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1 **Physiological and biochemical responses to thermal stress vary among**
2 **genotypes in the parasitic wasp *Nasonia vitripennis***

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4 Hidalgo Kevin^{*1,4}, Beaugeard Erika^{*1,5}, Renault David^{2,3}, Dedeine Franck¹, Lécureuil
5 Charlotte¹

6
7 * These authors contribute equally to the work

8
9 ¹ Institut de Recherche sur la Biologie de l’Insecte UMR 7261 CNRS – Université de Tours,
10 UFR Sciences et Techniques, Parc Grandmont, Tours, France

11
12 ² Université de Rennes 1, UMR CNRS 6553 Ecobio, Campus de Beaulieu, 263 Avenue du
13 Gal Leclerc, CS 74205 35042 Rennes Cedex, France

14
15 ³ Institut Universitaire de France, 1 rue Descartes, 75231 Paris Cedex 05, France

16
17 ⁴ Present address : In’Search, 367 rue Maria Gaucher, 45160 Olivet

18
19 ⁵ Present address : Centre d’Études Biologiques de Chizé (CEBC), UMR 7372 CNRS-
20 Université de La Rochelle, 79360 Villiers-en-Bois, France

21
22
23 **Abstract**

24 In ectotherm species such as insects, thermal fluctuations represent a major environmental
25 factor driving development, survival and reproduction of individuals. Reproductive traits are
26 particularly sensitive to heat stress that can induce a permanent sterility, or at least
27 hypofertility, of adult males. This study aims to compare physiological and biochemical
28 responses associated to male performances to an exposure of 24h to moderately high
29 temperature (36 °C) among three inbred lines of *N. vitripennis* (AsymC, Cor, Oul). Cor males
30 showed very specific metabolic adjustments compared to the two other lines. By contrast, Oul
31 males showed stronger phenotypic adjustment of its life cycle, and produced metabolic water
32 to compensate water loss by heat stress. Finally, AsymC males had probably more difficulties
33 to acclimate at 36 °C, even for a short period, as their adult longevity was significantly
34 reduced. Thus, the ability of developmental plasticity in *N. vitripennis* males exposed to heat
35 stress appears to be dependent of their genotypes.

36
37 **Keywords:** Heat stress; Metabolomic; Males; Reproduction/Sterility; Development;
38 Parasitoid

39

40 **List of abbreviations**

41 Cor: Corsica line

42 Oul: Oulu line

43 T0: pupae maintained under optimal condition at 25 °C

44 T10: pupae exposed for 10h at 36 °C and then returned at 25°C

45 T24: pupae exposed for 24h at 36 °C and then returned at 25°C

46 FM: fresh mass

47 DM: dry mass

48 PCA: principal component analysis

49 PC1-4: principal component axis 1 to 4

50 PPP: pentose phosphate pathway

51 ROS: reactive oxygen species

52 **Introduction**

53 In ectotherm species such as insects, thermal fluctuations represent a major
54 environmental factor driving development of individuals (Guderley, 2004; Keller and
55 Seehausen, 2012; Colinet et al. 2015). Many studies revealed that exposure to heat stress,
56 even for a short period, directly affects the locomotion, development, longevity, reproduction
57 and distribution of many insect species living in temperate latitudes (*e.g.* Clarke, A. and
58 Fraser, K.P.P., 2004; David et al., 1997; Folguera et al., 2011; Hance et al., 2007; Keller and
59 Seehausen, 2012; Schulte, 2015). Reproductive traits are particularly sensitive to both heat
60 and temperature fluctuations, even when fluctuations have slight effects on survival abilities
61 (Chirault et al., 2015; Jørgensen et al., 2006). Sperm and egg production both can be affected
62 by temperature, although several studies have reported differential effects of temperature on
63 male and female (Lei et al, 2016 ; Enriquez & Colinet, 2017). Heat stress during the juvenile
64 development may induce a permanent sterility, or at least hypofertility, of adults males, as
65 demonstrated in *Drosophila buzzatii* (Diptera: Drosophilidae – Jørgensen et al., 2006) and
66 *Nasonia vitripennis* (Hymenoptera: Pteromilidae) (Chirault et al., 2015). Such reproductive
67 repercussions are due to the alteration of spermatogenesis that produces less germ cells under
68 heat stress (Chirault et al., 2015; Lacoume et al., 2007; Nguyen et al., 2013; Rinehart et al.,
69 2000). Reproduction drains somatic energy reserves (*e.g.* carbohydrates and lipids), thus
70 limiting the energy available for biochemical systems that could protect cells from damages
71 (Harshman and Zera, 2007). Under stressful conditions, it is hypothesized that organisms may
72 reallocate a part of the energy reserves available for reproductive functions into cell
73 maintenance and protection.

74 At the cellular level, heat stress exposure can disrupt water and osmotic homeostasis,
75 therefore participating to cell desiccation (Benoit, 2010; Danks, 2000). Cell desiccation, in
76 combination with a warmer body temperature, is known to increase the probability of protein
77 and enzyme denaturation and unfolding, and can also affect phospholipid structures and
78 membrane fluidity, which can lead to phase transitions and unstable membranes (Overgaard
79 et al., 2005; Rozsypal et al., 2014). To protect cells against such adverse effects of heat stress,
80 accumulation of specific metabolites, the so-called compatible solutes, has often been
81 documented. These solutes include polyols, sugars or amino acids (Rathinasabapathi, 2000;
82 Yancey, 2005), which are acting as osmolytes by adjusting the osmolarity of body fluids to
83 maintain water and ion homeostasis. Osmoprotectants can also be produced to repair
84 damaged proteins and preserve cell membrane fluidity and structure (Hidalgo et al., 2013;
85 Yancey, 2005). The characterization of the biochemical modifications associated with heat

86 stress can be conveniently pictured by metabolomic approaches, as for instance done in
87 Diptera (*i.e.* *D. melanogaster*: Cooper et al., 2014; *Anopheles sp* (Culicidae): Hidalgo et al.,
88 2016, 2014) and in Hemiptera (*Trialeurodes vaporariorum* (Aleyrodidae): Hendrix and
89 Salvucci, 1998), among others.

90 The haplodiploid parasitoid wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae)
91 provides a unique model system for studying the physiological consequences of heat stress on
92 reproduction, at the organismal and cellular levels. In this species, recent studies revealed that
93 a quick exposure to heat stress during juvenile development disrupts spermatogenesis
94 (Chirault et al., 2016, 2015). Of particular, a 24-hour exposure at a range of moderately high
95 temperatures (34 to 38 °C) during the first pupal stage of *N. vitripennis* highly decreases (by
96 67% to 91%) the number of spermatozoa that a male produces (Chirault et al., 2015). In
97 haplodiploid species, like most Hymenoptera (Heimpel and de Boer, 2008), the disruption of
98 spermatogenesis can have dramatic effects on the subsequent offspring's sex ratio, which in
99 turn can profoundly affect population genetics and dynamics (Chirault et al., 2016, 2015).
100 This is especially true in *N. vitripennis* because (i) females are monandrous (Assem and
101 Werren, 1994) and (ii) the stock of spermatozoa is produced in one shot during the pupal
102 development (Chirault et al., 2016; Pennypacker, 1958). The effects of heat stress on the
103 quality and quantity of spermatozoa by *N. vitripennis* males can thus strongly alter population
104 dynamics. Finally, it is worth noting that *N. vitripennis* is a cosmopolitan species living in
105 several regions of the world, spanning a large range of temperature averages and fluctuations.
106 It is suspected that this distribution may have resulted in locally adapted populations, thus
107 suggesting that heat stress may differentially affect the reproductive capacities of *N.*
108 *vitripennis* individuals originating from distinct habitats.

109 Therefore, in this work we aimed to investigate how exposure to heat-stress (36 °C) during
110 juvenile development of *N. vitripennis* males (1) shapes life-history traits of *N. vitripennis*,
111 including fitness (*i.e.* adult longevity and sperm count), pupal development (*i.e.* development
112 time and mortality), morphological traits (*i.e.* body size and dry mass) or again physiological
113 ones (*i.e.* body water content and metabolome); and (2) depends on the genetic differences
114 among lines linked to their geographical origin. Thus, working on different lines of *N.*
115 *vitripennis* originated from distinct regions would allow highlighting possible different
116 plasticity abilities expressed by the different genotypes. If the responses to thermal stress have
117 a genetic basis, we expected to obtain (i) different trait values among lines when they are
118 placed at different temperatures, and (ii) line-specific responses to the stressful temperature.

119 By contrast, if the response to thermal stress have an environmental basis, we predicted the
120 effects of temperature to be similar across the three tested lines.
121

122 **Material and Methods**

123 **1) Origin of *Nasonia vitripennis* lines and rearing conditions**

124 Three lines of the parasitoid wasp *N. vitripennis* issued from the Louis van de Zande's
125 laboratory (Evolutionary Genetics laboratory – Groningen, Netherlands) were used in this
126 study. The first line, AsymC, is an inbred line whose genome was sequenced in 2010 (Werren
127 et al., 2010). The two other isofemale lines were chosen because their population of origin
128 live in very different climatic conditions, with a Mediterranean climate in Corsica and more
129 variable temperature in Finland. Indeed, they were established in 2009 by Paolucci et al.
130 (2013) from a female sampled in Corsica (France, 42°22'40.80''N, 8°44'52'80''E – hereafter
131 referred to as Cor), and from a female collected in Oulu (Finland, 65°3'40.16''N,
132 25°31'40.80''E – hereafter referred to as Oul). The three lines AsymC, Cor and Oul have
133 been reared under our laboratory conditions since October 2010 for AsymC, and since July
134 2015 for Cor and Oul. The insects were maintained under optimal climatic conditions
135 (Chirault et al., 2015; Werren and Loehlin, 2009) at 25 °C and under constant light into a
136 climatic chamber Strader phytronic. The lines were grown on *Calliphora sp* pupae (Diptera:
137 Calliphoridae).

138 As earlier studies revealed that exposure of juveniles to heat resulted in hypofertility or
139 permanent sterility in males, we focused on this sex. Because *N. vitripennis* reproduces by
140 arrhenotokous parthenogenesis, males were obtained by allowing virgin females to lay their
141 haploid eggs inside *Calliphora sp* pupae. Virgin females were collected during the beginning
142 of pupal stage, and were isolated into 2mL microtubes until adult emergence. At emergence,
143 two *Calliphora sp* pupae were proposed to each virgin female which could lay haploid eggs
144 inside these hosts. One female can oviposit 20 to 50 eggs per *Calliphora* pupae (Sykes, 2007).
145 Haploid males were collected from *Calliphora* pupae at the beginning of the white pupal
146 stage (*i.e.* 8-9 days after females laid at 25 °C). At collection, males were randomly
147 distributed into different Plexiglas plates containing 40 individual compartments.

148

149 **2) Experimental treatments**

150 Three experimental treatments were run for the three *N. vitripennis* lines: a control treatment
151 at 25 °C during insect lifelong (hereafter referred to as T0), and two heat treatments where
152 individuals were transferred to 36 °C in a climate room Sanyo incubator CFC Free at the
153 onset of the first pupal stage for 10h (hereafter referred to as T10) or 24h (hereafter referred to
154 as T24), before being returned to 25 °C.

155

156 3) Effects of the experimental treatments on wasps' life-history traits

157 3.1) Life cycle characteristics

158 For each experimental condition (three lines x three experimental treatments), adult
159 emergence and survival of male pupae individualized into Plexiglas plates were monitored
160 twice per day (*i.e.* every 12h). For each male, we calculated (1) the success of adult
161 emergence (proportion of pupae that survived and emerged after pupal development), (2) the
162 duration of pupal development, and (3) the adult longevity. Altogether, 1,367 males were
163 monitored for adult emergence (AsymC/T0 n=214 ; AsymC/T10 n=168 ; AsymC/T24 n=104
164 ; Cor/T0 n=224 ; Cor/T10 n=127 ; Cor/T24 n=97 ; Oul/T0 n=204 ; Oul/T10 n=128 ; Oul/T24
165 n=101), and 1,008 males were monitored for pupal development and longevity (AsymC/T0
166 n=166 ; AsymC/T10 n=152 ; AsymC/T24 n=71 ; Cor/T0 n=141 ; Cor/T10 n=98 ; Cor/T24
167 n=78 ; Oul/T0 n=134 ; Oul/T10 n=100 ; Oul/T24 n=68).

168

169 3.2) Allometric and physiological traits: inter-orbital distance, dry mass and body water 170 content

171 For experimental condition, the inter-orbital distance (*i.e.* minimal distance between the two
172 composed eyes) of *N. vitripennis* adult males was measured immediately after the death of
173 each male. In parasitoid wasps, there is evidence that the inter-orbital distance is positively
174 correlated to adult body size, and can be conveniently used as a good body size estimator
175 (Lacoume et al., 2006). Pictures of adult male heads were taken under a binocular microscope
176 Leica MZ6 and the inter-orbital distance was measured using the ImageJ software (Schneider
177 et al., 2012). A total of 290 males was used for this assay (AsymC/T0 n=31 ; AsymC/T10
178 n=30 ; AsymC/T24 n=31 ; Cor/T0 n=20 ; Cor/T10 n=20 ; Cor/T24 n=50 ; Oul/T0 n=20 ;
179 Oul/T10 n=30 ; Oul/T24 n=58).

180 Fresh mass (FM) of individual adult males was measured soon after adult emergence
181 for each line and each experimental treatment (Mettler Toledon microbalance, d = 0.01 mg).
182 Immediately after weighing, males were snap-frozen into liquid nitrogen, lyophilized for 72h
183 (Christ Alpha 1-4 LO plus) before being weighted again (dry mass, DM). Body water content
184 of wasps was obtained by the subtraction of FM and DM divided by DM. Altogether, 597
185 males were used for this part of the study (AsymC/T0 n=85 ; AsymC/T10 n=74 ; AsymC/T24
186 n=33 ; Cor/T0 n=102 ; Cor/T10 n=69 ; Cor/T24 n=39 ; Oul/T0 n=92 ; Oul/T10 n=68 ;
187 Oul/T24 n=35).

188

189 3.3) Reproductive trait: sperm count

190 For each experimental condition, the quantity of sperm of one-day-old adult males
191 (sexual maturity – Ruther et al., 2009) was measured in the seminal vesicles of six males.
192 Seminal vesicles of each *N. vitripennis* male were dissected under a binocular microscope,
193 and isolated in a drop of saline solution (128.3mM NaCl; 4.7mM KCl; 2.3mM CaCl₂) as
194 already described by Chirault et al. (2015). Spermatozoa were then extracted from seminal
195 vesicles, fixed with 70% ethanol and stained with a DAPI solution at 2E⁻⁰⁶g/mL to color
196 nuclei of sperm heads. Sperm nuclei were counted under a fluorescence microscope
197 (Olympus BX51, Japan). A total of 54 males was used to conduct this assay (AsymC/T0 n=6 ;
198 AsymC/T10 n=6 ; AsymC/T24 n=6 ; Cor/T0 n=6 ; Cor/T10 n=6 ; Cor/T24 n=6 ; Oul/T0 n=6 ;
199 Oul/T10 n=6 ; Oul/T24 n=6).

200

201 **4) Metabolic fingerprints of wasps under thermal stress**

202 To obtain information on early modification at the biochemical level after stress, for each line,
203 male pupae produced by virgin females were collected and immediately snap-frozen into
204 liquid nitrogen after they were exposed for 10 or 24h after pupation at 25 or 36 °C. Each
205 biological replicate (Six biological replicates per experimental condition) was made from a
206 pool of six pupae, achieving a minimal dry mass of 1 mg per replicate. Biological replicates
207 were lyophilized for 72h using a Christ Alpha 1-4 LO plus, and dry mass was measured with
208 a microbalance Sartorius (d = 0.001 mg). For the preparation of the samples, the protocol
209 described in Khodayari et al. (2013) and Hidalgo et al. (2015) was used. A 10µL volume of
210 arabinose (3 mM, internal standard) was added to each sample to monitor the reliability of the
211 metabolite quantification. A gas chromatograph-mass spectrometer (GC-MS) platform
212 (Thermo Fischer Scientific Inc, Waltham, MA) was used for metabolite quantification (report
213 to Hidalgo *et al.*, 2015 for the description of the GC-MS settings and chromatograms'
214 annotation). All samples were run under the SIM mode (electron energy: -70 eV) and the GC
215 was equipped with a 30 m fused silica column (TR-5MS, 95 % 200 dimethyl siloxane, 5 %
216 Phenyl Polysilphenylene-siloxane, I.D.: 0.25 mm). One microliter of each sample was
217 injected using the split mode (25:1). Randomized sample sequences were established for
218 sample injection, and chromatograms were deconvoluted using *XCalibur* v2.0.7. Standard
219 samples, consisting of 61 pure reference compounds at 1, 2, 5, 10, 20, 50, 100, 200, 500, 750,
220 1,000, and 1,500 µM were run. Finally, metabolite levels were quantified using the quadratic
221 calibration curves for each reference compound.

222

223 **5) Statistical analysis**

224 We compared the success of adult emergence among the three lines using a Chi2 test.
225 We analyzed the life history traits of *N. vitripennis* using a series of six general linear models.
226 In these models, we entered treatment (0, 10 or 24h at 36 °C), wasp line (AsymC, Cor or
227 Oul), and their interactions as explanatory factors; pupal development time, adult longevity,
228 inter-orbital distance, dry mass and body water of adults at emergence, or sperm count as
229 response variables. When required, pairwise comparisons between combinations of factors
230 were tested using model contrasts with p-values corrected for multiple testing using Tukey
231 correction. To fulfill homoscedasticity and Gaussian distribution of the model residuals, adult
232 longevity, inter-orbital distance and sperm count were square root-transformed, whereas body
233 water content was log transformed.

234 To examine the effects of exposure at 25 or 36 °C for 10 or 24h on the metabolome of
235 the wasps, a principal component analysis (PCA) was assessed on the metabolic dataset. For
236 this analysis, we conserved the first four axes that explained about 70% of the total variance
237 of the data. ANOVAs were performed on these axes to identify those explaining metabolic
238 variations among lines in response to thermal treatments (*i.e.* significance of interacting
239 factors “line*temperature*exposure_duration” and/or “line*temperature”). PC scores of both
240 metabolic (variables) and experimental group (factors) were plotted to identify which
241 metabolites contributed to the separation of the groups. Metabolites defined as significant
242 according to the “Mardia Criterium” (*i.e.* metabolites whose absolute value is 0.8 time larger
243 than the largest absolute value of the PC) were discussed.

244 We conducted all statistical analyses using the software R v3.5.1 loaded with the
245 packages car, carData, multcomp, and emmeans.

246

247 **Results**

248 Success of adult emergence (about $87.91 \pm 1.90\%$ (mean \pm se)) did not differ among
249 the three lines of *N. vitripennis* (χ^2 test: N=642 samples; $\chi^2=9.90$; df=15; P=0.83) chosen
250 because they live originally in different climatic conditions, among others things. It was not
251 influenced by exposures at 36 °C for 10h or 24h (χ^2 test: N=1367 samples; $\chi^2=24.18$; df=34;
252 P=0.89). By contrast, our analyses revealed a significant interaction for line and exposure
253 duration for adult longevity, sperm count, pupal development time, dry mass, body water
254 content and inter-orbital distance of *N. vitripennis* males. This line-exposure duration
255 interaction reveals the distinct responses of fitness parameters, allometric and physiological
256 adjustments of the three treated lines (**Table 1**). Without stress (T0), individuals from AsymC
257 showed the longest pupal development time, while adults from Cor were characterized by the
258 longest longevity and the highest sperm count (**Table2**).

259

260 **1) Effect of thermal stress on fitness parameters**

261 The longevity and sperm amount of males, considered as components of the fitness, of
262 the three lines were differentially altered by the thermal treatment. For longevity, the only
263 significant variation was recorded for AsymC males exposed for 24h at 36 °C (**Table 2**;
264 **Fig.1a**). AsymC males exposed to 24h-heat stress live significantly less long than control
265 male. Larger effects were observed for sperm count. Sperm count of AsymC males remained
266 unchanged at T10, but was significantly reduced, by about 90%, at T24 (**Table 2**; **Fig.1b**). In
267 Cor and Oul males, sperm count was reduced by 51 and 61%, respectively, at T10 as
268 compared to controls. Sperm counts were drastically reduced at T24 for Cor males, and
269 reached values very similar to that of AsymC and Oul (**Table 2**; **Fig.1b**).

270

271 **2) Effect of thermal stress on development, allometry and physiological parameters**

272 While developmental time of AsymC pupae exposed for 10h at 36 °C remained
273 similar to that of controls (T0), it was increased significantly in pupae that were exposed for
274 24h at 36 °C (**Table 2**; **Fig.1c**). An opposite pattern was observed for Oul and Cor pupae
275 whose developmental time was decreased by 15% after a 10h exposure at 36 °C (**Table 2**;
276 **Fig1c**), while it remained similar to controls for pupae exposed for 24h to heat stress.

277 Dry mass at emergence was decreased for males from AsymC, Cor and Oul from T10
278 experimental condition, with a reduction of 33, 23 and 33% of the dry mass as compared with
279 their respective controls (**Table 2**; **Fig.4d**). Dry mass at emergence of AsymC and Cor males

280 from T24 experimental condition was similar to that of controls (T0), while in this condition,
281 dry mass of Oul males was decreased by 24% as compared to controls T0 (**Table 2; Fig.1d**).

282 Body water content of Cor males was not affected by the thermal treatment, whatever
283 its duration (**Table 2; Fig.1e**), while body water content of AsymC had a tendency to increase
284 at T10 and was similar to control at T24. Body water content of Oul males significantly
285 increased (by 36%) at T10 as compared with T0 (**Table 2; Fig.1e**).

286 No change in inter-orbital distance was observed for AsymC males (**Table 2; Fig.1f**).
287 The inter-orbital distance was highly significantly decreased in Cor males from T24
288 experimental conditions, and also in males from Oul from both T10 and T24 experimental
289 conditions (**Table 2; Fig 1f**).

290

291 3) Metabolic profiling

292 Forty-six metabolites were detected and quantified from the 24 samples. Three
293 samples were discarded from the subsequent analyses, as they were detected as outliers
294 showing abnormal metabolite concentrations (Oul: one biological replicate with insects
295 exposed at 36 °C for 24h, and two others from the pupae exposed at 25 °C for 10 and 24h).
296 The first four PCs of the PCA explained about 70% of the total variation of the dataset (**Table**
297 **4**).

298 The response to thermal stress of the groups of metabolites loaded in PC1 and PC3
299 depended on the tested lines (see interactions between line and temperature in table 4). PC1
300 was mainly negatively loaded by valine, threonine, glycerol, glucose, glutamic acid,
301 phosphoric acid, leucine, glyceric acid, arabitol, isoleucine, inositol, serine, lysine,
302 ethanolamine, proline, phenylalanine, alanine and ornithine (**Figure 2**). For PC1, this
303 interaction reflected that pupae of Cor had more metabolites at 25°C than 36°C, whereas the
304 temperature did not affect this quantity in both AsymC and Oul (**Figure 2**). Conversely for
305 PC3, this interaction showed that the trade-off between mannose and the eight other
306 metabolites (trehalose, glycerol phosphate, malic acid, adonitol, erythritol, succinic acid, citric
307 acid and fumaric acid) was in favor of mannose at 36°C and of the other metabolites at 25°C
308 in the Cor line, whereas the temperature did not affect the outcome of this trade-off in AsymC
309 and Oul (**Figure 4**).

310 Interestingly, a triple interaction between line, temperature and exposure duration
311 shaped PC2 that was positively loaded by maltose and negatively by cadaverine and mannitol
312 (**Figure 3 and Table 5**). This reveals that exposure duration entailed a significant decrease in

313 PC2 (*i.e.* an increase in mannitol and cadaverine and a decrease in maltose) at 36°C in every
314 line, but that at 25°C, such a decrease only occurred in Cor (**Figure 3**).

315 PC4 was loaded negatively by the glucose-6-phosphate and positively by citrate
316 (**Figure 5**) and was determined by the variation of temperature and an interaction between
317 line and exposure duration (**Table 5**). Accordingly, PC4 was first constructed by the
318 temperature variation for AsymC samples, those exposed to 25 °C characterized by higher
319 amount of citric acid whereas those exposed at 36 °C characterized by higher amount of
320 glucose 6 phosphate (**Figure 5**). Second, PC4 was also constructed by the variation of Oul
321 pupae ageing of 24 hours with Cor pupae ageing of 10 hours, the first characterized by higher
322 amount of citric acid whereas the second were by higher amount of glucose 6 phosphate.

323

324 **Discussion**

325 The present work aim at comparing the effects of heat stress (36 °C) at the pupal stage
326 on the subsequent fitness components, life cycle, physiology and metabolic profiles of males
327 of *Nasonia vitripennis*.

328 *A common response to heat stress*

329 Our findings are consistent with the previous studies conducted on the AsymC, and
330 confirmed that a 24h exposure to heat stress during the early pupal stage disrupts
331 spermatogenesis and reduces spermatozoa amount of sexually-mature males (Chirault et al.
332 2015). Our results also report comparable conclusions for the two other *N. vitripennis* lines
333 (i.e., Cor and Oul) we used. In these two newly-tested lines, however, a shorter exposure to
334 heat stress (10h instead of 24h as applied before) also decreased significantly sperm number,
335 thus suggesting that spermatogenesis might be even more sensitive to heat stress than
336 previously thought in *N. vitripennis*. Indeed, a major sublethal consequence of heat stress
337 during testis development is the alteration of the reproductive capacity at emergence
338 confirming the susceptibility of this function at this particular development stage. It could be
339 interesting to measure response to heat stress at a different developmental stage to observe if
340 the reproductive function has the same sensitivity to temperature. The interaction of genetic
341 variation and environmental plasticity can be, in fact stage-specific (Freda et al, 2019). The
342 effects of both short (10h) and long (24h) period of heat stress had several additional negative
343 consequences on fitness (adult longevity), development (pupal development time), physiology
344 (dry mass, body water content, metabolic profiles), allometry (inter-orbital distance).
345 Altogether, these new results confirm that heat stress can be drastic for many biological
346 aspects of insects. As discussed previously, it is worth noting that such biological impacts at
347 an individual level might have drastic consequences at the population level, decreasing the
348 dynamics and effective size of populations. Indeed, this decrease in sperm count could induce
349 change in offspring sex ratios and consequently a decrease in the reproductive success of
350 females mated with heat-stressed males because only daughters are able to fly and disperse to
351 reproduce on new host patches. Thus, the next generation could have a lower parasitic activity
352 because only females lay their eggs in hosts.

353 A common metabolomic signature in response to heat stress was also observed, with the
354 concentrations of mannitol, cadaverine and maltose varying in the same way in AsymC, Cor
355 and Oul when pupae were exposed at 36 °C. It was already shown that adjustments in sugars
356 or amino acids during thermal-stressed pupal stage of insects participate to increase adult
357 survival (Mamai et al., 2014; Michaud et al., 2008; Overgaard et al., 2007). First, the maltose,

358 significantly decreased in wasps exposed at 36 °C. Maltose is a disaccharide formed by two
359 glucose molecules, and its hydrolysis may release free glucose further available as a
360 protecting compatible solute (Malmendal et al., 2006; Michaud et al., 2008). The increased
361 amount of both mannitol and cadaverine at 36 °C might suggest another adaptive response of
362 *N. vitripennis* to challenge the constraints linked to heat. In particular, such an increase might
363 help to protect cell integrity but such a role of mannitol and cadaverine need to be further
364 investigated in *N. vitripennis* wasps.

365 *Specificity of the lines*

366 Except for the success of adult emergence, all measured traits on *N. vitripennis* males
367 differ among the three lines reared at the control temperature of 25 °C. These inter-line
368 differences most probably have a genetic basis, because experiments were conducted in
369 highly controlled conditions, and all lines were previously maintained in the same laboratory
370 over many generations (since 2009). The three lines have been established from a single
371 female collected from geographically distinct populations. It is likely that these populations
372 have a distinct genetic background, and distinct life cycle characteristics, morphology and
373 physiology that optimize adaptive value matching the locally encountered environmental
374 conditions. The significant variation observed among lines might also partially result from the
375 limited genetic diversity within lines that are all highly homozygous due to inbreeding.

376 When maintained under controlled non-stressful conditions, AsymC is characterized by
377 the longest duration of pupal development. Eleven free amino acids (six essential: valine,
378 threonine, leucine, isoleucine, lysine, phenylalanine; and five non-essential: glutamic acid,
379 serine, proline, phenylalanine and alanine) were measured in higher amounts in AsymC as
380 compared with the two other lines. These observations may suggest a slightly lower metabolic
381 activity in specimens of AsymC in comparison to insects of Cor and Oul lines, thus reducing
382 the rate of use of amino acids for metamorphosis, in turn increasing the duration of pupal
383 development. The longest adult longevity and the highest sperm count were recorded in
384 insects of the Cor line. Such positive correlation between longevity and production of gametes
385 is in agreement with the Bowen-Atwood hypothesis presented by De Loof, “the Living and
386 dying for sex” (De Loof, 2011). This author argues that the factors regulating mitogenesis,
387 differentiation and cell death during development are also responsible for regulating aging
388 throughout life.

389 *Specific response to heat stress*

390 When subjected to heat stress, fitness and biological parameters of AsymC males were
391 particularly affected, as revealed by the significantly reduced adult longevity and sperm

392 count, increased duration of pupal development, decreased body water content and dry mass.
393 Similarly, several biological parameters were modified by a 10h-exposure at 36 °C in Oul
394 males. Of note, these insects exhibited a significant increase of their body water content,
395 which may result from an important mobilization of energetic resources in the heat-exposed
396 *N. vitripennis* males of Oul line (Landys et al., 2006). For example, Colinet et al. (2006)
397 showed that when exposed to a cold stress, the water content/dry mass ratio of *Aphidius*
398 *colemanni* (Hymenoptera: Aphidiinae) were increased because lipids were metabolized, in turn
399 releasing a non-negligible quantity of metabolic water. The use and reallocation of energy
400 resources after a heat stress were also discussed in *Holothuria scabra* (Aspidochirotida:
401 Holoturridae – Kühnhold et al., 2017), *Eleodes armata* and *Cryptoglossa verrucosa*
402 (Coleoptera: Tenebrionidae – Cooper, 1985), and in larvae of *P. vanderplanki* (Mitsumasu et
403 al., 2010). Catabolism of glycogen is also a possible strategy as it is able to produce until five
404 times its proper molecular mass in water molecules (Gibbs, 2002; Schmidt-Nielsen, 1990 in
405 Gibbs et al., 1997). Even if these assumptions need to be further investigated in Oul males,
406 this hypothesis is also supported by the fact that males cannot feed/drink during pupal stage,
407 suggesting that production of water has a metabolic origin.

408 Cor males displayed very specific metabolomic adjustments after an exposure to heat
409 stress, with adjustments of different metabolic pathways, including aminoacyl-tRNA
410 biosynthesis, valine, leucine and isoleucine biosynthesis, alanine, aspartate and glutamate
411 metabolism, galactose metabolism, arginine and proline metabolism, and Krebs cycle. We
412 propose that the decreased amounts of trehalose, glucose and other sugars observed in Cor
413 pupae exposed at 36 °C may have resulted from an increased energy production in these
414 insects, to enhance cell resistance mechanisms (Yancey, 2005). Glucose, for example, is
415 involved in the pentose phosphate pathway (PPP) which is often elicited in insects exposed to
416 stressful conditions, as this pathway produces many compatible solutes (Kruger and von
417 Schaewen, 2003; Timmermans et al., 2009). Phenylalanine and valine are also specifically
418 decreased in Cor males. These two amino acids are known to improve the hydrophobicity of
419 the insect cuticle under heat stress (Goltsev et al., 2009), as also suggested in females of *A.*
420 *gambiae* at the onset of the dry season (Hidalgo et al., 2014). Variation of these two amino
421 acids in Cor males may thus indicate their increased use for cuticle rearrangement (Neville,
422 1975 in Hidalgo, 2014; Stankiewicz et al., 1996; Truman & Riddiford, 2000 in Hidalgo,
423 2014). Although such a hypothesis needs further evidences, it could contribute to explain the
424 lack of thermal treatment incidence on body water content of Cor males.

425 The differences between lines could be the result of different energetic allocation
426 strategies. Indeed, most of the energy available in organism is used for basal metabolism
427 (maintenance), growth and reproduction. However, external factors such as temperature
428 fluctuations can affect the allocation process by increasing energetic demand in order to face
429 constraints induced by the new condition. Cor and Oul may allocate as a priority their
430 resources to somatic growth during the first 10h of stress, at the cost of the reproduction
431 function. Therefore, we observed a 50% reduction of the sperm stock in these two lines and a
432 reduction of pupal development time after 10h at 36 °C. This could be the result of an
433 increased rate of development as previously shown with higher temperature in *N. vitripennis*
434 (Grassberger and Frank, 2003; Voss et al., 2010) and other parasitoids (Lagos et al., 2001).
435 By contrast, AsymC may not reallocate energy during the first 10h but suffers the
436 consequences when stress persists after 24h. Therefore, AsymC males had few phenotypic
437 traits adjusted at T10, and showed a global increase developmental time and decrease of
438 sperm count and adult longevity at T24.

439

440 **Conclusion**

441

442 The three *N. vitripennis* lines showed different trait values and metabolic fingerprints
443 in response to heat stress, suggesting a genetic basis of their stress response strategies. Cor
444 males showed very specific metabolic adjustments compared to the two other lines, probably
445 an increase hydrophobicity to resist the stressful conditions. By contrast, Oul males showed
446 stronger phenotypical adjustment of its life cycle, and produced metabolic water to
447 compensate water loss by heat stress. Finally, AsymC males had probably more difficulties to
448 acclimate at 36 °C, even for a short period, as their adult longevity was significantly reduced.
449 Thus, the ability of developmental plasticity in *N. vitripennis* males exposed to heat stress
450 appears to rely on genotypes. The degree of tolerance and acclimation to heat stress varies
451 among genotypes. All together, these results suggest the existence of Genotype-by-
452 Environment Interactions for these traits, indicating that the propensity and degree of
453 responses to heat stress might be heritable. The populations of origin from which the three
454 insect lines come from live in very contrasted climatic conditions, with a Mediterranean
455 climate in Corsica, and more variable temperature in Finland. In a future study, it would be
456 interesting to explore whether the observed phenotypic variation of responses to thermal
457 stress has an adaptive basis in *N. vitripennis* and therefore that differences among lines
458 represent local adaptation along a latitudinal gradient.

459

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462 of the French National Research Agency (ANR). We would like to thank Marine Deluen for
463 her help in life cycle experimentations and Joël Meunier for his advices on statistical
464 analyses.

465

466 **Table legends**

467 **Table 1.** Results of the ANOVA analysis assessed on the different life history traits
468 among the three lines (AsymC, Cor and Oul) and different exposure (T0, T10 and T24h) of
469 the treatment at 36°C

470

471 **Table 2.** Mean traits values (\pm se) of each measured life history traits of pupae from
472 AsymC, Cor and Oul at 25 °C (T0), and after 10h (T10) or 24h (T24) at 36 °C. Different
473 letters indicate significant differences among lines for different treatment (T0, T10, T24).

474

475 **Table 3.** Resume of the first 4 PC axis variances assessed to analyze the metabolome
476 variation in *N. vitripennis* and their metabolite build contributions.

477

478 **Table 4.** Resume of the first 4 PC axis variances assessed to analyze the metabolome
479 variation in *N. vitripennis* and their metabolite build contributions.

480

481 **Table 5.** Results of ANOVA analysis on PCA scores of metabolite data.

482

483 **Figure legends**

484

485 **Figure 1.** Impact of thermal treatment on (a) adult longevity, (b) sperm count, (c) pupal
486 development time, (d) dry mass, (e) body water content, (f) inter-orbital distance of *N.*
487 *vitripennis* males for each line (AsymC, Cor, Oul). Control group (25°C, T0) is at 1 by
488 default. 36 °C-heat-stress treatments for 10h and 24h are represented as reductions (<1) or
489 increases (>1) compared to control group at 25 °C (T0). Error bars correspond to standard
490 error. Different letters indicate significant differences at $P < 0.05$, distinct for each line.

491

492 **Figure 2.** Left panel: barplot showing the different experimental group contributions
493 (scores) \pm se on PC1. Different letter above bars indicate significant differences at $\alpha = 0.05$.
494 Right panel: barplot showing the contributions of the 46 identified metabolites on PC1. Black
495 bars and * indicate metabolites that significantly influence the construction of PC1 according
496 to the Mardia Criterium.

497

498 **Figure 3.** Right panel: barplot showing the different experimental group contributions
499 (scores) \pm se on PC2. Different letter above bars indicate significant differences at $\alpha = 0.05$.
500 Left panel: barplot showing the contributions of the 46 identified metabolites on PC2. Black
501 bars and * indicate metabolites that significantly influence the construction of PC2 according
502 to the Mardia Criterium.

503

504 **Figure 4.** Right panel: barplot showing the different experimental group contributions
505 (scores) \pm se on PC3. Different letter above bars indicate significant differences at $\alpha = 0.05$.
506 Left panel: barplot showing the contributions of the 46 identified metabolites on PC3. Black
507 bars and * indicate metabolites that significantly influence the construction of PC3 according
508 to the Mardia Criterium.

509

510 **Figure 5.** Right panel: barplot showing the different experimental group contributions
511 (scores) \pm se on PC4. Different letter above bars indicate significant differences at $\alpha = 0.05$.
512 Left panel: barplot showing the contributions of the 46 identified metabolites on PC4. Black
513 bars and * indicate metabolites that significantly influence the construction of PC3 according
514 to the Mardia Criterium.

515 **Table**

516 Table 1

517

Factor	Adult longevity			Sperm count			Pupal development time			Dry mass			Body water content			Inter-orbital distance		
	Df	F value	P value	Df	F value	P value	Df	F value	P value	Df	F value	P value	Df	F value	P value	Df	F value	P value
Exposure duration	2	19.76	< 0.001	2	133.80	< 0.001	2	89.66	< 0.001	2	105.64	< 0.0001	2	11.76	< 0.0001	2	1600.37	< 0.001
Line	2	16.20	< 0.001	2	26.88	< 0.001	2	108.41	< 0.001	2	5.34	0.0050	2	0.81	0.4446	2	494.02	< 0.001
Exposure duration* Line	4	8.46	< 0.001	4	5.84	< 0.001	4	10.37	< 0.001	4	3.62	0.0063	4	2.85	0.0234	4	333.59	< 0.001

518

519 Table 2

520

Treatment	Adult longevity (h)			Sperm count in seminal vesicles			Pupal development time (h)		
	AsymC	Cor	Oul	AsymC	Cor	Oul	AsymC	Cor	Oul
T0	170.96 ± 3.33 bc	189.45 ± 4.56 d	177.22 ± 4.49 bd	9 366 ± 788 b	15 558 ± 1625 c	6 875 ± 770 b	118.77 ± 0.72 c	112.00 ± 1.26 b	110.06 ± 1.41 b
T10	184.74 ± 2.89 cd	180.49 ± 4.58 bd	161.04 ± 5.90 b	9 425 ± 630 b	7 677 ± 1 068 b	2 712 ± 608 a	114.24 ± 1.04 bc	92.81 ± 2.20 a	92.52 ± 1.72 a
T24	136.23 ± 5.35 a	175.54 ± 5.67 bd	162.53 ± 5.59 b	834 ± 324 a	1 729 ± 266 a	739 ± 363 a	126.42 ± 1.10 d	109.67 ± 1.41 b	109.44 ± 2.28 b

Treatment	Dry mass (µg)			Body water content (mg/mg of DM)			Inter-orbital distance (µm)		
	AsymC	Cor	Oul	AsymC	Cor	Oul	AsymC	Cor	Oul
T0	250.2 ± 8.1 de	256.5 ± 6.2 ef	279.9 ± 7.3 f	1.59 ± 0.07 be	1.55 ± 0.03 be	1.47 ± 0.03 be	420 ± 2.8 bc	423 ± 2.1 c	436 ± 4.1 c
T10	167.0 ± 6.4 a	197.1 ± 5.3 ab	181.1 ± 7.8 ab	2.04 ± 0.19 ae	1.70 ± 0.06 bce	2.03 ± 0.13 acd	425 ± 2.4 c	415 ± 3.5 bc	403 ± 4.9 b
T24	217.9 ± 8.9 bcd	237.9 ± 6.1 ce	210.2 ± 7.0 bc	1.42 ± 0.09 b	1.58 ± 0.06 bde	1.79 ± 0.10 abd	420 ± 4.2 bc	199 ± 4.0 a	204 ± 3.4 a

521

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523

524 Table 3

	PC1	PC2	PC3	PC4
Variance explained (%)	41.35	13.43	8.96	5.61
Cumulative variance explained (%)	41.35	54.78	63.74	69.34
Aspartic acid	-0.15	-0.15	0.11	0.11
Citric acid	-0.10	0.07	0.23	0.23
Fumaric acid	-0.11	-0.02	0.23	0.23
Gluconic acid_lactone	-0.02	-0.17	0.15	0.15
Glutamic acid	-0.20	0.05	0.01	0.01
Glyceric acid	-0.18	0.06	0.12	0.12
Lactic acid	-0.14	-0.16	0.20	0.20
Malic acid	-0.15	0.09	0.25	0.25
Phosphoric acid	-0.20	0.02	-0.11	-0.11
Pipecolic acid	-0.14	-0.21	0.03	0.03
Succinic acid	-0.14	0.07	0.23	0.23
Adonitol	-0.16	-0.02	0.24	0.24
Alanine	-0.17	0.00	0.03	0.03
Arabitol	-0.18	0.00	0.13	0.13
Cadaverine	-0.08	-0.29	0.01	0.01
Erythritol	-0.08	-0.20	0.24	0.24
Ethanolamine	-0.18	-0.04	-0.13	-0.13
Fructose	-0.06	-0.25	0.06	0.06
Fructose_6_phosphate	-0.12	0.23	-0.08	-0.08
GABA	-0.14	-0.21	-0.18	-0.18
Galactose	-0.11	-0.12	-0.09	-0.09
Glucose	-0.20	-0.07	-0.12	-0.12
Glucose_6_phosphate	-0.11	0.20	-0.13	-0.13
Glycerol	-0.20	-0.06	-0.10	-0.10
Glycerol_3_phosphate	-0.08	0.18	0.25	0.25
Glycine	-0.14	0.13	-0.08	-0.08
Inositol	-0.18	-0.14	-0.04	-0.04
Isoleucine	-0.18	0.17	-0.06	-0.06
Leucine	-0.19	0.07	-0.11	-0.11
Lysine	-0.18	-0.08	-0.12	-0.12
Maltose	-0.06	0.28	0.15	0.15
Mannitol	-0.05	-0.35	0.12	0.12
Mannose	-0.16	-0.03	-0.27	-0.27
Ornithine	-0.17	-0.09	-0.01	-0.01
Phenylalanine	-0.17	0.16	0.00	0.00

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Table 5

Factor	PC1 (41.35%)				PC2 (13.42%)				PC3 (5.61%)				PC4 (4.91%)			
	Sum Sq	ddl	F statistic	P-value	Sum Sq	ddl	F statistic	P-value	Sum Sq	ddl	F statistic	P-value	Sum Sq	ddl	F statistic	P-value
line	315.75	2	11.85	***	51.52	2	22.13	***	50.64	2	8.38	***	36.54	2	11.23	***
exposure_duration	1.32	1	0.10	0.75	91.34	1	78.47	***	7.93	1	2.63	0.11	11.42	1	7.02	**
temperature	61.60	1	4.62	*	191.58	1	164.59	***	0.40	1	0.13	0.72	19.15	1	11.77	***
line*exposure_duration	30.24	2	1.13	0.33	1.53	2	0.66	0.52	3.49	2	0.58	0.56	15.41	2	4.74	*
line*temperature	113.87	2	4.27	*	1.96	2	0.84	0.43	37.88	2	6.27	**	0.94	2	0.29	0.75
exposure_duration*temperature	0.14	1	0.01	0.92	16.18	1	13.90	***	1.64	1	0.54	0.47	0.94	1	0.58	0.45
line*exposure_duration*temperature	33.08	2	1.24	0.30	8.88	2	3.81	*	9.48	2	1.57	0.22	1.70	2	0.52	0.60

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536

	PC1	PC2	PC3	PC4
Variance explained (%)	41.35	13.43	8.96	5.61
Cumulative variance explained (%)	41.35	54.78	63.74	69.34
Aspartic acid	-0.15	-0.15	0.11	0.11
Citric acid	-0.10	0.07	0.23	0.23
Fumaric acid	-0.11	-0.02	0.23	0.23
Gluconic acid_lactone	-0.02	-0.17	0.15	0.15
Glutamic acid	-0.20	0.05	0.01	0.01
Glyceric acid	-0.18	0.06	0.12	0.12
Lactic acid	-0.14	-0.16	0.20	0.20
Malic acid	-0.15	0.09	0.25	0.25
Phosphoric acid	-0.20	0.02	-0.11	-0.11
Pipecolic acid	-0.14	-0.21	0.03	0.03
Succinic acid	-0.14	0.07	0.23	0.23
Adonitol	-0.16	-0.02	0.24	0.24
Alanine	-0.17	0.00	0.03	0.03
Arabitol	-0.18	0.00	0.13	0.13
Cadaverine	-0.08	-0.29	0.01	0.01
Erythritol	-0.08	-0.20	0.24	0.24
Ethanolamine	-0.18	-0.04	-0.13	-0.13
Fructose	-0.06	-0.25	0.06	0.06
Fructose_6_phosphate	-0.12	0.23	-0.08	-0.08
GABA	-0.14	-0.21	-0.18	-0.18
Galactose	-0.11	-0.12	-0.09	-0.09
Glucose	-0.20	-0.07	-0.12	-0.12
Glucose_6_phosphate	-0.11	0.20	-0.13	-0.13
Glycerol	-0.20	-0.06	-0.10	-0.10
Glycerol_3_phosphate	-0.08	0.18	0.25	0.25
Glycine	-0.14	0.13	-0.08	-0.08
Inositol	-0.18	-0.14	-0.04	-0.04
Isoleucine	-0.18	0.17	-0.06	-0.06
Leucine	-0.19	0.07	-0.11	-0.11
Lysine	-0.18	-0.08	-0.12	-0.12
Maltose	-0.06	0.28	0.15	0.15
Mannitol	-0.05	-0.35	0.12	0.12
Mannose	-0.16	-0.03	-0.27	-0.27
Ornithine	-0.17	-0.09	-0.01	-0.01
Phenylalanine	-0.17	0.16	0.00	0.00
Proline	-0.18	-0.04	-0.01	-0.01
Putrescine	-0.13	-0.13	-0.18	-0.18
Ribose	-0.14	0.05	-0.18	-0.18
Serine	-0.18	0.11	-0.18	-0.18
Sorbitol	-0.12	-0.12	0.13	0.13
Spermidine	-0.07	-0.03	0.03	0.03
Spermine	-0.10	0.25	0.17	0.17
Threonine	-0.21	0.11	-0.08	-0.08
Trehalose	-0.02	0.07	0.27	0.27
Valine	-0.21	0.06	0.01	0.01
Xylitol	-0.10	0.16	0.00	0.00

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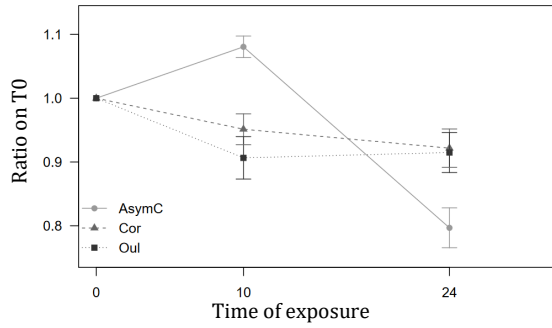
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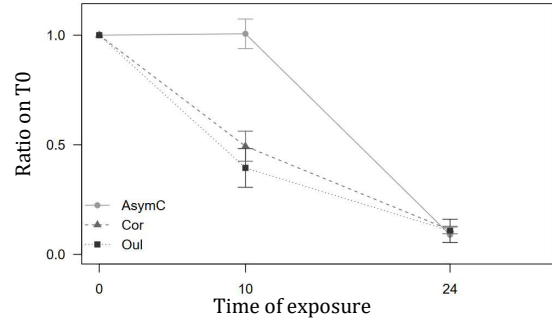
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Figure

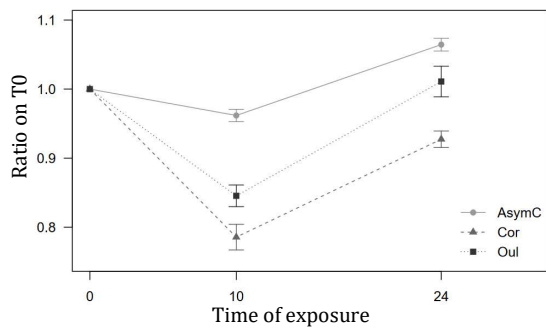
(a) Adult longevity



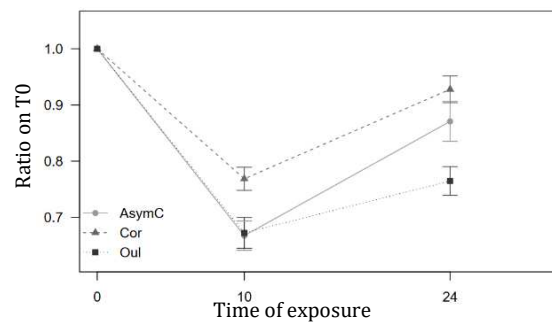
(b) Sperm count



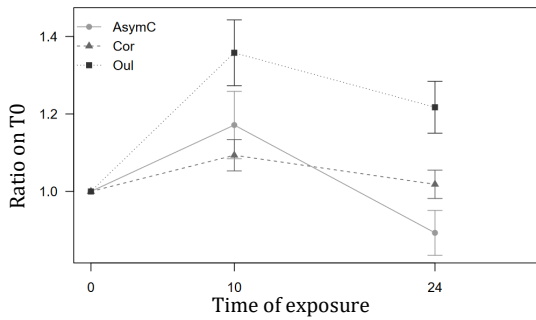
(c) Pupal development time



(d) Dry mass



(e) Body water content



(f) Inter-orbital distance

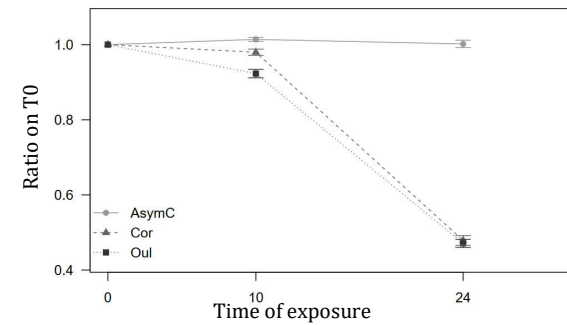


Figure 1.

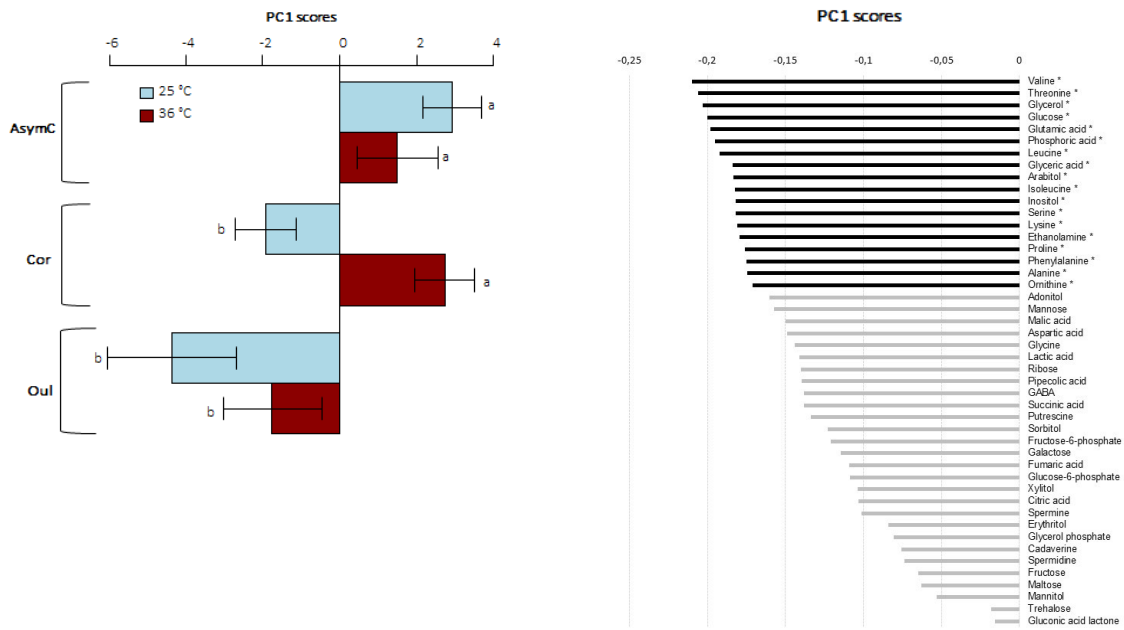


Figure 2.

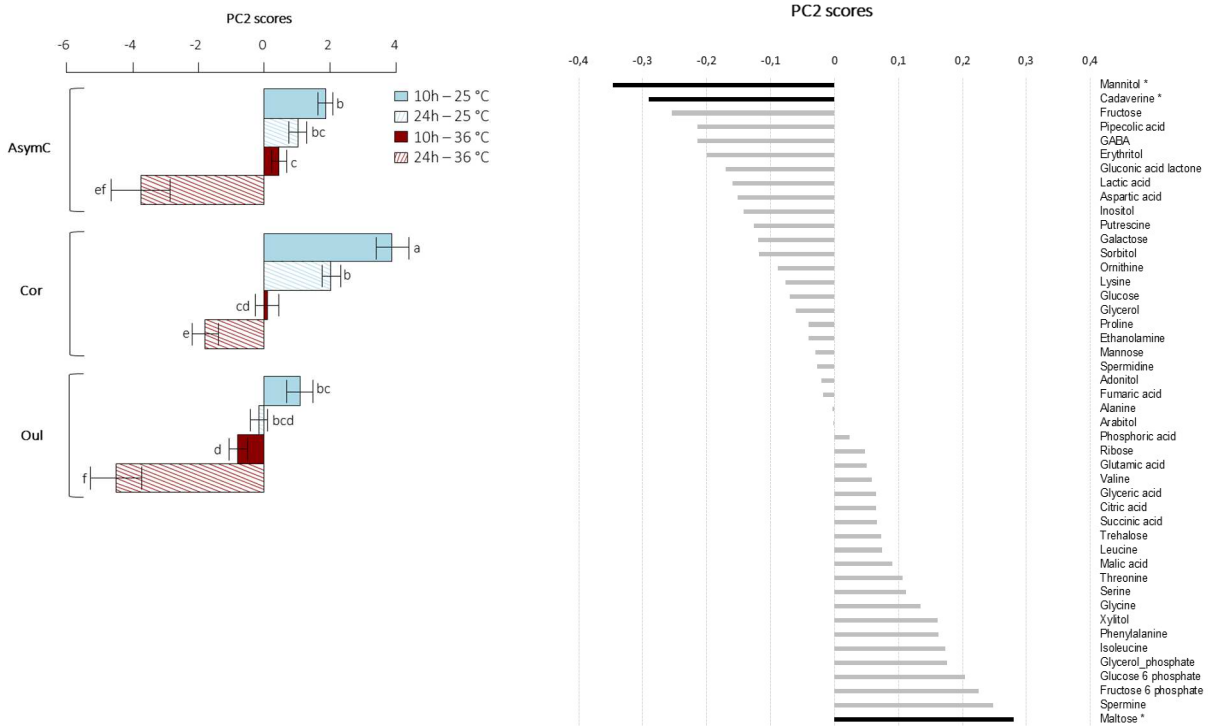


Figure 3.

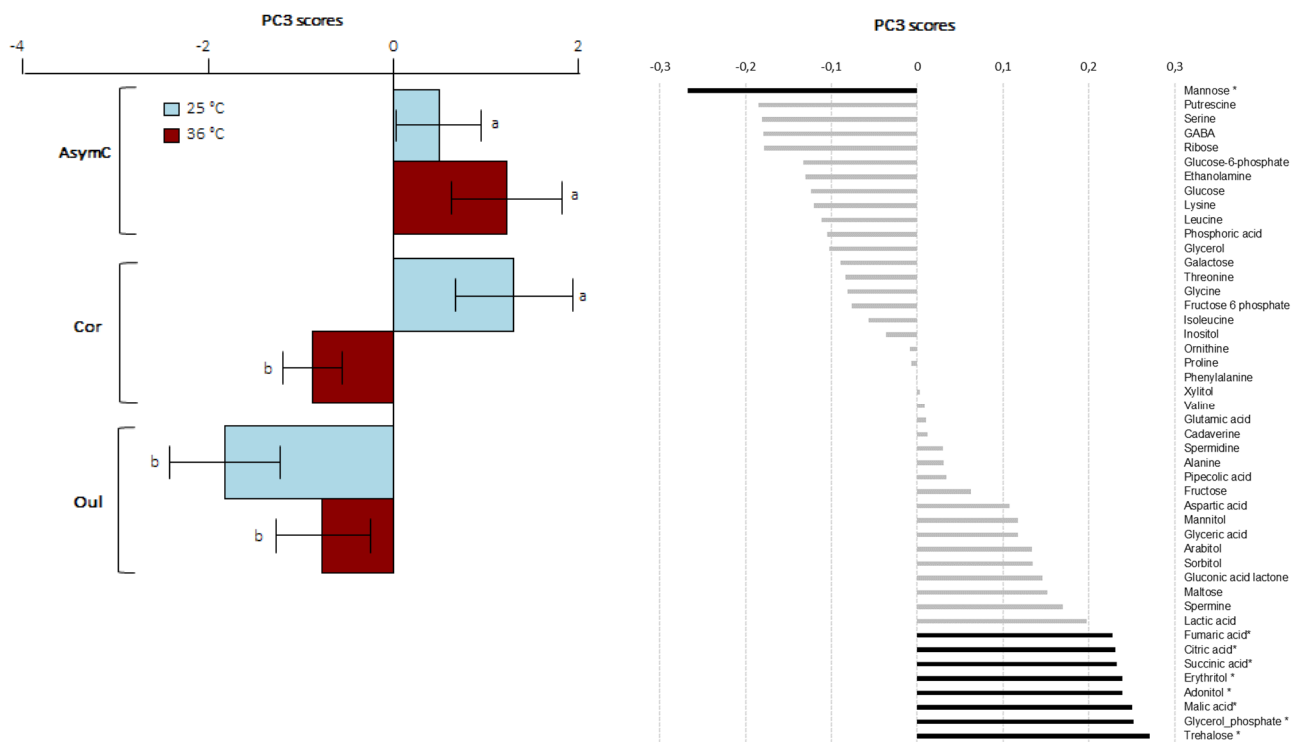


Figure 4

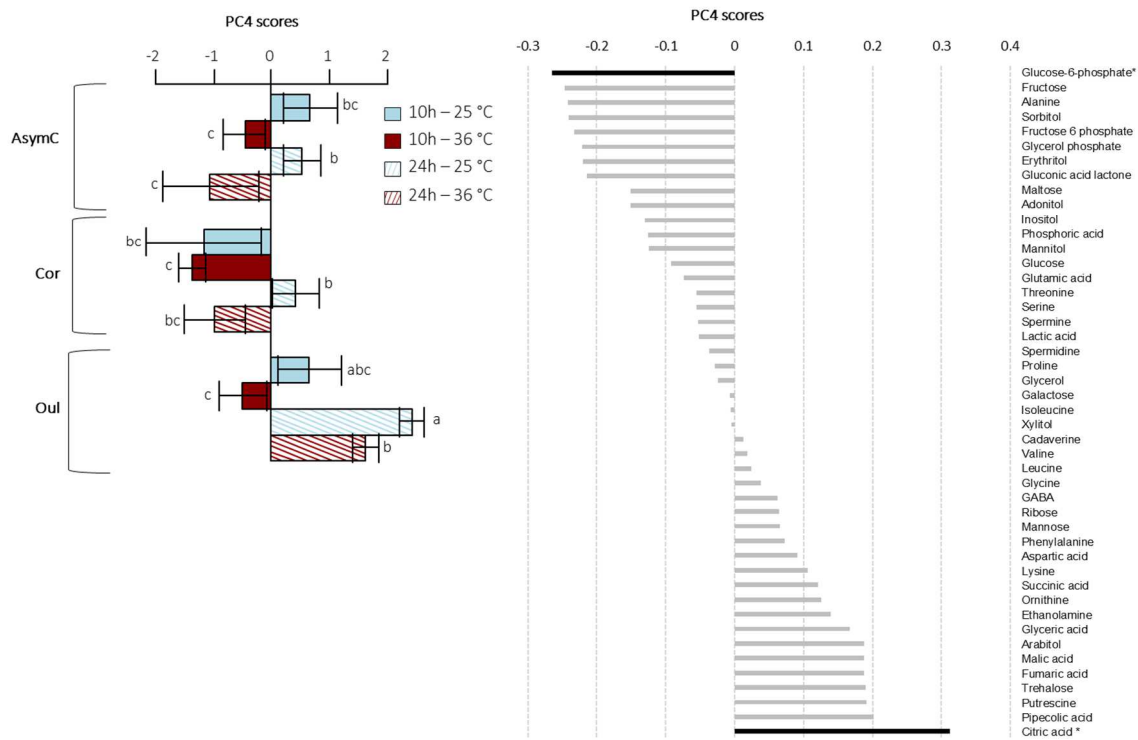


Figure 5.