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# Hot and cold waves decrease sperm production and bias sex ratio in the parasitoid wasp *Cotesia typhae* (Hymenoptera, Braconidae)

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## Abstract

Parasitoid wasps are haplodiploid, meaning that sperm stored by egg laying females are only used to produce daughters. Thus, the sex ratio of the offspring depends on the availability of sperm after mating. In these insects, males are sensitive to temperature at the pupal stage. This stress leads to subfertility due to a drastic reduction in the number of sperm produced and transferred to females. Experiments were conducted under controlled conditions on the parasitoid wasp *Cotesia typhae* (Hymenoptera, Braconidae), a natural enemy of the invading pest *Sesamia nonagrioides* (Lepidoptera, Noctuidae). At 25-27°C, sperm production was measured for 7 days, and found to reach a plateau at the third day of adult life. It leads to a final amount around 25,000 sperm per male. A male can successfully inseminate at least 10 females, producing predominantly female offspring. Sperm production decreased significantly after 1 day of pupal exposure to heat at 34 or 36°C and 7 days of cold at 0, 5 or 10°C. This highlights that both cold and heat are stressful. After mating with one male treated at 10 or 34°C, females store fewer sperm than the control, and produce fewer daughters. The sex ratio of the offspring is male biased when males experienced temperature stresses during development, like other parasitoid wasps. In the field, *C. typhae* populations would be affected by heat and cold, at least at the pupal stage. This lowers overwintering risk in case this biological agent was introduced in Europe. This risk is both economical, as companies seek to establish costly continuous production to sell beneficial insects, and ecological as the introduced population would not settle in the ecosystem. Lastly, the transport and storage of this insect of agronomic interest would need to consider temperature variations to ensure successful application.

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## Key words

40 Reproduction; temperature stress; male fertility; overwintering; biocontrol

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## Introduction

42 Parasitoid wasps are extensively studied for their ability to reduce insect pests in cultures and  
 43 storage, but also for their reproductive system. In these insects, sex determination is based on  
 44 haplodiploidy, whereby males are haploid and females, diploid. As only female offspring require the  
 45 use of sperm at oviposition, sex ratio is determined by the amount of sperm available to egg-laying  
 46 females (Quicke, 1997, Chevrier and Bressac, 2002). Virgin females only lay male offspring, which are  
 47 unable to control pests, and compromise biological control programmes. Therefore, sex ratio plays a  
 48 major role for the success of parasitoid wasps in pest management. In most species, the sex ratio is  
 49 female biased (Quicke, 1997), and whereas females usually mate only once (monoandry), males can  
 50 mate multiple times (polygyny). The sex ratio results from an evolutionary trade-off, as an excess of  
 51 males reduces egg laying and the control of targeted pests, and as a deficit in males results in  
 52 unmated or badly inseminated females that lay male-biased clutches, compromising the efficiency of  
 53 parasitism (Bressac et al., 2009; Chevrier et al., 2019). However, even when the proportion of males  
 54 appears optimal, a decreased quality of reproduction may occur due to the suboptimal fertility of  
 55 individual males. In many parasitoid wasps, so-called subfertile males produce a reduced amount of  
 56 sperm (Lacoume et al., 2007; Chevrier et al., 2019; Chirault et al., 2015; El Sabrout et al., 2021).

57 Insects are vulnerable to temperature variations because of their small size and ectothermic  
 58 physiology (Ma et al., 2021). Heat waves at the pupal stage can reduce sperm production and thus  
 59 induce sex ratio biases in the following generations. This thermal sensitivity was found in diverse  
 60 parasitoid wasps belonging to the families Pteromalidae (*Anisopteromalus calandrae* – Nguyen et al.,  
 61 2003; *Dinarmus basalis* – Lacoume et al., 2007; *Nasonia vitripennis* – Chirault et al., 2015), and  
 62 Braconidae (*Microplitis rufiventris* – El Sabrout et al., 2021).

63 In the field, insects experience temperature variations that go beyond standard ambient  
 64 temperature variation and they use many strategies to control the temperature actually felt, such as  
 65 individual behaviour (Ma et al., 2021) or specific morphology (Bota-Sierra et al., 2022). However,  
 66 most of these adaptations are not effective for immobile developmental stages, as are the pupae of  
 67 holometabolous insects.

68 Males are generally more sensitive to heat than females (Chen et al., 2018), and the pupa is a critical  
 69 stage because this is when spermatogenesis is most intensive (Chirault et al., 2016). Most insect  
 70 males survive a limited heat shock and may not become sterile. However, single heat periods induce  
 71 subfertility, as most males reproduce with lower performance than control males (Chevrier et al., 2019).

Moreover, in some parasitoid wasps, cold temperatures associated with short photoperiod induced a reproductive diapause of females (Chen et al., 2012). However, there is a lack of empirical evidence of the effect of lower temperatures on males.

*Cotesia typhae* (Hymenoptera, Braconidae) is a promising insect for the biocontrol of the maize pest *Sesamia nonagrioides* (Lepidoptera, Noctuidae) in Europe. *C. typhae* has been described as a specialized clade of *Cotesia sesamiae* in eastern Africa where it develops exclusively on *S. nonagrioides* on two associated plants (Kaiser et al., 2017; Gauthier et al 2018). In its native range, *C. typhae* is exposed to average temperatures between 20°C and 30°C with extremes varying between 15°C and 35°C (West Kenya, Kobodo, NASA Langley Research Center (LaRC), <http://power.larc.nasa.gov>). *C. typhae* is a gregarious species, and females lay between 80 and 100 eggs in their host (Benoist et al., 2020). After endoparasitic ovo-larval development, parasitoid larvae emerge from the host and spin their cocoon. The life cycle lasts 3 weeks at 27°C and adults live a few days in these laboratory conditions. The thermal resistance (CT min and CT max) of this insect remains to be investigated in the perspective of its introduction in Europe, where we expect heat shocks in summer amplified by climate change, and cold periods during winter.

We conducted laboratory experiments to characterize male fertility and to simulate summer and winter thermal variations on males at the pupal stage. The aim was to study a possible sensitivity to heat waves that are increasing in frequency due to climate change, and winter survival. This thermal sensitivity is also of major importance for storage and transport, in the perspective of production and commercialisation of *C. typhae* as a biological control agent. We focused on the main components of male fertility: sperm production by males, post-copulatory storage by females, and offspring sex ratio.

## Material and methods

### Insect production

The laboratory strain of the host *S. nonagrioides* originates from different localities of Southwest France and is refreshed yearly by adding field collected larvae. Eggs collected in the rearing cages were placed at 21°C for 9 days. After gentle wash with chlorine-based disinfectant (Daquin), rinse and dry, they were transferred into a baby bottle filled with artificial diet (adapted from Overholt et al., 1994). Bottles were placed in a rearing incubator at 26°C with 60-70 % RH and 16:8 LD cycle, until pupae formation to get adults for the rearing (5 weeks later), or until the formation of five-instar larvae (3-week-old) for parasitism.

The Wasp colony was initiated from individuals emerging from parasitized larvae collected in the field near Kobodo in Kenya (0.679S, 34.412E; West Kenya) in 2019. Experimental breeding of *C.*

*typhae* was performed in a containment laboratory at the Evolution, Génome, Comportement et Ecologie (EGCE, Gif-sur-Yvette, France) since 2015. Parasitized host larvae are placed in Petri dishes with artificial diet and kept at 27°C, 60-70% RH and 16:8 light-dark (LD) cycle in rearing incubators. After 10 days, host larvae are retrieved from the artificial diet and placed in folded disposal absorbent paper to provide a dry environment. The 3<sup>rd</sup> larval instars egress from the host larva 2 days later to pupate in silk cocoons, giving a larval development of 12 days. Cocoon masses are then collected and placed in a 15L bucket with access holes closed by foam plugs (cage) at 27 °C and 12h LD cycle until adult emergence (5 or 6 days). Honey droplets are deposited on the cage walls, and a paper ball soaked with water is placed in the centre of the cage, to provide food and water for the emerging adults.

#### Sperm in seminal vesicles and age

Experiments occurred under controlled conditions, at a temperature between 25 and 27°C. Host larvae were parasitized by virgin *C. typhae* females to obtain only male offspring. After egress from the host, parasitic larvae weave a silk cocoon and transform into pupae. Each day, some cocoons were opened to follow pupal development, and determine the day of emergence. After emergence, adult males were transferred to petri dishes by groups of 10 with a cotton ball saturated with water and a drop of honey as nutritional resources. They were maintained at 25-27°C with a 12h-12h light-dark cycle.

Males were collected at the age of -1 day (one day before emergence, n=25), 0 day (the day of emergence, n=30), 1 day (n=30), then 3 (n=17), 5 (n=19), and 7 days (n=6), and their reproductive apparatus isolated with fine forceps in a drop of saline solution. Following methodology of El Sabrout et al. (2021), seminal vesicles were isolated in saline solution on a microscope glass slide, their content spread out, then fixed in 4% paraformaldehyde, dried, post fixed with 99% ethanol, dried again, and labelled with DAPI. After rinsing with tap water, slides were mounted in Immu-Mount (Thermo Inc.) and observed under a fluorescence microscope with a UV filter and a x100 objective under immersion. All sperm on the slide were counted to avoid any bias due to dilution, which was impossible because in very short sperm insects sperm would lose their integrity when diluted (El Sabrout et al., 2021).

#### Male multiple mating

To perform controlled mating virgin males and females were obtained from individual cocoons isolated from cocoon masses and kept in tubes with a moistened cotton wool ball and a drop of honey at 25-27°C until the emergence. Ten males were separately placed with successive females until each male had mated with ten different females. The ten matings per male were performed over two days with 1–2-day-old males. The mated females were allowed to oviposit in *S.*

*nonagrioides* caterpillars one or two days later, and the number of male and female offspring were counted after the development of the next *C. typhae* generation to determine sex ratio calculated as females divided by the total offspring.

#### Cold and hot treatments

Heat treatments were applied on 960 male pupae inside their cocoons. They were from 16 host larvae parasitized by virgin *C. typhae* females for three successive repetitions, all from the same laboratory colony. Cocoons were collected 1 day after egress from the host - the pupa is white with red eyes - and transferred in petri dishes with a moistened cotton wool ball and a drop of honey. They were maintained for 24h in climatic chambers at 34, 36°C or 38°C with 60% relative humidity, then returned to 25-27°C until complete development and emergence. This protocol was applied in other parasitoid wasps (Chirault et al., 2015; El Sabrout et al., 2021). One set of cocoons per repetition was maintained at 25-27°C as a control.

Three repetitions of cold treatments were applied on 1980 male pupae inside their cocoons. They were from 28 host larvae parasitized by virgin *C. typhae* females. The protocol was the same as for heat treatments, but two durations have been tested, 24 hours and 7 days (168h). Preliminary experiments show that pupae do not survive after 2 hours at -5°C; therefore, only zero and positive temperatures were tested. Cold temperatures were 0°C, 5°C and 10°C. After the cold treatment, cocoons were returned to 25-27°C until emergence.

#### Sperm stored by females and egg laying

One virgin female was introduced in a petri dish with one virgin male and continuously observed until copulation. Then the females were isolated for at least 3 hours in a petri dish with a cotton ball saturated with water and either tested for egg laying or dissected for sperm count. All males were 0 day old (ie tested the day of their emergence) to avoid any bias due to ageing. The cold treatment was 7 days at the temperature of 10°C, chosen because it is representative of a mild winter in Europe, n=7 control females mated with experimental males were tested for sperm in spermathecae, n=11 females for egg-laying. At 25-27°C (for 1 day, control), n=35 control females mated with control males were tested for sperm in spermathecae, n=35 for egg-laying. For the 1-day hot treatment, 34°C was chosen because 36°C induced mortality (40%), and n=27 control females mated with heat treated males were tested for sperm in spermathecae, n=6 for egg-laying. The number of experimental males after a cold treatment is low because the cold stay extended the pupal period, which made it difficult to synchronise males with the availability of virgin young females to mate with them the day of their emergence. For 34°C, only 6 males were tested for their offspring after copulation because this test was conducted after showing that sperm numbers were dramatically decreased by a heat shock.

Spermathecae were considered for their sperm content. Sperm labelling by DAPI and counting methods were as for males. Ten spermathecae were photographed under a x100 objective to measure their size. Volume was calculated from length and width of this bulb-shaped organ.

Females that mated once with either a cold-treated (10°C), control or heat-treated (34°C) male were offered two hosts at 1–2-day interval for oviposition. Stinging behaviour was observed to prevent multiple parasitism. Stung hosts were isolated in a petri dish with food in the climate chamber until either the egress of *C. typhae* cocoons, host pupation (i.e. parasitism failed), or host death. When offspring emerged from their cocoons, females and males were counted to calculate the sex-ratio.

## Statistics

All statistics and graphs were performed with R studio (version 1.3.1093, RStudio Team, 2020). One- or two-way ANOVA followed by Tukey tests were applied to sperm counts of control and treated males when data conformed to ANOVA model assumptions (equal variance among conditions and normal distribution of residuals checked on diagnostic plots). Otherwise, non-parametric tests (Kruskall Wallis followed by pairwise Mann-Whitney tests) were performed. For each dataset (seminal vesicle or spermatheca content), factors tested were (1) temperature treatment and (2) age when relevant.

Sex ratio of offspring of the ten successive females mated with one male was analysed as a binomial variable and compared between mating ranks - encoded as a factor due to the lack of a discernible trend - using a mixed model (glmer) and male identity as a random effect. This model was compared to a simplest one that only take into account the male's identity using a Chisq-test. Spermathecae were measured with Image J (version 1.54, NIH, USA). Effective fertilization of mated females was first compared between control and temperature-treated males by comparing the ratio of clutches with females obtained in the different treatments. Chisq-test were applied, or Fisher exact tests when at least one cell of the table of expected values was below 5. Then, sex ratios of mixed clutches (comprising both males and females) were compared by Kruskal-Wallis tests followed by pairwise Student or Mann-Whitney tests, according to the conformity of the data to parametric tests. Offspring output of all progenies (mixed or male-only) were compared by ANOVA followed by Tukey tests. Means are presented  $\pm$  SD in the text.

## Results

### Sperm in seminal vesicles and age

Sperm increased in seminal vesicles of control males over several days (ANOVA,  $F_{5-122}=200.1$ ,  $p<0.001$ , Figure 1). After the 3<sup>rd</sup> day, sperm do not longer increase significantly. Sperm were present in seminal vesicles before emergence, and their maximum was over 20,000 after 3 days. At 7 days, counts were from surviving males since many of them died before. After 5 days in standard

conditions, 50% were alive and no male survived after 9 days. In the following experiments, 3 days will be considered as the maximum sperm stock for males.

### Male multiple mating

Ten males were each mated with ten different females successively for a total number of 100 females, and between 5 and 10 cocoon clutches were finally obtained for each male, corresponding to between 6 and 9 clutches for each mating rank. Six progenies contained no females, which could be due to a failure in sperm transfer during copulation and were discarded from statistical analysis. There was a significant effect of mating rank on the offspring sex ratio (Chisq=68.9, df=9, p-value <0.0001) with no evidence of sperm depletion. Based on the comparison of fixed effect estimates with rank one as a reference, the sex ratio was significantly higher in rank 2, lower in ranks 3 and 4, and not significantly different in ranks 5 to 10.

### Cold and hot treatments

Treatment at 38°C for the pupae was not considered because it resulted in the death of all males, and 36°C only led to 60% emergence. In all other temperature treatments, all males emerged and were alive at 3 days. For cold treatments, durations of 24h at either 0, 5 and 10°C induced no effect on sperm production in comparison with control males at both ages (ANOVA,  $F_{3,147}=1.46$ ,  $p=0.23$ ). Therefore, only 7-day durations were considered for further testing of cold temperatures.

For sperm counted in the seminal vesicles of virgin males after heat treatment at the pupal stage, all temperatures and all ages were significantly different (2-way ANOVA, Temperature  $F_{6,293}=62.3$ ,  $p<0.001$ ; Age  $F_{2,293}=180.4$ ,  $p<0.001$ ; Temperature + Age  $F_{12,293}=39.6$ ,  $p<0.001$ , figure 3). For all males, whatever the temperature treatment, sperm increased from one day before emergence to 3 days post emergence. Sperm productions in 25°C and 28°C treatments showed similar profiles, and were not significantly different (2-way ANOVA, Temperature + Age  $F_{2,78}=0.65$ ,  $p=0.42$ ). Exposure to cold for 7 days or heat for one day decreased sperm production. For cold treatments, age had an effect on sperm counts, but no difference could be observed between temperatures and the interaction between temperature and age is not significant (2 way ANOVA, temperature  $F_{2,127}=0.23$ ,  $p=0.63$ ; age  $F_{2,127}=24.5$ ,  $p<0.001$ ; Temperature + Age  $F_{4,127}=0.002$ ,  $p=0.97$ ). However, for heat, both temperature and age have an effect on sperm, and the interaction between temperature and age is significant; 34°C had less effect than 36°C (2 way ANOVA, Temperature  $F_{1,90}=20.71$ ,  $p<0.001$ ; Age  $F_{2,90}=136.68$ ,  $p<0.001$ ; Temperature + Age  $F_{2,90}=11.85$ ,  $p<0.001$ ).

### Mating

Sperm stored in spermathecae



Male were able to mate with females, whatever their temperature treatment at the pupal stage. Copulation times were not recorded (lasting some seconds), but no obvious difference was observed between males, except that few (not counted) treated at 34°C needed a longer time mounting or detaching from females. The reservoir of the spermatheca is a minute chitinized capsule, measuring 26.5 x 15.6  $\mu\text{m}$ ; its volume is 306  $\mu\text{m}^3$ , that is 3.06 E-4 nl (figure 4a). Sperm stored in spermathecae differed between heat treatment of males (Figure 4b, ANOVA,  $F_{2,70}=19.10$ ,  $p<0.001$ ). Mean sperm numbers stored by females after male treatment at either 10, 25 or 34°C were respectively  $83\pm15.1$ ,  $235\pm22.0$ ,  $61\pm18.0$  (numbers on figure 4b).

#### Egg-laying

Parasitic success can be defined on several level for any standard females mated with control or experimental males, while failures could be due to host resistance or death. The temperature treatment of males did not affect the development (and resistance) of the hosts or their death. A measure of parasitic success is the presence of parasitoid cocoons, from which males or females emerge. Male treatment did not impact overall offspring production (Figure 5, P1, ANOVA  $F_{2,49}=0.9$ ,  $p=0.4$ ; P2, ANOVA  $F_{2,49}=1.05$ ,  $p=0.36$ ). However, the reproductive success of males (father) is only reflected in the number female offspring, in haplodiploids. The number of unfertilized progeny, i.e. without females, increased when males faced with either cold or hot treatment during pupal development, but this change is not significant (P1,  $\text{Chisq}=3.3$ ,  $\text{df}=2$ ,  $p=0.19$ ,  $p=0.28$ ; P2, Fisher exact test,  $p=0.48$ ; hereafter P1 and P2 refer to the first and second hosts in which a female oviposited). However, the sex ratio of mixed progenies (containing females and thus reflecting the sperm use) differed between male treatments for both first and second egg-laying (Figure 6, P1, Kruskal Wallis  $\text{Chisq}=30.2$ ,  $\text{df}=2$ ,  $p<0.001$ ; P2, Kruskal Wallis  $\text{Chisq}=19.2$ ,  $\text{df}=2$ ,  $p<0.001$ ). Of note only two out of five clutches contained females for 34°C treated males at P2. Adding the outputs resulting from the first and second mating gave the overall production of female offspring, hence the minimum number of sperm actually used by each female. The mean values of sperm used per female are respectively 58 for 10°C stressed males, 78 for 25-27°C control males and 12 for 34°C stressed males, when considering only offspring with at least one female (Figure 6).

## Discussion

One of the challenges of establishing biological agents concerns their adaptability to new environmental conditions, including temperature variations. Here we found that males of the parasitoid wasp *Cotesia typhae* are strongly affected by heat or cold waves in the early pupal stage. Sperm production is reduced when pupae have been exposed to cold for 7 days or heat for one day. As a consequence post-copulatory sperm storage by females appeared reduced, and the resulting sex ratio of the offspring is strongly male biased, especially when males were exposed to heat. This sensitivity could compromise both winter and summer resistance of *C. typhae* in maize fields for controlling its host *S. nonagrioides*.

The physiological response of insects to heat is well documented, especially for pests under controlled conditions (Neven, 2000, Kirk Green et al., 2019). Generally, heat sensitivity was investigated through an ecophysiology approach (Gonzalez-Tokman et al., 2020; Harvey et al., 2020). It was found to result in an metabolic increase monopolising energy for individual survival to the detriment of other non-essential functions, such as reproduction, and in the overproduction of toxic metabolites, such as amines inducing oxidative stress. This led to the proposal of “thermal fertility limits” which is the highest temperature where reproduction is physiologically possible (Walsh et al., 2019), such limit being not restricted to insects. Unfortunately, this is poorly described for males, where consequences of heat seem to be variable. For instance, males of the moth *Plutella xylostella* are negatively affected by a short heat period, but apparently less than females (Zhang et al., 2013), however, sperm production was not considered in this experimental study. In *Drosophila suzukii*, males and females show a severe reduction of their reproduction after a heat stress, and males are more sensitive than females (Kirk Green et al., 2019). In this dipteran pest, sperm production was not actually quantified but the reduction of sperm was assessed by a whole examination of testes and seminal vesicles after nuclei staining.

A decrease of sperm production in parasitoid wasps after a heat period during pupal stage is well known for both ecto- and endo-parasitoids (Nguyen et al., 2013, Chirault et al., 2015, El-Sabrout et al., 2021) but the consequences of cold are less documented. Lacoume et al. (2007) showed that after a severe cold shock (-18°C during 1h) at the pupal stage, males of the Pteromalid *Dinarmus basalis* had 50% less sperm than controls but females mated to cold-shocked males produced as many daughters as those mated to control males. The only fitness reduction was the number of females inseminated by a male, which was 15 for controls and 11 for cold shocked males. In *C. typhae*, control males were able to inseminate at least 10 females with no apparent reduction in the production of daughters. Present results show that the sex ratio is mainly female biased, with on average 80% females, but 5% were not inseminated and 10% produced a balanced or male-biased sex ratio. In the closely related species *Cotesia congregata*, one male can inseminate up to 15 females, and the sex ratio gradually decreases along the rank of females (Bredlau et al., 2020).

Although heat- or cold-stressed males were not tested for successive mating here, the ability of males to inseminate multiple females should be reduced by temperature at the pupal stage, leading to females that are either virgin or sperm-constrained and cannot produce offspring with an optimal sex ratio.

Cold treatment experimented here could not be considered as a cold shock, because temperatures are null or positive, and the duration is much longer than a shock. Thus, physiological effects of cold could be compared to a chill torpor that is experienced in the field during overwintering. In *C. typhae* males, a 7day period at either 0, 5 or 10 °C did not induce diapause, because the development is interrupted and restarts as the temperature rises. This is a transitory state of dormancy at the immature stage of pupae. In insects, stage resisting to cold can differ between species (Dansk, 2002), as an example mosquito eggs tolerate cold periods better than other stages (Diniz et al., 2017). In parasitoid wasps, the induction of dormancy is very variable, triggered by the host, climatic conditions, light rhythm, and all parameters together (Polgar et al., 1995). Previous results show parasitoids may survive at the larval stage within the host body, as for three parasitoids species of the invading pest *Rhagoletis completa* (Tephritidae) (Rull et al., 2019). The resistance of *C. typhae* other stages as adults, eggs and larvae when they are inside the host, would require further investigations.

Heat and cold treatments of male pupae led to a decrease in sperm production in *C. typhae* males. A temperature dependant heat stress was showed, this decrease being higher after 36°C than 34°C. This phenomenon was also observed in other tested experimentally parasitoids (Nguyen et al., 2013, Chirault et al., 2015, El-Sabroun et al., 2021). In contrast, sperm production does not differ in males between a 0, 5 or 10°C exposure for 7 days, after which it is equally severely diminished. The physiological stress induced by cold does not appear to be temperature dependant; this suggests a physiological mechanism that is specifically triggered by lower temperatures and with the same intensity when it drops below a threshold. This do not preclude that an intermediate effect on males, as evidenced for heat, could be induced by less rigorous conditions that are encountered in temperate areas. Whereas physiological changes induced by heat in insects are well described (Gonzalez-Tokman et al., 2020; Kirk Green et al., 2019), cold stress induction is less documented, especially in relation to male reproduction.

The production of the second offspring is less than half that of the first. It shows that oocytes are limited, confirming that *C. typhae* females are strictly pro-ovigenic (Jervis et al., 2001), with ovaries producing oocytes in only one wave during preimaginal stages. Independently of the male treatments, the sex ratio does not change between 1<sup>st</sup> to 2<sup>nd</sup> egg laying, evidencing that females are not able to allocate sperm in the first egg laying even if the low availability of this reproductive resource results in a non-optimal progeny. This does not preclude subsequent mating from being less efficient, because in most parasitoid wasps, females mate only once and subfertile or sterile males

induce pseudo-virgin -females that receive fewer or even no sperm and lay only males (Damien and Boivin, 2005).

Sperm are produced in larger amount than needed for storage by females and production of female offspring. The spermatheca is small, as in the related species *C. congregata* (Bredlau et al., 2020), and contains fewer spermatozoa, ie 250 vs 1000 in *C. congregata*. Regarding sperm efficiency, females mated with control males lay on average 80 daughters, which is under the average 250 sperm stored in the spermathecae and represent 32% of sperm being used. In females mated with cold or heat sperm-decreased males, this ratio is respectively of 72% for 10°C treated males, and 20% for 34°C treated males (with a very reduced number of males tested). Such relatively high sperm efficiency was found in other parasitoid wasps (Bredlau et al., 2020; Chirault et al., 2015), evidencing that sperm stored by females is of optimal quality (Damien et al., 2002).

As a summary, cold and heat exposure of the pupal stage of *C. typhae* have negative effects on the production of sperm by males, this decreases sperm storage in females after mating, and results in constrained females that lay an offspring containing fewer females than the controls. Finally, *C. typhae* males are highly sensitive to temperature variations that could be encountered in Europe. The resulting stress is visible on sperm production and transfer. In practice, overwintering as cocoons is unlikely, and will result in subfertile males and subsequent strongly male biased sex ratios. Refrigerated transport of pupae is also not recommended.

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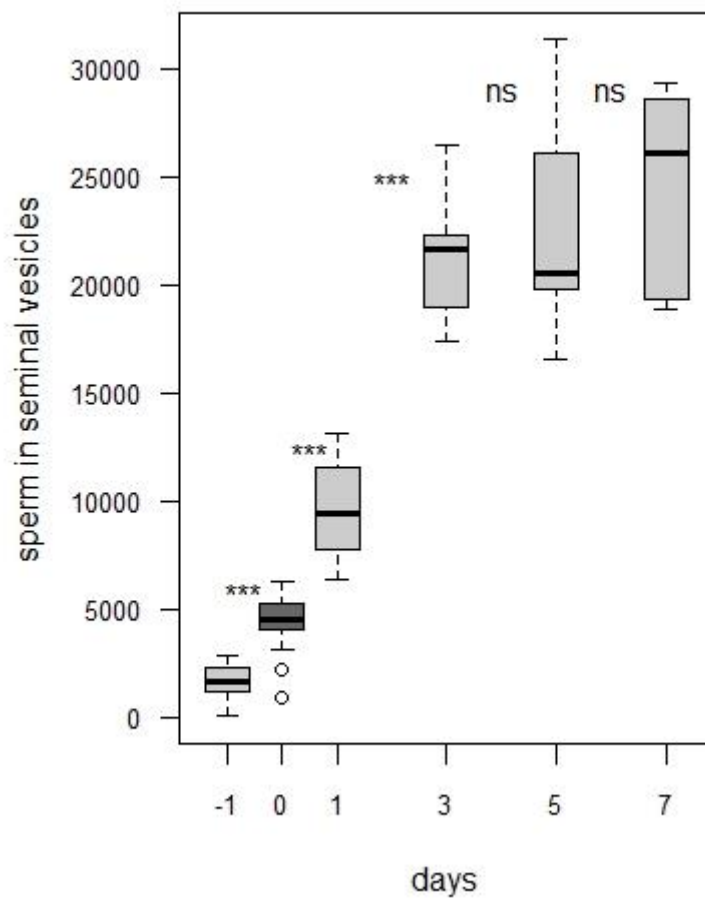
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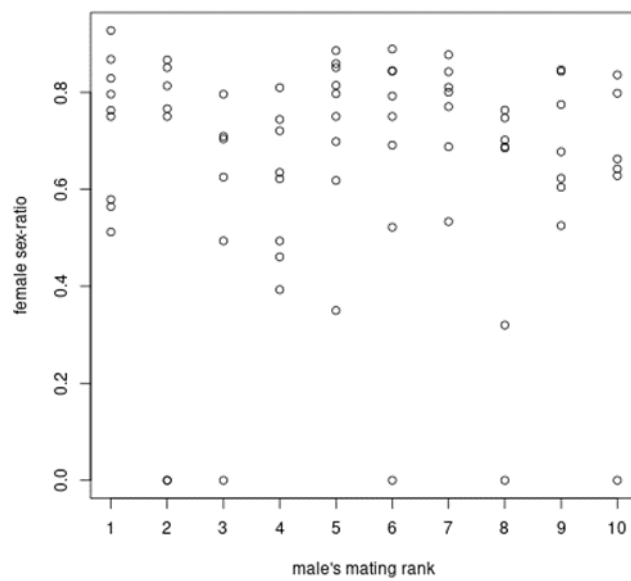


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466 Figure 1. Sperm in seminal vesicles along days in *Cotesia typhae*. Day of emergence is 0. All males  
 467 were virgin. Tukey tests significances for consecutive ages are indicated as \*\*\* when  $P < 0.001$  and ns  
 468 when  $p > 0.05$ . Numbers are respectively  $n = 25, 30, 30, 17, 19, 6$ .

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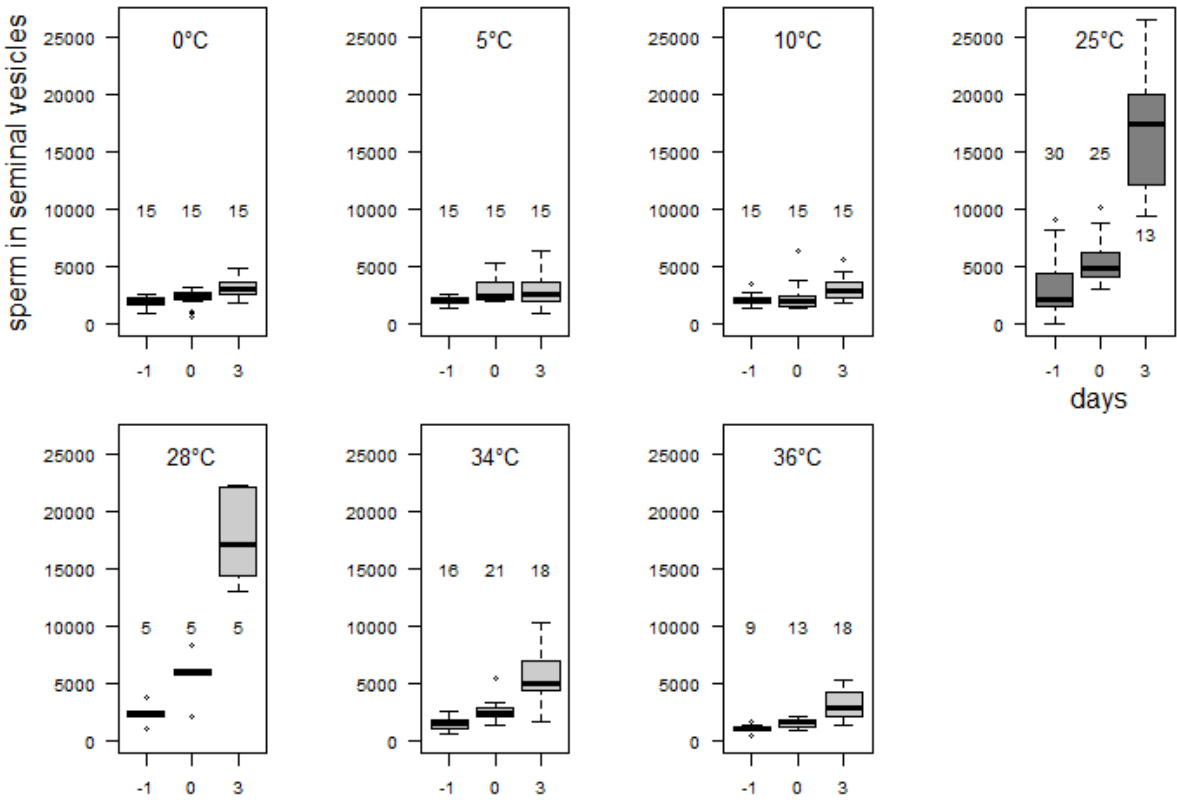




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471 Figure 2. Female sex-ratio (proportion of females in the offspring) according to male's mating rank,  
 472 statistics are in the text.

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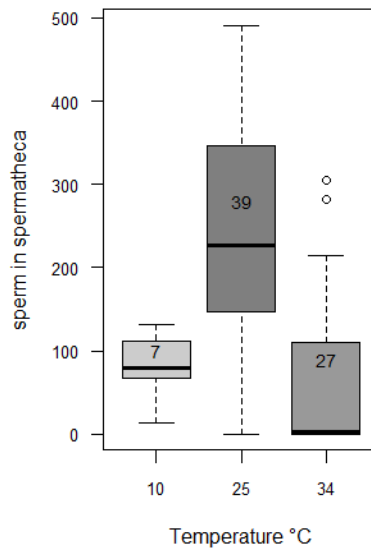
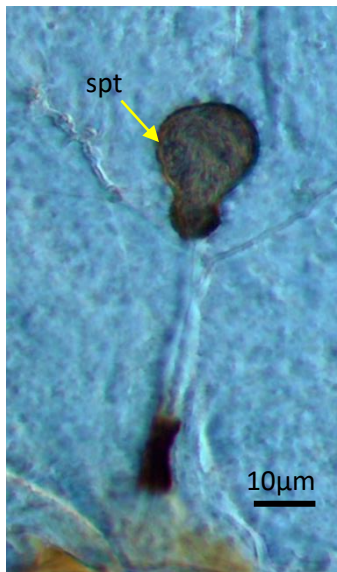
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Figure 3. Sperm in seminal vesicles of virgin males after temperature treatments at pupal stage. Pupae were exposed to 0, 5 or 10 °C for 7 days, then replaced back at 25-27°C until emergence. Others were placed at 25, 28, 34 or 36 °C for 1 day and then replaced back at 25-27°C until emergence. Sperm were counted one day before emergence (-1 day), the day of emergence (0 day) or 3 days after emergence (3 days). Sample sizes are on graphs, statistics are in the text.



483

484 Figure 4. Spermatheca of *C. typhae*. 3a. spermatheca under phase contrast at a x100 objective. Spt=  
 485 sperm reservoir. 3b. Sperm counted in spermathecae of once-mated females with one male treated  
 486 at the pupal stage at either 10°C for 7 days, 25-27°C (control) or 34°C during 1 day. Numbers in boxes  
 487 indicate the number of females.

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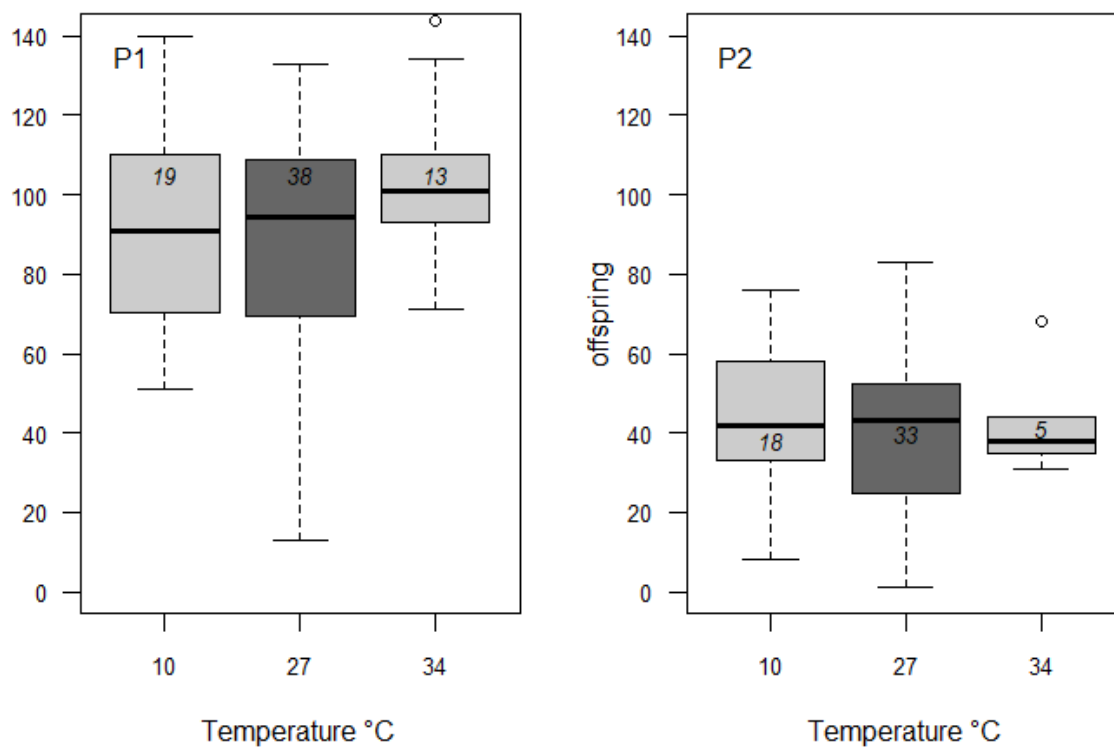
Male treatment	Host 1 P1					Host 2 P2				
	Host developme nt	Host death	cocoons	No female N and %	total	Host developme nt	Host death	cocoons	No female N and %	total
10 °C	3		19	10 – 53%	22	3		18	8 – 44%	21
27 °C	7	2	38	7 – 18%	47	9		33	4 – 12%	42
34 °C	3	1	13	9 – 69%	17	9	1	5	3 – 60%	15

490

491 Table 1. Fate of the stings of females of *C. typhae* mated with males that have undergone a cold or  
 492 hot period during the pupal stage. Host development means that parasitism was not successful, host  
 493 death occurred in some cases, cocoons are observed when larvae emerged from the host after a  
 494 complete development. The number and percentage of clutches containing no female offspring is  
 495 also provided as an indication of a failure in sperm transfer.

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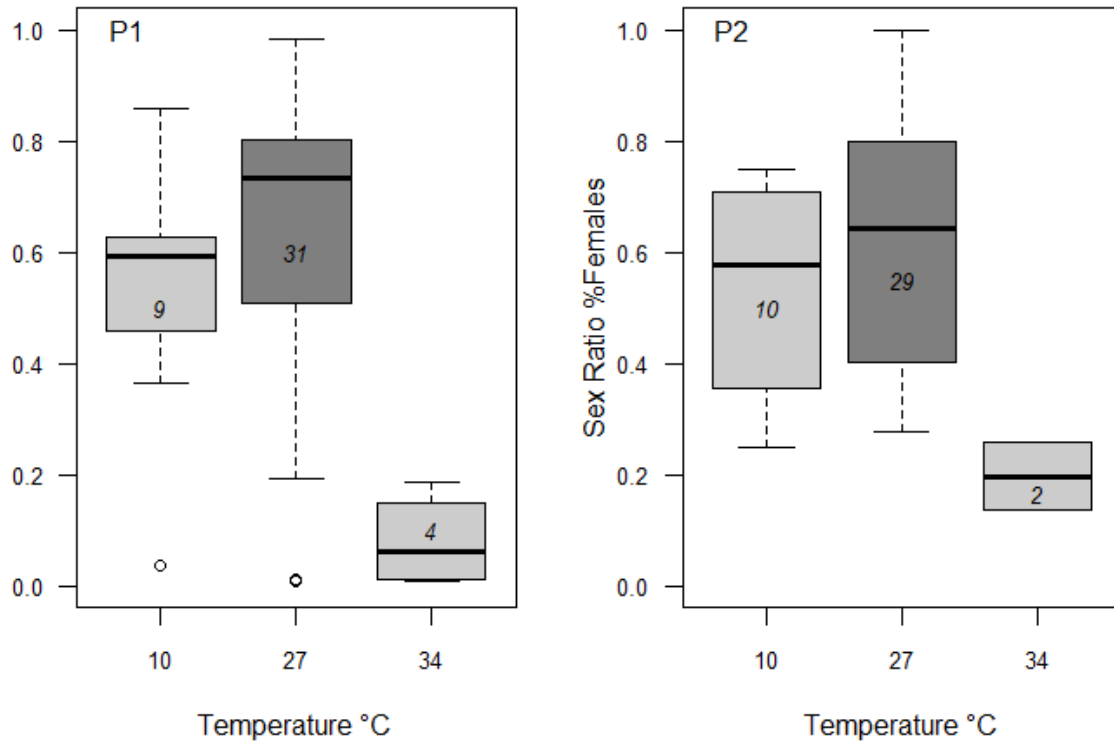
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500 Figure 5. Offspring output of *C. typhae* once-mated females with one male treated at the pupal stage  
 501 at either 10°C for 7 days, 25-27°C (control) or 34°C for 1 day. P1 is the first host, P2 the second,  
 502 numbers are in bars. There is no difference according to the state of the reproductive male (P1,  
 503 ANOVA  $F_{2,49}=0.9$ ,  $p=0.4$ ; P2, ANOVA  $F_{2,49}=1.05$ ,  $p=0.36$ ).

504



505

506 Figure 6. Sex-ratio, as females / total offspring, of *C. typhae* once-mated females with one male  
 507 treated at the pupal stage at either 10°C for 7 days, 25-27°C (control) or 34°C during 1 day. P1 is the  
 508 first host, P2 the second. Numbers are in bars. Sex ratios are statistically different for P1 (Kruskal  
 509 Wallis Chisq=30.2, df=2, p<0.001) and P2 (Kruskal Wallis Chisq=19.2, df=2, p<0.001). Data do not  
 510 include clutches containing no female offspring (see table 1). Sex ratios do not differ between first  
 511 (P1) and second (P2) egg-laying, for 10°C, Mann-Whitney, U=0.75, df=18, p=0.23; for 25-27°C, t test,  
 512 t=0.51, df=60, p=0.30; for 34°C, Mann-Whitney, U=0.64, df=5, p=0.27.

513