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Kevin Hidalgo, Erika Beaugeard, D Renault, Franck Dedeine, Charlotte Lécureuil. Physiological and biochemical responses to thermal stress vary among genotypes in the parasitic wasp *Nasonia vitripennis*. *Journal of Insect Physiology*, 2019, 117, pp.103909. 10.1016/j.jinsphys.2019.103909 . hal-02280456

**HAL Id: hal-02280456**

**<https://univ-rennes.hal.science/hal-02280456>**

Submitted on 25 Oct 2021

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

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## Abstract

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

**Keywords:** Heat stress; Metabolomic; Males; Reproduction/Sterility; Development; 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

## Introduction

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

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

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

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

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

## Material and Methods

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

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

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

### 2) Experimental treatments

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

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

#### 3.1) Life cycle characteristics

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

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

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

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

#### 3.3) Reproductive trait: sperm count



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

#### 4) Metabolic fingerprints of wasps under thermal stress

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

#### 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.

## Results

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

### 1) Effect of thermal stress on fitness parameters

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

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

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

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

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

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

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

### 3) Metabolic profiling

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

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

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

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

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

## Discussion

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

### *A common response to heat stress*

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

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

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

#### *Specificity of the lines*

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

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

#### *Specific response to heat stress*

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

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

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



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

## Conclusion

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

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460 **Acknowledgment**

461       This work benefited from the support of the FertiliWasp project ANR-14-CE21-0004-01  
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.

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## Table legends

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

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

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

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

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

## Figure legends

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

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

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

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

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

515 **Table**

516 Table 1

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	Adult longevity			Sperm count			Pupal development time			Dry mass			Body water content			Inter-orbital distance		
Factor	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

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519 Table 2

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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

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524 Table 3

	PC1	PC2	PC3	PC4
<b>Variance explained (%)</b>	<b>41.35</b>	<b>13.43</b>	<b>8.96</b>	<b>5.61</b>
<b>Cumulative variance explained (%)</b>	<b>41.35</b>	<b>54.78</b>	<b>63.74</b>	<b>69.34</b>
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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	PC1	PC2	PC3	539 PC4
<b>Variance explained (%)</b>	<b>41.35</b>	<b>13.43</b>	<b>8.96</b>	<b>5.61</b>
<b>Cumulative variance explained (%)</b>	<b>41.35</b>	<b>54.78</b>	<b>63.74</b>	<b>69.34</b>
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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Figure

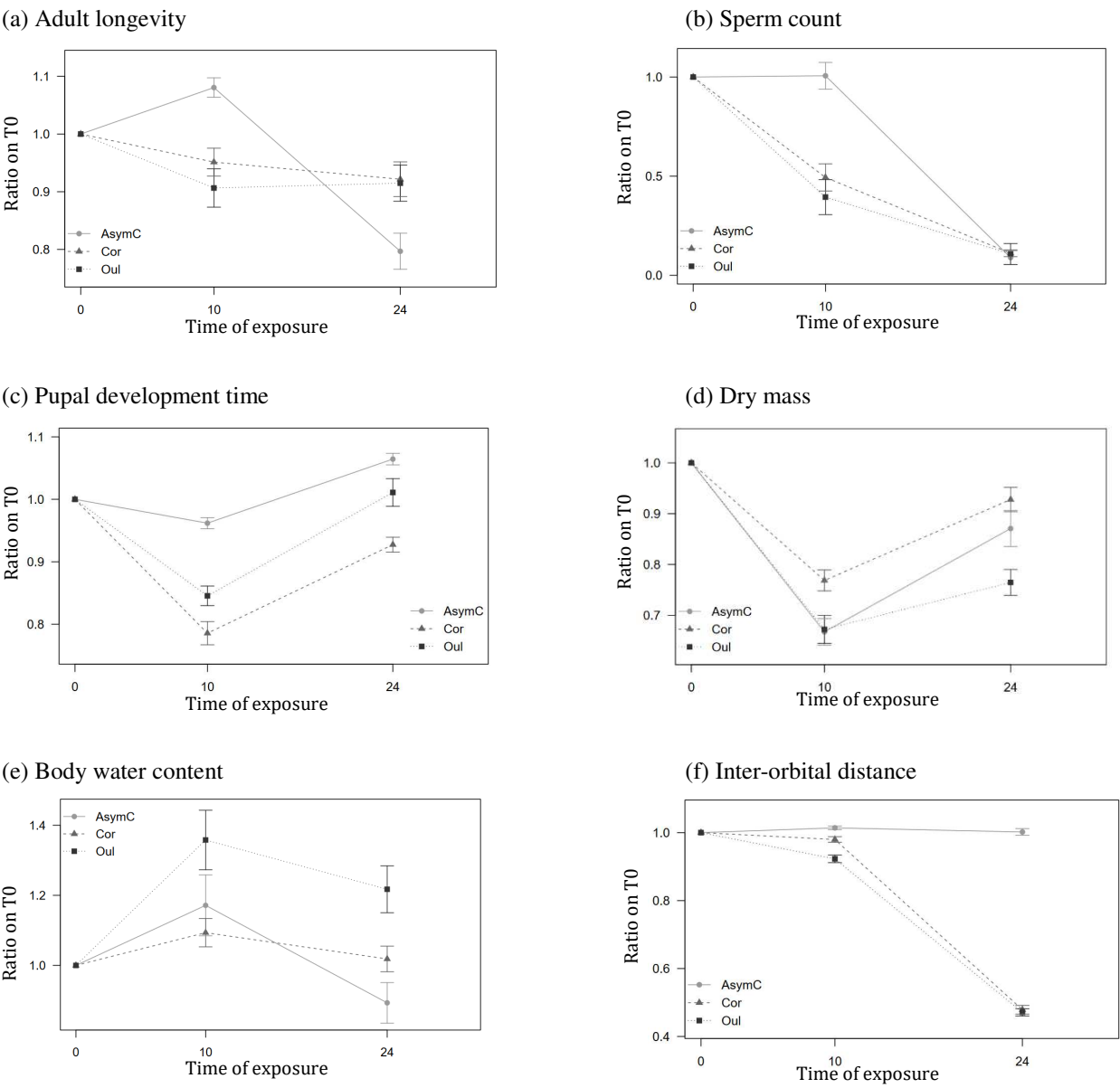
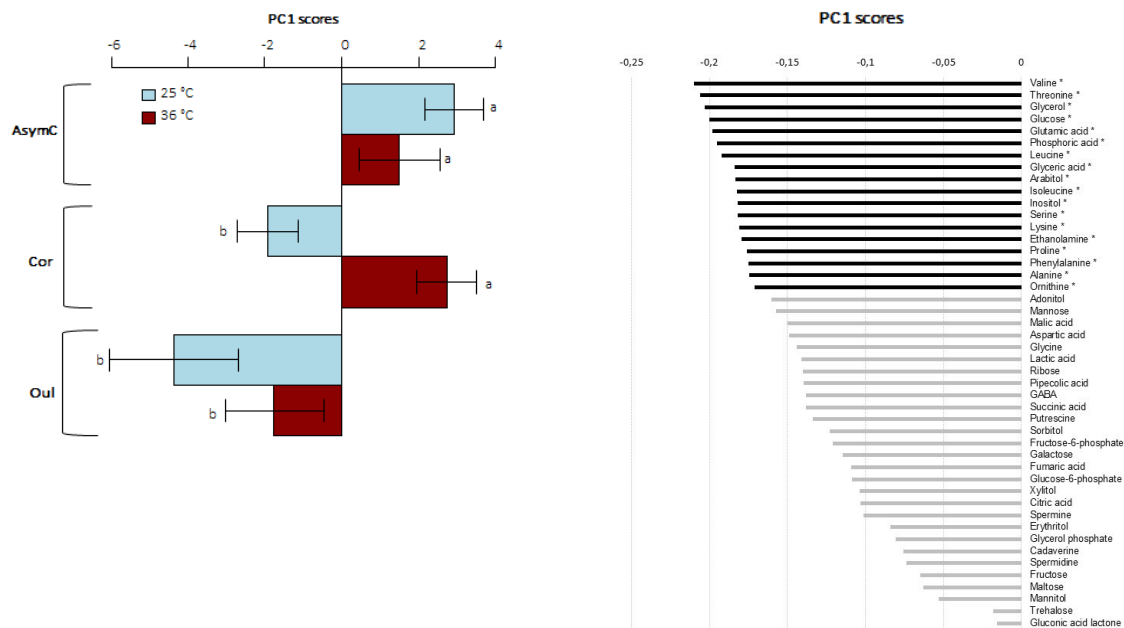
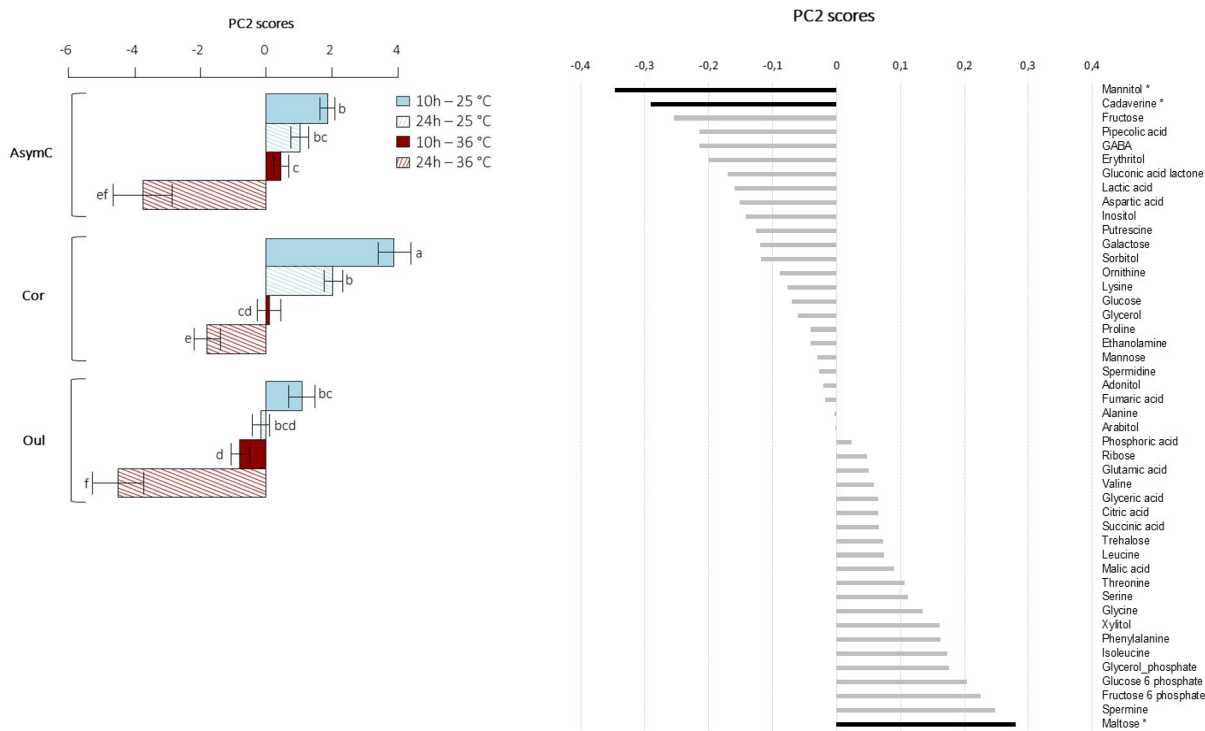


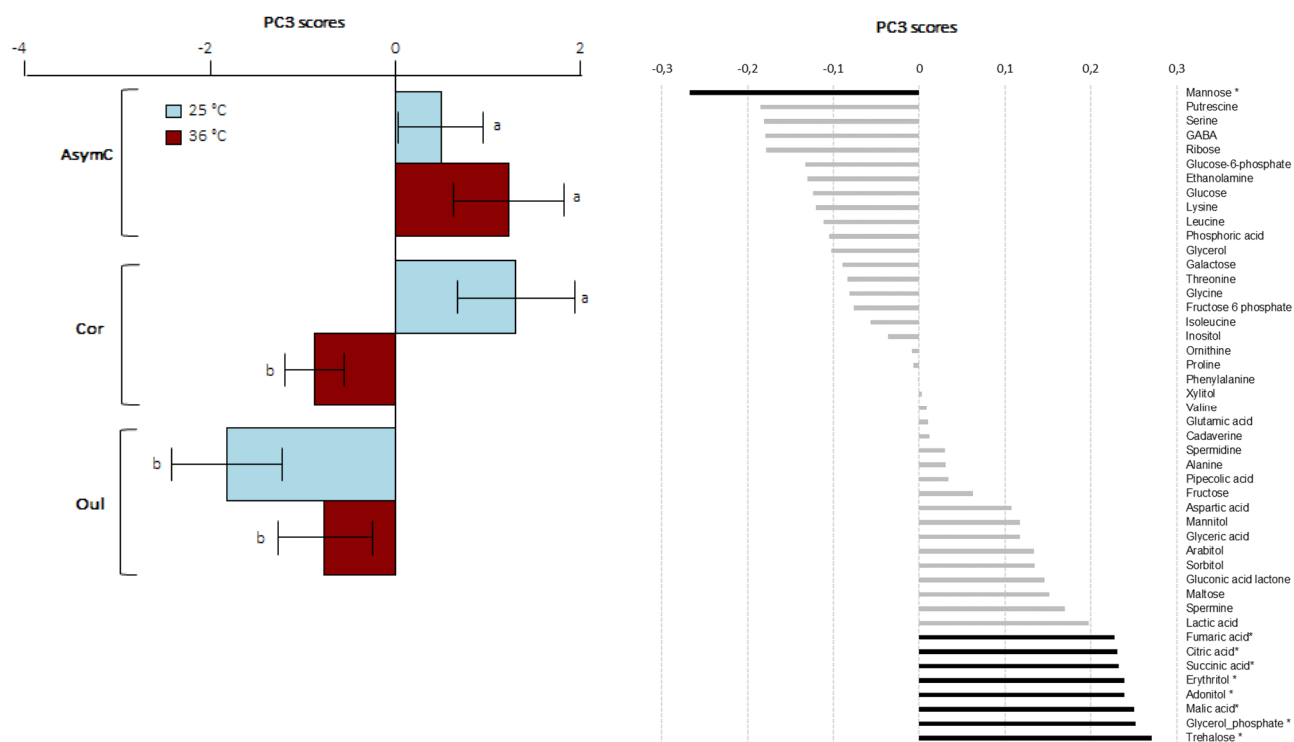
Figure 1.



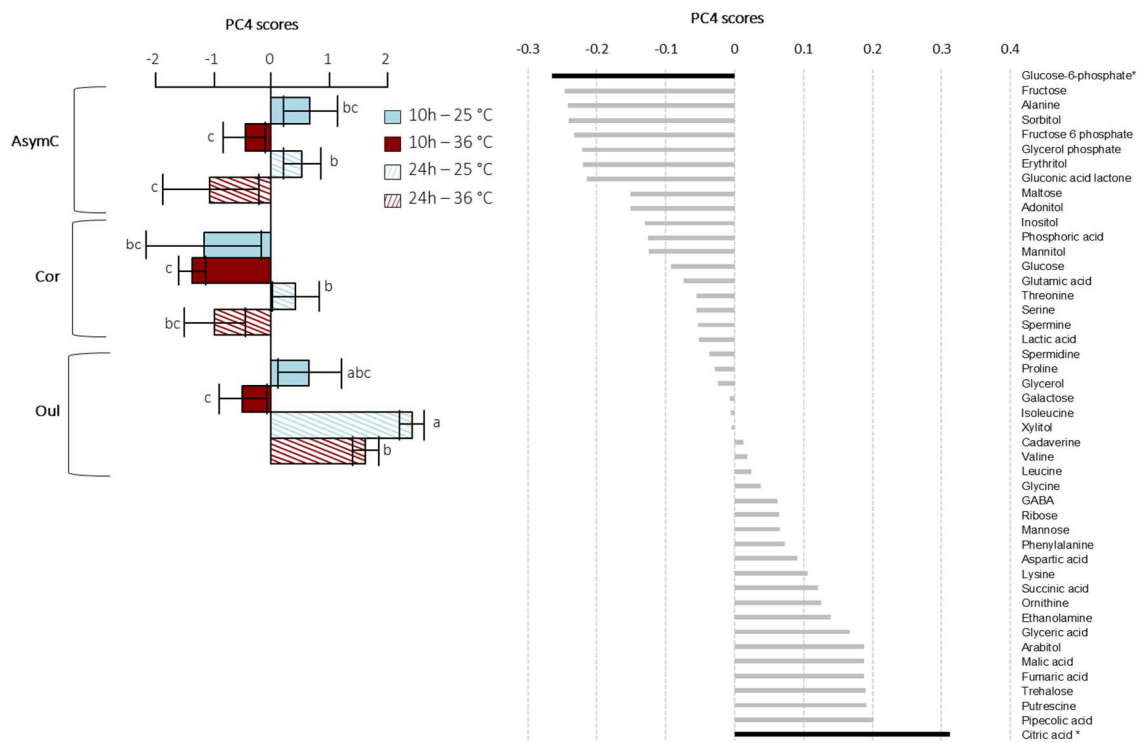
**Figure 2.**



**Figure 3.**



**Figure 4**



**Figure 5.**