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LOCAL ADAPTATION OF REPRODUCTIVE PERFORMANCE DURING THERMAL STRESS

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Abstract

Considerable evidence exists for local adaptation of critical thermal limits in ectotherms following adult temperature stress, but fewer studies have tested for local adaptation of sub-lethal heat stress effects across life history stages. In organisms with complex life cycles, such as holometablous insects, heat stress during juvenile stages may severely impact gametogenesis, having downstream consequences on reproductive performance that may be mediated by local adaptation, although this is rarely studied. Here, we tested how exposure to either benign or heat stress temperature during juvenile and adult stages, either independently or combined, influences egg-to-adult viability, adult sperm motility and fertility in high and low latitude populations of *Drosophila subobscura*. We found both population- and temperature-specific effects on survival and sperm motility; juvenile heat stress decreased survival and subsequent sperm motility and each trait was lower in the northern population. We found an interaction between population and temperature on fertility following application of juvenile heat stress; while fertility was negatively impacted in both populations, the southern population was less affected. When the adult stage was also subject to heat stress, the southern population exhibited positive carry-over effects whereas the northern population's fertility remained low.

Thus, the northern population is more susceptible to sub-lethal reproductive consequences following exposure to juvenile heat stress. This may be common in other organisms with complex life cycles and current models predicting population responses to climate change, which do not take into account the impact of juvenile heat stress on reproductive performance, may be too conservative.

Keywords: spermatogenesis, climate change, range margin, phenotypic plasticity, developmental acclimation, carry-over effects, evolution, inversions

Introduction

While a variety of abiotic factors are changing due to anthropogenic impacts on the climate, perhaps the most critical are increases in the mean and variability of temperature (IPCC, 2014). This is important because temperature has unique and profound effects on organismal biochemistry and physiology. Selection acts on the ability of organisms to function optimally within the range of temperatures that they routinely encounter and to minimize fitness costs during exposure to suboptimal temperatures (Angilleta, 2009). Ectotherms may be especially vulnerable to thermal stress given that their basic physiology is limited by ambient temperatures (Hochachka & Somero, 2002). Ectotherms also represent the vast majority of terrestrial biodiversity (Wilson, 1992) and so understanding how they will respond to increasing temperatures is of critical importance (Deutsch *et al.*, 2008).

For wide-ranging species that experience different thermal gradients in different environments, populations may exhibit variation in thermal adaptations and responses to suboptimal temperatures (e.g., Gardiner *et al.*, 2010; Yampolsky *et al.*, 2014). This may lead to local adaptation which predicts that populations inhabiting warmer climes will function better at higher temperatures relative to populations from cooler locations and vice versa (Angilletta *et al.*, 2003). Many studies have examined responses to temperature stress in terrestrial ectotherms and quantified local adaptation. In particular, critical thermal limits (CTL) - the temperatures at which the ability of an organism to remain active under extreme conditions breaks down - have received much attention. CTLs are thought to provide insight into species distributions, ecology and evolution, with upper thermal limits positively related to optimal performance temperatures (Terblanche *et al.*, 2007). However, recent reviews have emphasised that studies of local thermal adaptation have not focused on the most important life history traits, such as maintenance of fertility (Hoffmann, 2010). In previous studies, most of the traits measured to determine physiological failure are related to lethal effects (e.g. survival) or only indirectly related to fitness because they are general measures of mobility such as thermal knock-down. However, the latter may not be correlated with subsequent reproductive success. Moreover, reproductive processes, such as gamete production, are likely to be negatively affected at less extreme temperatures than loss of mobility and organismal death (Jørgensen *et al.*, 2006). The consequences of sub-lethal effects on reproductive performance may be exceptionally important in short lived organisms (i.e. many insects) because reversing thermal damage to reproductive processes may require a large proportion of total lifespan (Sinclair & Roberts, 2005). Thus, examining the consequences of thermal stress on reproductive performance is important in understanding how organisms will respond to climate change.

Not only should reproductive fitness traits be a focus, but different life history stages may experience different selection on these reproductive traits. Holometabolous insects typically experience radically different juvenile and adult thermal niches and therefore are expected to exhibit different susceptibilities to thermal stress (Angilleta, 2009; Hoffmann, 2010; Kingsolver *et al.*, 2011). Vagile stages will have the opportunity to take advantage of environmental heterogeneity via behavioural flexibility whereas developmental stages that are physically restricted have little or no ability to move to less stressful environments.

The effects of thermal stress on reproductive performance may be particularly relevant for males. For example, in *Drosophila*, spermatogenesis is predicted to be more sensitive to thermal stress than oogenesis (David *et al.*, 2005). Given that sperm production begins during the non-vagile juvenile stage in many insects (Nijhout, 1998), such that mature sperm have already left the testes at the time of emergence from the pupa (Wigglesworth, 1965), thermal stress during this time may represent a profound episode of selection subject to local adaptation. Relatively few studies in holometabolous insects have examined how thermal stress during earlier life cycle stages could affect subsequent organism physiology (Kingsolver *et al.*, 2011). While recent work has begun to examine this, studies tend to measure egg-to-adult viability (Kristensen *et al.*, 2015; Rohde *et al.*, 2016) but only rarely the survivors' subsequent reproductive performance (Chirault *et al.*, 2015; Zhang *et al.*, 2015a; b) and even more rarely in the context of local adaptation (Fragata *et al.*, 2016). Some studies assess physiological responses following relatively short heat shock (Chirault *et al.*, 2015; Zhang *et al.*, 2015a; b) but others ask what the fitness consequences are after longer term exposure to mildly stressful temperatures during development and whether this changes after laboratory adaptation (Fragata *et al.*, 2016).

Understanding the extent to which exposure to sub-lethal thermal extremes during early life-history stages subsequently impacts survival and reproduction later in life is critical to understanding the evolutionary potential and putative constraints on thermal adaptation (Bowler & Terblanche, 2008). Positive carry-over effects (also known as developmental acclimation) are one way that the non-vagile stages could mitigate the cost of thermal stress in subsequent life-cycle stages, and may represent an important component of thermal tolerance adaptation (Sgrò *et al.*, 2016). Such effects occur when exposure to thermal stress at an earlier life cycle stage results in increased resistance/tolerance to extreme temperatures at later stages (Angilleta, 2009) and may vary across geographic gradients, suggesting local adaptation (de Jong *et al.*, 2010; Scharf *et al.*, 2010). In contrast, negative carry-over effects occur when stress during development negatively impacts fitness components at the adult stage (Bacigalupe *et al.*, 2007; Gaston & Spicer, 2013; Schiffer *et al.*, 2013; O'Connor *et al.*, 2014). Two knowledge gaps exists in this literature, however. Most studies examining carry-over effects have ignored reproductive traits, which may be more sensitive to thermal stress. Moreover, when performed, these studies have examined response to cold stress (Sgrò *et al.*, 2016). However, capacity to deal with thermal changes is likely to be reduced as upper critical limits are reached. This is because the range of variation in resistance to thermal stress is considerably less at high temperatures than at cold, both between species (Gaston & Chown, 1999; Addo-Bediako *et al.*, 2000) and within species (Gaston & Chown, 1999; Terblanche *et al.*, 2007).

In this study, we test for local adaptation of survival and reproductive traits in a southern and a northern population of the holometabolous ectotherm, *Drosophila subobscura*, manipulating heat stress during either juvenile or both juvenile and adult stages. We focus on this species as it is a model for research on local adaptation to increasing temperatures (Balanyá *et al.*, 2006). We predict that the southern population will have greater reproductive fitness after continuous application of heat stress during the juvenile stage whereas the northern population will have either greater or similar fitness to the southern population at permissive temperatures. Moreover, because traits related to reproduction should exhibit thermal impacts before more frequently measured traits, such as survival, we predict the signal of local adaptation will be stronger for reproductive traits compared to survival following heat stress. Subsequent application of adult heat stress is predicted to affect reproductive performance differently between the populations with the southern population exhibiting positive, and the northern population exhibiting negative, carry-over effects.

Material and Methods

Fly stocks

Stocks of *D. subobscura* originated from wild-caught individuals collected in August-September 2011 from Uppsala, Sweden (59˚85'N, 17˚63'E) and from Valencia, Spain (39˚51'N, 0˚42'W), in the same season, with outbred laboratory populations established in mesh cages (50cm^3) at 18˚C under a 12h light/dark cycle. Subsequently, isofemale lines were established in August 2012 from these two populations (representing ca. 8 overlapping generations) from a single female per line and kept at a population size of greater than 50 at 18˚C. The 30 year historical average maximum temperature of the hottest month at each site is 23˚C in Uppsala and 31˚C in Valencia and previous work across the distribution of this species has suggested that 18˚C is a non-thermally stressful temperature for all populations (Santos, 2007; Castañeda *et al.*, 2013). Previous work found that males become sterile at 25˚C or even lower (Krimbas, 1993) and our preliminary experiments suggested that this temperature elicited substantial egg-to-adult mortality.

A maximum of twelve isofemale lines from the Valencia population and four isofemale lines from the Uppsala population were used in the experiments, 3-7 months after they were established (representing 3-6 generations), as Valencia exhibits a more diverse set of chromosomal inversion polymorphisms (Krimbas, 1993) and we wanted to capture as much genetic variation as possible. While isofemale lines may produce inbreeding effects, levels of fertility and sperm motility under benign conditions are relatively high (see Results) and our statistical models specified overdispersion to account for isofemale line variation. Flies were kept in vials containing a maize, sugar and agar food mix with additional live dry yeast for all experiments, and kept under a 12h light/dark cycle. In all experiments, light CO_2 anaesthesia was used to collect and sex flies. Flies were collected as virgins, stored for 6 days to become reproductively mature (Holman *et al.*, 2008) and then used in experiments.

Egg-to-adult viability

Male and female pairs from each isofemale line were put in separate vials and kept at 18 ˚C. Vials were inspected each day, and when more than 30 eggs had been laid (ca. 2 days), the pair was removed, the final number of eggs counted, and vials maintained at either 18˚C or 23.5˚C. We hereafter refer to the 18˚C treatment as benign (B) and the 23.5˚C treatment as stressful (H). We aimed for a minimum of ten vials per isofemale line per population per temperature treatment (total sample sizes: B, Upp = 37 vials; H, Upp = 67; B, Val = 102; H, Val = 192). The vials were kept at one of these two constant temperatures through development from egg to imago, and the numbers of eggs that emerged as adults were counted.

All analyses were performed using R (v. 3.2.2, the R Foundation for Statistical Computing, 2016). For each trait, the maximal model was simplified by step-wise removal of non-significant factors, as judged by change in Akaike Information Criterion. For ease of comparison among traits, data in figures are presented relative to the mean performance of the Valencia population at the benign temperature, after back transformation from the logit scale. For viability we used a generalized linear models (GLM) of the proportion of survivors, with quasibinomial error structure to account for over-dispersion and a logit link function. Temperature [B, H], population [Val, Upp]) and their interaction, to detect local adaptation (Kawecki & Ebert, 2004), were included in the analysis. The number of eggs was log transformed and treated as a covariate in order to account for density effects.

Sperm motility

Spermatogenesis is predicted to be more strongly affected by thermal stress than oogenesis. Male sterility is frequently quantified in *Drosophila* as lack of motile sperm (Coyne & Orr, 1989) and here we examined the seminal vesicles for the presence of motile sperm following either benign or stressful developmental temperatures. Flies were generated as described above. Male flies were collected on the day of eclosion and kept in vials of less than 20 flies for six days at their developmental temperature (B or H) to reach sexual maturity. Seminal vesicles of a minimum of ten males per line per population per temperature treatment were dissected (Snook, 1998), gently squashed on a slide under a cover slip in phosphate buffered saline, and sperm motility assessed (we aimed for a minimum of 10 males per line per population per temperature treatment; total sample sizes: B, Upp = 46; H, Upp = 73; B, Val = 126; H, Val = 223). Males were considered sterile (coded as 0) if no sperm, or very few motile sperm, were present; males were considered fertile (coded as 1) if motile sperm were present. This response was analysed using a quasibinomial GLM as for viability (but with no covariate).

Fertility

An ultimate measure of fertility is the ability to produce progeny. Here we were interested to measure the consequences of the thermal environment at both developmental and adult stages on fertility. We had four treatments for each population: both juvenile and adult temperatures were benign (BB); both were stressful (HH); juvenile temperature was stressful but adult temperature was benign (HB); or juvenile temperature was benign and adult temperature stressful (BH). Flies were generated as described above, being reared throughout development at either the benign or stressful temperature and upon eclosion, virgin males and females were collected and separated by sex into vials of less than 20 individuals for six days to reach sexual maturity and were randomly assigned to either remain as adults at their developmental temperature or to switch to the alternative temperature. At 6 days post-ecolsion, a male and female were placed in a vial and monitored for larvae production (we aimed for a minimum of 5 pairs per line per population per temperature treatment; total sample size: for Uppsala, BB = 14, HH = 19, HB = 22, BH = 21; for Valencia, BB = 50, HH = 48, HB = 89, BH = 73). Pairs were transferred from the first vial after five days and placed in another vial for a further five days. Pairs were considered fully sterile if no larvae were produced in either vial (coded as 0); pairs were considered fully fertile if larvae were produced in both vials (coded as 2); and pairs were considered partially fertile if larvae were produced in only one of the two vials (coded as 1). This response was analysed using a binomial GLM (0, 1 or 2 successes out of 2 trials) because no over-dispersion was detected. The temperature treatment in this case had four levels (BB, BH, HB, HH). We tested for the predicted carry-over effects by making the appropriate contrast: the effect of HH relative to HB was expected to depend on population (HH>HB in Val, HB>HH in Upp).

Results

Temperature and population, but not their interaction, significantly influenced viability from egg to adult (Fig. 1a) and sperm motility (Fig. 1b; Table 1; interaction term, viability: df = 1, deviance = 0.065, p = 0.98; interaction term, sperm motility: df = 1, deviance = 1.042, p = 0.31). Higher temperatures resulted in decreased survivorship and sperm motility compared to lower temperature. Uppsala had lower viability and sperm motility than Valencia at both temperatures.

In contrast, temperature, population and their interaction significantly influenced fertility (Fig. 2, Table 1). Stressful temperatures experienced only during development (HB, Fig. 2) had a greater consequence on fertility than only experiencing stressful temperatures as an adult (BH, Fig. 2) and this affect was stronger on the Uppsala population than the Valencia population. We predicted that the southern population would exhibit positive, whereas the northern population would exhibit negative, carry-over effects. To test these predictions, we performed two directional (and thus, one-tailed) t-tests, contrasting the effect of HH relative to HB (HH>HB in Val, HB>HH in Upp). There was no difference in fertility for Uppsala between stress in the juvenile stage only compared with stress in both stages (t_{328} = 0.99, P = 0.16). However, in Valencia, while heat stress negatively impacted fertility during any stage, fertility was higher in individuals that experienced heat stress in both juvenile and adult stages compared to heat stress only during the juvenile stage (t_{328} = 1.74, P = 0.041).

Discussion

In this study, we aimed to reveal local adaptation of reproductive performance traits under sub-lethal heat stress, with a particular emphasis on heat stress during the juvenile stage. Furthermore, we aimed to evaluate whether acclimation to heat stress during development impacted the late life response to these temperatures. We found juvenile heat stress and population impacted egg-to-adult viability and sperm motility, with the northern population having lower fitness than the southern population. We found fertility exhibited local adaptation; although both populations were negatively impacted during juvenile heat stress, the southern population was less effected. We also found positive carry-over effects in fertility. Heat stress exposure during the adult stage, after juvenile heat stress exposure, increased fertility in the southern, but not northern, population. Overall, the northern population was significantly more susceptible to juvenile and adult thermal stress across all fitness traits measured. Our work fills a gap in research on temperature effects that has largely been dominated by experiments focusing on CTLs of non-reproductive traits, primarily following adult exposure to thermal, particularly cold, stress. In this work, we have linked reproductive performance based responses to chronic sub-lethal heat stress, particularly at the juvenile stage, to local adaptation. This work will provide novel insights of how populations may respond to such thermal variation and may be applicable across a variety of other animals. The generality of the results is likely because many animals will lose fertility at temperatures much lower than fatal thermal limits (Jørgensen *et al.*, 2006) and because, for many invertebrates, gamete production begins during juvenile stages.

As predicted, we found no evidence of local adaptation in juvenile survival although northern males had lower egg-to-adult viability. Likewise, sperm motility was negatively impacted primarily by juvenile temperature and weakly by population, with northern males having lower sperm motility overall than southern males but no evidence of local adaptation. Sperm motility is a standard measure of male sterility but is frequently measured as a binary trait, and thus may not reveal subtle changes in sperm performance following thermal stress. The lower general fitness of the Uppsala population may reflect range margin effects (Kawecki, 2008), consistent with the decreased variation in chromosomal inversion polymorphisms as latitude increases (Krimbas, 1993). Decreased genetic variation has been suggested to occur at range margins as a consequence of some combination of Allee effects, genetic drift and the lack of sufficient gene flow into populations at range limits to fuel adaptive potential (Hoffmann & Blows, 1994; Bridle & Vines, 2007). Alternatively, local adaptation may be decreased due to gene swamping, with dispersal from (typically larger) more central populations continually introducing alleles into range margins that are more appropriate for maximising fitness elsewhere (Hoffmann & Blows 1994). The situation in *D. subobscura* is more complicated given the presence of extensive variation in chromosomal inversion polymorphisms that prevent recombination in inversion heterozygotes. While there is gene flow within the same inversion arrangement (Simões *et al*., 2012; Pegueroles *et al*., 2013; Pratdesaba *et al*., 2015), there is substantial evidence that inversion polymorphisms themselves are under selection (Balanyá *et al*., 2006; Santos *et al*., 2016).

We did, however, demonstrate local adaptation to the thermal environment in our other measure of fertility, as predicted. Fertility was negatively impacted by application of juvenile heat stress, particularly for Uppsala compared to Valencia pairs. Juvenile heat stress resulted in positive carry-over effects on fertility in Valencia, but not Uppsala, when adults also experienced heat stress. These results indicate that northern populations are more susceptible to sub-lethal reproductive consequences following exposure to juvenile heat stress, and that such exposure does not buffer reproductive performance against subsequent adult heat stress.

The effects of thermal stress on male and female reproductive function in the fertility experiment cannot be separated. Now that we have demonstrated local adaptation in fertility, future work should decompose the sex-specific effects of juvenile thermal stress on gamete performance in more physiological detail, such as quantifying apoptosis of oocytes and the effect of thermal stress on sperm number and length. Female *D. subobscura* are monogamous (Smith, 1956; Holman *et al*., 2008; Fisher *et al*., 2013) but, as with other *obscura* group species, males are sperm heteromorphic, with males producing short, non-fertile sperm (parasperm) and longer, fertile sperm (eusperm; Snook & Karr, 1998). In work on the closely related polyandrous species, *D. pseudoobscura*, parasperm protect brother eusperm from a hostile female reproductive tract (Holman & Snook, 2008). Sperm precedence favours the last male, but after lengthy exposure to cold stress, sperm precedence reverses to favour the first male (Giraldo-Perez *et al.*, 2016). Whether, and the extent to which intra-ejaculate interactions and ejaculate-female reproductive tract interactions change as a consequence of either juvenile or adult heat stress and whether this is impacted by mating system is unknown.

We have previously found, across six *D. subobscura* populations from a 20° latitudinal cline in Europe (of which the Valencia and Uppsala populations represent the southern- and northern-most population respectively), a gradient in gene expression in which adult males from southern populations have higher expression of spermatogenesis genes than males from northern populations, under benign (18℃) conditions ((Porcelli *et al.*, 2016). Given that females are monandrous, sperm competition cannot explain increased investment in spermatogenesis in lower latitude populations. As investment in spermatogenesis is less in Uppsala than Valencia, it may be that northern males are not able to compensate as much as southern males following juvenile heat stress. In support, we found evidence of developmental acclimation in Valencia, but not Uppsala, populations.

If fertility reduction following juvenile heat stress is male-driven, then population fitness can recover only if females remate with a fertile male. While females are naturally monogamous, previous work has shown that *D. subobscura* females will remate if they do not receive a functional ejaculate during copulation, a phenomenon termed pseudo-polyandry (Fisher *et al.*, 2013). Thus, mitigating male sterility and recovering population fitness may be possible if females can find a fertile mate, although whether females will remate after receipt of a partially-fertile ejaculate is unknown. Moreover, finding a fertile mate may be more difficult in northern, more susceptible populations, because of the lower density of range margin populations (including the *D. subobscura* Uppsala population; Krimbas, 1993). However, if oogenesis is also damaged during juvenile heat stress, then even pseudo-polyandry cannot rescue population fitness. The combination of higher latitude populations being more susceptible to juvenile heat stress, decreasing both survival and measures of reproductive performance (sperm motility and fertility), northern populations investing less in spermatogenesis, and having low population density, makes high latitude *D. subobscura* populations susceptible to extinction as both average temperature and the frequency of extreme heat events increase (IPCC, 2014).

While this work is specific for *D. subobscura*, such results may be quite common. Many organisms spend a portion of their life cycle as relatively immobile juveniles, so thermal stress during this life cycle stage may have profound negative fitness consequences beyond survival because gamete production typically starts during juvenile stages. Such subsequent negative fertility effects on adults should compound reductions in population size, which can then have substantial downstream effects via, for example, genetic drift (Polechova & Barton, 2015). As range margin populations tend to have less genetic diversity to begin with, and the range of variation in resistance to thermal stress is considerably less at high temperatures than at cold (Gaston & Chown, 1999; Addo-Bediako *et al.*, 2000;

Terblanche *et al.*, 2007), sub-lethal effects on reproductive traits may make these populations even more susceptible as the climate continues to get warmer and the frequency of extreme heat events increases. Local adaptation may provide some resilience to climate change and we found such local adaptation for juvenile heat stress and reproductive performance in a southern population. Variation in organismal tolerance throughout the life cycle should be incorporated in models that predict impacts of climate change. To date, heavy reliance has necessarily been placed on predictions based on latitudinal trends in upper and lower critical or lethal thermal limits (Lancaster, 2016) derived from data compilations (e.g. Addo-Bediako *et al.*, 2000; Sunday *et al.*, 2011). A recent study on lizards, with a sessile embryonic stage, developed a life cycle model for demographic consequences based on microclimates at high spatio-thermal resolution and experimentally derived embryonic thermal tolerances (Levy *et al.*, 2015). Levy and colleagues found that models that ignored embryonic consequences (egg-to-adult viability) severely underestimated the demographic effects of (previously unrecognised) thermal events. Such detailed analysis that also incorporates any subsequent fertility effects remains to be done. Given that we show substantial effects beyond egg-to-adult survival, models without this consideration may prove even more unduly conservative.

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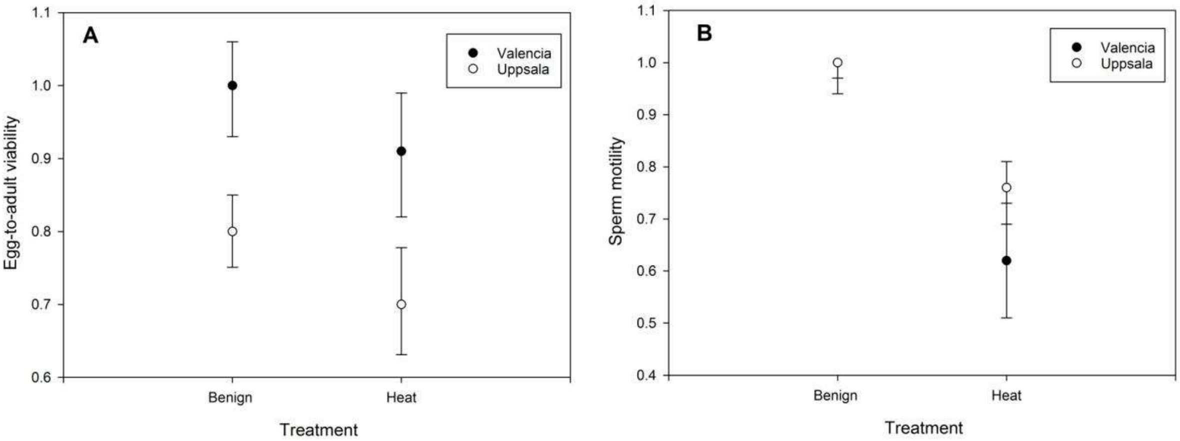
Table 1. Generalized linear model of the effects of temperature, population and their interaction on (a) egg-to-adult viability, (b) sperm motility and (c) fertility. *p* values are reported either exactly or < 0.001. For egg-to-adult viability, the starting number of eggs was also included in the model. Non-significant terms have been dropped. Residual deviance is included where the quasibinomial model was used.

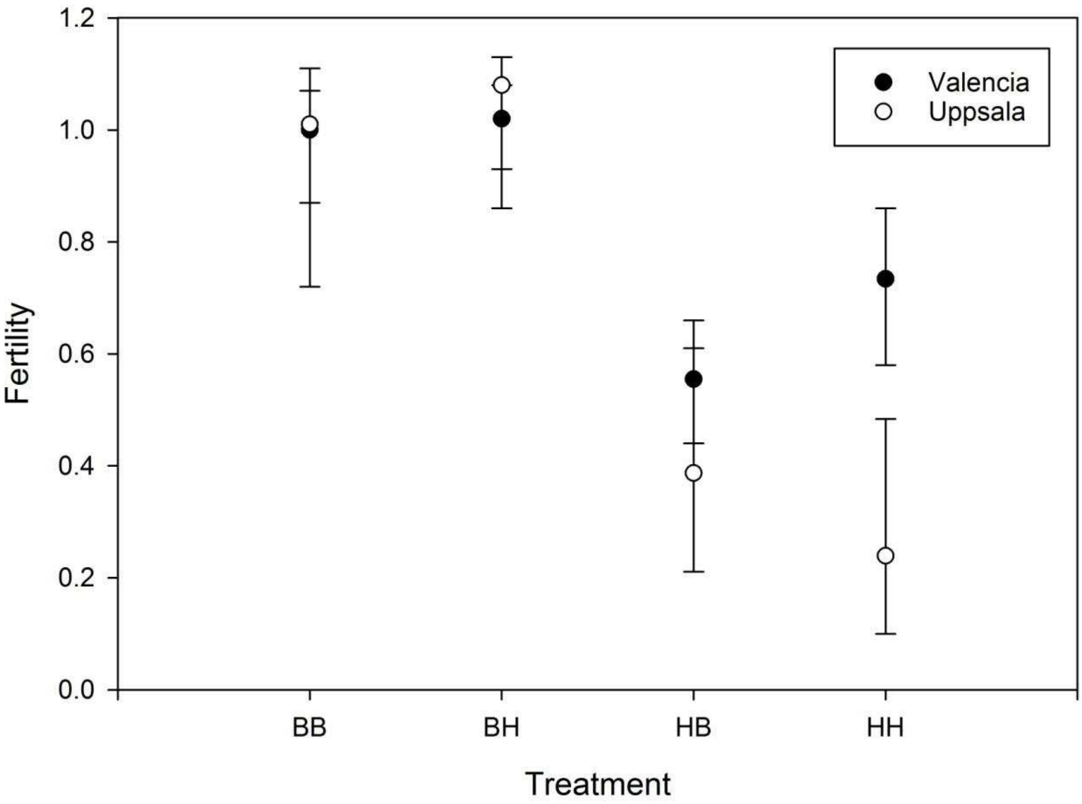
source	d.f.	Deviance	Deviance ratio	*p*
(a) egg-to-adult viability				
log(eggs)	1	67.232	6.1864	0.01
Temperature	1	302.528	27.8375	< 0.001
Population	1	59.993	5.5203	0.01
Residual	394	4937.1		
(b) sperm motility				
Temperature	1	76.95		<0.001
Population	1	4.49		0.03
(c) fertility				
Temperature combination	3	148.731	28.77	<0.001
Population	1	11.224	6.5128	0.01
Temperature*Population	3	14.657	2.8348	0.038
Residual	328	587.43		

Figure legends.

Figure 1. The effect of juvenile heat stress and population influence a) egg-to-adult viability and b) sperm motility. Mean ± SE relative to Valencia (southern population) at the benign temperature, following back transformation from the logit scale.

Figure 2. Heat stress during the juvenile life cycle stages negatively impacts fertility in Valencia (southern population) pairs significantly less than Uppsala (northern population) pairs and subsequent adult heat stress improves fertility in Valenica, but not Uppsala, pairs. B= benign and H = heat stress; BB = benign temperature for both juvenile and adult stages, BH = benign temperature during juvenile stages but heat stress as adults, HB = heat stress during juvenile stages but not during the adult stage; HH = heat stress during juvenile and adult stages. Mean ± SE relative to Valencia at the benign temperature for BB, following back transformation from the logit scale.


A
Egg-to-adult viability
1.1
1.0
0.9
0.8
0.7
0.6
Benign
Heat
Treatment
● Valencia
○ Uppsala
B
Sperm motility
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
Benign
Heat
Treatment
● Valencia
○ Uppsala


Fertility
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BB
BH
HB
HH
Treatment
● Valencia
○ Uppsala