to male–female interactions (see also Bjork *et al.*, 2007). Thus, what constitutes an optimal sperm phenotype will depend upon several layers of environmental heterogeneity and consequently, there may be no single optimal sperm phenotype (Morrow *et al.*, 2008). Despite this, our data point towards an optimal sperm phenotype; males reared at 27 °C had the longest sperm and performed best in sperm competition trials. This could be a consequence of ~200 generations of selection to 27 °C and a relatively constant socio-sexual environment, such that the population has evolved towards a reproductive optimum under very homogeneous conditions. Cross-population sperm competition assays provide some evidence that male *C. maculatus* are adapted to the prevailing female reproductive environment (Brown & Eady, 2001), although culturing populations for several generations at different environmental temperatures would reveal whether sperm size and function evolve in response to developmental temperature (i.e. natural selection).

At a mechanistic level, the reduction in the sperm competitiveness of males reared at the temperature extremes could be a direct consequence of these males transferring fewer sperm at copulation. Indeed, van Lieshout *et al.* (2013) found that male *C. maculatus* exposed to heat shock (50 °C for 50 min) during the pupal phase of development transferred smaller ejaculates and obtained lower P_2. However, like van Lieshout *et al.* (2013), we argue that the reduction in P_2 in response to environmental perturbation during development is unlikely to be solely a consequence of reduced sperm transfer. In *C. maculatus,* an approximate 90% reduction in the number of sperm transferred at copulation has been shown to have no detectable effect on P_1 and only a marginal effect on P_2 (P_2 dropped from ~0.9 to ~0.78; Eady, 1995). In the present study,

males reared at 17 and 33 °C inseminated more sperm than the sperm limited males of Eady (1995), yet P_1 and P_2 were substantially reduced.

A more important determinant of P_1 and P_2 in this species might be sperm size; Rugman-Jones and Eady (2007) found the smaller sperm of *C. maculatus* to be less competitive than the larger sperm of the closely related *Callosobruchus subinnotatus* during heterospecific sperm competition. Thus, the smaller sperm of the 17 and 33 °C males could account for their reduced competitive ability. This would require experimental confirmation as plasticity in molecular, physiological and behavioural traits associated with reproduction (Eberhard, 1996; Simmons, 2001; Fox *et al.*, 2006) could also affect the outcome of sperm competition.

It is worth noting that in the present study the largest males (i.e. those reared at 17 °C) did worst in terms of pre- and post-copulatory sexual selection (males reared at 17 °C were less likely to mate and experienced lower P_1 and P_2). Body size is frequently used as a proxy for male condition in sperm competition studies (Pitnick *et al.*, 2009). However, when developmental conditions are unknown, size may well be a poor indicator of male condition as developmental trade-offs between somatic growth/maintenance and reproduction may necessitate reduced investment in reproductive homoeostasis (Angilletta *et al.*, 2003). The nature of these trade-offs appears to differ with regard to whether the organism faces high- or low-temperature stress (hence large body size in response to low-temperature stress and small body size in response to high-temperature stress; Atkinson, 1994) and thus may explain why intraspecific studies tend to find no association between body size and sperm length (Pitnick *et al.*, 2009; but see Simmons & Kotiaho, 2002; Amitin & Pitnick, 2006).

The resurgence of interest in ecological development (Sultan, 2007; Pfennig *et al.*, 2010) may provide a valuable perspective to understand the remarkable diversity of primary reproductive traits. There is growing awareness that developmental outcomes (phenotypes) are dependent upon complex chains of regulatory events that are informed by both internal and external cues (Sultan, 2007). Phenotypic plasticity exposes these regulatory pathways to natural and, in the case presented here, sexual selection. This plasticity might promote both the origin of novel phenotypes and divergence among populations (Lande, 2009; Pfennig *et al.*, 2010; Espinosa-Soto *et al.*, 2011; Gomez-Mestre & Jovani, 2013). Thus, the interplay between ecology and development may prove to be pertinent to the rapid and divergent evolution of primary reproductive traits.

Acknowledgments

We are very grateful to Carl Soulsbury, Tom Pike and two anonymous referees for commenting on this manuscript.

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Received 3 March 2014; revised 6 May 2014; accepted 7 May 2014

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