Gut microbiota of *Drosophila subobscura* contributes to its heat tolerance but is sensitive to transient thermal stress

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Running title: Heat tolerance and gut microbiota

Word count: Abstract (177 words); Main text (5072 words).

References: 70.

Figures: 4.

Tables: 2.

Supplementary material: 3 figures and 2 tables.

Abstract

Global warming impacts the animal's fitness, leading to increasing extinction risk in ectotherm species. Gut microbiota can contribute to host physiology leading to an increase of resistance to abiotic stress conditions. Temperature has profound effects on ectotherms and gut microbiota can influence cold and heat tolerance of ectotherm species. Additionally, the gut microbiota is sensitive to environmental temperature, which induces changes in its composition and diversity. Here, we investigated the role of the gut microbiota on heat tolerance of *Drosophila subobscura*, comparing the knockdown time between conventional and axenic, and we also assessed the impact of heat stress on the diversity and community structure of the gut microbiota, comparing non-stressed and heat-stressed flies. Our findings provide evidence that gut microbiota influences heat tolerance, and that heat stress modifies the gut microbiota at taxonomical and structural levels. These results demonstrate that gut microbiota contributes to heat tolerance but it is also highly sensitive to transient heat stress, which could have important effects on host fitness, population risk extinction, and vulnerability of ectotherms to current and future climatic conditions.

Keywords: *bacterial microbiota; climate change; heat stress; stress resilience; thermal resistance.*

40

41 **Introduction**

42 Gut microbiota influences multiple features of the host's biology, including nutrient
43 acquisition, immune response, metabolism, behavior, and life-history traits (Broderick and
44 Lemaitre, 2012; Douglas, 2018a; Hoyer and Fenton, 2018). In general, gut microbiota
45 influences phenotypic variation exhibited by host organisms, which can contribute speeding
46 up the adaptive response under changing and fluctuating environments (Alberdi et al.,
47 2016; Macke et al., 2017; Romano, 2017). However, environmental variability, ranging
48 from benign to stressful conditions, could impact the composition and diversity of the gut
49 microbiota, altering its contribution to host phenotypic variability and modifying the
50 functional relationship between hosts and the gut microbiota (Sepulveda and Moeller,
51 2020).

52 Among multiple environmental factors, temperature has profound effects on
53 physiology, behavior, and performance of ectotherms, because body temperature of
54 ectotherms is influenced by the environmental temperature (Angilletta, 2009). The ongoing
55 climate change is expected to impose strong selection pressures on natural populations of
56 ectotherms (Hoffmann and Sgrò, 2011), and ectotherms can display genetic and non-
57 genetic mechanisms that contribute to host thermal tolerance (Kokou et al., 2018). Indeed,
58 recent evidence demonstrates that microbiota impacts on thermal performance of ectotherm
59 species (Renoz et al., 2019; Sepulveda and Moeller, 2020). For instance, obligatory
60 endosymbionts contribute to aphid performance at high temperatures (Dunbar et al., 2007;
61 Zhang et al., 2019), whereas facultative endosymbionts also confer tolerance to high
62 temperature in aphids (Montllor et al., 2002; Russell and Moran, 2006) and *Drosophila*
63 (Gruntenko et al., 2017). Additionally, it has been demonstrated that microbiota also

influences cold and heat tolerance of ectotherm species (Ziegler et al., 2017; Henry and Colinet, 2018; Kokou et al., 2018; Moghadam et al., 2018; Raza et al., 2020).

On the other hand, several studies have also explored the impact of temperature on host microbiota, indicating that gut microbiota is sensitive to environmental temperature (Wernegreen, 2012; Sepulveda and Moeller, 2020). Temperature induces changes in the composition and diversity of the gut microbiota, which could have important consequences on host phenotype and fitness (Wernegreen, 2012; Alberdi et al., 2016). For example, small ectotherms reared at high temperatures show an increase in the abundance of bacteria belonging to the Proteobacteria phylum (Li et al., 2018; Moghadam et al., 2018; Horváthová et al., 2019). Indeed, *Drosophila melanogaster* flies acclimated in warm conditions showed a higher abundance of *Acetobacter* bacteria (Proteobacteria) and a lower abundance of *Leuconostoc* bacteria (Firmicutes) in comparison to cold-acclimated flies (Moghadam et al., 2018). On the other hand, several studies have demonstrated that bacterial diversity and richness decrease when hosts are exposed to warm conditions (Kokou et al., 2018; Moghadam et al., 2018).

In order to investigate the role of the gut microbiota on heat tolerance of *D. subobscura*, in the present study, we compared the heat tolerance between conventional (non-manipulated) and axenic (germ-free) flies across different heat stressful temperatures. Additionally, we assessed the impact of heat stress on the diversity and community structure of the gut microbiota of *D. subobscura*, comparing non-stressed and heat-stressed flies. To study the gut microbiota of *D. subobscura*, we used a metabarcoding approach based on the amplicon sequencing of the bacterial 16S rRNA gene.

Material and Methods

***Drosophila* sampling and maintenance**

Adult *D. subobscura* flies were collected at the locality of Valdivia (southern Chile: 39° 48' S, 73° 14' W) and separated by sex. Females were individually placed in plastic vials with David's killed-yeast *Drosophila* medium (David 1962) to establish isofemale lines. At the next generation, 100 isofemale lines were randomly selected, and adult flies were dumped into an acrylic cage to set up one large outbred population, which was maintained in a climatic chamber (Bioref, Pitec, Chile) at 21 ± 1 °C and a photoperiod 12L:12D. Maintaining conditions were similar along with the experiments and the population cage was maintained on a discrete generation, controlled larval density regime (Castañeda et al., 2015).

Preparation of axenic and conventional flies

Axenic (germ-free) flies were obtained by using dechorionated eggs (Koyle et al., 2016). Eggs (≤ 18 h-old) were collected from Petri dishes containing fly media placed within the population cage and dechorionated as follow: 3 washes with 0.5% hypochlorite sodium solution per 2 min each wash, 3 washes with 70% ethanol solution per 2 min each wash, and 3 washes with autoclaved water per 2 min each wash. Dechorionated eggs were transferred to 50-ml Falcon tubes containing autoclaved *Drosophila* media at a density of 50 eggs/tube. The procedure to obtain axenic flies was performed under sterile conditions in a flow laminar chamber. For conventional (non-manipulated microbiota) flies, eggs were collected from the same Petri dishes used previously, washed four times with autoclaved water, and transferred to 50-ml Falcon tubes containing autoclaved *Drosophila* media at a density of 50 eggs/tube.

Elimination of bacteria in axenic flies was corroborated by testing the amplification of bacterial DNA. Medium samples and 10 flies were randomly collected from tubes containing axenic flies. From both types of samples, DNA was extracted using the GeneJet kit (Thermo-Fisher) following the protocol to extract DNA from negative-Gram and positive-Gram bacteria. Then, a PCR was performed to amplify the bacterial DNA using specific primers for the 16S rRNA gene: 341F (5'-CCT ACG GGN GGC WGC AG-3') and 805R (5'-GGA CTA CHV GGG TWT CTA AR-3') (Fadrosh et al., 2014). The PCR mix contained 0.02 U DNA polymerase (Invitrogen), 1X PCR buffer, 0.2 mM dNTPs, 1 μ M of each primer, 0.5 μ M MgCl₂, and 0.5 μ l template DNA. PCR cycle conditions were set up following the recommendations of Caporaso et al. (2010b): denaturation at 94 °C for 3 min; 35 amplification cycles at 94 °C for 45 s, 52 °C for 1 min and 72 °C for 70 s; and a final extension at 72 °C for 10 min. PCR products were loaded on a 2% agarose gel stained with Sybr Safe (Invitrogen). DNA extractions from conventional flies were used as bacteria-positive controls. Thus, effective bacterial elimination was considered effective when any amplification band was visualized in the agarose gel.

Heat tolerance of axenic and conventional flies

Axenic and conventional virgin flies of both sexes at the age of 4 days-old were individually placed in capped 5-mL glass vials, which were attached to a rack with capacity to contain 60 capped vials. In each rack, we placed 15 axenic females, 15 axenic males, 15 conventional females, and 15 conventional males. Each rack was immersed in a water tank at a specific static temperature: 35, 36, 37, and 38°C. Temperature ($\pm$ 0.1 °C) was controlled by a heating unit (Model ED, Julabo Labortechnik, Seelbach, Germany). Each static assay

was photographed using a high-resolution camera (D5100, Nikon, Tokyo, Japan) and photos were taken every 3 sec. Photos for each assay were collated in a video file, which was visualized to score the knockdown time (i.e. time at which each fly ceased to move (Castañeda et al., 2019).

Heat stress exposure

Petri dishes with fly medium were placed within the population cage for collecting eggs. Eggs (≤ 18 h-old) were transferred to vials at a density of 40 eggs/vial. After eclosion, virgin flies were separated by sex and transferred to new vials. At the age of 4 days, 100 females and 100 males were transferred to empty vials at a density of 25 flies/vial, and vials were closed with moistened stoppers to avoid fly desiccation. Vials were split into two groups: non-stressed and heat-stressed flies. Non-stressed flies were transferred to a climatic chamber (Bioref, Pitec, Chile) at 21 ± 1 °C for 3 h; whereas heat-stressed flies were placed in a water bath at 34 °C for 1 h; temperature (± 0.1 °C) was controlled by a heating unit (Model ED, Julabo Labortechnik, Seelbach, Germany). This temperature was chosen because it has been previously used to induce thermal stress in *D. melanogaster* (Hoffmann et al., 2003) and *D. subobscura* (Calabria et al., 2012). Then, heat-stressed flies were transferred to a climatic chamber (Bioref, Pitec, Chile) at 21 ± 1 °C for 2 h for recovery from heat stress (no fly died after stress).

DNA extraction and amplicon sequencing

Flies of each thermal stress treatment (non-stressed and heat-stressed flies) and sex were pooled into groups of 5 flies each: 10 pools of non-stressed females, 10 pools of non-

stressed males, 10 pools of heat-stressed females, and 10 pools of heat-stressed males. To eliminate superficial bacteria, each pool was washed 3 washes with 0.5% hypochlorite sodium solution per 2 min each wash, 3 washes with 70% ethanol solution per 2 min each wash, and 3 washes with autoclaved water per 2 min each wash. Then, each pool was transferred in a Petri dish with sterile 1X PBS solution, where intestines of flies were removed and transferred to Eppendorf tubes with ice-cold sterile 1X PBS solution.

Genomic DNA was extracted from pooled guts using the GeneJet kit (Thermo-Fisher) following the protocol for negative-Gram and positive-Gram bacteria. Then, the V3-V4 hypervariable region of the 16S rRNA gene was amplified using a dual-indexing approach according to Fadrosch *et al.* (2014). Amplicon PCR was performed using modified 341F and 805F primers, which contained: 1) a linker sequence to bind amplicons to the Nextera XT DNA indexes; 2) a 12 bp barcode sequence to multiplex samples; 3) a 0 to 5 bp "heterogeneity spacer" to increase the heterogeneity of amplicon sequences; and 4) 16S rRNA gene universal primers (Table S1). The amplicon PCR mix had a final volume of 12.5 µl: 6.5 µl ultrapure water; 5 µl 2X Hot Start PCR Master Mix (Invitrogen); 0.25 µl 1 µM forward primer; 0.25 µl 1 µM reverse primer; and 0.5 µl template genomic DNA. Amplicon PCR cycle conditions were set up as follows: denaturation at 94 °C for 3 min, 35 amplification cycles at 94 °C for 45 s, 52 °C for 1 min, 72 °C for 70 s, and a final extension at 72 °C for 10 min. Amplified reactions were purified using an enzyme mix (exonuclease I and Fast AP, Invitrogen) to eliminate free primers and dNTPs, and then loaded on a 2% agarose gel stained with Sybr Safe (Invitrogen) to visualize the PCR products.

PCR product was quantified by fluorescence using the Quan-iT PicoGreen dsDNA kit (Invitrogen) and then, all samples were standardized at the lowest DNA concentration samples (7.78 ng/µl). The primer design allowed to multiplex 23 samples into two different

sets of Nextera XT DNA indexes (Illumina Corporation, San Diego, CA). The index PCR had a final volume of 50 µl: 5µl amplicon PCR, 5 µl indexes (N701 and S502 for library 1, and N707 and S506 for library 2), 25 µl 2X Kapa Taq HotStart DNA Polymerase (Invitrogen), and 10 µl ultrapure water. Index PCR cycle conditions were set up as follows: denaturation at 94 °C for 3 min, 8 amplification cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were cleaned using the AMPure XT Bead kit (Beckman Coulter, Brea, CA) and quantified using the Qubit Fluorometer and Qubit dsDNA HS assay kit. Library 1 had a concentration of 37.2 ng/µl and library 2 had a concentration of 46.2 ng/µl, and both libraries were diluted at a concentration of 4 nM. Libraries were sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA) and the MiSeq Reagent v3 (600 cycles). Sequencing was performed at AUSTRAL-omics Sequencing Core Facility at Universidad Austral de Chile.

Metabarcoding analysis

After sequencing, 3,061,220 sequences were obtained. Raw sequence quality was inspected using FastQC (Andrews, 2010) and then filtered for a Q-value higher than 28 and sequences longer than 150 bp using the script Reads_Quality_Length_distribution.pl (Bálint et al., 2014). Forward and reverse filtered sequences were paired using PANDASeq with a minimum overlap of 5 bp (Masella et al., 2012). Paired-end sequences were trimmed to remove forward/reverse barcodes, heterogeneity spacers, and 16S rRNA gene primers. Quality-filtered and trimmed sequences were analyzed using QIIME v1.9.1 (Caporaso et al., 2010a). An open-reference OTU-picking strategy was used to generate operational taxonomic units (OTUs) using *usearch* v6.1 algorithm to cluster OTUs at 97 % of

nucleotide identity. Taxonomy assignment was performed using *uclust* method (Edgar, 2010) against the Greengenes 16S rRNA gene database at 97% pairwise identity (version 13.8; McDonald *et al.*, 2012) as database reference. Finally, representative OTU sequences we aligned using PyNast and used to build a phylogenetic tree using FastTree. After this procedure, we retained 1,559,937 sequences assigned to 1,263 OTUs. After this, we performed two filtering steps: (1) remove mitochondrial-, chloroplast-, *Spiroplasma*-, and *Wolbachia*-related sequences; and (2) remove OTUs comprising less than 100 sequences. Retained sequences (total = 1,538,400 sequences : range = 746 – 59,210 sequences) and OTU number (total = 135) by sample are reported in Table S2. For diversity analyses, samples were rarified at 12,000 sequences according to the rarefaction curve (Fig. S1), which resulted in the removal of 4 samples (1FDRD, 2FDRD, 1FBRB, and 2FBRB; see Table S2).

Statistical analyses

Assumptions of normality and homoscedasticity were checked for knockdown time. Then, knockdown time was analyzed using a two-factor analysis of variance (ANOVA) with microbiota condition (axenic and conventional flies) and sex (females and males) as main effects. Knockdown time at 35 and 36 °C showed a normal distribution, but knockdown time at 37 and 38 °C were squared-root transformed. We also tested the difference between survival curves of axenic and conventional flies at each static assays using the G-rho family test (long-rank test) using the *survival* R package (Therneau, 2020), and survival curves were plotted using the *survminer* R package (Kassambara *et al.*, 2020).

We also analyzed the effects of heat stress, sex, and its interaction on the bacterial abundance, diversity indexes, and community structure of the gut microbiota. First, relative

abundance at phylum and family taxonomical levels were obtained using *phyloseq* (Mcmurdie and Holmes, 2013) and *microbiome* (Lahti et al., 2017) packages for R, and then relative abundances were compared using a generalized linear model (GLM) assuming a quasibinomial distribution. Second, we analyzed the OTU relative abundances between non-stressed and heat-stressed flies for each sex by separated using the package *DESeq2* for R (Love et al., 2014). *DESeq2* uses a negative-binomial model for count data, taking into account the zero-skewed distribution of the microbiome dataset. Significant differences between groups in OTU relative abundance were considered when the adjusted-*P* value (False Discovery Rate correction, FDR) was lower than 0.05. Third, OTU richness and Shannon diversity were estimated using the *microbiome* package for R (Lahti et al., 2017), whereas the phylogenetic diversity was estimated using QIIME. Diversity indexes were analyzed using a two-way ANOVA and *a posteriori* comparisons were performed using a Bonferroni t-test. Finally, we estimated the weighted-UniFrac distances among samples using QIIME, which was used as input to compare the bacterial community structure between thermal stress treatment (non-stressed and heat-stressed flies) and sexes (females and males). Bacterial community analysis was performed through a permutational multivariate analysis of variance (PERMANOVA) using *vegan* package for R (Oksanen et al., 2020)

All statistical analyses were performed using R version 4.0.3 (R Core Team, 2020) and RStudio version 1.3.959 (RStudio Team, 2020), and plots were made using *ggpubr* (Kassambara, 2020a) and *rstatix* (Kassambara, 2020b) packages for R.

Results

Gut microbiota and heat tolerance

As expected, knockdown time decreased when exposure temperature increased: the higher the exposure temperature, the shorter the knockdown time (Fig. 1). Evaluating the effect of the microbiota condition on heat tolerance at each static assay, we found that axenic flies exhibited a significantly lower heat tolerance than conventional flies in the assays at 35 °C (Table 1; Fig. 1A). For heat tolerance assayed at 36 °C, we found that ANOVA showed a marginally significant difference between axenic and conventional flies (Table 1), but the log-rank test showed significant differences between survival curves of axenic and conventional flies (Fig. 1B). On the other hand, thermal tolerance between axenic and conventional flies did not differ at 37 and 38 °C (Table 1; Fig. 1C and 1D, respectively). On the other hand, we did not find differences in heat tolerance between sexes at any thermal assay (Table 1). Finally, we only detected a significant interaction between the microbiota condition and sex at 35 °C (Table 1).

Gut microbiota composition

Gut microbiota of *D. subobscura* was dominated by bacteria belonging Actinobacteria (mean relative frequency $\pm$ SE = 0.09 ± 0.03), Bacteroidetes (mean relative frequency $\pm$ SE = 0.0002 ± 0.00004), Firmicutes (mean relative frequency $\pm$ SE = 0.66 ± 0.04), and Proteobacteria (mean relative frequency $\pm$ SE = 0.24 ± 0.04). In general, we found that the relative abundance of these phyla depended on heat stress and fly sex (Fig. 2). Actinobacteria increased their abundances from 0.2% in non-stressed females to 38.5% in heat-stressed females (GLM: $t = -16.14$, $P = 9.6 \times 10^{-12}$; Fig. S2A); whereas the increase was more moderated in male flies (GLM: $t = -2.49$, $P = 0.02$; Fig. S2A). Bacteroidetes relative abundance showed a significant interaction between heat stress and sex (GLM: $t =$

3.23, $P = 0.003$), with males showing an important reduction of Bacteroidetes abundance between non-stressed and heat-stressed flies (GLM: $t = 5.58$, $P = 3.3 \times 10^{-5}$; Fig. S2B) in comparison to female flies (GLM: $t = 2.26$, $P = 0.04$; Fig. S2B). Similarly, Proteobacteria abundance analysis showed a significant interaction between heat stress and sex (GLM: $t = 7.77$, $P = 4.8 \times 10^{-9}$; Fig. S2C): females showing similar abundance between non-stressed and heat-stressed flies (GLM: $t = -1.69$, $P = 0.11$), whereas heat stress induced an important reduction of Proteobacteria abundance in males (GLM: $t = 7.92$, $P = 4.2 \times 10^{-5}$). On the other hand, Firmicutes abundance showed an interaction response between heat stress and sex (GLM: $t = -14.38$, $P = 5.2 \times 10^{-16}$; Fig. S2D): females exposed to heat stress displayed a decrease in Firmicutes abundance (GLM: $t = 29.18$, $P = 5.8 \times 10^{-16}$), whereas heat stress induced an increase of Firmicutes abundance from 38.2% in non-stressed males to 89.4% in heat-stressed males (GLM: $t = -7.99$, $P = 3.7 \times 10^{-7}$).

Among the most abundant bacterial families (abundance higher than 1%) associated with the gut of *D. subobscura*, we found Acetobacteraceae (mean relative frequency $\pm$ SE = 0.19 ± 0.03), Dermabacteraceae (mean relative frequency $\pm$ SE = 0.09 ± 0.03), Halomonadaceae (mean relative frequency $\pm$ SE = 0.02 ± 0.003), Lactobacillaceae (mean relative frequency $\pm$ SE = 0.45 ± 0.04), and Leuconostocaceae (mean relative frequency $\pm$ SE = 0.21 ± 0.05). Particularly, we focused on the relative abundances of acetic-acid and lactic-acid bacteria (AAB and LAB, respectively). We found a significant interaction between heat stress and sex for AAB (Acetobacteraceae) abundance (GLM: $t = 6.97$, $P = 4.8 \times 10^{-8}$; Fig. S3A), which is explained because whereas AAB abundance increased in heat-stressed females compared to non-stressed females (GLM: $t = -7.20$, $P = 1.5 \times 10^{-6}$) non-stressed males showed a higher abundance than heat-stressed males (GLM: $t = 6.05$, P

$= 1.3 \times 10^{-5}$). On the other hand, LAB families exhibited contrasting responses to heat stress. Lactobacillaceae abundance showed a significant interaction between heat stress and sex (GLM: $t = -2.80$, $P = 0.008$; Fig. S3B): heat-stressed females a lower abundance of Lactobacillaceae than non-stressed females (GLM: $t = 17.93$, $P = 1.8 \times 10^{-12}$); whereas non-stressed and heat-stressed males showed similar Lactobacillaceae abundances (GLM: $t = 1.67$, $P = 0.11$). On the other hand, Leuconostocaceae abundance also showed a significant interaction between heat stress and sex (GLM: $t = -5.45$, $P = 4.4 \times 10^{-6}$; Fig. S3C): non-stressed females showed a higher Leuconostocaceae abundance than heat-stressed females (GLM: $t = 2.37$, $P = 0.03$); whereas heat-stressed males harbored a higher Leuconostocaceae abundance than non-stressed males (GLM: $t = -4.85$, $P = 0.0001$).

Analysis of OTU abundance (total = 135) showed that 42 OTUs significantly decreased their abundance in heat-stressed flies (blue circles in Fig. 3), whereas 51 OTUs significantly increased their abundance in heat-stressed flies (red circles in Fig. 3), and 42 OTUs did not change their abundance between non-stressed and heat-stressed flies (black circles in Fig. 3).

Gut microbiota diversity

OTU richness (Table S2) was significantly lower in heat-stressed flies than in non-stressed flies and not different between sexes, but we found a significant interaction between heat stress and sex (Table 2; Fig. 4A): thermal stress reduced the OTU number by 13.3% for female flies (Bonferroni t-test: $P = 0.002$), whereas this reduction was in 39.4% for male flies (Bonferroni t-test: $P = 6.2 \times 10^{-7}$). On the other hand, Shannon diversity (Table S2) showed non-significant individual effects associated with heat stress and sex, but a

significant interaction was found (Table 2; Fig. 4B): non-stressed females harbor a lower diversity than heat-stressed females (Bonferroni t-test: $P = 2.4 \times 10^{-10}$), but non-stressed males showed higher diversity than heat-stressed males (Bonferroni t-test: $P = 0.005$). Finally, phylogenetic diversity (Table S2) was significantly lower in heat-stressed flies than in non-stressed flies and not different between sexes, but we found a significant interaction between heat stress and sex (Table 2; Fig. 4C): non-stressed and heat-stressed females showed a similar phylogenetic diversity (Bonferroni t-test: $P = 0.18$); whereas heat-stressed males harbor a lower phylogenetic diversity than non-stressed females (Bonferroni t-test: $P = 5.5 \times 10^{-10}$). Effects of heat stress and sex on the gut microbiota structure of *D. subobscura* showed a significant effect of heat stress ($F_{1,32} = 73.35$, $P = 0.001$, $R^2 = 0.19$), sex ($F_{1,32} = 33.48$, $P = 0.001$, $R^2 = 0.09$), and a significant interaction between heat stress and sex ($F_{1,32} = 238.96$, $P = 0.001$, $R^2 = 0.63$). Heat stress had effects the gut microbiota structure of female and male flies, with each group clustering separately (Fig. 4D).

Discussion

Global warming impacts the animal's fitness, leading to an increased extinction risk in ectotherm species (Deutsch et al., 2008; Huey et al., 2012). Gut microbiota can contribute to host physiology leading to an increase of resistance to abiotic stressful conditions (Ferguson, 2018; Henry and Colinet, 2018). In the present work, we have studied the association between the gut microbiota and thermal physiology of *D. subobscura*, representing the first characterization of the gut microbiota for this species. Our findings provide evidence that gut microbiota influences heat tolerance, and that heat stress modifies the gut microbiota at taxonomical and diversity levels. These results demonstrate the

sensitivity of the gut microbiota to transient heat stress, which can have negative impacts on host fitness.

Gut microbiota and heat tolerance

Several studies have evaluated the role of the gut microbiota on cold and heat tolerance in ectotherms, finding different results. For instance, Henry and Colinet (2018) found that the gut microbiota contributes to cold tolerance, but they found no differences in heat tolerance between axenic and conventional flies of *D. melanogaster*. In the same line, Raza et al. (2020) found that the gut microbiota increases the tolerance to low temperatures in the dipteran *Bactrocera dorsalis*. On the other hand, a recent study found that the composition of the gut microbiota influences the heat tolerance of the western fence lizard (*Sclerophorus occidentalis*), with a positive association between the abundance of genus *Anaerotignum* (Firmicutes) and heat tolerance (Moeller et al., 2020). The different results about the role of the gut microbiota on heat tolerance could be due the fact that heat tolerance depends on the methodology employed to measure it (Chown et al., 2009; Rezende et al., 2011; Castañeda et al., 2015), which can blurry physiological effects of different experimental treatments. To have a better approach to the thermal tolerance landscape (Rezende et al., 2014), we measured the heat tolerance at different temperatures from 35 °C (chronic thermal stress) to 38 °C (acute thermal stress). Interestingly, we only found a significant effect of the gut microbiota on heat tolerance when it was assayed at the lowest experimental temperature (35 °C): axenic flies tolerate this temperature for an average of 14.9 min, whereas conventional flies withstand for 25.5 min. A plausible explanation for these findings is the impact of gut microbiota on the host nutritional status (Ridley et al., 2012; Douglas, 2018a), which in turn determines the heat tolerance in ectotherms (Moghadam et al., 2018;

Henry et al., 2019; Moeller et al., 2020). If this is true, thermal assays at high experimental temperatures (37 or 38 °C) occur so fast (7 and 3 min, respectively) that they cannot detect differences between axenic and conventional flies. Whereas, if gut microbiota impacts on nutritional status, conventional flies can withstand longer heat stress than axenic flies, but this difference can be only detected when there is enough time for energy reserves to be used in costly, resistance mechanisms such as heat tolerance (Feder and Hofmann, 1999; Calabria et al., 2012). Among these mechanisms, there is evidence that gut microbiota can influence the expression of heat-shock proteins in the gut epithelium (Liu et al., 2014; Arnal and Lalle, 2016), which represents a key response to mitigate cellular damage during thermal stress (Sørensen et al., 2003; Calabria et al., 2012).

Gut microbiota composition

Recent studies have provided clear evidence about the impact of temperature on the gut microbiota of ectotherms (see Sepulveda and Moeller (2020) for a review). In general, these studies have used thermal acclimation (i.e. > 2 weeks) to evaluate changes in the gut microbiota composition and they have found that in warm temperatures, vertebrate ectotherms show a progressive decrease of bacteria belonging Firmicutes (Bestion et al., 2017; Fontaine et al., 2018), whereas warm-temperature acclimation led to an increase of the relative abundance of Proteobacteria invertebrate ectotherms (Berg et al., 2016; Moghadam et al., 2018; Horváthová et al., 2019). Here, we studied the effect of transient heat stress on the gut microbiota of *D. subobscura*, but we found a very different response of the bacterial composition when flies were exposed at 34 °C for 1 h. We found that the impact of heat stress led to an increase in the abundance in 37.8% of total OTUs, whereas 31.1% of OTUs decreased their abundance after heat stress. Interestingly, this short

exposure to heat stress changed the gut microbiota composition differentially for each sex: heat stress induced a reduction in Firmicutes relative abundance, and an increase in Actinobacteria abundance; whereas for males, we observed an increase of Firmicutes and a decline of Proteobacteria abundances. Our findings suggest that temperature-induced changes in the gut microbiota of ectotherms can occur as fast as hours (present work), days (Sun et al., 2017), or weeks (Moghadam et al., 2018), which can explain the difference between our results and the expected increase of Proteobacteria in warm-acclimated ectotherms. Additionally, this difference can be explained by the fact that we analyzed the impact of temperature on the gut microbiota in both sexes, whereas other studies have assessed this impact only using only males (Moghadam *et al.*, 2018 and Horváthová *et al.*, 2019).

At the family level, we found that the gut microbiota of *D. subobscura* was dominated by acetic-acid (*Acetobacteraceae*) and lactic-acid (*Lactobacillaceae* and *Leuconostocaceae*) bacteria, which is a common characteristic in *Drosophila* species (Douglas, 2018b). Regarding the effect of temperature on the bacterial family composition, *D. melanogaster* acclimated in warm conditions show a higher abundance of *Acetobacter* bacteria (AAB) and lower abundance of *Leuconostoc* bacteria (LAB) in comparison to cold-acclimated flies (Moghadam et al., 2018). Interestingly, this temperature-induced response of the gut microbiota composition under laboratory conditions matches wild populations, where low-latitude populations of *D. melanogaster* showed a higher AAB abundance and lower LAB abundance compared to high-latitude populations (Walters et al., 2020). Here, we found that AAB and LAB abundance changed with thermal stress, but these changes depended on the fly sex. In general, thermal stress reduced the *Acetobacteraceae* (AAB) and *Lactobacillaceae* (LAB) abundances, but the relative

abundance of *Leuconostoceae* (LAB) increased in heat-stressed flies. Differences between *D. melanogaster* and *D. subobscura* can be explained: (1) because they have traditionally fed on different diets, which is known to impact the gut microbiota composition (Jehrke et al., 2018; Obadia et al., 2018); or (2) just because they diverged around 40 million years ago (Gibbs and Matzkin, 2001), resulting in different evolutionary histories under different environmental contexts. Therefore, comparative studies are needed to understand the thermal plasticity of the gut microbiota in a wider range of *Drosophila* species.

Gut microbiota diversity

Temperature also has important effects on the diversity and structure of the gut microbiota in ectotherms (Sepulveda and Moeller, 2020). In general, warm conditions lead to a decrease of OTU number (richness) and diversity of gut microbiota in ectotherms (Bestion et al., 2017; Kokou et al., 2018; Li et al., 2018). Here, we found that heat stress induces a reduction in OTU number and phylogenetic diversity decreased in heat-stressed flies, being these effects more important in males than females. Similar response of richness and phylogenetic diversity is not surprising because commonly both diversity indices are highly correlated (Tôrres and Diniz-Filho, 2004). Conversely, Shannon diversity increased in heat-stressed females but decreased in heat-stressed males, which can be associated with changes in the abundance of some phyla in response to heat tolerance. Indeed, non-stressed females and heat-stressed males showed a more similar community structure compared to the other groups. This can be explained because the gut bacterial community of the non-stressed females was dominated by Firmicutes (88.4%) and Proteobacteria (11.2%), which was relatively similar for heat-stressed males: Firmicutes (89.7%) and Proteobacteria (9.8%). Changes in the gut microbiota diversity are common in ectotherms exposed to

warm conditions and it could be explained because some bacterial species have higher thermal tolerance or they can tolerate better indirect effects of heat stress such as the production of reactive oxygen species by hosts as a response to heat stress (Lian et al., 2020). However, our study had some limitations to explain the proximal causes about changes in bacterial abundances and future steps should be focused to explore the resistance mechanisms in members of the gut microbiota.

Conclusions

Temperature induces changes in the gut microbiota of ectotherms, regardless of how long organisms have been exposed to warm conditions. Here, we demonstrate that these changes are different for both sexes, and future studies should assess the sexual dimorphism in gut microbiota responses to abiotic and biotic factors. These changes in gut microbiota have consequences on the physiological mechanism as thermal resistance, which can impact on host fitness, population risk extinction, and vulnerability of ectotherms to current and future climatic conditions. Research about the role of gut microbiota on the adaptive response to climate change is a new venue and future research needs to balance mechanistic approaches to understand the host-microbiota interactions and holistic approaches to know the role of gut microbiota on the ecology and evolution of ectotherms.

Author's contributions

A.J. performed experiments, analyze the dataset and approved the final version of the manuscript. L.E.C. conceived the original idea, designed experiments, conducted bioinformatic and statistical analyses, provided funds for all experiments, and wrote the manuscript.

464

465 **Acknowledgments**

466 We thank Andrea Silva from AUSTRAL-omics Sequencing Core Facility (Universidad
467 Austral de Chile) for her advice and support during amplicon sequencing. A.J. thanks an
468 internal scholarship of the Master Program in Genetics (Facultad de Ciencias, Universidad
469 Austral de Chile).

470

471 **Competing interests**

472 There are no conflicts of interest with respect to this manuscript or the reported data.

473

474 **Data accessibility**

475 Data available from Figshare: <https://figshare.com/s/07258a71c3074fa59b1e>. For copyright
476 reasons, amplicon sequences will be available once the manuscript has been published.

477

478 **Funding**

479 This work was funded by FONDECYT 1140066 (Fondo Nacional de Investigación
480 Científica y Tecnológica, Chile) and ENL-09/18 (Vicerrectoría de Investigación y
481 Desarrollo (VID), Universidad de Chile) grants.

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Table 1. Results of the analysis of variance about the effects of microbiota condition (axenic and conventional flies), sex (female and male flies), and its interaction on heat tolerance (measures as the knockdown time in static assays) of *Drosophila subobscura*.

Trait	Microbiota condition	Sex	Interaction
Knockdown time at 35°C	$F_{1,116} = 43.43$ $P = 1.35 \times 10^{-9}$	$F_{1,116} = 1.54$ $P = 0.22$	$F_{1,116} = 4.15$ $P = 0.04$
Knockdown time at 36°C	$F_{1,116} = 3.47$ $P = 0.07$	$F_{1,116} = 0.004$ $P = 0.95$	$F_{1,116} = 1.16$ $P = 0.28$
Knockdown time at 37°C	$F_{1,116} = 0.24$ $P = 0.63$	$F_{1,116} = 3.18$ $P = 0.08$	$F_{1,116} = 1.92$ $P = 0.17$
Knockdown time at 38°C	$F_{1,116} = 0.31$ $P = 0.58$	$F_{1,116} = 0.45$ $P = 0.50$	$F_{1,116} = 1.63$ $P = 0.20$

Table 2. Results of the analysis of variance about the effects of heat stress (non-stressed and heat-stressed flies), sex (female and male flies), and its interaction on bacterial diversity indices associated with the gut microbiota of *Drosophila subobscura*.

Diversity index	Heat stress	Sex	Interaction
OTU number (richness)	$F_{1,32} = 124.81$ $P = 1.41 \times 10^{-12}$	$F_{1,32} = 3.11$ $P = 0.09$	$F_{1,32} = 33.43$ $P = 2.04 \times 10^{-6}$
Shannon's diversity (H')	$F_{1,32} = 0.41$ $P = 0.53$	$F_{1,32} = 3.43$ $P = 0.07$	$F_{1,32} = 63.19$ $P = 4.50 \times 10^{-9}$
Phylogenetic diversity (Faith's index)	$F_{1,32} = 93.37$ $P = 5.21 \times 10^{-11}$	$F_{1,32} = 1.0$ $P = 0.33$	$F_{1,32} = 43.50$ $P = 1.96 \times 10^{-7}$

Figure legends

Figure 1. Relative survival (measured as knockdown time) of axenic (red) and conventional (green) flies estimated at different static assays (A: 35 °C, B: 36 °C, C: 37 °C, and D: 38 °C) in *Drosophila subobscura*. Survival curves (lines) were estimated using a Kaplan-Meier model, and shaded areas indicate 95% confidence interval of each survival curve.

Figure 2. Relative abundance of bacterial composition (phylum) of the gut microbiota of non-stressed and heat-stressed female (A) and male (B) of *Drosophila subobscura*. Each color represents a bacterial phylum.

Figure 3. Volcano plot illustrating bacterial OTUs that showed significantly higher abundance in non-stressed flies (blue circles) or in heat-stressed flies (red circles) of *Drosophila subobscura*. OTUs that exhibit similar abundances in non-stressed and heat-stressed flies are represented by black circles.

Figure 4. Bacterial diversity found in guts of non-stressed (blue) and heat-stressed (red) flies of *Drosophila subobscura* (females: circles, and males: triangles): (A) richness (OTU number); (B) Shannon's diversity; (C) phylogenetic diversity (Faith's index); and (D) bacterial community structure for gut microbiota using a non-metric multidimensional scaling (NMDS), which is based on weighted-UniFrac distances among samples. Box plots show median and interquartile range (IQR) and whiskers represent the 1.5*IQR. Symbols above boxplots denote non-significant (ns) or significant differences between non-stressed

717 (blue) and heat-stressed flies (red) obtained from generalized-linear models (*: $P < 0.05$;

718 **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$).

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Figure 1

Figure 3

Figure 4

Supplementary Material

Figure S1. Rarefaction plots indicating OTU number and sequencing depth after rarefaction at 12,000 sequences. Samples were taken from non-stressed female (orange), non-stressed male (green), heat-stressed female (lightblue), and heat-stressed male (purples) flies of *D. subobscura*.

Figure S2. Relative abundances of the phyla (A) Actinobacteria, (B) Bacteroidetes, (C) Proteobacteria, and (D) Firmicutes associated with the guts of non-stressed (blue) and heat-stressed (red) flies (females and males) of *Drosophila subobscura*. Box plots show median and interquartile range (IQR) and whiskers represent the 1.5*IQR. Symbols above boxplots denote non-significant (ns) or significant differences between non-stressed (blue) and heat-stressed flies (red) obtained from generalized-linear models (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$).

Figure S3. Relative abundances of the bacterial families (A) Acetobacteraceae, (B) Lactobacillaceae, and (C) Leuconostocaceae associated with the guts of non-stressed (blue) and heat-stressed (red) flies (females and males) of *Drosophila subobscura*. Box plots show median and interquartile range (IQR) and whiskers represent the 1.5*IQR. Symbols above boxplots denote non-significant (ns) or significant differences between non-stressed (blue) and heat-stressed flies (red) obtained from generalized-linear models (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ****: $P < 0.0001$).

Table S1. Modified 341F and 805F primers for bacterial 16S rRNA gene used in this study. Each primer contains: a linker sequence to bind amplicons to the Nextera XT DNA indexes (black letters); a 12 bp barcode sequence to multiplex samples (red letters); a 0 to 5 bp "heterogeneity spacer" (blue letters), and 16S rRNA gene universal primers (green letters).

Primer	Illumina linker sequence – 12 bp barcode sequence – Heterogeneity spacer – 16S rRNA primer
16S_For_A	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – CCTAAACTACGG – CCTACGGGNGGCWGCAG
16S_For_B	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – TGCAGATCCAAC – T – CCTACGGGNGGCWGCAG
16S_For_C	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – CCATCACATAGG – GT – CCTACGGGNGGCWGCAG
16S_For_D	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – GTGGTATGGGAG – CGA – CCTACGGGNGGCWGCAG
16S_For_E	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – ACTTTAAGGGTG – ATGA – CCTACGGGNGGCWGCAG
16S_For_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG – GAGCAACATCCT – TGCGA – CCTACGGGNGGCWGCAG
16S_Rev_A	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – CCTAAACTACGG – GACTACHVGGGTATCTAATCC
16S_Rev_B	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – TGCAGATCCAAC – A – GACTACHVGGGTATCTAATCC
16S_Rev_C	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – CCATCACATAGG – TC – GACTACHVGGGTATCTAATCC
16S_Rev_D	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – GTGGTATGGGAG – CTA – GACTACHVGGGTATCTAATCC
16S_Rev_E	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – ACTTTAAGGGTG – GATA – GACTACHVGGGTATCTAATCC
16S_Rev_F	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG – GAGCAACATCCT – ACTAC – GACTACHVGGGTATCTAATCC

766 **Table S2.** Information for each sample about the thermal stress and sex of *Drosophila*
767 *subobscura* included in this study. Number of sequences, removal after rarefaction at
768 12,000 sequences, and richness (OTU number), Shannon diversity, and phylogenetic
769 diversity estimates for each sample are provided. NA means non-available estimate because
770 sample was removed after rarefaction (see Material and Methods section).

Sample ID	Sex	Thermal stress	Sequences	Sample removed after rarefaction	Richness (OTU number)	Shannon diversity	Phylogenetic diversity
1FDRD	Female	Heat-stressed	746	Yes	NA	NA	NA
2FDRD	Male	Heat-stressed	860	Yes	NA	NA	NA
2FBRB	Male	Non-stressed	5748	Yes	NA	NA	NA
1FBRB	Female	Non-stressed	6596	Yes	NA	NA	NA
2FCRC	Female	Heat-stressed	12496	No	90	2.0783283	5.14382
1FCRC	Male	Heat-stressed	13042	No	62	0.7040019	4.18603
1FARA	Male	Non-stressed	15864	No	105	1.9065749	6.05509
2FARA	Female	Non-stressed	17341	No	83	1.203535	4.47469
2FBRD	Male	Heat-stressed	24885	No	72	1.5965272	4.23032
2FCRD	Male	Heat-stressed	28514	No	74	1.6631817	4.33094
2FCRB	Male	Non-stressed	33992	No	99	2.0364269	5.64249
2FCRA	Female	Non-stressed	35376	No	88	1.26127	4.64091
2FDRC	Female	Heat-stressed	38526	No	77	1.9712162	4.80308
2FBRC	Female	Heat-stressed	39676	No	87	2.045393	4.62237
2FARC	Female	Heat-stressed	40144	No	78	2.0240818	4.80176
1FCRA	Male	Non-stressed	41559	No	103	1.8614536	5.54767
2FERC	Female	Heat-stressed	41693	No	81	2.0365678	4.91643
2FDRB	Male	Non-stressed	42681	No	99	1.8427923	5.61006
2FBRA	Female	Non-stressed	44190	No	88	1.2241704	4.83322
2FARD	Male	Heat-stressed	45337	No	75	1.7221588	4.37624
2FERB	Male	Non-stressed	45609	No	99	1.8698738	5.42088
1FDRC	Male	Heat-stressed	45911	No	56	0.6206609	3.63735
2FERA	Female	Non-stressed	46086	No	99	1.3249281	5.09436
2FDRA	Female	Non-stressed	46127	No	98	1.2309471	5.04824
1FERD	Female	Heat-stressed	47339	No	82	2.0531454	4.62203
1FERB	Female	Non-stressed	48321	No	86	1.4479118	4.73222
1FBRC	Male	Heat-stressed	48350	No	53	0.5368444	3.75234
1FARC	Male	Heat-stressed	49183	No	52	0.5356601	4.04646
1FERA	Male	Non-stressed	49422	No	101	1.9705597	5.80055

1FCRD	Female	Heat-stressed	49884	No	79	2.044334	4.64284
1FERC	Male	Heat-stressed	50290	No	54	0.4892269	3.95857
1FBRD	Female	Heat-stressed	50902	No	73	1.9746317	4.1562
1FDRB	Female	Non-stressed	51134	No	98	1.366587	5.00307
2FARB	Male	Non-stressed	51351	No	107	1.9728177	5.8345
1FCRB	Female	Non-stressed	53051	No	99	1.3970142	5.49794
1FDRA	Male	Non-stressed	53163	No	100	1.8445902	5.66431
1FARD	Female	Heat-stressed	53901	No	69	2.0003884	3.73369
1FBRA	Male	Non-stressed	54842	No	95	1.8485612	5.60939
2FERD	Male	Heat-stressed	55058	No	64	1.6865227	3.8092
1FARB	Female	Non-stressed	59210	No	87	1.3002729	4.92085

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