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Heat shock sensitivity of adult male fertility in the parasitoid wasp *Anisopteromalus calandrae* (Hymenoptera, Pteromalidae)

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ABSTRACT

In insects, decreased reproduction is a sublethal consequence of high temperatures, with males being more sensitive to this in many species. In hymenoptera, arrhenotokous parthenogenesis means that female offspring are produced using sperm and are thus diploid, while males are haploid. Consequently, sperm stocks in males and females (after copulation) are a key regulator of the sex ratio. *Anisopteromalus calandrae* is a parasitoid wasp in which males can suffer from subfertility due to a drastic decrease in sperm count after exposure to high temperatures during a critical early pupal stage. However, in this species spermatogenesis continues during adulthood, therefore the heat sensitivity of adult males remains to be studied. Laboratory studies were conducted on virgin and previously mated young adult males under control (30 °C) and heat shock (10 min at 48 °C) conditions to exhaust their initial sperm stock. After heat shock, in both virgin and already mated males, the individual sperm potential was half that of controls. Both groups continuously produced sperm, but sperm stock of heat shocked males' never reached that of the controls. Heat shock reduced survival at 10 days only in previously experienced males but had no impact on the mating ability in competition for a female compared to controls. Despite a reduced sperm count, heat shocked males had fully fertile spermatozoa. Such a physiological response to heat shock in a species with continuous sperm production could be of major interest for both wild populations in a context of temperature variations and parasitoid wasps introduced for agronomical purposes.

1. Introduction

Temperature is considered as the major abiotic factor which has a profound effect on the survival, behavior, fitness and the life history of insects (Bale, 2002; Denlinger and Lee, 1998; Denlinger and Yocum, 1998). Most studies on temperature-dependent performance have focused on the "zone of effective temperature" (Girish, 1965), namely the temperature range in which insects are active. Very few studies exist on the influence of extreme temperatures which occur under natural conditions, for example, the minimum and maximum effective temperature in winter and summer (but see Niedermayer et al., 2013). Insects are especially vulnerable to such extremes because of their small size and ectothermic physiology, and temperature plays a vital role in survival and reproductive success (Lee et al., 1996; Rinehart et al., 2000). When temperatures exceed an insect's optimum temperature range, there are three mutually exclusive results, either survival in optimal conditions, sublethality with a decrease of some vital functions, or death (Denlinger and Yocum, 1998; Mutamiswa et al., 2017).

Compared to lethal consequences, the non-lethal effects of heat stress have received less attention. Sublethal tests include determining critical thermal limits (stress temperature and exposure duration) and time to recovery after chill-coma (Andersen et al., 2015; Alford et al., 2016). From an ecological point of view, sublethal tests are considered more relevant than mortality because the temperature variations used are closer to field conditions (Alford et al., 2016; Verberk et al., 2016).

Several studies have shown that insects surviving exposure to sublethal high temperatures may exhibit impaired development, hatching rhythm, morphology, longevity, fecundity, fertility and over-all fitness; damage results from temperature exposure (Proverbs and Newton, 1962; Gonen, 1977; Arbogoast, 1981; Saxena et al., 1992; Mahroof et al., 2005a, 2005b; JØrgensen et al., 2006; Xie et al., 2008; Cui et al., 2008; Niedermayer et al., 2013). Among traits currently known to be affected by heat stress, reproductive processes are often diminished by less severe conditions than those causing mortality (Fasolo and Krebs, 2004; Walsh et al., 2019).

In hymenoptera, sperm stocks in both males and females (after copulation) are of major importance because sex determination relies on arrhenotokous parthenogenesis. As males are produced from haploid oocytes and only female offspring emerge from diploid eggs, sperm stock is a key regulator of sex ratio (Nguyen et al., 2013). However, rare

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are the studies that have measured sperm stocks as a means of quantifying male reproductive capacity (Snook et al., 2000; Lacoume et al., 2007, 2009; Nguyen et al., 2013; Chirault et al., 2015). Sperm production might be only in the pre-imaginal stages (larvae and pupae), or throughout those immature stages, as well as in the adult; males could be respectively named prospermatogenic, when the sperm reserves are full at emergence, or synspermatogenic, when spermatogenesis continues into adulthood (Boivin et al., 2005). However, most insect parasitoids are between these two extremes and are classified according to a spermatogenic index based on the proportion of the maximum production of sperm over their lifetime (Boivin et al., 2005).

A heat period of some days during pupal development of males is known to cause injury to testes and sperm stock (Chihrane and Laugé, 1994, 1997; Krebs and Loeschcke, 1994; Scott et al., 1997; Rinehart et al., 2000; Rohmer et al., 2004; Chirault et al., 2015) reducing fertility and consequently reproductive success of female mates. However, the experimental heat stress has to occur at a critical phase of spermatogenesis to affect male fertility (Giojolas and Catala, 1993; Nguyen et al., 2013; Chirault et al., 2015). In most insects, spermatogenesis starts at an early stage (larvae) and is maximal in pupae when spermatozoa begin to mature and are not yet stored in seminal vesicles (Chihrane and Laugé, 1994; Lacoume et al., 2007, 2009; Nguyen et al., 2013). In prospermatogenic species, young adult males no longer have functional testis, thus only heat stress occurring during early stages results in male sub-fertility (*Nasonia vitripennis*, Chirault et al., 2015). On the other hand, in synspermatogenic species, adult males surviving heat shock would be expected to demonstrate sub fertility because they have functional testes.

Anisopteromalus calandrae (Howard) (Hymenoptera: Pteromalidae) is a common and cosmopolitan parasitoid wasp in tropical and subtropical areas. It is known as a biological control agent against bruchid (Coleoptera, bruchidae) pests in seed stocks (Van den Assem et al., 1984; Smith, 1992, 1993; Ahmed, 1996; Choi et al., 2001; Tuda and Shimada, 2005). In *A. calandrae*, the reproductive capacity of males (i.e. male fertility) is well-known under laboratory conditions: mating capacity, sperm reserve, sperm stored in spermatheca of females, outcome of sperm competition, and offspring obtained after one mating (Do Thi Khanh et al., 2005; Kasamatsu and Abe, 2015). Sperm stocks in both males and females are small (a few thousands in males, some hundreds in females), and the fertilizing efficiency of stored sperm is high [0.75, representing the expected fertilization of one spermatozoon stored in the female spermatheca (Do Thi Khanh et al., 2005)]. Furthermore, *A. calandrae* is a synspermatogenic species; spermatogenesis continues in adult males, resulting in old virgin males having about 1.75 times more spermatozoa than young virgins (Bressac et al., 2009).

Studies have shown that high temperatures during the pupal stage have temperature dependent deleterious effects (Nguyen et al., 2013). One clear result was that only the quantity of sperm was diminished without their quality being impaired by pupal heat stress, demonstrated by the fact that female offspring were obtained after mating of males emerging from heat-stressed pupae.

To summarize, *A. calandrae* is a laboratory model in which adult males have active spermatogenesis, subfertility in response to a heat period at the pupal stage, and in which sperm count is a main determinant of the sex ratio of the mate's offspring.

The question tested here in a species with continuous spermatogenesis, was whether the testes of adult males were sensitive to temperature and whether the latter has an impact on the stock of sperm immediately and some days after heat shock. Males have mature sperm in their seminal vesicles on emerging (Do Thi Khanh et al., 2005), thus heat shock in adults could act on both immature and mature gametes. The only way to remove most of those mature sperm is by successive copulations (Bressac et al., 2009). Under controlled conditions, either virgin or previously mated adult males underwent heat treatments. Quantification of their sperm stocks, one day after heat shock and nine days later, gave interesting information about testis heat sensitivity. A second question was whether those males had access to females, a major component of male fertility. Therefore, heat shocked males and controls were also tested for their mating capacities, both with and without competition, to assess their functional sub fertility or complete sterility. The two groups were compared in relation to their survival, maturation of sperm stock, access to females and subsequent offspring sex ratio. Results indicate that heat shock in adult males led to limited sub fertility because sperm stock was reduced, while measured fitness did not differ from controls.

2. Materials and methods

2.1. Insects

Anisopteromalus calandrae (Howard) (Hymenoptera, Chalcidoidea, Pteromalidae). Since 2000, the strain collected from cowpea stocks in Ivory Coast (Africa) has been reared in the laboratory (Do Thi Khanh et al., 2005; Lacoume et al., 2009) on larvae of *Callosobruchus maculatus* (Coleoptera: Bruchidae), which develop in cowpea seeds (*Vigna unguiculata*), and maintained in a climatic room corresponding to standard conditions (12 h light/12 h dark, temperature 28–30 °C, relative humidity 50–55%).

To obtain only males, eggs of virgin *A. calandrae* females were allowed to develop under controlled conditions (Nguyen et al., 2013).

2.2. Heat shock adjustment

Preliminary tests were conducted to identify the sublethal parameters (temperature and time of exposure) under which a decrease in male reproduction could be measured while optimizing individual survival and obtaining less than 50% mortality.

Preliminary treatments involved pools of 10 males in a glass tube. Tubes were put in a temperature controlled water bath adjusted to 45, 46, 47, 48, 49 and 50 °C respectively for 1 h and 90 males were tested for each temperature. After heat shock, males were kept in a climatic room under standard conditions (see above). Mortality counts were then recorded 72 h later.

According to these preliminary results, 48 °C was identified as the highest sublethal temperature. It induced 50% mortality when applied for 1 h. Consequently, this temperature was applied to subsequent 1-day-old virgin males for only 10 min to obtain heat shocked males without any mortality. Anesthesia was not used when transferring males, and only soft brush handling was applied when necessary.

2.3. Experimental males

Four sets of 1-day-old males were prepared. Two control sets (non-heat shocked males) were considered: (1) just emerged 1-day-old males (virgin control males, CM) and (2) males having a sexual experience. These previously experienced males (PEM) were 1-day-old males which had been in contact with five virgin females for 4 h. This time was sufficient for one male to copulate with five females (checked by the presence of sperm in the spermathecae of all females; Do Thi Khanh et al., 2005; Bressac et al., 2009). Control males were transferred into glass tubes, maintained under standard conditions for 10 min, and then removed from the tube to mimic conditions of experimental males (see below).

Heat shocked males were 1-day-old males maintained 10 min at 48 °C in glass tubes in a heat controlled water bath. Two sets of males were also considered: (1) virgin heat shocked males (HSM) and (2) previously experienced heat shocked males (PEHSM) which had been in contact for 4 h with five virgin females 1 h prior to heat shocking.

Some males from each treatment were maintained by groups for two days and others for 10 days in the climate chamber (see above), in a petri dish with a 20% saturated sucrose solution.

According to heat treatment, mating experience and age, there was

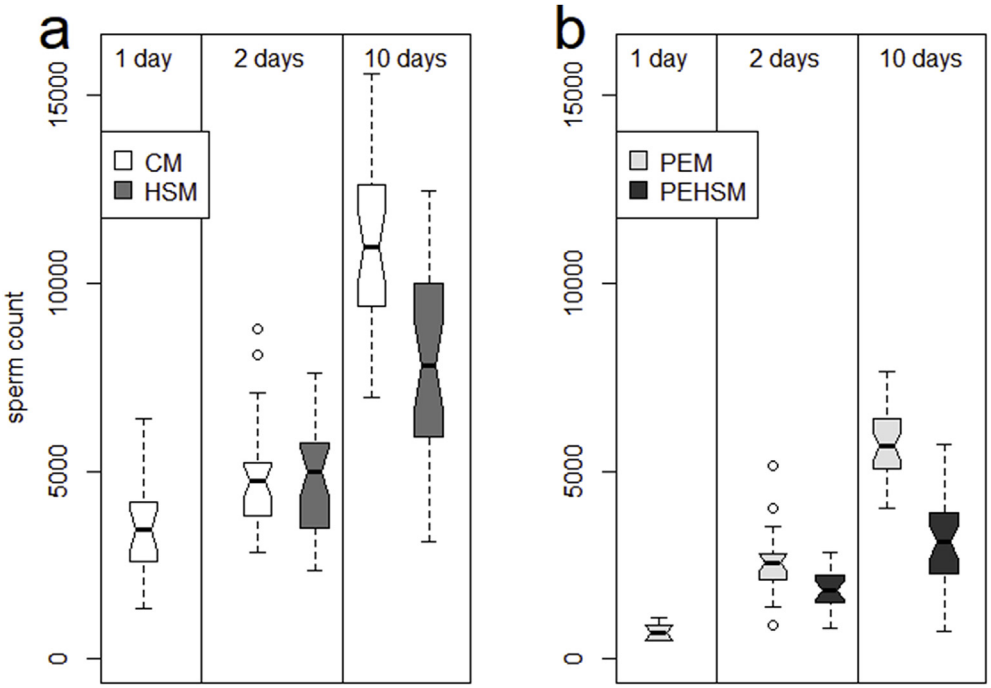

Fig. 1. Sperm counts in seminal vesicles of *A. calandrae* males after different treatments and times. **a**. virgin males, CM were virgin males without heat treatment, i.e. 30 °C (according to Nguyen et al., 2013), HSM were virgin males which were heat shocked for 10 min at 48 °C. **b**. Males mated with 5 females, PEM were previously mated males which were not heat shocked, PEHSM were previously mated males which were heat shocked for 10 min at 48 °C, see materials and methods for protocols. Box plots show median (solid line), as 75% upper and 25% lower quartiles (box). Bars are standard deviations (upper and lower bars), open circles are outlier data and notches indicate the density on both sides of the median. Numbers and detailed statistics are in the text and Table 1.

a total of ten sets: two controls, and eight sets of experimental males, the numbers for each set are given in the results (N = 20 to 30 for each experimental set).

2.4. Sperm counts

Males from each of the two control and the eight experimental sets were dissected and the sperm counts in their seminal vesicles were recorded. A similar quantity of sperm was contained in the two seminal vesicles (Lacoume et al., 2009), therefore only one of the two seminal vesicles was dissected and the sperm dispersed in a drop of saline solution (128.3 mM NaCl, 4.7 mM KCl, 2.3 mM $CaCl_2$). Spermatozoa were exhaustively counted under a fluorescence microscope after ethanol fixation and DAPI staining (Bressac and Chevrier, 1998). This sperm count was multiplied by two to obtain the total sperm reserve for each male.

2.5. Male fitness

In addition to sperm counts, fitness of males was compared through complementary traits: (1) survival (CM, HSM, PEM and PEHSM), (2) access to females (only for males with sperm made at the adult stage, 10-day-old PEM and PEHSM) and (3) progeny sex ratio (10-day-old PEM and PEHSM) after one mating.

To measure their survival without mating, several series of males were isolated in plastic tubes under standard conditions and fed with a 20% saturated sucrose solution; N = 148 to 176 males per treatment. The percentage of males surviving after two and 10 days was recorded.

Access to females was tested through competition between two previously experienced males, one 10-day-old PEM (n = 30) and one 10-day-old PEHSM (n = 30). Both males were put into a 30 mm diameter Petri dish, and then a 2 h-old virgin female was added. To distinguish between the two males, a white lacquer (Marabuwerke GmbH & Co.) spot was put on the thorax of one male 24 h before the competition, marks were alternated between sets of males (Lacoume et al., 2007). The winning male and copulation duration were recorded. Pairs were separated immediately after the first copulation. The loser of the competition was immediately offered another 2 h-old virgin female for a single copulation. As previously, the copulation time was recorded and pairs were separated immediately after copulation.

To determine progeny sex ratio, females mated by both first and second males were individually placed in an oviposition box with seven bruchid-infested cowpea seeds, renewed daily, and a source of sucrose solution (20% in water). Each seed contained one to three 18-day-old hosts (last instar larvae) (Chevrier and Bressac, 2002). Egg-laying was carried out until the females died. After complete development of parasitoids, sex ratios [females/(males + females)] were recorded as a measure of male fertility. Females producing less than 10 offspring or dying within seven days were discarded from the analyses because they were considered artifacts, being far from the usual fertility of this species in the laboratory (Lacoume et al., 2009); this represented one female mated with a PEM, and three with PEHSM.

2.6. Statistical analysis

Sperm counts were analyzed using a generalized log-linear model corrected for under dispersion with a quasi-Poisson distribution of errors. Explanatory variables were age, heat shock and sexual experience. The model was simplified following a backward stepwise procedure after examination of the estimates until the minimal adequate model. The effect of each factor on sperm counts was analyzed using an ANOVA with a Chi^2 post hoc test. Tukey contrasts were used for multiple comparisons of means. Then interactions between heat shock and age, and heat shock and sexual experience were calculated respectively.

Male fitness was compared by Fisher's exact test for frequencies (survival, percentage of first copulating males, and offspring sex ratios) and by Mann-Whitney for quantitative data after confirming the non-normality of data distribution using Shapiro-Wilk tests (copulation durations in seconds). Additionally, both daughter production and sex ratio were tested for a correlation with copulation duration for both PEM and PEHSM to reveal an influence on the sperm use by egg laying females.

Statistics were performed with R. Rcmdr package was used for logistic regression.

Table 1
Summary of results of the final generalized linear model showing the effects of heat shock, sexual experience, age and their significant interactions on the sperm stock in *A. calandrae* adult males.

	Df	Deviance	Resid. Df	Resid. Dev	Pr (> Chi^2)
NULL	255	475,294			
Age	3	178,434	252	296,861	< 2.2e-16
Sexual experience	1	153,174	251	143,687	< 2.2e-16
Heat shock	1	29,401	250	114,286	< 2.2e-16
Age x Heat shock	1	8494	249	105,792	3.59e-06
Sexual experience x Heat Shock	1	5923	248	99,869	0.0001091

3. Results

3.1. Sperm counts in experimental males

Sperm stocks of males according to heat shock and sexual experience are presented in Fig. 1. Regardless the status (CM, HSM, PEM, PEHSM), the male sperm stock increased with age.

Effects of both age and sexual experience on sperm counts were dependent on heat shocks (Table 1). The decrease in sperm count after heat shock differed between virgin and experienced males (significant interaction). Moreover, it was age-dependent (significant interaction). However, with no heat shock, the deficit in sperm counts due to the sexual experience of males did not depend on their age (non-significant interaction, $F_{1,\ 247} = 6.8$, $P = 0.90$).

Heat shock had stronger impact on the stock of spermatozoa in sexually experienced males than in virgins (Fig. 1). In 10-day-old males, the stock was reconstituted to 71% in virgin males compared to 52% in sexually experienced males. Regarding sperm stock at 10 days of age, the deficit caused by heat treatment of adult males was 29% (7775 in HSM vs 10921 in CM). This caused by previous mating increased to 47% (5836 in PEM vs 10921 in CM). Interestingly, when both heat shock and mating occurred, it resulted in a sperm deficit of 72% (3087 in PEHSM vs 10921 CM), which was close to 76%, i.e. the sum of 29% and 47%.

To summarize, heat shock and initial experience reduced sperm stocks, whereas age increased them (Fig. 1 and Table 1). After 10 days, males that had successively multiply mated and had been heat shocked had only a third of the sperm stock of virgin control males.

3.2. Male fitness

Survival after two and 10 days is shown in Fig. 2. Whatever the status and treatment, 100% of males survived after two days. After 10 days, survival did not significantly decrease in either virgin or previously experienced males. However, when males had been heat shocked on the first day, survival was significantly reduced. The survival of virgin males decreased by about 20% compared to two-day-old males (81.3 *vs* 100%, not significantly different, Fisher's exact test, $p = 0.41$) and by about 50% in previously experienced males (55.3 *vs* 100%, significantly different, Fisher's exact test, $p < 0.01$) (Fig. 2).

Regarding male-male competition of 10-day-old males, PEM copulated no more frequently than PEHSM (60% *vs* 40% Fisher exact test against a 50% success, $p = 0.6042$). Moreover, there was no difference either in copulation times (21.6 ± 1.9s for PEM vs 17.5 ± 1.3s for PEHSM, Mann Whitney, $W = 580$, $p = 0.055$) or in progeny sex ratio of females mated with the winner of the male-male competition (female sex ratio 0.72 for PEM *vs* 0.66 for PEHSM, Fisher's exact test, $p = 0.81$). Finally, sex ratios were not related to copulation duration (Pearson correlation, $R^2 = 0.032$).

For losers of first male-male competition, second chance male mating ability was 100% in the two categories and there was no difference in the offspring of females mated with either type of male (female sex ratio 0.72 for PEM *vs* 0.69 for PEHSM, Fisher's exact test, $p = 0.91$).

4. Discussion

Male fertility as measured by the number of sperm produced in *Anisopteromalus calandrae* was modified by ageing, sexual experience and heat shocking at the adult stage. A heat shock of 48 °C for 10 min in young adult males did not completely prevent sperm production, and sperm were fertile, but the individual sperm potential was half that of controls. This does not indicate sterility, but to our knowledge is the first evidence of male sub-fertility induced by heat in an adult parasitoid wasp. Moreover, the sensitivity of testes to heat stress was not amplified in young males that had had previous sexual experience. Furthermore, heat shock alone did not reduce survival of males, or their access to females when competing with a control challenger, and mating with one female was successful and produced offspring with a typical sex ratio (Lacoume et al., 2009).

Adult *A. calandrae* males showed an increase in their sperm stock regardless of the test conditions, even after multiple sperm transfer to successive females (Bressac et al., 2009). This evidences continuous sperm release, which certainly results from continuous spermiogenesis. Such prolonged spermiogenesis has also been observed in *Dinarmus basalis* (Pteromalidae, Lacoume et al., 2007), and *Cotesia congregata*, Braconidae (Uzbekov et al., 2017) in males maintained under their optimal conditions.

Our results show that heat shock in adults decreased reproductive performance of male *A. calandrae* and particularly reduced the number of sperm produced without stopping sperm production. The fact that physiological consequences were observed shows that heat shock was perceived as a stress. The reduction in male fertility due to heat shock is consistent with studies in the pupae of the parasitic wasp *Trichogramma brassicae*, Trichogrammatidae (Chihrane and Laugé, 1994) and *Drosophila melanogaster* (Krebs and Loeschcke, 1994) suggesting that the reduction in male fertility through heat shock at an immature stage is most likely due to direct injury to male germ lines, leading to the production of atypical sperm or empty seminal vesicles (Chihrane and Laugé, 1997). In other studies, heat shock increased the time needed to produce spermatophores in male *Orchesella cincta*, Collembola (Zizzari and Ellers, 2011), inducing a disadvantage for heat shocked males compared to controls.

In the present study, only male sperm production was affected by the temperature causing no consequences for competitive mating ability. It is possible that the high temperature treatment did not affect all the physiological functions of males, such as breathing, the nervous system and general behavior (Neven, 2000; Wojda, 2016) which otherwise would have made males weaker and less likely to mate with females. The fact that measured fitness was not reduced may also result from the present experimental design, which did not test successive mating of males throughout their lives or competition in natural situations involving numerous males and females emerging simultaneously in seed stocks (Ahmed, 1996). In this insect, it was shown that male fecundity (i.e. offspring production) was closely correlated to the sperm count in seminal vesicles (Bressac et al., 2009), with males having fewer sperm giving rise to fewer offspring. Thus, heat shocked adult male offspring would be under represented in the next generation in cases of multiple mating females.

Increased mortality in heat shocked males was only significant after sexual experience. This suggests that sexual experience rendered males less resistant. This could not be due to extra metabolic costs of spermatogenesis because increased sperm count with ageing was lower in previously mated males than in virgins (53% in mated vs 71% in virgin males). In the literature there is a lack of references on male metabolic investment (Snook, 2005), and additional experiments are needed to highlight the physiological cost of spermatogenesis in insects. There is plasticity in the response of *A. calandrae* to heat stress and sperm

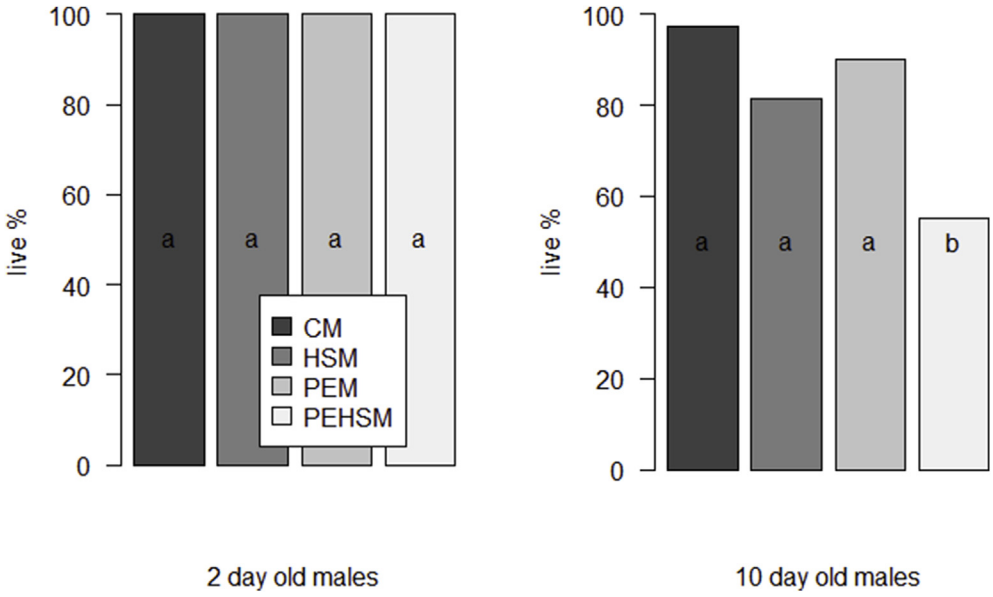

Fig. 2. Survival of experimental males after 2 (left) and 10 days (right) without mating. CM were virgin males which had no heat treatment, i.e. 30 °C (according to Nguyen et al., 2013), HSM were virgin males which were heat shocked for 10 min at 48 °C. Regarding sexually experienced males (mated with five females), PEM were not heat shocked, PEHSM were heat shocked for 10 min at 48 °C, see materials and methods for protocols. In each series N = 148 to 176 at the beginning of the experiment (day 0). Letters indicate series that are significantly different following a Fisher's exact test ($p < 0.05$).

production is able to resist such stress conditions. In both sets of males with no previous sexual experience (virgins), the number of sperm in the seminal vesicle did not differ at two days of age, but it became significantly different after nine days. The amount of sperm stored in seminal vesicles of non-heat shocked males was more than twice that in heat shocked individuals. Moreover, we found the same results in the sexually experienced males. Such a difference in sperm production may be the consequence of many physiological changes after high temperature treatment. It could be a negative effect due to the whole organism's response to heat shock (Zizzari and Ellers, 2011) or it may be related to heat shock proteins which are energetically costly to produce (Koehn and Bayne, 1989; SØrensen et al., 2003). Other studies have shown that heat shock proteins are synthesized in response to several forms of stress (Denlinger and Lee, 1998; SØrensen et al., 2003). As yet, there is a lack of data linking sperm production and specific physiological mechanisms, but *A. calandrae* males represent a suitable model to investigate such mechanisms.

In *A. calandrae*, heat shock applied at an early pupal stage dramatically decreased the sperm supply (Nguyen et al., 2013). A three-day period at 38 °C caused a decrease of 93% in the sperm stock. This physiological sensitivity was temperature dependent because reduction in fertility was less after periods at 36 °C and no effect was observed at 32 °C. As observed here on adults, heat stressed males at the pupal stage had a reduced lifespans. However, their sperm fertility was not affected. High temperature effects on both pupae and adult males are similar, namely a reduction in sperm production, showing that testes in both life stages are sensitive to heat stress.

Such consequences of heat stress could have considerable effects on the protection of seed stocks with *A. calandrae* as a bruchid biocontrol agent. Although only females are of interest for laying eggs on the pest larvae, a decrease in male fertility would result in sperm-limited females which would lay male-biased generations. The question of the sensibility of females remains. As in most organisms, heat stresses on reproduction are sex specific (Iossa, 2019).

Heat periods are also used in industry to kill pest insects in flour and seed stocks (Porto et al., 2017). It may also be deleterious for beneficial insects such as parasitoid wasps which can control such pests if they recontaminate the stock. However, it was shown that parasitized hosts such as aphids survive better after heat shock than non-parasitized hosts (Trotta et al., 2018). In both of these studies, heat had consequences on host survival, and issues involving sublethal effects on the control of insect pests by other insects require further investigation.

To conclude, a short period of 48 °C for 10 min on adult *A. calandrae* males caused delayed increased mortality and lowered the sperm production. Fertility of heat stressed adult males did not decrease for the first mating, but the reduced sperm stock predicts a disadvantage of heat shocked males over successive copulations. Such a physiological response to heat stress in unpredictable environments has to be considered in natural and cultured populations of parasitoid wasps subjected to high temperatures as adults. This could be of major interest in a context of global climate change (Walsh et al., 2019).

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