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1 Hot and cold waves decrease sperm production and bias sex ratio in 2 the parasitoid wasp *Cotesia typhae* (Hymenoptera, Braconidae)

3

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16

17 Abstract

18 Parasitoid wasps are haplodiploid, meaning that sperm stored by egg laying females are only used to
19 produce daughters. Thus, the sex ratio of the offspring depends on the availability of sperm after
20 mating. In these insects, males are sensitive to temperature at the pupal stage. This stress leads to
21 subfertility due to a drastic reduction in the number of sperm produced and transferred to females.
22 Experiments were conducted under controlled conditions on the parasitoid wasp *Cotesia typhae*
23 (Hymenoptera, Braconidae), a natural enemy of the invading pest *Sesamia nonagrioides*
24 (Lepidoptera, Noctuidae). At 25-27°C, sperm production was measured for 7 days, and found to
25 reach a plateau at the third day of adult life. It leads to a final amount around 25,000 sperm per
26 male. A male can successfully inseminate at least 10 females, producing predominantly female
27 offspring. Sperm production decreased significantly after 1 day of pupal exposure to heat at 34 or
28 36°C and 7 days of cold at 0, 5 or 10°C. This highlights that both cold and heat are stressful. After
29 mating with one male treated at 10 or 34°C, females store fewer sperm than the control, and
30 produce fewer daughters. The sex ratio of the offspring is male biased when males experienced
31 temperature stresses during development, like other parasitoid wasps. In the field, *C. typhae*
32 populations would be affected by heat and cold, at least at the pupal stage. This lowers
33 overwintering risk in case this biological agent was introduced in Europe. This risk is both economical,
34 as companies seek to establish costly continuous production to sell beneficial insects, and ecological
35 as the introduced population would not settle in the ecosystem. Lastly, the transport and storage of
36 this insect of agronomic interest would need to consider temperature variations to ensure successful
37 application.

39 Key words

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

41 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).

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

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

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

94

95 [Material and methods](#)

96 [Insect production](#)

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

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

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

116

117 Sperm in seminal vesicles and age

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

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

135 Male multiple mating

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

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

144 Cold and hot treatments

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

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

159 Sperm stored by females and egg laying

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

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

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

183 [Statistics](#)

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

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

203 [Results](#)

204 [Sperm in seminal vesicles and age](#)

205 Sperm increased in seminal vesicles of control males over several days (ANOVA, $F_{5-122}=200.1$,
206 $p<0.001$, Figure 1). After the 3rd day, sperm do not longer increase significantly. Sperm were present
207 in seminal vesicles before emergence, and their maximum was over 20,000 after 3 days. At 7 days,
208 counts were from surviving males since many of them died before. After 5 days in standard

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

211

212 Male multiple mating

213 Ten males were each mated with ten different females successively for a total number of 100
214 females, and between 5 and 10 cocoon clutches were finally obtained for each male, corresponding
215 to between 6 and 9 clutches for each mating rank. Six progenies contained no females, which could
216 be due to a failure in sperm transfer during copulation and were discarded from statistical analysis.
217 There was a significant effect of mating rank on the offspring sex ratio (Chisq=68.9, df=9, p-value
218 <0.0001) with no evidence of sperm depletion. Based on the comparison of fixed effect estimates
219 with rank one as a reference, the sex ratio was significantly higher in rank 2, lower in ranks 3 and 4,
220 and not significantly different in ranks 5 to 10.

221

222 Cold and hot treatments

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

228 For sperm counted in the seminal vesicles of virgin males after heat treatment at the pupal stage, all
229 temperatures and all ages were significantly different (2-way ANOVA, Temperature $F_{6,293}=62.3$,
230 $p<0.001$; Age $F_{2,293}=180.4$, $p<0.001$; Temperature + Age $F_{12,293}=39.6$, $p<0.001$, figure 3). For all males,
231 whatever the temperature treatment, sperm increased from one day before emergence to 3 days
232 post emergence. Sperm productions in 25°C and 28°C treatments showed similar profiles, and were
233 not significantly different (2-way ANOVA, Temperature + Age $F_{2,78}=0.65$, $p=0.42$). Exposure to cold for
234 7 days or heat for one day decreased sperm production. For cold treatments, age had an effect on
235 sperm counts, but no difference could be observed between temperatures and the interaction
236 between temperature and age is not significant (2 way ANOVA, temperature $F_{2,127}=0.23$, $p=0.63$; age
237 $F_{2,127}=24.5$, $p<0.001$; Temperature + Age $F_{4,127}=0.002$, $p=0.97$). However, for heat, both temperature
238 and age have an effect on sperm, and the interaction between temperature and age is significant;
239 34°C had less effect than 36°C (2 way ANOVA, Temperature $F_{1,90}=20.71$, $p<0.001$; Age $F_{2,90}=136.68$,
240 $p<0.001$; Temperature + Age $F_{2,90}=11.85$, $p<0.001$).

241 Mating

242 Sperm stored in spermathecae

243 Male were able to mate with females, whatever their temperature treatment at the pupal stage.
244 Copulation times were not recorded (lasting some seconds), but no obvious difference was observed
245 between males, except that few (not counted) treated at 34°C needed a longer time mounting or
246 detaching from females. The reservoir of the spermatheca is a minute chitinized capsule, measuring
247 26.5 x 15.6 μm ; its volume is 306 μm^3 , that is 3.06 E-4 nl (figure 4a). Sperm stored in spermathecae
248 differed between heat treatment of males (Figure 4b, ANOVA, $F_{2,70}=19.10$, $p<0.001$). Mean sperm
249 numbers stored by females after male treatment at either 10, 25 or 34°C were respectively 83 \pm 15.1,
250 235 \pm 22.0, 61 \pm 18.0 (numbers on figure 4b).

251 Egg-laying

252 Parasitic success can be defined on several level for any standard females mated with control or
253 experimental males, while failures could be due to host resistance or death. The temperature
254 treatment of males did not affect the development (and resistance) of the hosts or their death. A
255 measure of parasitic success is the presence of parasitoid cocoons, from which males or females
256 emerge. Male treatment did not impact overall offspring production (Figure 5, P1, ANOVA $F_{2,49}=0.9$,
257 $p=0.4$; P2, ANOVA $F_{2,49}=1.05$, $p=0.36$). However, the reproductive success of males (father) is only
258 reflected in the number female offspring, in haplodiploids. The number of unfertilized progeny, i.e.
259 without females, increased when males faced with either cold or hot treatment during pupal
260 development, but this change is not significant (P1, $\text{Chisq}=3.3$, $\text{df}=2$, $p=0.19$, $p=0.28$; P2, Fisher exact
261 test, $p=0.48$; hereafter P1 and P2 refer to the first and second hosts in which a female oviposited).
262 However, the sex ratio of mixed progenies (containing females and thus reflecting the sperm use)
263 differed between male treatments for both first and second egg-laying (Figure 6, P1, Kruskal Wallis
264 $\text{Chisq}=30.2$, $\text{df}=2$, $p<0.001$; P2, Kruskal Wallis $\text{Chisq}=19.2$, $\text{df}=2$, $p<0.001$). Of note only two out of five
265 clutches contained females for 34°C treated males at P2. Adding the outputs resulting from the first
266 and second mating gave the overall production of female offspring, hence the minimum number of
267 sperm actually used by each female. The mean values of sperm used per female are respectively 58
268 for 10°C stressed males, 78 for 25-27°C control males and 12 for 34°C stressed males, when
269 considering only offspring with at least one female (Figure 6).

270

271 Discussion

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

280 The physiological response of insects to heat is well documented, especially for pests under
281 controlled conditions (Neven, 2000, Kirk Green et al., 2019). Generally, heat sensitivity was
282 investigated through an ecophysiology approach (Gonzalez-Tokman et al., 2020; Harvey et al., 2020).
283 It was found to result in an metabolic increase monopolising energy for individual survival to the
284 detriment of other non-essential functions, such as reproduction, and in the overproduction of toxic
285 metabolites, such as amines inducing oxidative stress. This led to the proposal of “thermal fertility
286 limits” which is the highest temperature where reproduction is physiologically possible (Walsh et al.,
287 2019), such limit being not restricted to insects. Unfortunately, this is poorly described for males,
288 where consequences of heat seem to be variable. For instance, males of the moth *Plutella xylostella*
289 are negatively affected by a short heat period, but apparently less than females (Zhang et al., 2013),
290 however, sperm production was not considered in this experimental study. In *Drosophila suzukii*,
291 males and females show a severe reduction of their reproduction after a heat stress, and males are
292 more sensitive than females (Kirk Green et al., 2019). In this dipteran pest, sperm production was not
293 actually quantified but the reduction of sperm was assessed by a whole examination of testes and
294 seminal vesicles after nuclei staining.

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

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

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

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

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

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

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

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

361

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373

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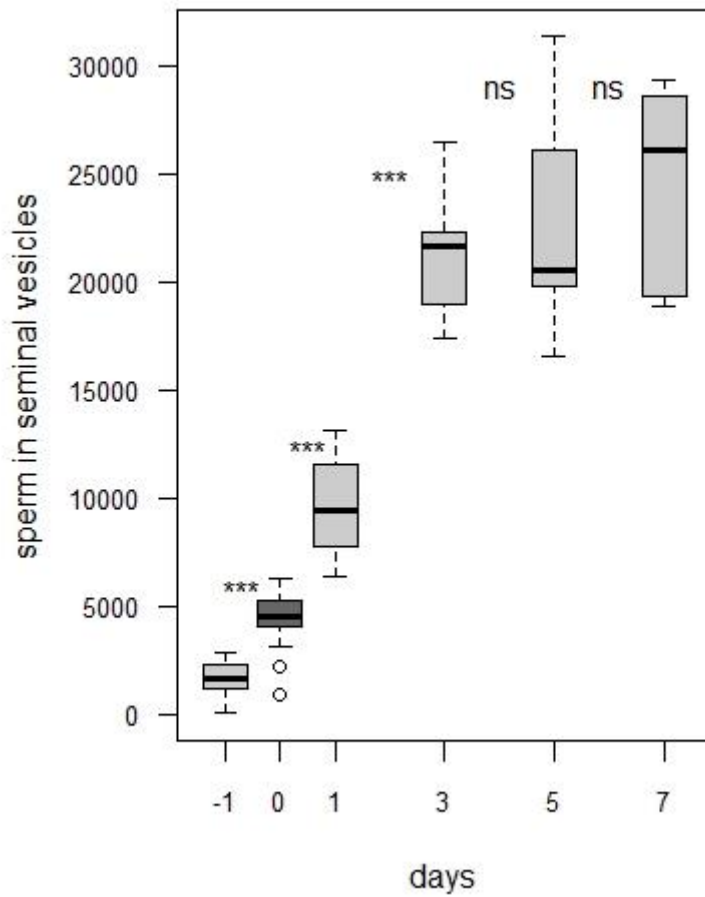
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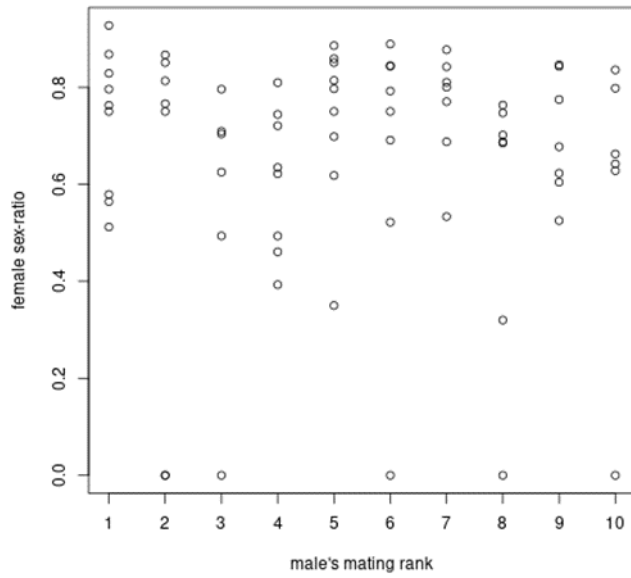
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465

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 $n = 25, 30, 30, 17, 19, 6$.

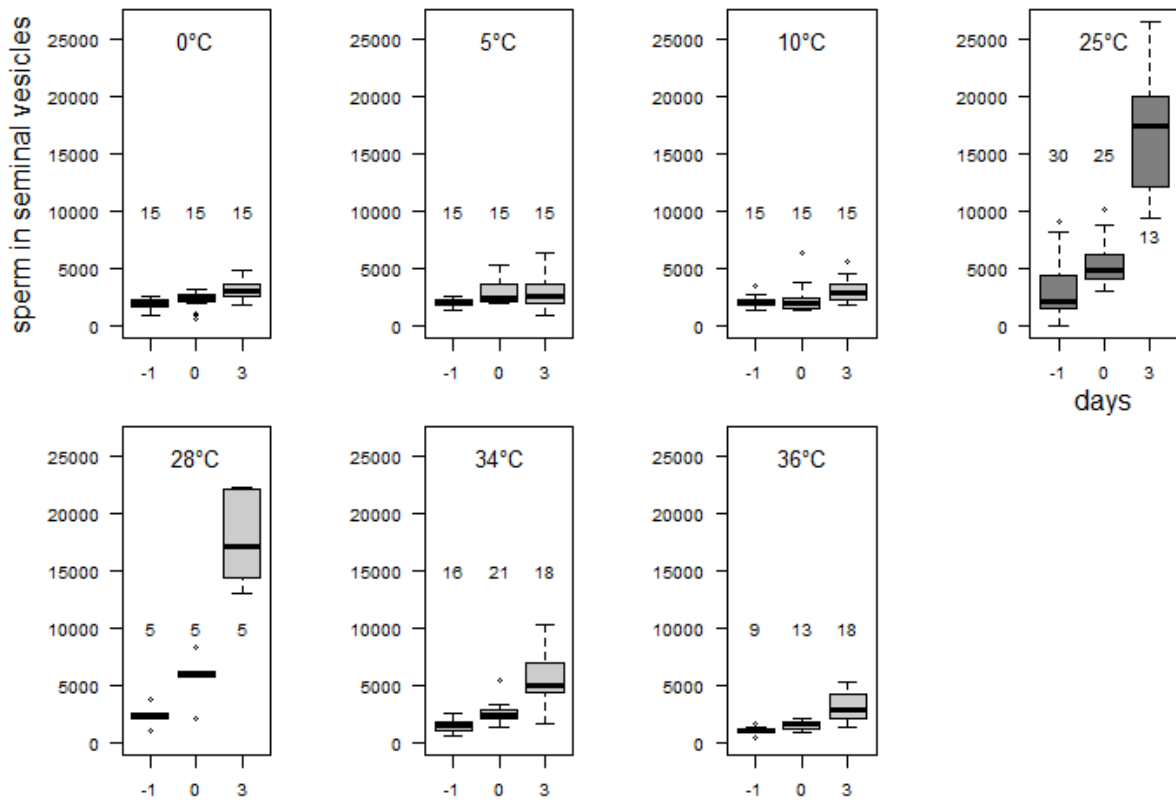
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471 Figure 2. Female sex-ratio (proportion of females in the offspring) according to male's mating rank,
 472 statistics are in the text.

473



475

476 Figure 3. Sperm in seminal vesicles of virgin males after temperature treatments at pupal stage.

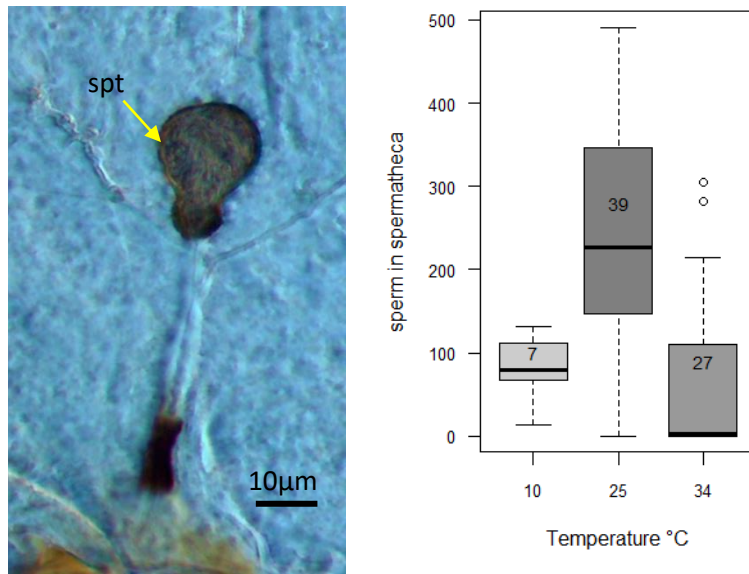
477 Pupae were exposed to 0, 5 or 10 °C for 7 days, then replaced back at 25-27°C until emergence.

478 Others were placed at 25, 28, 34 or 36 °C for 1 day and then replaced back at 25-27°C until

479 emergence. Sperm were counted one day before emergence (-1 day), the day of emergence (0 day)

480 or 3 days after emergence (3 days). Sample sizes are on graphs, statistics are in the text.

481



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.

488

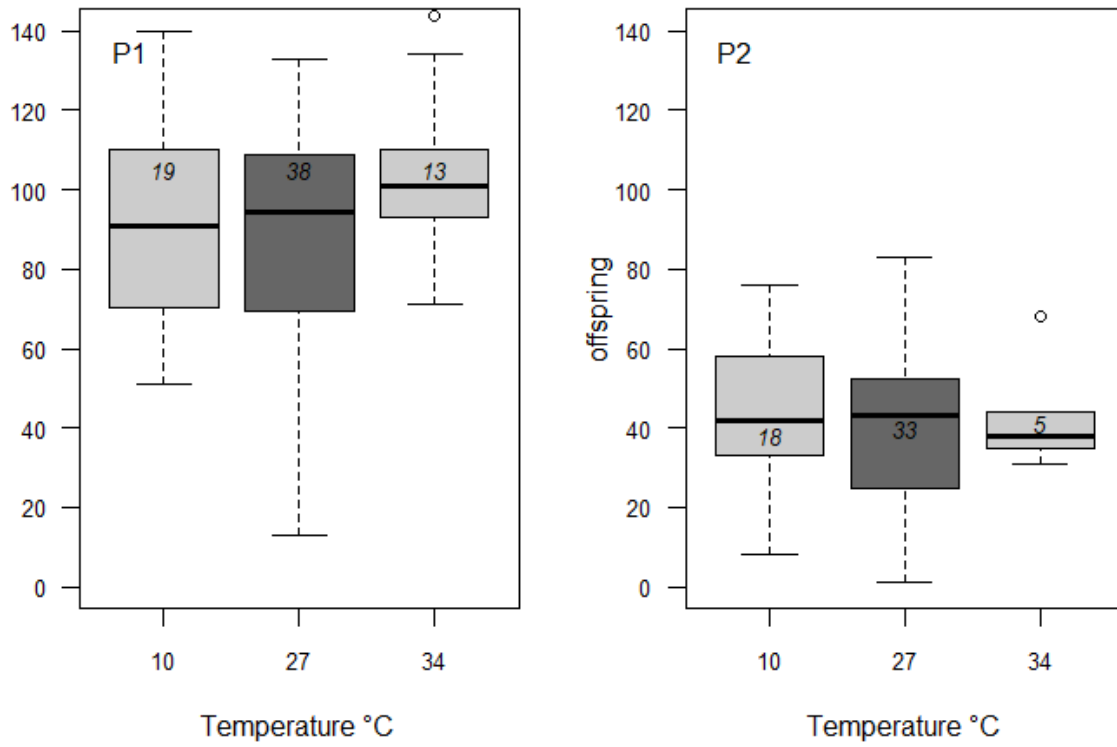
Male treatment	Host 1 P1					Host 2 P2				
	Host development	Host death	cocoons	No female N and %	total	Host development	Host death	cocoons	No female N and %	total
10 °C	3		19	10 – 53%	22	3		18	8 – 44%	21
27 °C	7	2	38	7 – 18%	47	9		33	4 – 12%	42
34 °C	3	1	13	9 – 69%	17	9	1	5	3 – 60%	15

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.

496

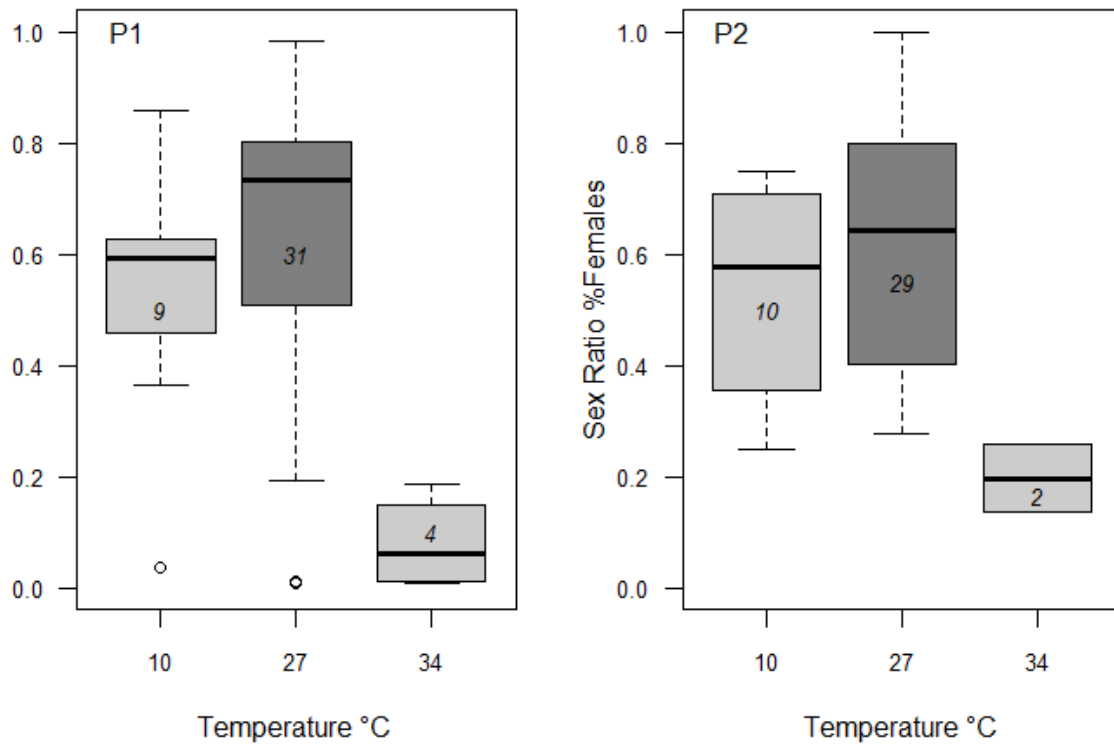
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500 Figure 5. Offspring output of *C. typhae* once-mated females with one male treated at the pupal stage
 501 at either 10°C for 7 days, 25-27°C (control) or 34°C for 1 day. P1 is the first host, P2 the second,
 502 numbers are in bars. There is no 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 $\text{Chisq}=30.2$, $\text{df}=2$, $p<0.001$) and P2 (Kruskal Wallis $\text{Chisq}=19.2$, $\text{df}=2$, $p<0.001$). 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, $U=0.75$, $\text{df}=18$, $p=0.23$; for 25-27°C, t test,
 512 $t=0.51$, $\text{df}=60$, $p=0.30$; for 34°C, Mann-Whitney, $U=0.64$, $\text{df}=5$, $p=0.27$.

513