Paternal-effects in a terrestrial ectotherm are temperature dependent but no evidence for adaptive effects.

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Running headline: Temperature-mediated paternal effects
ABSTRACT

1. Global rising of average temperatures and increase in extreme climatic events may largely impact animal survival and reproduction. Yet, how variation in temperature may affect male fertility, in particular ejaculate traits, and whether this can in turn affect offspring fitness, is seldom addressed. Paternal effects may be of key importance as they could impact the rate and direction of evolutionary change in response to climate change.

2. We tested the effects of temperature experienced by males on sperm traits, and asked whether the paternal environmental temperature affected offspring phenotype. We further explored the potential for paternal effects to be adaptive, which would occur when offspring fitness increased under the same environmental conditions experienced by the fathers. We exposed male field crickets to high or low temperatures at two life stages, either throughout development or as adults, and tested sperm traits (number and quality) and offspring fitness (hatching success and survival). We further assessed sperm traits in offspring, after they had also been exposed to the same or different temperature experienced by their father.

3. We found that temperature affected sperm traits depending on the life-stage of individuals. When the exposure was given during adulthood, males exposed to high temperature produced less sperm and of lower quality compared to males exposed to the lower temperature, while if exposure was given during development males exposed to high temperature produced more sperm and of better quality compared to males exposed to low temperatures. Offspring fitness was significantly affected by paternal temperature, evidence for anticipatory paternal effects on sperm traits was not found.

4. Our study indicates that temperature can mediate cross-generational effects, and that paternal effects may be mediated by changes in temperature and therefore much more widespread in nature than previously assumed.
Keywords: Paternal effects, anticipatory effects, sperm traits, temperature, cross-generational effects, crickets
INTRODUCTION

The increasing evidence for global rising of average temperatures, as well as the increased frequency and severity of extreme events such as heat waves (Meehl & Tebaldi 2004; Stone, Hess & Frumkin 2010; Hartmann et al. 2013; IPCC 2013), have led scientists to investigate how temperature may affect cellular, physiological and behavioural traits in a variety of animal and plant species. Our understanding of how the changes in temperature associated with climate change will affect reproductive processes, and how these may in turn determine evolutionary responses to climate change, is nevertheless still limited. This is surprising, as even small effects on reproductive performance may largely affect an individual’s reproductive success and its trans-generational fitness, through effects on offspring phenotype. Moreover, the majority of studies focussed on effects of temperature on female fecundity, while effects on male fertility remain largely overlooked. There are three main reasons why temperature-induced effects on male fertility are important. First, sperm are more likely to suffer from temperature stress than any other type of cell; second, reduced male fertility affects female reproductive output; and third, there is a small but growing body of evidence suggesting that environmentally-induced changes in ejaculate components may affect offspring quality (Crean & Bonduriansky 2014).

Sperm cells are known to be extremely susceptible to high temperatures due to their intrinsic structure; indeed, temperature-induced declines in sperm number and quality (motility and viability) have been reported in humans and in a variety of other animal taxa (Reinhardt, Dobler & Abbott 2015). Importantly, even small changes in sperm traits can affect male fertility: sperm traits such as number and quality are key determinants of male reproductive success in many species, including those where females mate multiply and therefore sperm must outcompete rival male’s sperm to fertilise the eggs (e.g. Gage et al. 2004; Denk et al. 2005; Garcia-Gonzalez & Simmons 2005b; Gasparini et al. 2010).
Temperature thereby also has the potential to affect offspring fitness, as recently documented for other environmentally-induced changes in sperm phenotype, such as variation in social environment and diet (Crean & Bonduriansky 2014; Marshall 2015; Evans et al. 2017).

Cross-generational environmental effects represent another form of phenotypic plasticity (called transgenerational plasticity) where the parental environment (maternal and/or paternal) affects the phenotype of the offspring (Mousseau & Fox 1998). Transgenerational plasticity is emerging as a critical factor in ecological and evolutionary processes, contributing to variation in offspring phenotype that likely influences the strength and direction of selective processes beyond the classical Mendelian inheritance (Bonduriansky & Day 2009). Although the extent and significance of maternal effects as a source of phenotypic variation has been long recognized (Mousseau & Fox 1998; Mousseau et al. 2009), the same is not true for the effects mediated by fathers (i.e. paternal effects), with perhaps the exception of species where males provide paternal care (Crean & Bonduriansky 2014). It is indeed generally assumed that in species where males contribute nothing but gametes, paternal effects should be weak, if not absent. Nevertheless, recent studies showed that variation in offspring performance can be attributable to paternal effects alone (Crean & Bonduriansky 2014). Despite recent progress in understanding the evolutionary consequences of environmentally-induced paternal effects (Bonduriansky & Head 2007; Crean, Dwyer & Marshall 2013; Zajitschek et al. 2014; Marshall 2015), we lack understanding of the potential for temperature changes to mediate such effects. Empirical studies are therefore required to understand the role of paternal effects as potentially powerful evolutionary force in mediating responses to climate change.

Paternal effects might also be adaptive, provided that the paternal effects on offspring phenotype improves offspring fitness and/or survival (Crean & Bonduriansky 2014) a notion that remains largely untested (but see Crean, Dwyer & Marshall 2013). Paternal effects may
act similarly to adaptive maternal effects, which exist when mothers improve offspring’s fitness by affecting their phenotype non-genetically (adaptive maternal effect hypothesis, Mousseau & Fox 1998). Typically, these adaptive effects are referred to as ‘anticipatory’ effects’ and have been demonstrated in several species as maternal or parental effects (Marshall & Uller 2007). Importantly, this would require cross-generational temporal autocorrelations in environmental conditions, such that parents can ‘prepare’ their offspring to the environment they will experience, which is likely the case for environmental temperature. The adaptive paternal effect hypothesis would predict that changes to ejaculate/sperm phenotype generated by the environment (i.e. temperature) results in offspring having higher fitness when exposed to the same environment of their fathers compared to offspring exposed to a different environment. Transgenerational phenotypic plasticity is of fundamental importance in evolutionary ecology, as it may provide individuals with the ability to cope with environmental changes, and ultimately adapt to climate change. Studies assessing the potential for paternal transgenerational adaptation to temperature change are still lacking, representing a severe oversight in climate change studies.

The effects of environmental temperature are particularly relevant for ectotherms (Zeh et al. 2012) due to their well-documented sensitivity to temperature changes (Deutsch et al. 2008; Dell, Pawar & Savage 2011). In terrestrial ectotherms such as arthropods and reptiles, metabolic rates are temperature-dependent (Gillooly et al. 2001; Dillon, Wang & Huey 2010) and, as a consequence, many fundamental processes, including those involved in reproduction, are affected even by the smallest variation in environmental temperature (Willmer 1991). For example, exposure of males to high temperatures has already been shown to decrease sperm number in the pseudoscorpion Cordylochernes scorpioides (Zeh et al. 2012), and affect sperm morphology in the dung fly Scathophaga stercoraria (Hellriegel & Blanckenhorn 2002). However, despite the important role of ectotherms, and in particular
insects, in food chains and terrestrial ecosystems, only a handful of studies have investigated within and cross-generational effects of temperature, and none have tested the potential of adaptive paternal effects.

Here we present a comprehensive study testing the effects of temperature on ejaculate traits on males and their offspring, using an ectotherm widely used in both ecological and sexual selection studies, the Mediterranean field cricket (Gryllus bimaculatus). This species has been reported to breed over a broad temperature range (between 15 °C - 35 °C, Doherty 1985 and citations therein), but the effect of temperature on ejaculate traits important in determining fertilization success, such as sperm number and viability (Garcia-Gonzalez & Simmons 2005b), has never been considered. Our experimental design consisted of exposing males to either of two different experimental temperatures (24 °C or 28 °C). These temperatures were chosen as they were within the natural range of temperature experienced by this species in the wild (see Methods). Males were exposed to the given temperature at two different stages of their lifecycle, during development or during adulthood, to understand the life-stage where temperature effects are of key importance. We then tested: i) the effects of temperature on ejaculate traits (sperm numbers and quality), ii) the fitness consequences of these effects on male reproductive success (i.e., within-generational effects) and offspring phenotype (i.e., cross-generational effects), and, using a split brood design, iii) the potential for adaptive anticipatory paternal effects.

MATERIALS AND METHODS

Cricket maintenance

The crickets used in our study were the second generation of a population originating from wild-caught animals collected in Capalbio, Tuscany (Italy), in 2015, where temperature
ranges between 17 °C and 31 °C (min-max, based on data collected during the breeding season in 2014, 2015 and 2016). Stock populations of crickets were maintained in a climate room under a constant temperature (26 °C) and humidity (65%) at a 14:10 hour light: dark cycle at the Ludwig Maximilian University of Munich (Germany). Animals were kept in several large plastic tanks (35×27×20 cm, L×H×W) equipped with gravel and carton shelters and maintained at similar density (approx. 30 individuals) and sex ratio (1:1 female:male). Animals were provided with *ad libitum* access to water and food (dry bird food and fresh apple slices). Prior to the start of the experiment we added several small plastic cups containing moist soil to the stock populations to provide females a substrate for egg laying. Cups were removed after one week and any deposited eggs left to hatch.

**Overview of the experimental design**

Three-days old nymphs collected from stock populations were randomly assigned to one of two temperature treatments (hereafter ‘temperature’; High (H): 28 °C or Low (L): 24 °C) and one of two treatments differing in the life stage at which males were exposed to the given temperature treatment (hereafter ‘exposure age’; throughout development (DEV) or upon reaching adulthood (AD)). We thus created four treatment groups in total: two groups in which males experienced high or low temperatures throughout development (DEV-H and DEV-L), and two groups where males were exposed to temperature manipulations only after becoming sexually mature (AD-H and AD-L) (see ‘Treatment - Fathers ’ below). After treatment application, spermatophores were collected from males and assessed for sperm quality and number (see ‘Sperm assays’ below). Males were then paired with virgin females raised under standard conditions, who were left to lay eggs. Offspring hatching success and their survival to 7 weeks of age was assessed; male offspring were tested for sperm quality
and number as detailed for their fathers (see ‘Treatment- offspring’ below). For a visual overview of the experimental design see Fig. 1.

**Treatments - Fathers**

Males in the DEV treatment groups were reared inside plastic tanks (as described above) at 24 °C (L) or 28 °C (H) from an age of 3 days onwards. Temperature was controlled using a controlled temperature room (CTR) set at 24 °C; the high temperature treatment was obtained by placing the appropriate tanks on top (but not in direct contact) of 20W thermo mats (Lucky reptile, Germany). The efficacy of the practical arrangement to obtain the two desired temperatures was confirmed by recording temperatures in a pilot experiment (4-week period, mean temperature ± SE: 28.5 ± 0.2 °C, median: 28.4 °C, range 26.8-29.8 °C, N= 18). From 4 weeks of age onwards, crickets were inspected every second day. Just before the last moult to adulthood, males were isolated into individual plastic containers (10×10×9 cm) to ensure virginity and to standardise mating history. Developmental time (number of days from birth to adulthood) was recorded. Food and water were provided *ad libitum* as described for the stock populations. Males in the AD treatment groups were raised under the same conditions but at a standard temperature of 26 °C (i.e., same as the stock population), and were assigned either the low or high temperature treatment (as described above) following their final moult into adulthood (i.e. once sexually mature).

For each male (total = 200 males) we assessed sperm traits (sperm number and viability, see details below) three times (12, 16 and 20 days after maturation). Immediately prior to sperm measurements, males were weighed to the nearest 0.01 g using a digital scale (KERN, PKT) to obtain a measure of body mass.
Sperm assays

To control for the age of sperm cells on the day before the sperm assay, each male was checked and any spermatophore present removed from its genital pouch. On the day of the assay, the newly formed spermatophore was collected and placed into 200µl of Beadle saline (128.3 mM NaCl, 4.7 mM KCl and 23 mM CaCl₂). The evacuating tube was cut with scissors and the spermatophore was left to release its contents for 10 minutes (Simmons & Beveridge 2011).

Sperm number. Sperm number was estimated using an automated cell counter (CASY Schärfe-System, Reutlingen, Germany). An aliquot (100µl) of the ejaculate was mixed with a known volume of CASY-ton solution (0.99 mL), an isotonic and iso-osmotic electrolyte, and subsequently placed in the automated cell counter. The cell counter estimates the total number of cells in each sample from three automatized consecutive measurements.

Sperm viability. An aliquot (100µl) of the diluted ejaculate was stained with the LIVE/DEAD® sperm viability kit (Invitrogen, Molecular Probes Inc, Eugene, OR, USA) following previous protocols (Tuni et al. 2016). Each sample was then viewed under a florescence microscope (Olympus BX61; Olympus, Tokyo, Japan) and the number of live (stained green) and dead (with a damaged membrane, stained red) sperm cells recorded. 500 sperm cells were scored in total per sample.

Mating

After the last sperm assay was completed, each male was paired overnight with a non-experimental, virgin female. The virgin females used here were raised in standard conditions as stock populations (at 26° C) and the mating occurred at standard temperature (26° C). We employed this design, with females reared under standard conditions and mating environment, to evaluate the importance of paternal temperature while excluding female’s
environmentally-induced effects (Behrens et al. 1983), such as those associated with oviposition temperature (e.g. Fischer, Brakefield & Zwaan 2003). The following morning, the female was placed in a separate tank equipped with shelter and an oviposition cup to lay eggs again at standard temperature (26 °C).

Hatching success and offspring survival

To score hatching success, the oviposition cups were moved after one week to a different container and checked every second day for emerged nymphs. At this stage, we scored each female-male pair for success (hatched) or failure (unhatched) in producing viable offspring. Where offspring were produced, 60 nymphs were randomly selected from each brood 3 days post-hatching, and split over 2 containers (30 nymphs each) equipped with shelter, food and water. All nymphs were kept in identical conditions and temperature (26 °C) to the stock population and their mothers, to avoid our estimation of paternal effects being confounded by the effect of temperature during the nymphal stage. To estimate nymph survival rates, we recorded the number of live offspring at 7 weeks post-hatching.

Treatments - offspring

The next part of the experiment consisted of a split brood design using offspring from a subsample of broods (n=25 families per treatment). Unfortunately, the number of families with viable offspring varied substantially between the two DEV groups (see Results section), so that we could not test the DEV group’s offspring. We therefore applied the split-brood approach only to male offspring from the AD treatment (AD-H n= 25 families, AD-L n= 25 families). Male offspring were isolated before the last moult (as detailed above for the AD paternal generation). After the last moult, half of the males from each brood were randomly assigned to the high and the other half to the low temperature treatment (hereafter, ADO-H
and ADO-L respectively, where O indicates offspring generation). We tested an average of 6.9 ± 2.1 SD male offspring per full-sib family (range 1-14), for a total of 362 offspring. Sperm number and sperm viability were assessed as described for the paternal generation but only once, at 20 days following the last moult.

**Statistical analyses**

All analyses were conducted using R v 3.3 (R Development Core Team 2016). Data collected for the fathers were analysed using mixed-effects models with the R package ‘lme4’ and included ‘exposure age’ (DEV or AD), ‘temperature’ (L or H) and their interaction as fixed effect factors. We further include male ID in the model to account for non-independence of the data collected from the same male (three spermatophores were collected for each male). P-values for the fixed effects were obtained using the “Anova” function in the package “car”. The number of sperm produced was analysed using a generalized linear mixed-model (GLMM) with a negative binomial distribution to account for overdispersion, while the sperm viability was analysed assuming a binomial distribution and included an observation-level factor to account for overdispersion. A linear mixed model was used for male body weight. Hatching success was analysed with a general linear model (GLM) with binomial distribution and nymphs survival was analysed using a GLM with a quasibinomial distribution. When significant interactions were found between the two categorical factors (exposure age and temperature) we subsequently run two separated models for each exposure age subsample, to further interpret the role of temperature at each life stage (throughout development or adulthood).

Data collected from the offspring generation were analysed using mixed-effects models (GLMM with Poisson distribution for sperm production and binomial for sperm viability, LMM for male body weight) with temperature (H or L), paternal treatment (AD-H or AD-L),
and their interactions as fixed effect factors. Body weight was log-transformed. Father identity was included as a random effect to account for the non-independence of data collected from full sibs. An observation-level factor was added to both sperm production and sperm viability models to account for overdispersion. The distribution of residuals was checked for each model to confirm model assumptions were met.

For the offspring generation, we used ‘relative’ fitness traits, calculated following (Burgess & Marshall 2011), where the traits measured in male offspring (sperm quantity, sperm quality and male body weight) were calculated relative to the mean trait from all fathers independently of their temperature treatment. Being $t_{OFF(L)}$ and $t_{OFF(H)}$ the trait considered in the offspring kept at Low and High temperature respectively, and FAT(L) or FAT(H) the temperature treatment of the fathers, we calculated the relative trait as follow: $t_{OFF(L)}$, FAT(L)/$\bar{t}_{OFF(L), FAT(L,H)}$ and $t_{OFF(H)}$, FAT(H)/$\bar{t}_{OFF(H), FAT(L,H)}$. The relative nature of the new traits standardises fitness differences between temperatures, so that an effect of the interaction between paternal temperature and offspring temperature would indicate that offspring exposed to a certain temperature was favoured differently accordingly to their father temperature treatment, and provide evidence for adaptive paternal effects.

### Results

#### Fathers Sperm traits

There was a significant effect of temperature ($\chi^2=33.254$, $P<0.001$), exposure age ($\chi^2=81.166$, $P<0.001$) and their interaction ($\chi^2=85.405$, $P<0.001$) on the number of sperm produced. During adulthood, males exposed to a high temperature (AD-H) produced less sperm compared to those exposed to a low temperature (AD-L) ($\chi^2=86.974$, $P<0.001$), while the opposite occurred in males exposed since development ($\chi^2=11.409$, $P<0.001$) (Fig. 2a, Table S1 and S2a in Supporting Information).
Similarly, sperm viability was affected by temperature ($\chi^2 = 4.922, P = 0.027$), exposure age ($\chi^2 = 14.391, P < 0.001$) and their interaction ($\chi^2 = 9.516, P = 0.002$), and showed the same trend found for sperm number (AD-H < AD-L and DEV-H > DEV-L; Table S1, S2b, Fig. 2b). Analysed separately by exposure age, sperm viability was significantly affected by temperature when the exposure started during development ($\chi^2 = 13.249, P < 0.001$) but the difference was not significant when exposure started when males were adults ($\chi^2 = 0.0538, P = 0.816$).

**Body weight & developmental time**

Males did not differ in their weight according to temperature ($\chi^2 = 1.868, P = 0.172$) or exposure age ($\chi^2 = 0.366, P = 0.545$), but there was a significant interaction between duration of exposure age and temperature ($\chi^2 = 8.712, P = 0.003$) (Table S1, S2c, Fig. 2c). Males exposed to a high temperature during development were larger than the ones exposed to low temperature ($\chi^2 = 8.418, P < 0.001$), while body weight was not significantly affected by temperature when exposure started during adulthood ($\chi^2 = 1.6605, P = 0.197$).

Male developmental time was affected by temperature ($F_{1,203} = 527.851, P < 0.001$) and exposure age ($F_{1,203} = 85.635, P < 0.001$), and there was a significant interaction between duration of exposure age and temperature ($F_{1,203} = 463.287, P < 0.001$). When analysed separately, males exposed to high temperature throughout development reached maturity significantly faster than males exposed to low temperature ($F_{1,117} = 1079.8, P < 0.001$), while in the adulthood group males there was not differences between males exposed to high or low temperature as expected as the temperature treatment was not imposed until after maturity ($F_{1,86} = 1.6488, P = 0.203$).

**Hatching success and offspring survival**
Hatching success was affected both by paternal temperature ($\chi^2=26.349, P<0.001$) and paternal exposure age ($\chi^2=25.952, P<0.001$) and there was a significant interaction between the two factors ($\chi^2=6.112, P=0.013$). Hatching success was higher for the offspring of male crickets that had been exposed to higher temperatures throughout development ($\chi^2=31.361, P<0.001$), but there was no significant difference in the hatching success for the offspring of those males exposed as adults to higher or lower temperatures ($\chi^2=0.7033, P=0.402$). The lowest hatching success occurred in females mated with DEV-L where only 8 females produced viable offspring (clutches hatched/tot: DEV-H 31/35, DEV-L 8/33, AD-H 35/38, AD-L 38/44, Table S1, S3a).

Offspring survival was not affected by paternal temperature ($F_{1,81}=2.315, P=0.132$) or paternal exposure age ($F_{1,81}=0.001, P=0.975$), but there was a significant interaction between the two factors ($F_{1,81}=12.339, P<0.001$; Table S1, S3b, Fig. 3). When males were exposed throughout development, offspring survival was higher for those males exposed to higher temperatures ($F_{1,28}=8.653, P=0.006$), while in the group of males exposed as adults, offspring survival was lower for those males exposed to higher temperatures ($F_{1,53}=7.263, P=0.009$).

**Offspring**

*Sperm traits*

Sperm assays were conducted on a total of 394 male offspring (ADO-H n= 179, ADO-L n=215) obtained from rearing offspring from 53 families (fathers’ treatment: AD-H n= 28 families, AD-L n= 25 families). Data on sperm production were obtained for 363 males and data on sperm viability for 309 males. There was an effect of temperature ($\chi^2=3.893, P=0.048$) on sperm production, but no effect of paternal temperature treatment ($\chi^2=2.136, P=0.144$) or their interaction ($\chi^2= 0.297, P=0.586, Table S4a, Fig. 4a$). Similarly, sperm viability was significantly affected by temperature ($\chi^2= 20.265, P<0.001$), but there was no
effect of paternal temperature treatment ($\chi^2_1 = 0.777, P=0.378$) or their interaction ($\chi^2_1 = 0.646, P=0.421$). As we found for the paternal generation (see above), males exposed to high temperature produced fewer and less viable sperm (Table S4b, Fig. 4b).

Relative trait analysis revealed that offspring within each temperature treatment were not favoured differently according to their fathers’ temperature treatment (treatment × paternal treatment: sperm production $\chi^2_1 = 1.059, P=0.589$; sperm viability $\chi^2_1 = 1.405, P=0.495$, Fig. S1a,b). This relative trait analysis therefore confirmed what we found using analyses of absolute trait values.

**Body weight**

Male offspring body weight was affected by the temperature that they themselves were exposed to, with males exposed to low temperature having on average lower weight ($\chi^2_1 = 8.340, P=0.004$), but it was not affected by paternal temperature ($\chi^2_1 = 2.353, P=0.125$); the interaction was also not significant ($\chi^2_1 = 1.128, P=0.288$, table S4c, Fig. 4c).

Using relative weight analysis, there was no evidence that offspring within each temperature treatment were favoured differently according to the paternal temperature treatment (treatment × paternal treatment: $\chi^2_1 = 1.285, P=0.526$, Fig. S1c) and offspring of fathers subjected to different temperature did not differ in their relative weight (paternal temperature: $\chi^2_1 = 2.351, P=0.125$).

**DISCUSSION**

Our study provides empirical evidence that deviations in temperature from standard conditions greatly affect sperm traits and male reproductive success, and fitness components of offspring. Our results also reveal the contrasting effects that temperature can have on the expression of sperm traits depending on the life stage of individuals. High temperatures
promoted sperm production and sperm quality if males were exposed throughout their development, while it reduced sperm production and quality if males were treated as adult. Our finding of reduced sperm production and quality following adult male exposure to high temperatures is in line both with studies showing detrimental effects of in vitro temperature manipulation applied directly to sperm (post-ejaculation) (Reinhardt, Dobler & Abbott 2015), and studies showing decreasing ejaculate quantity and quality following exposure of males to high temperatures (e.g. Zeh et al. 2012; Breckels & Neff 2013, but see Adriaenssens et al. 2012). Males reared at a high temperature during development, by contrast, produced larger amounts, and sperm of higher viability than their counterparts grown at a lower temperature. Interestingly, these males were also larger at maturity than males exposed to a high temperature as adults. This finding was unexpected, as individuals reared at high temperature are predicted to develop faster, resulting in smaller adult body size (temperature-size rule) (Atkinson 1994), which likely translates into smaller testes size and hence decreased sperm production. For example, in the beetle Callosobruchus maculatus (Vasudeva, Deeming & Eady 2014), the yellow dung fly Scatophaga stercoraria (Blanckenhorn & Henseler 2005), and the pseudoscorpion Cordylochernes scorpiodes (Zeh et al. 2012), males reared at high temperatures have smaller testes and produced fewer sperm compared to those reared at lower temperatures. Our results instead showed the opposite pattern. Among various possible explanations, two are particularly relevant here, one non-adaptive and one adaptive (Angilletta Jr, Steury & Sears 2004). First, it is possible that the exposure to low temperature during development constrained the development and maturation of testes leading to the observed differences in sperm traits between males developing at different temperatures. The second explanation involves evolutionary trade-offs between life history traits. Exposure to high temperature during development triggered males to develop faster and these males may have consequently invested more heavily in early reproduction, compared to the slower-
developing individuals at low temperatures. Hence, by altering male development time through our temperature treatment, we may have triggered such a response; an interesting possibility that requires further investigation. Overall, our results indicate that temperature effects can profoundly differ according to when the temperature manipulation, and that caution should therefore be used when designing studies investigating effects of temperature.

We found evidence for cross-generational paternal effects, as temperature experienced by fathers impacted with variation in both hatching success and offspring fitness. This adds to the small but growing body of evidence on the prevalence of paternal effects (Crean, Dwyer & Marshall 2013; Zajitschek et al. 2014; Evans et al. 2017; Zajitschek, Zajitschek & Manier 2017). Males exposed to a high temperature as adults produced offspring with lower survival compared to their low-temperature treated conspecifics. The opposite pattern was found in males exposed to a high temperature during development, which at high temperatures produced offspring with higher survival compared to males raised at lower temperature. During the time males and females were mated, females might have been able to detect temperature-induced changes in male body size or behaviour, and therefore differentially allocated resources into the resulting offspring. However, it is unlikely that the observed effects on offspring has be confounded by differential maternal allocation (Sheldon 2000). A previous study in this species has shown that within the temperatures we used there was no difference in the singing behaviour of males (Doherty 1985). In addition, even if we found difference in body size among males in different treatments, this should not have affected female choice or resource allocation, as in a previous study in this population, and under similar laboratory conditions, females did not show a preference based on male body size (Tuni et al. 2016). The pattern found in offspring hatching success and survival reflects the one found for sperm traits (number and quality) and indicates that the effect of temperature on sperm traits is the most likely mediator of the difference we observed in the offspring
generation. Our study therefore provides experimental evidence for cross-generational paternal effects mediated by temperature in a resource-free mating system. These findings add to the body of evidence that environmentally induced effects on ejaculate traits can result in cross-generational effects with far-reaching evolutionary implications. While the cross-generational consequences of environmentally-induced maternal effects are well known, studies on temperature-mediated paternal effects are rare, despite the large interest in understanding the impact of climate change at an evolutionary scale.

While the identification of the mechanistic basis of the association between environmentally induced changes in sperm and offspring fitness was outside the scope of the current study, we nevertheless discuss various plausible mechanisms here. Temperature may have affected sperm or other components of the ejaculate, and therefore offspring effects may have been mediated by changes in sperm structure, RNA, or modification of ejaculate components, such as seminal fluid (Bonduriansky & Day 2009; Crean & Bonduriansky 2014; Holman & Price 2014; Crean, Adler & Bonduriansky 2016). Since variability in sperm quality (Simmons & Beveridge 2011) and offspring viability (Garcia-Gonzalez & Simmons 2005a; Garcia-Gonzalez & Simmons 2007) has been attributed to the effects of seminal fluid in field crickets, further studies focusing on modifications in seminal fluid quantity or composition induced by temperature may be a fruitful direction to investigate the mechanisms underlying the observed effects.

Whether paternal effects alone can be adaptive is largely unknown (Crean & Bonduriansky 2014; Holman & Price 2014). Can paternal effects constitute an anticipatory effect, so that offspring fitness is increased under the same environmental conditions to which fathers were exposed? The only study that tested this anticipatory effect has been conducted in a broadcast spawning ascidian (Styela plicata) where Crean and colleagues (2013) exposed males to different population densities. They found that offspring had a higher hatching
success and increased survival in the same density-environment experienced by the fathers.

In our study, we tested whether offspring performed better (in terms of reproductive traits and body size) in the temperature their fathers were exposed to as adults. To address this question, we assigned male offspring to high or low temperature during adulthood, as we did for their paternal generation. We found that temperature had a similar effect on sperm traits as described for the paternal generation, further corroborating our findings of the strong effect of temperature on sperm traits in this species. Nevertheless, we found no evidence for an anticipatory effect, as the relative fitness traits (sperm quality, sperm production, and body weight) of offspring kept at the same temperature as their father did not differ from that of offspring kept at a different temperature. We cannot exclude that adaptive effects occur under different circumstances, for example under different temperatures. We decided to use a range of temperatures (24° and 28°) that were deemed ecologically relevant to this species to thereby simulate naturally occurring temperature variability during the lifecycle of an ectotherm such as the cricket (see Methods). Using more extreme temperatures may allow adaptive paternal effects, if any, to arise, but in that case the ecological relevance of findings should be interpreted with caution. Contrary to favourable laboratory conditions, in the wild changes in temperature are likely to affect organisms indirectly, as through, for example, their effects on water and food availability. The combination of multiple environmental effects (such as temperature and water/food availability) may therefore exacerbate the effects imposed solely by the manipulation of temperature. Future studies would benefit from considering multiple environmental factors concurrently.

In designing experiments on transgenerational adaptive effects (maternal or paternal or both), it would be ideal to quantify the predictability of the environmental factor manipulated -in our case temperature- in the wild, to understand whether the temperature that parents experience is a good predictor of the temperature their offspring will experience too.
Unfortunately, we do not have that information. If the temperature experienced by the fathers predicts the temperature the offspring will be exposed to, the best design would be the one planned for the developmental group (both fathers and offspring exposed to the same temperature from development) but that could not be completed due to the low offspring viability of the DEV-L group. However, we should not underestimate the relevance of the adult treatment of our design that tested the anticipatory effects by exposing both fathers and offspring to the manipulated temperature during adulthood only. Global warming is not only affecting the average temperature, but it is also associated with an increase in intensity and frequency of local extreme events, such as heat waves, that are short in time and do not imply autocorrelation in the temperature experienced at different life stages or among generations. This also applies to diverse environmental factors (abiotic or biotic), and caution should be taken in interpreting results of such experimental design in the light of the predictability in time, and space in the case of species with offspring dispersal, of the temperature manipulation in the wild (Burgess & Marshall 2014). In the future we should ideally identify the predictability of environmental temperature (both heat waves and average temperature) for the offspring generation, to design the most appropriate experiment mimicking what is more likely to occur in the wild.

In summary, we demonstrated that temperature exposure affects sperm traits in *G. bimaculatus* in a complex fashion, with high and low temperature having opposite effects depending on which life stage the exposure was administered. Our findings provide evidence for cross-generational effects of temperature-mediated paternal effects but did not provide support for adaptive paternal transgenerational plasticity, at least for the traits that we measured. This is an important point, as this study is only the second that ever tested the adaptive paternal effects hypothesis and the first to look at it from a climate change prospective. Importantly, paternal effects may nevertheless adaptively influence the ability of
offspring to cope with the environment, but this may be evident only in conjunction with maternal effects or under harsher conditions (e.g. diet-restriction). Our results thereby shed new light on how temperature affects male reproductive traits and their trans-generational implications, providing new insights into factors influencing how populations may face environmental changes.

AUTHORS’ CONTRIBUTIONS
CG and CT conceived the experiment. CG, ND and CT design the experiment. CG, CL and CT collected the data. CG analysed the data. CG and CT led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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DATA ACCESSIBILITY
Data deposited in Dryad Digital Repository (xxx).


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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article.

Appendix S1: Supplementary tables and figures
Figure Legends

**Figure 1.** Schematic representation of the experimental design and predictions for offspring fitness. Males were given Low (blue) and High (red) temperatures either from (A) development (DEV), or (B) adulthood (AD) onwards. After the treatment, sperm number and quality were assessed and the male paired with a virgin female (see text for details). Hatching success and offspring survival at 7 weeks was assessed, and once adult, male offspring of AD groups were exposed to Low and High temperatures. Male offspring sired by males of DEV-groups were not tested due to reproductive failure in DEV-L group (see results).

**Figure 2.** Effect of Low and High temperatures and exposure age (developmental-DEV and adult-AD) on (a) sperm numbers and (b) sperm viability, and (c) male body weight. Means ± SE.

**Figure 3.** Effect of Low and High temperatures and exposure age (developmental-DEV and adult-AD) on offspring survival. Means ± SE.

**Figure 4.** Effect of Low and High temperatures and paternal treatment (Low and High temperatures) on (A) sperm numbers and (B) sperm viability, and (C) male body weight. Means ± SE.
Figure 1

(A) Developmental treatment:  
Paternal condition  
Sperm traits assessment  
Hatching success & survival  
Offspring condition  
Sperm traits assessment

(B) Adult treatment:  
DEV-L  
DEV-H  
AD-L  
AD-H
Figure 3

[Diagram showing survival at 7 weeks for two groups: DEV and AD, with categories Low and High for each group.]
Figure 4

A) Sperm number (x10^6)

B) Sperm viability

C) Body weight (g)