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# Susceptibility to cold suggests low risk of establishment of a tropical parasitoid attacking the corn pest *Sesamia nonagrioides*

Taiadjana Fortuna, Mélissa Anne, Manuel Le Gonnidec, Rémi Jeannette, Christophe Bressac, François Rebaudo, Florence Mougel, Laure Kaiser

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1 **Assessing the effects of low temperatures on the risk of establishment of the non-native**  
2 **biological control agent *Cotesia typhae***

3

4 Taiadjana M. Fortuna<sup>1\*</sup>, Mélissa Anne<sup>1</sup>, Manuel Le Gonnidec<sup>1</sup>, Rémi Jeannette<sup>1</sup>, Christophe  
5 Bressac<sup>2</sup>, François Rebaudo<sup>1</sup>, Florence Mougel<sup>1</sup>, Laure Kaiser<sup>1</sup>

6

7 <sup>1</sup>Université Paris-Saclay, CNRS, IRD, UMR Évolution, Génomes, Comportement et  
8 Écologie,

9 91190 Gif-sur-Yvette, France.

10 <sup>2</sup>UMR 7261 CNRS, Institut de Recherche sur la Biologie de l'Insecte, Faculté des Sciences et  
11 Techniques, Université de Tours, Tours, France

12

13 \*Author for Correspondence: taiadjana.marquesfortuna@ird.fr

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15 *Keywords:* insect, parasitoid, exotic species, development, survival, risk assessment

16 **Abstract**

17 Cold is a limiting factor for the distribution and performance of many terrestrial insects,  
18 including crop pests and their natural enemies, which are sometimes introduced in non-native  
19 areas. The European Union regulation requires that prior to the introduction of non-native  
20 insects used for biological control, the risk of winter survival and establishment in the  
21 environment should be estimated to avoid undesirable non-target effects. Here, we evaluate the  
22 lower thermal thresholds of survival, development and activity of the tropical species *Cotesia*  
23 *typhae* Fernandez-Triana (Hymenoptera: Braconidae), a larval parasitoid of the moth, *Sesamia*  
24 *nonagrioides* Lefèbvre (Lepidoptera: Noctuidae), an important corn pest in Southern Europe.  
25 *C. typhae* is highly host specific and very efficient in greenhouse conditions, and its impact on  
26 non-target species is low. It is thus a promising biocontrol agent against *S. nonagrioides*.  
27 Different developmental stages of the parasitoid were exposed to different constant  
28 temperatures (ranging from 5° to 27°C), exposure durations, and host physiological stages. Our  
29 results revealed that this tropical species is highly susceptible to cold, with high mortality rate  
30 occurring at all developmental stages at temperatures below 15°C. Thresholds of egg-larval and  
31 pupal development were close to 15°C, and a drastic decrease of adult locomotor activity was  
32 also observed below this temperature. Males were more susceptible to cold than females since  
33 their longevity was significantly reduced at 5°C, and fewer males were formed when host larvae  
34 were exposed to 10°C. Given established critical thermal limits for all the developmental stages  
35 of *C. typhae*, we estimated the probability of winter survival of *C. typhae* in France, based on  
36 the percentage of winters with harmful temperatures in the last 20 years. Under current climate  
37 conditions, this probability is low, which reinforces environmental innocuity of *C. typhae*  
38 introduction as a biological control agent against *S. nonagrioides*. Quantification of life history  
39 traits associated with thermal biology in a tropical species will also allow the construction of  
40 models describing host-parasitoid population dynamics and design optimal pest management  
41 programs.

## 42 1. Introduction

43 Most insects are ectotherms, maintaining their body temperature in equilibrium with the  
44 ambient microclimate temperature. Thus, abiotic factors, such as temperature and humidity,  
45 affect insect performances (*e.g.* survival, development, fecundity, locomotion), which may  
46 affect population dynamics over longer timescales (Wallner, 1987). Temperature constraints,  
47 therefore, the seasonal activity and the geographic distribution of the insects, and can directly  
48 or indirectly affect the dynamics of native insect populations, the spread and impact of invasive  
49 pests, and the success of species introduced for biological control (Bale and Hayward, 2010;  
50 Hance et al., 2007).

51 Low temperatures are one of the key limiting factors for the distribution and performance of  
52 many terrestrial insects (Bale, 1996; Sinclair et al., 2003). Cold is considered as a key  
53 component of overwintering stress in temperate, polar and alpine habitats, as the majority of  
54 insects die from the effects of cold (Bale and Hayward, 2010). However, depending on the  
55 environments in which species have evolved, temperature tolerance may differ. For instance,  
56 basal tolerance of cold is greater in the temperate and alpine species than in the tropical ones  
57 (Chown and Terblanche, 2006). Species of tropical and Mediterranean origin usually have high  
58 mortality whenever insects experience temperatures close to or below their developmental  
59 threshold, *i.e.* between 10°C and 0°C (Hatherly et al., 2005). Hence, cold-susceptible species  
60 need to have a high level of thermal protection to overwinter in cool temperate climates.

61 The effects of cold on the survival of insects may also differ according to their trophic level.  
62 Insect parasitoids, for instance, as they develop in or on hosts on which they are dependent to  
63 complete their development, are intrinsically dependent on the ecology and physiology of their  
64 hosts to survive. Several life-history traits in parasitoids are impacted by cold exposure. When  
65 exposed to decreasing temperatures, insects enter a temporary cold stupor zone, in which  
66 feeding and mating behaviours are strongly reduced, and may enter chill coma, in which  
67 movement stops (Boivin et al., 2006). Cold can also affect behaviours linked to host location  
68 and learning in parasitoids (van Baaren et al., 2005), and negatively affect adult size, adult  
69 longevity, fecundity and sex allocation (Hance et al., 2007). One adaptation of insects to  
70 extreme temperatures is diapause, which allows resistance to low temperatures by a series of  
71 physiological adaptations (Bale, 2002). In parasitoids, diapause often takes place in the host,  
72 but not always (see Hao et al., 2013; Nealis, 1985). Hence, cold adaptation in parasitoids,  
73 although similar to those of other ectotherms, is often constrained by the responses of their hosts  
74 and its physiological conditions (Hance et al., 2007).

75 Insect parasitoids have been used as successful biological control agents of crop pests  
76 worldwide (Brodeur et al., 2018). Understanding how cold impacts the third trophic level is of  
77 fundamental and applied importance, especially when using parasitoids in biological control  
78 programs. Parasitoids of the *Cotesia flavipes* species complex have been used worldwide as  
79 biological control agents of lepidopteran stemborers attacking important crops (Kfir et al., 2002;  
80 Parra and Coelho, 2019; Polaszek and Walker, 1991). *Cotesia typhae* Fernandez-Triana  
81 (Hymenoptera: Braconidae), belonging to *C. flavipes* complex, is native to eastern sub-saharan  
82 Africa, and is a gregarious koinobiont endoparasitoid specialised on larvae of *Sesamia*  
83 *nonagrioides* Lefèbvre (Lepidoptera: Noctuidae) (Kaiser et al., 2017, 2015). In different  
84 countries of its native range, the temperature varies between 9° and 38°C (NASA LaRC, 2019).  
85 *S. nonagrioides*, of African origin, is an important maize pest in southern Europe, where it  
86 overwinters at late larval stages. At the beginning of spring, larvae resume activity and pupate  
87 (Galichet et al., 1982). Due to the strict specificity of *C. typhae* for *S. nonagrioides* and its  
88 efficiency on European host populations (Kaiser et al., 2017), this parasitoid species is a  
89 promising biocontrol agent.

90 The use of exotic arthropods for the biological control of insect pests as intentional  
91 introductions may be associated with environmental risks. One of them is based on the fact that  
92 some of these released organisms under certain circumstances may become “invasive”. To  
93 overcome these concerns, there has been a focus in Europe, over the last 20 years, on conceiving  
94 an environmental risk assessment process for exotic biocontrol agents, with the aim of ensuring  
95 environmental safety of released organisms (Bale et al., 2008; van Lenteren et al., 2003). Within  
96 the generic template proposed for risk assessment (van Lenteren et al., 2003), the risk of  
97 establishment is considered to be one of the first assessments to be conducted for species that  
98 will be released in temperate and colder climates (Bale and Hayward, 2010). Other risks, such  
99 as the attack of non-target species and the dispersal capacity of the biocontrol agent, are also  
100 included in this risk assessment template.

101 Therefore, our main question is whether *C. typhae* can survive during several months in  
102 overwintering larvae of *S. nonagrioides*, pupate before host pupation, and reproduce during its  
103 short adult life. Species within the *Cotesia flavipes* complex share the same biology and have a  
104 close ecological range, and they are all subtropical species (Fujie et al., 2018; Muirhead, 2010;  
105 Polaszek and Walker, 1991). *C. typhae* was formally considered as a population of *Cotesia*  
106 *sesamiae*, and both species are sympatric (Kaiser et al., 2015). Thus, thermal responses are  
107 expected to be close among these congener species, which help setting temperature range to

108 test the response of *C. typhae* to cold. Lower Lethal Temperatures (LLT) of *C. sesamiae* adults  
109 range from 0 to 5°C for a period of exposure of 4h, while LLT of *C. flavipes* adults vary between  
110 -9 and -1°C for the same period of exposure (Mutamiswa et al., 2018). Other developmental  
111 stages of *C. flavipes* are, however, less cold tolerant. For instance, *C. flavipes* show no  
112 development (egg to adult) at 10 and 15°C, and the threshold temperature among different  
113 strains of this species is between 9.6 and 11.1°C (Smaniotto et al., 2019). *Cotesia chilonis*,  
114 another species of the *C. flavipes* complex, is unable to develop (egg to adult) at 5 and 10°C  
115 (Galichet, 1979). Based on the tropical origin of *C. typhae* and on the thermal tolerances of its  
116 congeners, we expect that the LLT of this species at any developmental stage ranges between  
117 5 and 15°C.

118 Here, we investigate thermal thresholds of survival and development of the different stages of  
119 *C. typhae*, and adult activity. These thresholds depend on duration of exposure, therefore, egg-  
120 larval and pupal stages have been exposed to different combinations of temperatures and  
121 duration to determine lower critical thermal limits. The influence of the host physiological stage  
122 (diapause) was also tested. Thus, we exposed *C. typhae* at temperatures ranging from 5 to 27°C,  
123 the highest being the lab-rearing condition taken as control. Given established critical thermal  
124 limits for all the developmental stages of the parasitoid, risk of winter survival is discussed  
125 relative to a map of France showing the percentages of winter presenting thermal conditions  
126 below these temperature thresholds. Determining the lower thermal limits of *C. typhae* is an  
127 important step to document the risk of establishment of this species in the introduced  
128 environment during winter conditions and its potential impact on non-target species in the long  
129 term. Knowledge on the thermal biology of this natural enemy will help as well to predict host-  
130 parasitoid population dynamics, and design more optimal pest management programs.

131

## 132 **2. Material and methods**

### 133 **2.1. Biological material**

134 *Cotesia typhae* has been reared in a containment laboratory at the Evolution, Génome,  
135 Comportement et Ecologie (EGCE, Gif-sur-Yvette) since 2015. The strain used in this study  
136 was initiated from individuals emerging from parasitized larvae collected in the field near  
137 Kobodo in Kenya (0.679S, 34.412E; West Kenya) in 2019. A strain of the host, *S. nonagrioides*,  
138 is also reared in the laboratory since 2011 and enriched yearly, by overwintering larvae  
139 collected in maize residues in southwest France (Gironde, Haute Garonne, Landes, Pyrénées  
140 Atlantiques, and Tarn et Garonne).

141

## 142 **2.2. Rearing conditions and methods**

143 *Parasitoid*: After being parasitized by *C. typhae*, host larvae are placed in Petri dishes with  
144 artificial diet adapted from Overholt et al. (1994) and kept at 27°C, 60-70% RH and 16:8 light-  
145 dark (LD) cycle in rearing incubators. Endoparasitic egg-larval development lasts for 12 days  
146 and ends by the emergence of the 3<sup>rd</sup> larval instars from the host larva to pupate in silk cocoons.  
147 One day before expected emergence, host larvae are retrieved from the artificial diet and placed  
148 in folded toilet paper to provide a dry environment for the formation of parasitoid cocoons.  
149 Cocoon masses are then collected and placed in a 15L bucket with access holes closed by foam  
150 plugs (cage) at 27 °C and 12:12 LD cycle until adult emergence (5 or 6 days). Honey droplets  
151 are deposited on the cage walls, and a paper ball soaked with water is placed in the center of  
152 the cage, to provide sugar and water for the emerging adults. At 27°C, the full life cycle of *C.*  
153 *typhae* takes 19 days. The temperature is then lowered to 24 or 21°C to lengthen adult parasitoid  
154 life expectancy, depending on the parasitism day. Forty-eight hours before parasitism, 3-week-  
155 old host larvae are placed in a container with fresh maize stems to produce faeces, which attract  
156 female wasps and stimulate egg laying. Then in a containment room at 24 °C, a host is presented  
157 to a female wasp until the ovipositor insertion is observed. At each generation, thirty host larvae  
158 are individually parasitized to obtain about 20-25 cocoon masses.

159 *Host*: Eggs of *S. nonagrioides* are collected and placed at 21°C during 9 days. They are, then,  
160 gently washed in a chlorine-based disinfectant (Daquin®), rinsed with distilled water, wiped  
161 and transferred into a baby bottle filled with artificial diet. Bottles are placed in a rearing  
162 incubator at 26°C with 60-70 % RH and 16:8 LD cycle, until pupae formation to get adults for  
163 the rearing (5 weeks later), or until the formation of five-instar larvae (3-weeks old) for  
164 parasitism.

165

## 166 **2.3. General experimental conditions**

167 Diapause induction of *S. nonagrioides* was done by setting the photoperiod to a short daylight  
168 cycle of 10:14 LD from the egg stage to mimic fall and winter under French latitudes. At these  
169 conditions, the theoretical beginning of the developmental arrest correspond to 4-weeks.  
170 Therefore, for the different experiments some parasitized larvae were 4-6 weeks old (no or short  
171 developmental arrest), or 3-4 months old (2-3 months of developmental arrest). Such short day-  
172 length without temperature decrease induces developmental arrest in about 50-60% of the

173 larvae when they reach their last stage. It is not a true diapause because they continue feeding,  
174 but they do not undergo pupal moult. Although for simplification we will use the term diapause  
175 here.

176 To assess the effect of low temperatures on the probability of establishment of *C. typhae* in  
177 France, the following range of temperatures was chosen: 5, 10, 15, 20 and 27°C (rearing  
178 temperature used as control), unless stated otherwise. Temperatures were chosen based on  
179 winter temperatures in mid-southwest France, where *S. nonagrioides* causes most corn damage.  
180 The tests were conducted using 5L incubators kept in an air-conditioned room. Photoperiod  
181 was set to 10:14 LD thanks to LED lighting, and temperature was continuously monitored with  
182 data accessible directly on a server (Rebaudo and Benoist, 2019). Parasitoids were exposed to  
183 the tested temperatures either during their egg-larval development inside the host-larva, or  
184 during their pupal development inside their silk cocoon, or at their adult stage.

185

#### 186 **2.4. Exposure of parasitized host larvae to cool temperatures**

187 The effect of temperatures on *C. typhae* egg-larval development was assessed by exposing  
188 parasitized host-larvae for two different durations: 14 days, which is close to the mean egg-  
189 larval development duration at the rearing temperature (27°C), or 50 days, to approach winter  
190 conditions. At the end of these exposures the parasitized larvae were placed back at 27°C. For  
191 all the experiments unless stated otherwise, host-larvae were surveyed until one of the following  
192 outcomes was observed: parasitoid larvae emergence from the host body and cocoon formation,  
193 host pupation or host death. One day after cocoon formation, the cocoon masses were placed in  
194 a tube closed with a foam plug until adult emergence, if any. The emerged adults with their  
195 cocoon masses were then kept at -20°C until counting progeny size and dead cocoons.

##### 196 **2.4.1. Effect of duration and temperature exposure on parasitoid egg-larval development**

197 Four-week-old host larvae were parasitized and placed by 3 or 4 per Petri dish with artificial  
198 diet at each temperature: 5, 10, 15, 20 and 27 °C, a few hours after parasitism to let the hosts  
199 bury into the artificial diet, and were then transferred to 27 °C after 14 or 50 days. Twenty-two  
200 larvae were set per temperature and duration. Twelve non-parasitized larvae (of the same age)  
201 were placed under each temperature for 50 days and then transferred to 27 °C, to assess host  
202 mortality due to low temperature without parasitism. The artificial diet was changed if dried or  
203 contaminated by fungi.

204 Since parasitoid and host mortality was high when exposed at 5 or 10°C for 14 days, possibly  
205 because host larvae were not totally in diapause at time of cold exposure, the experiment was



206 repeated using diapausing host larvae (3-month-old). Three groups of 21 parasitized and 7 non-  
207 parasitized hosts were placed respectively at 5, 10 and 27°C during 14 days, and transferred to  
208 27°C afterwards. The effect of temperature and exposure duration on developmental outcomes  
209 of hosts were analysed, as well as the duration of parasitoid larval development according to  
210 temperature and duration.

#### 211 **2.4.2. Lethal duration of exposure at 10 °C**

212 The first experiment showed that there was no or rare parasitoid larvae emerging from the host  
213 when this one was exposed at 10 °C for 14 days and then placed at 27°C. To determine the  
214 lethal duration of exposure at 10°C, groups of 27 parasitized and 6 non-parasitized hosts were  
215 then exposed for 2, 5, 8, 11 or 14 days at 10 °C and then transferred to 27 °C. The host larvae  
216 used in this experiment were 6-weeks old (in diapause). After transfer to 27°C for cocoon  
217 formation, the number of cocoons per masse and pupal mortality were counted, as well as the  
218 number of adults emerged and the sex ratio. At temperatures lower than 27°C, it was not  
219 possible to know when parasitoid larvae would emerge, so host larvae were left in the artificial  
220 diet, instead of being placed in toilet paper for cocoon formation (rearing procedure), until the  
221 outcomes described above. Some cocoon masses were, therefore, formed inside the artificial  
222 diet, which is detrimental to cocoon formation due to humidity, resulting in the mortality of *C.*  
223 *typhae* larvae that were unable to spin their cocoons. From these masses, only the occurrence  
224 of larvae emergence was used for analysis, but not the number of cocoons within each mass.

#### 225 **2.4.3. Egg-larval developmental temperature threshold**

226 Dissection of some hosts exposed at each temperature for 14 days suggested that the threshold  
227 for larval development was between 15 and 20°C. To determine this threshold 16 parasitized  
228 and 7 non-parasitized host larvae were exposed at 15, 17 or 19°C for 14 days, frozen and then  
229 dissected. The host larvae used were 4-months old (in diapause). Dissections were done in a  
230 glass petri dishes over ice, to maintain the host frozen, which facilitates the cuticle and digestive  
231 tract removing. The host-larva extremities were then cut with curved scissors, and the cuticle  
232 was cut in the dorso ventral axis and removed with a fine tweezer. The digestive tract was also  
233 removed, as well as the host fat, if parasitoid larvae were clearly visible. If larvae were not  
234 visible, a binocular magnifier was used to observe *C. typhae* larvae and eggs (egg length is  
235 approximately 100 µm). A picture was then taken using a camera fixed to the binocular to  
236 measure larval size. For each dissection, pictures of a few larvae were used as reference size.  
237 The size of parasitoid larvae were then determined using the reference size and drawing the

238 measurement along the larval body with ImageJ software. The parasitoid larval size inside host  
239 larvae exposed to the different temperatures was then analysed.

240

## 241 ***2.5. Exposure of the parasitoid pupal stage to cool temperatures***

### 242 ***2.5.1. Effect on pupal mortality and developmental duration***

243 One-day-old cocoons were exposed at 5, 10, 15, 20 or 27 °C for 7 days (pupal duration lasts 7  
244 days at 27°C) and photoperiod of 10:14 LD, and then transferred (or maintained at) to 27°C  
245 until adult emergence. Prior to the exposure, the cocoon masses were split in two, to place the  
246 half-masses at different temperatures and limit possible impact of inter-individual variability of  
247 developmental time on the results. Cocoons were placed in tubes closed with foam plugs. Eight  
248 or nine half-masses were exposed per temperature. Occurrence and date of adult emergence  
249 were noted. Absence of emergence, 14 days after transfer to 27°C, was noted as cocoon death.  
250 Pupal mortality and pupal development time at the different exposure temperatures were then  
251 analysed.

### 252 ***2.5.2. Effect on female fecundity***

253 Ten females from different cocoon masses exposed to different temperatures were dissected to  
254 count the number of eggs produced in their ovaries. The dissections were realised on a  
255 microscope slide with two droplets of physiological solution and using fine tweezers. Firstly, a  
256 female was placed in a droplet and the reproductive system was removed by pulling the  
257 ovipositor while maintaining the female by its first abdominal segment. If unsuccessful, the  
258 cuticle had to be removed with tweezers. The reproductive tract is then placed in a new droplet  
259 of physiological solution. The ovaries are then separated from the rest of the reproductive tract,  
260 and they are opened with a thin entomological pin (0,1 mm diameter). A droplet of diluted  
261 methylene blue is then added to colour the eggs and facilitate egg counting. The preparation is  
262 photographed and eggs counted using ImageJ software. The number of eggs in the ovaries of  
263 *C. typhae* females was then analysed in function of temperature exposure.

264

## 265 ***2.6. Exposure of adult parasitoids to cool temperatures***

### 266 ***2.6.1. Effect on survival***

267 Newly emerged males and females were isolated in tubes with honey droplets on the wall and  
268 a cotton wool soaked with water at the bottom. Tubes were closed with foam plugs and placed  
269 in incubators at a given constant temperature: 5, 10, 15, 20 or 27°C. Twenty adults of each sex  
270 were placed per temperature. To check adult survival, tubes were observed daily at room

271 temperature (21°C). All tubes were taken from the incubators at the same time, and those  
272 coming from 5 and 10°C were checked at the end, *i.e.* after about 15 min spent at 21°C, to  
273 differentiate between alive and dead individuals.

### 274 **2.6.2. Effect on locomotor activity**

275 An actimeter (TriKinetics, Waltham, MA, USA) was used to monitor the activity of *C. typhae*  
276 adults. The actimeter is a system consisting of a vertical plate containing 32 holes (cells) each  
277 crossed by infrared beams, called a monitor, in which tubes (8 cm length and 7 mm inner  
278 diameter) with insects can be inserted perpendicularly to the plate. When an insect move along  
279 the tube, it passes across the monitor and cuts the infrared beam. The actimeter detects the  
280 number of times the infrared beam is cut off and the insect activity can be inferred by the  
281 number of movements along the actimeter tube. Each detection is transmitted computationally  
282 by the DAMSystem collection® software (TriKinetics, Waltham, MA, USA). The number of  
283 detections per minute in each cell is automatically recorded on a txt file. Before the beginning  
284 of the experiment, each insect tube inserted in a monitor was closed by two Q-tip heads, one of  
285 which is soaked with water. A drop of honey is placed on the tube wall at the opposite side of  
286 the wet Q-tip head. Two females or two males aged (1-3 days old) were introduced per tube,  
287 and twelve tubes of each sex were inserted in a monitor (24 cells in total). Each monitor was  
288 placed in an incubator at constant temperatures: 10, 12.5, 15, 20 or 27°C for 6 hours. Since  
289 some adult activity was still observed at 10°C, a second experiment was performed, where  
290 monitors were placed respectively at 5, 7.5, 10 or 27°C. The number of movements of *C. typhae*  
291 adults exposed to the different temperatures was then analysed.

### 292 **2.6.3. Effect on female egg-laying activity**

293 To test if *C. typhae* females were able to lay eggs at low temperatures, two *S. nonagrioides*  
294 larvae (3-weeks old) were exposed to a group of eight *C. typhae* females (1-2 days old) in a  
295 Petri dish that was placed either at 5, 7.5, 10, 12.5 or 15°C. Five replicates were set up per  
296 temperature. To ensure that both hosts and parasitoids were at the exposure temperature before  
297 female parasitoids could access the host larvae, they were placed separately in a Petri dish at  
298 the given temperature for 1 hour. The host larvae were then transferred quickly into the Petri  
299 dish of the female parasitoids. The exposed larvae were collected 2h later, and kept at -80°C  
300 before detection of *C. typhae* parasitism by PCR. As females of braconid parasitoids inject  
301 symbiotic viral particles (bracovirus) in the host hemolymph during oviposition, we can use  
302 these virulence gene markers to verify if a given host is parasitized. The host's DNA was then  
303 extracted from the hemolymph with the NucleoSpin Tissue kit (Macherey-Nagel™) following

304 the manufacturer's protocol. PCR amplifications were performed for the virulence marker CrV1  
305 using the specific forward (5'-AGAACCGATTTGCTCCCGTT-3') and reverse primers (5'-  
306 ACTGCTTTCTAGGTGCTTCAGA-3') designed in our lab. The PCR program was 95 °C for  
307 5min, 40 cycles of 95 °C for 1min, 50 °C for 1min, 72 °C for 1min, followed by 72 °C for 5 min.  
308 Successful PCR amplifications were confirmed by 2% (w/v) agarose gel electrophoresis. The  
309 presence of marker bands in the gel indicates an oviposition by *C. typhae* females.

310

### 311 **2.7. Percentage of winters with cold temperatures in France**

312 From the results obtained, we considered that winters were harmful for the development of *C.*  
313 *typhae* when they presented at least 14 consecutive days with an average daily temperature  
314 lower than 10°C. The percentage of these winters in France was estimated using temperature  
315 meteorological data from (Météo France SYNOP, 2023) for the 42 meteorological stations  
316 located in metropolitan France at a resolution of 3 hours from 2000 to 2020. These probabilities  
317 were represented in a map together with the known distribution of *S. nonagrioides* inferred by  
318 damages on maize fields in France (Thibord, 2017). These estimations were realised using R  
319 Software version 4.2.2 (R Core Team, 2022).

320

### 321 **2.8. Statistical analyses**

322 On developmental outcomes of hosts and proportion of successfully parasitized hosts, Chi-  
323 squared tests and Fisher exact tests were used for all comparisons, with *P*-values adjusted for  
324 multiple comparisons. Quantitative variables were analysed using Kruskal-Wallis followed by  
325 pairwise Wilcoxon tests and *P*-values adjusted with Benjamini-Hochberg or Bonferroni  
326 corrections. Adult longevity fulfilled the conditions (residuals homoscedasticity and normality)  
327 for running two-way ANOVA (temperature and sex factors) and Tukey HSD tests were used  
328 for pairwise comparisons. The significance threshold was set at 0.05 for adjusted p-value. All  
329 statistical analyses were performed in R Software version 4.2.2 (R Core Team, 2022) using  
330 base and car packages. Plots were realised using ggplot2 (Wickham, 2016), ggpubr  
331 (Kassambara, 2023) and scales (Wickham and Seidel, 2022) packages and tables were  
332 generated with the table1 package (Rich, 2023).

333

## 334 **3. Results**

### 335 **3.1. Exposure of parasitized host larvae to cool temperatures**

336 **3.1.1. Effect of duration and temperature exposure on parasitoid egg-larval development**

337 The effect of temperature was highly significant on the developmental outcomes of host larvae  
338 exposed for 14 days ( $P < 0.001$ ; Table 1A). After the transfer of parasitized larvae at 27°C, no  
339 cocoons were formed from parasitized host larvae exposed at 5°C (Figure 1). At 10°C, complete  
340 egg-larval parasitoid development could occur after transfer, as seen from host-larva bodies  
341 bearing traces of parasitic larval emergence, but cocoon formation was observed only once  
342 (4.5%). The other temperatures allowed complete egg-larval development (after the transfer)  
343 with about 50, 60 and 70% of parasitized larvae allowing cocoon formation at 15, 20 and 27°C  
344 respectively (Figure 1). Thus, 14 days' exposure at 5°C killed the internal egg-larval parasitoid  
345 but not the host, since 75% of parasitized host larvae developed into pupae.

346 Given winter length under temperate latitude, we also exposed parasitized larvae for 50 days at  
347 the same temperature range. Non-parasitized larvae were exposed similarly as a control group  
348 because we expected host mortality during long exposure at low temperature. The effect of  
349 temperature was highly significant on the developmental outcomes of host larvae, parasitized  
350 ( $P < 0.001$ ; Table 1A) or not parasitized ( $P = 0.001$ ; Table 1A). At 5 and 10°C there was no sign  
351 of parasitoid development, without larvae emergence from the host, after the transfer at 27°C  
352 (Figure 1). At 5°C this could be partially explained by host mortality, which was significantly  
353 higher in parasitized larvae compared to non-parasitized ones ( $P = 0.01$ , Table 1B). But this was  
354 not the case at 10°C where host larvae were able to complete their development until the pupal  
355 stage (after the transfer), being parasitized or not (Table 1B). Thus, 50 days of exposure at 5°C  
356 killed both host and parasitoid, and host mortality was increased by parasitism, whereas such  
357 long exposure at 10°C killed only the endoparasitic larvae, but not the host.

358 Comparing the outcome of parasitism between 14 and 50 days' exposure for each temperature  
359 confirmed a significantly higher host mortality during long exposure at 5°C ( $P < 0.001$ ; Table  
360 1B) and 10°C ( $P < 0.016$ ; Table 1B), and showed that the probability of cocoon formation after  
361 exposure to 15° and 20°C were not significantly different between exposure durations (Table  
362 1B).

363 *S. nonagrioides* spend winter as diapausing larvae, which may influence survival of the  
364 endoparasitoid at low temperature. Using 3-month-old larvae (in diapause), we tested the effect  
365 of the two temperatures critical for the egg-larval parasitoid development. Thus, we observed  
366 the developmental outcome of parasitized and non-parasitized host larvae after a 14-day  
367 exposure to 5° and 10°C followed by the transfer at 27°C, and using 14 days' exposure at 27°C  
368 as control (Figure 2). As expected, there was a drastic difference in the developmental outcome

369 of parasitized host larvae between the control and the cool temperatures (5°C:  $P=0.003$ ; and  
370 10°C:  $P<0.001$ ), but cold exposure had no effect on the mortality of non-parasitized hosts  
371 (Figure 2). There was no significant difference in the developmental outcome of parasitized  
372 hosts between 5 and 10°C (Figure 2). No parasitoid cocoons were found after the 10°C  
373 exposure, and only one host presented signs of emerged parasitoid larvae after being transferred  
374 at 27°C (Figure 2). However, after the 5°C treatment, 3 hosts allowed egg-larval development,  
375 one of them presented only signs of emerged larvae, but the other two allowed forming two  
376 small cocoon masses, with 14 and 49 individuals respectively (in rearing progeny size is on  
377 average 70, (Benoist et al., 2020). Among the 49 adults of *C. typhae* that emerged from one  
378 host larva exposed at 5°C, we let four females oviposit, and only one was able to produce  
379 progeny. Comparison of the outcome of host larvae parasitized at 4-weeks versus 3-months old  
380 pointed out that host larvae in diapause confer little protection of the egg-larval stage of *C.*  
381 *typhae* when they are exposed to cold ( $P=0.025$ ), but using these hosts showed that egg-larval  
382 survival was not impossible even after an exposure to 5°C (Figure 2). This comparison also  
383 revealed that parasitized host larvae exposed to cold (5 and 10°C) suffered more mortality when  
384 they were older (>50%, Figure 2) and diapausing at the time of parasitism than when they were  
385 younger (>25%, Figure 1) ( $P=0.007$ ).

### 386 **3.1.2. Lethal duration of exposure at 10°C**

387 The preceding results showed that over a total of 38 parasitized host larvae exposed at 10°C for  
388 14 days, regardless of their age and state, only one allowed formation of cocoons, and few (5)  
389 were formed (Figure 1 and 2). These results indicated that parasitoids had died in the host during  
390 their egg-larval development. The next objective was to determine, therefore, the lethal duration  
391 of 10°C exposure.

392 The proportion of 6-week-old hosts successfully parasitized (meaning with formation of  
393 cocoons) was significantly decreased when exposed at 10°C for 11 or 14 days ( $P<0.001$ ), but  
394 exposures lower than 11 days had no effect on the probability of cocoon formation (Table 2).  
395 Among 13 parasitized hosts exposed at 10°C for 14 days only one produced cocoons (Table 2),  
396 confirming high egg-larval mortality at this exposure duration. This only mass of cocoons was  
397 not statistically compared to the others. On average the masses formed after 11 days of exposure  
398 were 4 to 5 times smaller ( $P<0.001$ ; Table 2) and the pupal mortality was higher than those  
399 exposed for less days ( $P<0.001$ ; Table 2). There was also a female-biased sex-ratio of emerging  
400 adults when host larvae had been exposed for 11 or 14 days in comparison with other exposure  
401 durations ( $P<0.001$ ; Table 2).

### 402 **3.1.3. Egg-larval developmental duration and threshold**

403 The duration of egg-larval development was recorded as the time elapsed between *C. typhae*  
404 oviposition into the host larvae and the egression of *C. typhae* mature larvae from the host body.  
405 This analysis was done using the 4-week-old larvae exposed at different temperatures for 14  
406 and 50 days. For each exposure condition, egg-larval developmental duration was significantly  
407 higher than in the control condition at 27°C (P<0.001; Table 3). This duration was longer when  
408 exposure temperature decreased (Table 3). When parasitized host larvae were kept at 10 and  
409 15°C, parasitoid egression from the host body and pupal moult occurred only after host larvae  
410 had been transferred to 27°C and continued their development. When parasitized host larvae  
411 were exposed at 10°C for 14 days, the parasitoid egg-larval development lasted for 28 days  
412 (Table 3). Since at 27°C it lasts for about 14 days, this means that egg-larval development of  
413 *C. typhae* is blocked at 10°C. When host larvae were exposed at 15°C for 14 and 50 days, the  
414 duration of egg-larval development was equal to 25 and 56 days respectively, meaning that the  
415 development started slowly at this temperature, because otherwise, it would have lasted for 28  
416 and 64 days respectively (Table 3). At 20°C, full egg-larval development lasted for 25 days  
417 without being transferred at 27°C, meaning that 20°C permits pupal moult of *C. typhae*.

418 These results indicated that the thermal threshold of parasitic egg-larval development was close  
419 to 15°C. To better characterise this threshold and developmental rate in function of temperature,  
420 dissections of parasitized host larvae were done after 14 days of exposure at 15, 17 and 19°C.  
421 These dissections showed that at 15°C parasitoids were still in the egg stage in some hosts,  
422 while in the first larval stage in others (Figure 3A and 4). However, very few eggs were  
423 observed because their small size make them difficult to distinguish from other dissection  
424 debris. At 17°C, no parasitoid eggs were observed, only larvae slightly more developed (in first  
425 or second stage; Figure 3B) than at 15°C. At 19°C, only second and third (last) stage larvae  
426 were observed (Figure 3C). Measurements of parasitic larvae at different temperature exposures  
427 confirmed that their size increased with temperatures after 14 days' exposure (Figure 4, KW =  
428 99.201, P<0.001).

429

## 430 **3.2. Exposure of the parasitoid pupal stage to cool temperatures**

### 431 **3.2.1. Effect on pupal mortality**

432 Pupal cocoons of the parasitoid were exposed to temperatures ranging between 5° and 27°C for  
433 7 days, and then transferred to 27°C. Absence of adult emergence 14 days after the transfer  
434 indicated pupal death since pupal development lasts for 7.3±0.7 days at 27°C (Kaiser et al.,

435 2017). The pupal mortality was significantly different between the exposure temperatures  
436 (KW=32.53, df=4, P<0.001). After cocoon exposure at 5 or 10°C, mean mortality was about  
437 90%, and it decreased to about 50% at 15°C, and to about 10% at 20 and 27°C (Figure 5). Thus,  
438 as for the egg-larval stage, temperatures lower than 15°C are highly lethal for *C. typhae* pupae.

### 439 **3.2.2. Effect on pupal developmental duration**

440 Duration of pupal development increased significantly when lowering exposure temperatures  
441 (KW=38.029, df=4, P<0.001). However, at low temperatures (5 and 10°C), developmental  
442 duration measured after the transfer to 27°C was equivalent to the development time of control  
443 pupae always kept at 27°C, around 7 days (Figure 6). This result indicates that pupal  
444 development was arrested at 5° and 10°C and only started after the transfer at 27°C. At 15 and  
445 20°C, pupal development took longer than control nymphs, but it was completed faster after  
446 being transferred (Figure 6). Therefore, only few parasitoid adults are able to develop when  
447 exposed at 5 and 10°C at the pupal stage and it only happens after being transferred to  
448 temperature above 10°C. The threshold for pupal development was thus comprised between 10  
449 and 15°C, like the threshold for egg-larval development.

### 450 **3.2.3 Effect of female fecundity**

451 The number of eggs in the ovaries of *C. typhae* females was significantly lower after pupal  
452 exposure at 10 and 15°C than after exposure at 5, 20 and 27°C (P<0.001; Table 4). The amount  
453 of eggs was reduced by 21 and 25% at 10 and 15°C respectively, compared to the highest value  
454 observed at 20°C. Relatively high fecundity observed after pupal exposure at 5°C may result  
455 from the fact that the females that emerged from the only surviving cocoon mass at this  
456 temperature had the highest fitness, but then the same phenomena should have been observed  
457 at 10°C since only 14% individuals reached adulthood at this temperature. A trade-off between  
458 fecundity and survival is often observe in insects (Flatt and Heyland, 2011), thus small sample  
459 size due to high mortality may prevent reliable estimation of fecundity of surviving females.

460

## 461 **3.3. Exposure of adult parasitoids to cool temperatures**

### 462 **3.3.1. Effect on survival**

463 Adults were kept individually at the same range of temperatures used for pre-imaginal stages.  
464 Mean longevity varied significantly between 5 and 12 days depending on both temperature and  
465 sex (P<0.001; Table 5). For both sexes, minimal longevity was observed at 27°C (Figure 7).  
466 Males seemed to benefit the most from temperatures between 10 and 15°C, with a mean  
467 longevity above 10 days. Above and below these temperatures, their longevity diminished



468 greatly. Females, on the other hand, appeared to be more tolerant to lower temperatures,  
469 showing no significant differences in longevity between 5, 10, 15, and 20 °C (Figure 7).

### 470 **3.3.2. Effect on locomotor activity**

471 *C. typhae* activity (number of movements of 2 females or 2 males in glass tubes) increased with  
472 the temperature (KW=126; P<0.001). We noticed a drastic collapse in activity between  
473 individuals exposed to the control temperature (27°C) and the tested temperatures (Figure 8).  
474 Thus, at 5°C the median activity was null and at 7.5, 10, 12.5, 15°C was equal to 14, 40, 13, 86  
475 movements respectively, which were all significantly lower than the median number of  
476 movements observed at 20°C (498 movements) and 27°C (1232 movements). Males were  
477 significantly less active than females at 7.5°C (P=0.031), 10°C (P=0.016), 20°C (P<0.001), and  
478 27°C (P<0.001).

### 479 **3.3.3. Effect on female egg-laying activity**

480 PCR diagnosis of *C. typhae* parasitism in host larvae revealed that even at temperatures severely  
481 undermining locomotor activity (7.5, 10, 12.5 and 15°C), females of *C. typhae* were still capable  
482 of laying their eggs in a host. The only temperature in which we did not observe any trace of  
483 oviposition was 5°C (Figure 9).

484

### 485 **3.4. Percentage of winters with cold temperatures in France**

486 The analysis of meteorological data since the last twenty years in France, shows that 80 to 100%  
487 (97% ± 1% on average) of the winters have temperatures equal or inferior to 10°C during 14  
488 consecutive days in the regions where *S. nonagrioides* is present (Figure 10). This result means  
489 that very few winters (3%) would be on average permissive to allow *C. typhae* development  
490 under cold temperatures, but it depends on the region. In more littoral regions in the west and  
491 southwest of France, as in Charente Maritime, Gironde and Landes, some milder winters might  
492 allow *C. typhae* development.

493

## 494 **4. Discussion**

495 The aim of this paper was to investigate the cold-susceptibility of the tropical species *C. typhae*  
496 in the perspective of estimating its chances to survive winter and to establish if it is introduced  
497 in France as a biocontrol agent against the corn stemborer *S. nonagrioides*. This is why we  
498 focused on testing susceptibility to cold temperatures for prolonged periods occurring during

499 French winters, rather than investigating freeze susceptibility, that had been documented on  
500 sympatric sister species, *C. sesamiae* and *C. flavipes*, which appeared to be clearly freeze-  
501 susceptible (Mutamiswa et al., 2018). The experiments conducted on *C. typhae* in the present  
502 study revealed that this species was highly susceptible to cold, because high mortality occurred  
503 at all developmental stages when insects were exposed at temperature below 15°C. Thresholds  
504 of egg-larval and pupal development were close to 15°C, and a drastic decrease of adult  
505 locomotor activity was also observed from this temperature and below.

506 Cold susceptibility is directly linked to geographic distribution of species. Tropical and  
507 Mediterranean species usually experience high mortality when exposed to temperatures below  
508 their developmental threshold, between 10°C and 0°C (Hatherly et al., 2005). Our results  
509 confirm this finding and suggest a strong adaptation of *C. typhae* to the temperature in its native  
510 range, ranging from 9 to 38°C (Kenya, Ethiopia and Tanzania), and varying more locally  
511 between 15 and 32°C with a mean temperature of 22°C (West Kenya, Kobodo; (NASA LaRC,  
512 2019). Data obtained for *C. typhae* are consistent with the cold-susceptibility reported for its  
513 sympatric sister species. (Smaniotto et al., 2019) found egg-larval development of *C. flavipes*  
514 at 18°C but not at 15 or 10°C. They did not expose the pupal stage to these non-permissive  
515 temperatures. (Galichet, 1979) reported 100% of *C. chilonis* egg-mortality when parasitized  
516 host larvae (*Chilo suppressalis*) were exposed at 5 and 10°C. There was, however, a variation  
517 in cold-susceptibility of the different stages of *C. chilonis*. The first and second larval stages  
518 were more resistant to cold, showing 66% of mortality at 2, 5 and 10°C, when parasitized host  
519 larvae were exposed to these temperatures after parasitoid embryonic development.  
520 Nevertheless, the last parasitoid larval stage was as susceptible as the eggs to 10°C. Complete  
521 larval development occurred only at 15°C. The pupal stage was less cold tolerant because no  
522 development occurred at 15°C. Thus, in the three tropical sister species *C. flavipes*, *C. chilonis*  
523 and *C. typhae*, immature stages appear to have their Lower Lethal Temperature between 10 and  
524 15°C.

525 In *C. flavipes* and *C. chilonis*, adults were tested for their chill resistance, but not their longevity  
526 at cold temperatures above zero. In *C. typhae*, there was no significant adult longevity  
527 difference between 10, 15 and 20°C. Shorter longevity observed at 27°C was probably related  
528 to high locomotor activity of the insects, which decreased rapidly with the temperature, and  
529 was already fourteen times lower at 15°C. At 5°C, adults looked in a chill coma state, they laid  
530 motionless looking dead, with disorganised legs and wings, and 10-15 min at room temperature  
531 were necessary to observe movements upon stimulation with the tweezers. Inability to move

532 in cold temperature can be explained by cell membrane depolarization disturbing the  
533 transmission of the neuronal influx (Overgaard and MacMillan, 2017). Males were more  
534 susceptible to cold than females since their longevity was significantly reduced at 5°C, and they  
535 were less active than females at 7.5° and 10°C. We also observed that fewer males of *C. typhae*  
536 were formed when host larvae were exposed to lethal conditions (10°C during 11 and 14 days).  
537 In addition, Bressac et al., (submitted for publication) showed that *C. typhae* spermatogenesis  
538 was drastically reduced when male pupae were exposed at 10, 5 or 0°C. This is a general pattern  
539 in parasitoids, especially in arrhenotokous hymenopteran species. Interferences in their normal  
540 reproductive process are known to occur, when immatures or adults are exposed to low  
541 temperatures. Sex ratios, for instance, are often affected, because at low temperatures males are  
542 sterile and the activity of either sex is reduced, preventing mating, which results in a male-based  
543 progeny (Denlinger and Lee, 2019; King, 1987). This has been confirmed for *C. typhae*  
544 (Bressac et al., submitted for publication). The fact that there were more females emerging from  
545 larval hosts exposed at 10°C after 11 days of exposure, shows that *C. typhae* males are already  
546 less cold resistant at an immature stage.

547 Regarding the impact of pupal exposure, female *C. typhae* were also more resistant than males,  
548 because they showed a decreased fecundity but to a lesser extent, producing about 20% less  
549 eggs at 10 and 15°C, whereas males from pupae exposed at 10°C produced 80% less  
550 spermatozoa (Bressac et al., submitted for publication). The fact that some host larvae were  
551 parasitized at 7.5 and 10°C remains surprising. However, although at these temperatures female  
552 parasitoids have a drastic decrease in activity, they remain as active as at 15°C, which also  
553 allows host parasitism. Yet, it is possible that opening the incubator to place host larvae in the  
554 Petri dish containing *C. typhae* females may have caused a transient rise of temperature  
555 sufficient to allow the female parasitoids to oviposit. The rise of temperature was however too  
556 short for females kept at 5°C for which chill-coma was induced.

557 We wondered if survival of *C. typhae* consecutive to cold exposure could have been higher if  
558 the protocols had included a progressive exposure to cold, because cold acclimation can  
559 increase cold tolerance of chill-susceptible insects (*i.e.* Gerber et al., 2021), although it has been  
560 little investigated in tropical species (Chowanski et al., 2015). In the experiments of Galichet  
561 (1979) with *C. chilonis* during three winters in outdoor conditions, the timing of exposure of  
562 parasitized host larvae allowed for parasitoid acclimation to winter temperatures, but the  
563 probability to observe adult parasitoids in spring was quite low, varying from 0 to 10%  
564 (Galichet, 1979). Such findings suggest that cold acclimation did not increase cold hardiness of

565 *C. chilonis*, and this may well apply to its sympatric sister species *C. typhae*. Another factor  
566 that might favour winter survival in *Cotesia typhae* and establishment of this species in France  
567 would be diapause. In the *Cotesia* genus, ability to diapause has been shown for three species  
568 experiencing winter, *C. rubecula* (Nealis, 1985), *C. melanoscella* (Nealis et al., 1996) and *C.*  
569 *vestalis* (Alvi and Momoi, 1994; Hao et al., 2013). Such diapause is induced by day-length  
570 decrease perceived at the parasitoid larval stage and it takes place in the prepupal stage, inside  
571 the silk cocoon. In our experimental conditions, egg-larval development of *C. typhae* was not  
572 submitted to day-length decrease, but this was the case in the outdoor experiments with *C.*  
573 *chilonis*, and the few prepupae formed in spring did not diapause and experienced severe  
574 mortality (Galichet, 1979). Pre-pupae diapause has not been reported in any of the species of  
575 the *flavipes* complex, although some of their hosts diapause during off-crop season (Sokame et  
576 al., 2019) or during austral winter in altitude (Chinwada et al., 2003), and *Cotesia* infestation  
577 resumes in the next generation, which may suggest possible parasitoid diapause. There are  
578 therefore no elements in favour of diapause ability in *C. typhae*, which host *S. nonagrioides* is  
579 available all year long in the parasitoid's native habitat.

580 We cannot rule out, however, the possibility that rare *C. typhae* individuals could survive  
581 winter. Our data revealed inter-individual variability of cold tolerance, few individuals were  
582 able to survive exposures at 5 and 10°C at the egg-larval and the pupal stage. If some of these  
583 individuals were able to reproduce, would it lead to a slow establishment of *C. typhae* in France,  
584 possibly associated in the long term to natural selection of more cold tolerant individual  
585 genotypes, and possibly facilitated by global warming? Genetic variation of cold resistance  
586 traits was found within a beetle species, which establish populations that successfully survive  
587 cold climates during winter months (Zhao et al., 2018). Significant differences in allele  
588 frequencies of genes associated to climate adaptation were also found between populations of  
589 the mosquito *Aedes aegypti* (Kramer et al., 2023), showing adaptation of a tropical species to  
590 cooler climates.

591 Nevertheless, based on the results obtained in this study the probability that *C. typhae* establish  
592 surviving winter populations seems to be quite low under current climate conditions. We  
593 considered that French winters were harmful for the development of *C. typhae* when they  
594 presented at least 14 consecutive days with an average daily temperature lower than 10°C.  
595 When matching the distribution of *S. nonagrioides* in France, regions where *C. typhae* could be  
596 introduced as a biological control agent, we see that in most regions 100% of the winters fulfil  
597 this condition. However, littoral regions in the west can have milder winters (10-20% in the last

598 20 years) with cold temperatures for shorter periods of time, which can be more permissive for  
599 *C. typhae* development and establishment at short term. Nonetheless, we should consider that  
600 these estimated percentages of harmful winters represent average daily temperatures and  
601 temperatures in these periods can be much lower, even in cases where 14 consecutive days are  
602 not observed. Future studies using a modelling approach on life history traits of *C. typhae* in  
603 function of temperatures and climatological data during winters will help predict the survival  
604 capacity of *C. typhae* per region and determine its probability of establishment in the French  
605 environment.

606 In addition, several barriers based on the *C. typhae* life cycle support its high susceptibility to  
607 cold temperatures. First, *C. typhae* is a pro-ovigenic short-lived species, females can lay all  
608 their eggs in 2-3 host larvae, beginning the day after their emergence (Benoist et al., 2020).  
609 Thus, the adult stage is clearly not the one that could pass winter. Second, contrary to what we  
610 expected, there was an increased mortality of diapausing larvae of *S. nonagrioides* when  
611 parasitized by *C. typhae*, even if non-parasitized diapausing larvae were more resistant to cold  
612 (5 and 10°C) than younger larvae. Generally, in insects, diapause enhances cold stress tolerance  
613 by the initiation of a suite of stress response mechanisms that facilitate overwintering survival  
614 (Bale and Hayward, 2010; Denlinger, 2002). However, *S. nonagrioides* in diapause did not  
615 protect *C. typhae* immature stages. This indicates that host larvae parasitized late in the season  
616 at the beginning of diapause will have smaller survival capacity, reducing the overwintering  
617 population of *S. nonagrioides*. Third, the mortality rate of *C. typhae* immature stages measured  
618 under laboratory conditions at cold temperatures might be underestimated. In our protocol,  
619 cold-exposed parasitized host larvae and parasitoid cocoons were transferred to control  
620 temperature (27°C) to measure parasitoid survival. However, this wide change of temperature  
621 does not occur under natural conditions during winter, so it is very likely that at normal winter  
622 temperatures in France the mortality of *C. typhae* will be higher due to repeated exposure to  
623 cold temperatures without permissive temperature in between. Fourth, the last larval stage of  
624 *C. typhae* that could emerge from diapausing hosts in early spring are likely to experience a  
625 high mortality before completing pupal development. This high pupal mortality in the  
626 beginning of spring was shown in the sister species *C. chilonis*. At this moment, the  
627 temperatures in France were not sufficiently high to complete the pupal stage (Galichet, 1979).  
628 Fifth, parasitoid adults that could possibly emerge after cold exposure during immature stages  
629 are prone to have abnormalities, such as problems in tissue differentiation, altered hormonal  
630 milieu and expression of mutations (Denlinger and Lee, 2019), which can strongly decrease

631 their reproductive success. The exposure of parasitoid immature stages to low temperatures can  
632 also decrease longevity of surviving adults (Rundle et al., 2004). Although *C. typhae* adults  
633 showed a relatively higher longevity compared to control temperature when exposed to cold  
634 temperatures, their longevity may have decreased if immatures stages had been exposed to cold,  
635 which we did not measure in our experimental setup. Finally, knowing that during winter *S.*  
636 *nonagrioides* diapauses at the last larval stage, with almost no feeding activity and on which *C.*  
637 *typhae* have reduced parasitism success (Kaiser et al., 2017), parasitoid females that could  
638 emerge will be faced with depleted host availability on non-attractive plant residues. Laboratory  
639 studies on non-target species showed that only few species were parasitized *in planta* choice  
640 tests, representing a very low risk of *C. typhae* towards these species (Fortuna et al in prep.). In  
641 addition, most of these species pass the winter at the egg stage, which make them inaccessible  
642 for *C. typhae* parasitism during winter.

643 At individual level, the results presented in this study suggest also a common physiological  
644 response to cold of the different parasitoid stages. Several cellular and molecular mechanisms  
645 have been documented in insects. At the cellular level, cold-susceptible insects die from  
646 exposure to cold temperatures above zero due to loss of ion and water balance linked to  
647 alterations in the function, composition and structure of their cytoplasmic membrane, especially  
648 severe at the gut and Malpighian tubules level (Overgaard and MacMillan, 2017). At the  
649 molecular level, transcriptomic analyses run in different insect orders have pointed out  
650 numerous genes and proteins involved in responses to cold temperatures (Des Marteaux et al.,  
651 2017; Dunning et al., 2014; Kramer et al., 2023; Toxopeus et al., 2019) and their functions are  
652 being progressively identified (De Jong and Saastamoinen, 2018; Guz et al., 2014; Poikela et  
653 al., 2021; Zhang et al., 2020). For instance, polymorphism is an amino acid in the gene *flightin*,  
654 known to have an important function in insect flight and locomotion, was associated with chill  
655 coma recovery in a Nymphalid butterfly (De Jong and Saastamoinen, 2018). Similarly, the  
656 comparison of *vrille* gene sequence variation to cold tolerance traits among different  
657 populations of *Drosophila*, suggested that this key circadian clock gene has an essential role in  
658 species acclimation and cold resistance (Poikela et al., 2021). In general, such knowledge will  
659 help better understand the mechanisms underlying cold susceptibility through the study of  
660 candidate gene variations.

661  
662 In conclusion, this study revealed that the Lower Lethal Temperature of *C. typhae* immature  
663 stages was quite high, between 10° and 15°C, and only rare individuals might become adults in

664 spring, but with a low probability to find suitable hosts. Such findings support environmental  
665 innocuity of *C. typhae* introduction in France, and confirm that annual releases are required to  
666 control the European corn stemborer *S. nonagrioides*, a major maize pest in France and other  
667 Mediterranean countries. Future experimental release of *C. typhae* in field trials will allow  
668 assessing its ability to establish following winter, by monitoring its larvae emergence from  
669 overwintering *S. nonagrioides* larvae, before a new annual release of *C. typhae*. Beyond the  
670 potential agronomic application of *C. typhae*, this study contributes to the quantification of life  
671 history traits associated with thermal biology in a tropical species, which will allow the  
672 construction of models describing the host-parasitoid population dynamics, and the design of  
673 optimal pest management programs for an efficient and sustainable biological control of crop  
674 pests.

675

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856 **Tables**

857 **Table 1.** Statistical results on the effect of temperature (A) and exposure duration (B) on host  
 858 developmental outcomes, and the effect of parasitism on host mortality (B). The following  
 859 developmental outcomes of the host were tested depending if they were or not parasitized:  
 860 parasitoid larvae emergence and cocoon formation, host pupation or host death. Four-week-old  
 861 host larvae were used in this experiment. Pearson's chi-squared test with simulated p-value  
 862 (10000 Monte Carlo simulations) was used for all comparisons. Monte Carlo simulations were  
 863 used because the sample size was not large enough to follow Chi-squared law. Significant  
 864 effects are noted with \*(P<0.05), \*\*(P<0.01), \*\*\*(P<0.001).

865

A)	Parasitized host exposed 14 days	Parasitized host exposed 50 days	Non-parasitized host exposed 50 days
<i>Effect of temperature on developmental outcomes of hosts, within each exposure duration</i>	X <sup>2</sup> = 65.463 p-value < 1e-4 ***	X <sup>2</sup> = 99.258 p-value = 1e-4 ***	X <sup>2</sup> = 19.933 p-value = 4e-4 ***

866

B)	Temperature (°C)				
	5	10	15	20	27
<i>Effect of exposure duration (14 or 50 days) on developmental outcomes of parasitized hosts</i>	X <sup>2</sup> = 25.956 p-value = 1e-4 ***	X <sup>2</sup> = 9.326 p-value = 0.016 *	X <sup>2</sup> = 2.4 p-value = 0.558	X <sup>2</sup> = 2.4 p-value = 0.511	
<i>Effect of parasitism on mortality of the host before larvae emergence</i>	X <sup>2</sup> = 8.656 p-value = 0.010 **	X <sup>2</sup> = 0.861 p-value = 0.464	X <sup>2</sup> = 1.879 p-value = 0.298	X <sup>2</sup> = 0.597 p-value = 1	X <sup>2</sup> = 1.535 p-value = 0.3391

867

868 **Table 2. Descriptive statistics of parasitic success traits depending on duration of host exposure**  
 869 **at 10°C.** Fisher exact test and Kruskal-Wallis test followed by Wilcoxon tests (p-value adjusted with  
 870 the Benjamini-Hochberg correction) were used for comparisons between exposure durations. Main  
 871 significant effects are noted with \*\*\* (P<0.001), and superscript letters indicate significant differences  
 872 between exposure durations. Asterisk in black corresponds to one cocoon mass that was not used for  
 873 analyses due to the small sample size.

		Duration of exposure (days)					
		2	5	8	11	14	
<b>Number of parasitized hosts</b>		(N=27)	(N=27)	(N=27)	(N=26)	(N=13)	<b>Test: Fisher exact test</b>
<b>Percentage of successfully parasitized host (formation of cocoons)</b>		74,1 <sup>a</sup>	81,5 <sup>a</sup>	85,2 <sup>a</sup>	38,5 <sup>b</sup>	7,7 <sup>b</sup>	P-value = 3.2e-07 ***
<b>Number of masses analyzed</b>		(N=19)	(N=20)	(N=21)	(N=10)	(N=1)	<b>Test: Kruskal-Wallis</b>
<b>Number of cocoons per masse</b>	Mean (±SD)	91.5 (28.4)	103 (23.0)	76.7 (28.5)	18.0 (14.1)	22.0 (NA)	KW= 29.847 df = 4 p-value =
	Median	97.0 <sup>ab</sup>	104 <sup>b</sup>	79.0 <sup>a</sup>	10.0 <sup>c</sup>	22.0*	5.257e-06 ***
<b>Pupal mortality (%)</b>	Mean (±SD)	12.4 (8.24)	5.99 (4.73)	10.3 (8.55)	32.9 (18.7)	22.7 (NA)	KW= 21.233 df = 4 p-value =
	Median	12.8 <sup>a</sup>	4.76 <sup>b</sup>	7.53 <sup>ab</sup>	30.0 <sup>c</sup>	22.7*	2.847e-04 ***
<b>Number of adults emerged</b>	Mean (±SD)	81.4 (28.9)	97.2 (22.0)	68.4 (28.6)	13.3 (11.5)	17.0 (NA)	KW= 30.747 df = 4 p-value =
	Median	90.0 <sup>ab</sup>	99.0 <sup>b</sup>	75.0 <sup>a</sup>	7.00 <sup>c</sup>	17.0*	3.447e-06 ***
<b>Sex ratio (% female)</b>	Mean (±SD)	59.8 (30.9)	68.1 (20.8)	62.9 (29.0)	88.4 (18.5)	94.1 (NA)	KW= 15.449 df = 4 p-value =
	Median	73.1 <sup>a</sup>	73.9 <sup>a</sup>	73.3 <sup>a</sup>	95.2 <sup>b</sup>	94.1*	3.855e-03 ***

875 **Table 3. Mean and median duration of parasitoid larval development depending on temperature**  
 876 **and duration of host exposure.** Larval development was considered as the time between female  
 877 oviposition and the egression of parasitoid larvae from the host body. Four-week-old host larvae were  
 878 used in this experiment. The non-parametric Kruskal-Wallis test followed by Wilcoxon tests (p-value  
 879 adjusted with the Benjamini-Hochberg correction) were used for comparisons between temperatures  
 880 within each exposure duration. Main significant effects are noted with \*\*\*( $P < 0.001$ ), and superscript  
 881 letters indicate significant differences in developmental duration within exposure conditions.

882

Temperature (°C)	14 days exposure				Test: Kruskal- Wallis	50 days exposure			Test: Kruskal- Wallis	
	10	15	20	27		15	20	27		
Number of masses	(N=7)	(N=13)	(N=14)	(N=31)		(N=12)	(N=17)	(N=31)		
<i>Duration of larval development (days)</i>	Mean (±SD)	29.1 (±3.27)	25.2 (±0.67)	19.3 (±1.54)	14.9 (±2.77)	KW = 51.874 df = 3 p-value = 3.186e-11 ***	55.7 (±2.39)	25.4 (±1.49)	14.9 (±2.77)	KW = 48.374 df = 2 p-value = 3.132e-11 ***
	Median	28.0 <sup>a</sup>	25.0 <sup>b</sup>	19.0 <sup>c</sup>	14.0 <sup>d</sup>		55.5 <sup>a</sup>	25.0 <sup>b</sup>	14.0 <sup>c</sup>	

884 **Table 4. Effect of temperature exposure at the pupal stage on female fecundity.** Fecundity was  
 885 measured as the number of eggs counted inside ovaries of dissected females exposed at different  
 886 temperatures for 1 week during pupal stage. Kruskal-Wallis test followed by Wilcoxon tests (p-value  
 887 adjusted with the Benjamini-Hochberg correction) were used for comparisons between temperatures.  
 888 Superscript letters indicate significant differences between exposure temperatures.

		Temperature (°C)					
		5	10	15	20	27	
Number of females dissected		(N=11)	(N=10)	(N=10)	(N=10)	(N=19)	<b>Test: Kruskal-Wallis</b>
Number of eggs	Mean (±SD)	170 (18.0)	144 (13.8)	137 (20.6)	182 (25.6)	170 (20.4)	KW = 24.644, df = 4
	Median	167 <sup>a</sup>	139 <sup>b</sup>	134 <sup>b</sup>	189 <sup>a</sup>	176 <sup>a</sup>	p-value = 5.933e-05 ***

889

890

891 **Table 5.** Effect of temperature and sex on longevity of males and females of *C. typhae* analysed by  
 892 two-way anova (model I).

Source	df	SS	MS	F	Pr > F
Model	9	892.50	99.17	6.27	<0.001 ***
sex	1	18.79	18.79	1.19	0.277
temperature	4	551.62	137.91	8.72	0.000
sex*temperature	4	322.08	80.52	5.09	0.001

893



894 **Figure legends**

895 **Figure 1. Developmental outcome of four-week-old host larvae, parasitized or not,**  
896 **exposed 14 or 50 days at different temperatures.** Vertical bars represent percentages of each  
897 developmental outcome. Parasitized larvae at 27°C correspond to control condition for  
898 comparison.

899 **Figure 2. Developmental outcome of three-month-old host larvae, parasitized or not,**  
900 **exposed 14 days at low and control temperatures.** Vertical bars represent percentages of each  
901 developmental outcome.

902 **Figure 3. *Cotesia typhae* larvae observed in dissected host larvae exposed at different**  
903 **temperatures during 14 days.** Parasitoid first larval instar observed at 15°C (A) and second  
904 larval instars observed at 17°C (B) and 19°C (C).

905 **Figure 4. Egg-larval measurements of parasitoids found in hosts exposed to different**  
906 **temperatures during 14 days.** In parentheses are indicated the number of parasitoids measured  
907 (on top of boxplots) and the number of dissected hosts for each temperature (on the x axis).  
908 Letters indicate a significant difference of larvae size at the 0,05 threshold by the Wilcoxon-  
909 Mann-Whitney test, P-value adjusted with Benjamini-Hochberg correction.

910 **Figure 5. Pupal mortality after one-week exposure at different temperatures.** Letters  
911 indicate significant differences in percentage of mortality between exposure temperatures  
912 (Wilcoxon-Mann-Whitney tests, p-value adjusted with the Benjamini-Hochberg correction). In  
913 parenthesis is indicated the number of cocoon half-masses exposed at each temperature.

914 **Figure 6. Pupal development time after one-week exposure at different temperatures.**  
915 Letters indicate significant differences in developmental time between exposure temperatures  
916 (Wilcoxon-Mann-Whitney tests, p-value adjusted with the Benjamini-Hochberg correction). In  
917 parenthesis is indicated the number of cocoon half-masses exposed at each temperature with  
918 adult emergence.

919 **Figure 7. Effect of temperature on female and male longevity.** Bars indicate the mean  
920 longevity in days and vertical lines the standard error to the mean. Letters indicate significant  
921 difference at  $P < 0.05$  for each sex (Tukey HSD multiple comparison tests). In parentheses is  
922 indicated the number of parasitoid adults measured (on the x axis).

923 **Figure 8. Adult locomotor activity in function of temperature.** Boxplots represent the  
924 median and dispersion of the number of movements in the actimeter during 6 hours of test

925 (n=32 tubes per temperature, each containing either 2 males or 2 females). Letters indicate a  
926 significant difference at the 0.05 threshold by the Wilcoxon-Mann-Whitney test, P-value  
927 adjusted with Bonferroni correction.

928 **Figure 9. Electrophoresis gel of PCR amplification on a polydnavirus locus of *C. typhae***  
929 **(CrV1 gene)** extracted from *S. nonagrioides* larvae left with females of *C. typhae* at several  
930 temperatures from 5° to 15°C. Positive samples present a band at 500 bp. T+ represents the  
931 positive control and corresponds to a *S. nonagrioides* larvae parasitized by *C. typhae*.

932 **Figure 10. Probability of occurrence of harmful winters for the development of *Cotesia***  
933 ***typhae*.** For each of the 42 Meteo France weather stations represented by a square, and over 21  
934 years (2000-2020), we calculated the probability of having 14 days consecutives with an  
935 average daily temperature below a threshold of 10 degrees during the winter season. The  
936 probability is represented by a colour gradient from red (0%) to blue (50%) to green (100%).  
937 The gray background area in the map represents the distribution range of *Sesamia nonagrioides*  
938 in France, extracted from Thibord et al. 2017.