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Effect of brief exposures of anesthesia on thermotolerance and metabolic rate of the spotted-wing fly, *Drosophila suzukii:* differences between sexes?

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Abstract

The spotted-wing fly, *Drosophila suzukii,* is a world-wide pest insect for which there is increasing interest in its physiological traits including metabolism and thermotolerance. Most studies focus only on survival to different time exposures to extreme temperatures, mainly in female flies. In addition, it has not been tested yet how anesthesia affects these measurements. We analyzed the effects of anesthesia by brief exposures to cold, anoxia by CO_2 or N_2 on three standard thermotolerance assays, as well as the aerobic metabolic rate in both sexes. For heat tolerance we measured CTmax by thermolimit respirometry, and CTmin and chill-coma recovery time for cold tolerance. Aerobic metabolism was calculated by CO_2 production of individual flies in real time by open flow respirometry. Results showed that females have a significantly higher $\dot{V}CO_2$ for inactive (at 25°C) and maximum metabolic rate than males. This difference is mainly explained by body mass and disappears after mass correction. Males had a more sensitive MR to temperature than females showed by a significantly higher Q_{10} (2.19 vs. 1.98, for males and females, respectively). We observed a significantly lower CTmin ($X^2=4.27$, P=0.03) in females (3.68±0.38°C) than males (4.56±0.39°C), although we did not find significant effects of anesthesia. In contrast, anesthesia significantly modifies CTmax for both sexes ($F_{3,62}=7.86$, P<0.001) with a decrease of the CTmax in cold-anesthetized flies. Finally, we found a significantly higher CTmax in females (37.87±0.07ºC) than males (37.36±0.09ºC). We conclude that cold anesthesia seems to have detrimental effects on heat tolerance, and females have broader thermotolerance range than males, which could help them to establish in invaded temperate regions with more variable environmental temperatures.

Key words: critical thermal temperatures, open-flow respirometry, chill-coma recovery time, invasive species, thermal sensitivity

Abbreviations:
CTmin = Critical thermal minimum
CTmax = Critical thermal maximum
STP = Standard Temperature and Pressure
RQ = Respiratory quotient
ADS = Absolute differential sum
MR = Metabolic rate
MMR = Maximum metabolic rate
$\dot{V}CO_2$ = rate of CO_2

1. Introduction

Animals are exposed to a wide variety of stressors. Among them the lack of oxygen or anoxia and extreme temperatures such as cold shocks and heat waves are some of the most common ones found in nature. Some of these are not only stressors to which animals are exposed during their lives but are also used as anesthetic agents for insect's handling. In order to perform various tasks, such as sex sorting, phenotyping, dsRNA injection, tissue sampling or place an insect inside an experimental device among others, immobilization of small flying insects is often necessary (Ashburner, 1989). Various forms of anesthesia are available for this purpose, with cold and anoxia exposure being the most commonly employed (*e.g.,* Nilson et al., 2006; Toxopeus et al., 2016; Macmillan et al., 2017 to mention only a few). The former consists of placing adults in a cold environment or on a cold surface to cause loss of motor control. The latter consists of placing insects in a saturated atmosphere of carbon dioxide or other gas (like N_2) replacing the O_2 from air, which causes anoxia resulting in narcotic coma (Ashburner, 1989). It is known that these procedures are not innocuous; effects of anesthesia on multiple traits have been well documented in insects, including *Drosophila*. For example, exposure time from 25 to 180 minutes of cold anesthesia also affects cold tolerance in a dose-dependent manner in *D. melanogaster* (Macmillan et al., 2017). Similarly, exposure time from 2.5 to 10 minutes of carbon dioxide anesthesia increased the time to recover from a chill coma in a dose-dependent manner (MacMillan et al., 2017). In addition, it has been shown that anoxia by other gasses, for example, gaseous nitrogen (N_2), can affect cold tolerance even more than CO_2 (Nilson et al., 2006). Regarding the anesthesia effects on metabolism, Colinet and Renault (2012) showed that a 7-min exposure to CO_2 leads to an alteration of the metabolic profiles up to 14 h after the use of anesthesia.

Long-term exposures to cold or CO_2 also influenced thermotolerance and life-history traits. On the one hand, exposure to chronic cold (72 h at 4°C) affects fertility, as females produce fewer and smaller offspring, having a male-biased sex ratio in *D. melanogaster* (MacAlpine et al., 2011). Cold exposure may also induce rapid cold-hardening responses that can affect the insect's thermotolerance (Lee et al., 1987), including *D. melanogaster* (Kelty and Lee, 2001). On the other hand, it has been shown that exposure to CO_2 decreases sensitivity to glutamate at the neuromuscular junction producing loss of motor control in *D. melanogaster* (Badre et al., 2005). Anoxia by CO_2 also produces a drop in hemolymph pH (Badre et al., 2005; Nicolas and Sillans, 1989), heart-beat ceases and oxygen supply markedly deteriorates (Badre et al., 2005). These effects have negative consequences on the fly as reduced longevity, fecundity (Perron et al., 1972), and mating success (Barron, 2000). Carbon dioxide exposure also affects climbing and flight capacity (Bartholomew et al., 2015),

and eliminates the effect of heat hardening (Milton and Partridge, 2008). Although there has been extensive research on the impact of stressors on insects, including *D. melanogaster*, our understanding of other *Drosophila* flies, such as *Drosophila suzukii*, remains limited. Therefore, we cannot assume beforehand that the effects of anesthesia on these species will be similar, as significant variations have been observed in species-specific susceptibility to chemicals related to fermentation (Kim et al., 2018), desiccation tolerance, and insecticide permeability (Wang et al., 2020). *D. suzukii*, known as the spotted-wing fly, is a fruit fly originated from Asia (Matsumura, 1931), and it has recently become a world-wide pest (Asplen et al., 2015). Females can lay eggs under the skin of preharvest fruit thanks to its saw-like ovipositor (Kanzawa, 1939; Mitsui et al., 2006) producing high economic losses due to fruit damage (Goodhue et al., 2011). Increasing scientific interest caused an avalanche of work on the biology of *D. suzukii*, including reproductive biology (Kirk Green et al., 2019; Toxopeus et al., 2016; Wallingford et al., 2016), population dynamics (Kirk Green et al., 2019), and thermotolerance (Dalton et al., 2011; Enriquez and Colinet, 2017; Jakobs et al., 2015; Zerulla et al., 2015). Special interest has been raised on *D. suzukii* cold tolerance, as this species has the capacity for overwintering (Panel et al., 2020), which results in the success of the fly at colonizing temperate regions (de la Vega and Corley, 2019; dos Santos et al., 2017; Ørsted and Ørsted, 2019; Ørsted et al., 2021). Some of these studies used brief CO_2 anesthesia before measuring cold tolerance (Jakobs et al., 2015; Toxopeus et al., 2016), taking necessary precautions through good practices in anesthesia use, such as allowing for a 48-hour or longer recovery period for the effects of anesthesia to diminish. In many cases, it may not be possible to comply with these timeframes, and if not careful, the residual effects of anesthesia could modify the measurements. As a consequence, knowledge about the effects of a brief exposure to anesthesia on thermotolerance of *D. suzukii* is essential.

The aim of the study was to investigate the effects of brief exposures to different types of anesthesia, such as cold exposure, anoxia by N_2, or CO_2, on the thermotolerance and metabolic rate of *D. suzukii* immediately after anesthesia. This research is particularly important in situations where it is not feasible to wait for extended periods, ranging from hours to 2-3 days like in Jakobs et al. (2015) and Toxopeus et al. (2016), after anesthesia application to conduct the desired measurements. There are scenarios where insects are in an experimental setup where they cannot be kept for extended periods before conducting measurements, for instance when researchers aim to measure thermotolerance and metabolic rate in newly emerged adult flies. For that, after anesthetizing the flies, we immediately measured CTmax by thermolimit respirometry and cold tolerance by CTmin using a dynamic method, as well as chill coma recovery time (CCRT), two traits widely used

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as a cold tolerance indicators (David et al., 1998; Andersen et al., 2015; Overgaard and MacMillan, 2017; Mensch et al., 2017; Mensch et al., 2021). We also measured the inactive metabolic rate at 25ºC, thermal sensitivity (Q_{10}) and the maximal metabolic rate (MMR) by measuring CO_2 emission as a proxy for aerobic metabolism in *D. suzukii* after exposure to anesthesia. We predict that the brief exposure to anesthetics will not affect the analyzed characteristics of metabolism and thermotolerance.

2. Materials and methods

2.1. Insect breeding and maintenance.

A 1-year-old laboratory stock culture of *D. suzukii* flies was used (Gandini et al., 2023). It was established from a field collection of flies on plum trees in Luján, Argentina (34°34′41″S, 59°05′14″W), in December 2019. The stocks of *D. suzukii* were maintained with cornmeal standard medium at 25ºC with 12/12 hours light/dark cycle (lights on at 08:00 am). Composition of the medium was: 9.5 g of agar, 24 g of saccharose, 28 g of dextrose, 24 g of yeast, 56 g of cornmeal, 21 ml of nipagin per 100 ml of water (Petino Zappala et al., 2019). Male and female adults were collected upon emergence and maintained in vials with food for 2 days. Visually sex sorting was done without anesthesia by taking advantage of the wing's black spots of males. After measurements, sex was confirmed using a stereo microscope.

2.2. Anesthesia Treatments

For anesthesia treatments, all measurements were performed within 2-3 minutes after anesthesia exposure. In the case of cold anesthesia, flies were placed inside a 1.5 ml microcentrifuge tube in a freezer at -22ºC for 1 minute, which produced a drop in the temperature inside the Eppendorf to less than 1°C with a ramping rate of *ca.* -19°C min⁻¹ (see Figure S1 and Table S1 for temperature dynamics). For anoxia treatments, flies were exposed to 1 min anoxia (CO_2 or N_2). For doing that, flies were placed in a 4 ml chamber (Sable Systems International (SSI), Las Vegas, NV, USA), and gas (CO_2 or N_2) was passed at a very high and constant flow rate (*ca.* 800 ml min⁻¹). The high flow rate together with the small volume of the chamber allows the gas to displace atmospheric air almost immediately causing anoxia on the insect. No anesthesia was applied for the control group. Finally, flies were weighed after each trial on an analytical balance (resolution 0.1 mg; Mettler Toledo, Columbus, OH, USA).

2.3. Critical Thermal Minimum (CTmin)

CTmin of *D. suzukii* was measured using a dynamic method. The protocol performed was modified from Mensch et al. (2021). As sexes can be easily recognized, one male and one

female were simultaneously measured on a metallic plate (SSI-Pelt-plate, Las Vegas, NV, USA) under a 6.5 cm diameter Petri's dish. Petri dishes´ walls were painted with fluon® to avoid flies from climbing. The plate was connected to a temperature controller (Pelt-5; SSI) programmed with a temperature profile that started with 10 minutes at 25ºC followed by a temperature ramp that decreased at 0.25°C min^{-1}. CTmin was defined as the temperature at which insects lose motor control (Terblanche et al., 2006), and it was determined visually when insects fell to the side or in a ventral position upwards, that is, they can't stay upright on their six legs. At least ten replicates for each sex and anesthesia combination were performed.

2.4.   Chill Coma Recovery Time (CCRT)

Flies were placed in a 1.5 ml microcentrifuge tube with cotton on the top floating in a polyethylene container filled with water and ice to maintain temperature at *ca.* 0ºC for 14 h. After that, flies were individually placed on each of the wells of an ELISA plate, and time they took to recover their stand-up position (up-right on six legs) was recorded up to a maximum of 90 min at room temperature (*ca.* 25ºC).

2.5.   Open-flow respirometry

To measure real time CO_2 production and activity in unrestrained individual *D. suzukii*, we used the high-resolution TR-2 Sable System International (SSI; Las Vegas, NV, USA) flow-through respirometry system with a Li-Cor (LI-6251) CO_2 infrared analyzer (resolution 0.1 ppm CO_2) attached to an AD-2 Activity Detector (SSI) (Rolandi et al. 2014). Briefly, air free of CO_2 and H_2O was drawn at a flow rate of *ca.* 50 ml min^{-1} STP by a SS4 sub sampler (SSI), which unites a pump, needle valve and a linearized mass flow meter, through 3 mm diameter low-permeability Bev-A-Line tubing and a RC-M precision miniature respirometer chamber (volume *ca.* 13 ml; SSI). Specimen temperatures were controlled to ± 0.2°C by a SSI's Pelt-5 temperature controller and SSI's PTC-1 Peltier Effect cabinet. In order to equilibrate the temperature of the respirometer chamber with that inside cabinet, the air flow passed through a copper coiled tube (*ca.* 6.5 meters long) placed inside the cabinet. The temperature inside the respirometer chamber was measured by a thermocouple attached to a SSI TC-2000 thermocouple meter (accuracy 0.2 and resolution 0.01°C). The analog outputs from the analyzers measuring CO_2, insect´s activity, temperature of the chamber and air flow rate were connected to an A/D converter (SSI UI-2, 16 bits basic accuracy = 0.05%). Data was acquired at 1 Hz, and it was digitized and processed using SSI ExpeData data acquisition and analyzes software (SSI v. 1.8.2).

2.5.1.   Critical Thermal Maximum (CTmax)

CTmax of *D. suzukii* were measured using thermo-limit respirometry (Lighton and Turner, 2004). This methodology provides a way to objectively determine CTmax. Flies were measured individually with a similar protocol to de la Vega et al. (2015), after the equilibration phase (5 minutes at 25ºC) started a ramp of increasing temperature at rate of 0.25ºC min^{-1} until the CTmax was reached. CTmax was defined as the temperature at which the fly loses motor control (Terblanche et al., 2006), and was objectively determined by calculating the absolute difference sum (ADS) of the activity, this is the cumulative sum of the absolute difference between all the adjacent data points (Lighton and Turner, 2004; Rolandi et al., 2018). The inflection point of the ADS residuals indicates the point at which the insect ceases motor activity and, therefore, when it reaches its CTmax (Figure 1). An average of 12 flies for each anesthesia and sex were measured.
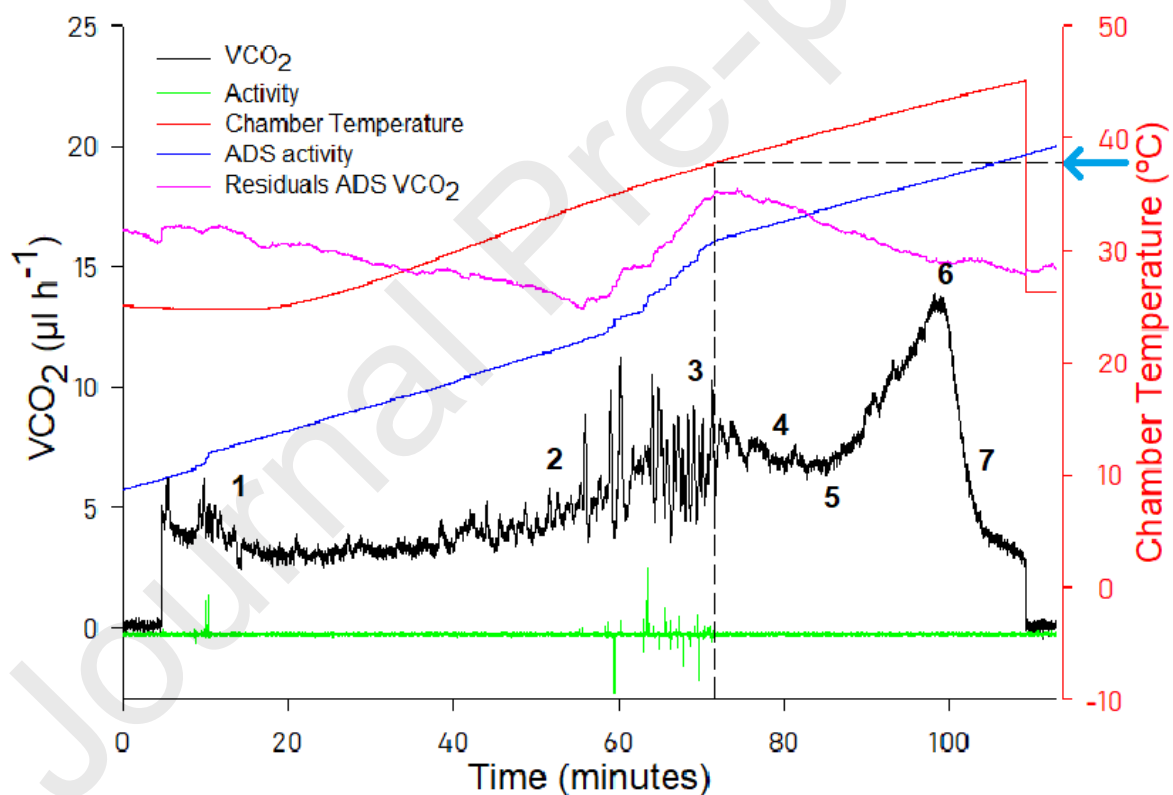

Figure 1. Example of thermo-limit respirometry recording of a male control fly of *D. suzukii.* Dashed lines and blue arrow show CTmax point. Numbers indicates $\dot{V}CO_2$ response phases: 1) equilibration phase, 2) ramping phase, 3) premortal plateau phase, 4) mortal fall phase, 5) valley phase, 6) post-mortal peak phase and 7) exponential decay phase (see Lighton and Turner (2004) and de la Vega et al. (2015) for details)

2.5.2. Metabolic rate

The following corrections and conversions were made from the CTmax data recordings: (1) CO_2 baselines were subtracted assuming a linear drift; (2) CO_2 in ppm was converted to μL h^{-1} ($\dot{V}CO_2$) (for formulae see: Lighton, 2018), (3), CO_2 emission rate in μL h^{-1} was converted to energy units of μW assuming the dissipative catabolism of glucose (RQ=1), which was measured for fed *D. melanogaster* (Wang et al. 2008; Brown et al. 2022), and (4) activity was converted to ADS and data stored into a new channel. Following these corrections and conversions, temporal changes in $\dot{V}CO_2$ patterns were analyzed following Lighton and Turner's description in 2004. This response consists of seven phases (shown in Figure 1): following (1) the equilibration phase during which $\dot{V}CO_2$ was constant, and it was used to calculate the inactive MR at 25°C (2) ramping began, $\dot{V}CO_2$ increased exponentially with increasing temperature. This second phase was used to estimate the sensitivity of metabolic rate to temperature by calculating the Q_{10}. The exponential rise in $\dot{V}CO_2$ ended in (3) a 'premortal plateau' phase, during which $\dot{V}CO_2$ was kept high and did not increase with temperature. Average values of $\dot{V}CO_2$ during this 3rd phase was used for calculation of the MMR. A steep decline in $\dot{V}CO_2$ then occurred (4) during which 'mortal fall' both spiracular control and activity abruptly ceased (at this point flies reach their CTmax); this was followed by (5) 'postmortal valley', a low point in post-mortal $\dot{V}CO_2$. After this, $\dot{V}CO_2$ rose again into (6) the 'postmortal peak', before slowly declining (7) with a classic exponential decay which progressed, if the recording was allowed to continue for long enough, back to baseline levels.

From the mean $\dot{V}CO_2$ trace of equilibration phase (phase 1 from Figure 1), we corroborated no activity, and then calculated for each fly the inactive metabolic rate (MR) at 25ºC and the maximum metabolic rate (MMR) from 'premortal plateau' phase (phase 3 from Figure 1). To eliminate the mass dependence of inactive MR and MMR, we divided them in μW by live mass of the flies in g raised to the 0.856 power (equation 1), which is the interspecific mass scaling exponent for tracheate arthropods (Lighton et al., 2001), allowing male and female flies to be directly compared.

$$Mass - independent\ MR = MR\ /\ mass^{0.856} \qquad (1)$$

While temperature is increasing MR follows it in a phase called ramping phase (phase 2 from Figure 1). We calculated and analyzed slopes and intercepts during the rise of $\dot{V}CO_2$. As a measurement of MR temperature sensitivity, we calculated Q_{10}, which is the change in

the rate of a process in response to a 10°C variation. It arises from the Arrhenius equation and shows the exponential relationship of the rate of a process as a function of temperature (equation 2).

$$MR_2 = MR_1 * Q_{10}^{\left(\frac{T2-T1}{10}\right)} \qquad (2)$$

Q_{10} values were calculated and compared between males and females. When a fly is exposed to the ramping increase of temperature it tends to increase movement in order to escape to heat stress, this movement raises the $\dot{V}CO_2$ production. For this reason, it is important to analyze if these changes in motor activity are related to the slope of increasing $\dot{V}CO_2$. So, we calculated an activity index by subtracting the start value to the last value of ADS activity during ramping phase and then this number was divided by the total seconds of ramp duration and multiplied by 60 (ADS min^{-1}). This allows us to compare motor activity during this phase independently of the time duration of the phase.

2.6. Statistical Analysis

Data were analyzed in R program ver. 4.1.1 (R Core Team, 2021) on Rstudio interface (ver. 1.3.1073). CTmin data were analyzed using a maximum likelihood approach for fitting a mixed effects model using *lmer* function in *lme4* package (Bates et al., 2014). Models included sex, anesthesia (levels: control, cold, CO_2 and N_2), and their interactions as fixed factors, and day of assay as a random factor. For CTmax, we performed a general linear model with sex, anesthesia and its interaction as fixed effects. In both cases, we did model selection using Akaike Information Criterion (AIC), resulting in the models that excluded mass of the flies as an additive factor.

In general, recovery time data do not follow a normal distribution. For this reason, CCRT data were fitted to a Gamma distribution using a generalized linear model from *glmmTMB* library (Kristensen and Maechler, 2022). The model included fixed factors: sex, anesthesia and their interaction. This method has the advantage of avoiding transforming data using math functions, for example log function, allowing to analyze and conclude on raw data. The effects of sex and anesthesia treatment on the thermotolerance measurements were tested using ANOVA. As for the critical temperatures, based on AIC, mass of the flies was not included as an additive factor in the model. Assumptions of the generalized linear models were checked using DHARMa package (Harting, 2020).

The temporal response of $\dot{V}CO_2$ and ADS min^{-1} values were analyzed using generalized linear models fitted to a gamma distribution (or a general linear model in the case of the

intercept of the ramping phase) as previously mentioned. All MR measurements and ADS min^{-1} values were compared using ANOVA with anesthesia, sex and their interaction as fixed factors.

3. Results

Anesthetized flies of both sexes with CO_2 and N_2 had similar recovery times (Mean ± SEM, 170 ± 8.89 s for CO_2 and 182 ± 6.86 s for N_2). Group anesthetized with cold took approximately 1 minute longer to recover than the other two treatments (Mean ± SEM, 240 ± 9.53 s, see Table S2 and S3), and this difference was statistically significant ($F_{2,30}$=19.35, P<0.001, see Table S2 and S3). No significant differences were found in the recovery times between sexes or in the interaction between sex and anesthesia (see Table S2 and S3).

3.1. Effects of sex and anesthesia on cold tolerance of *D. suzukii*

For CTmin analysis, no effect of anesthesia was found (Table S4). Interestingly, females showed a significantly lower CTmin (*i.e.*, higher cold tolerance) than males (Figure 2 and Table S4). CTmin values (mean ± SEM) were 3.68 ± 0.38°C for females, and 4.56 ± 0.39°C for males (Figure 2). For CCRT analysis, the females tended to recover faster than males, although this difference was not significant (X^2=2.71, P=0.09). Females recovered in a meantime of 25.82 ± 0.92 min, while males in 27.76 ± 0.78 min (mean ± SEM). No interaction between sex and anesthesia was found (Supplementary Table S5).
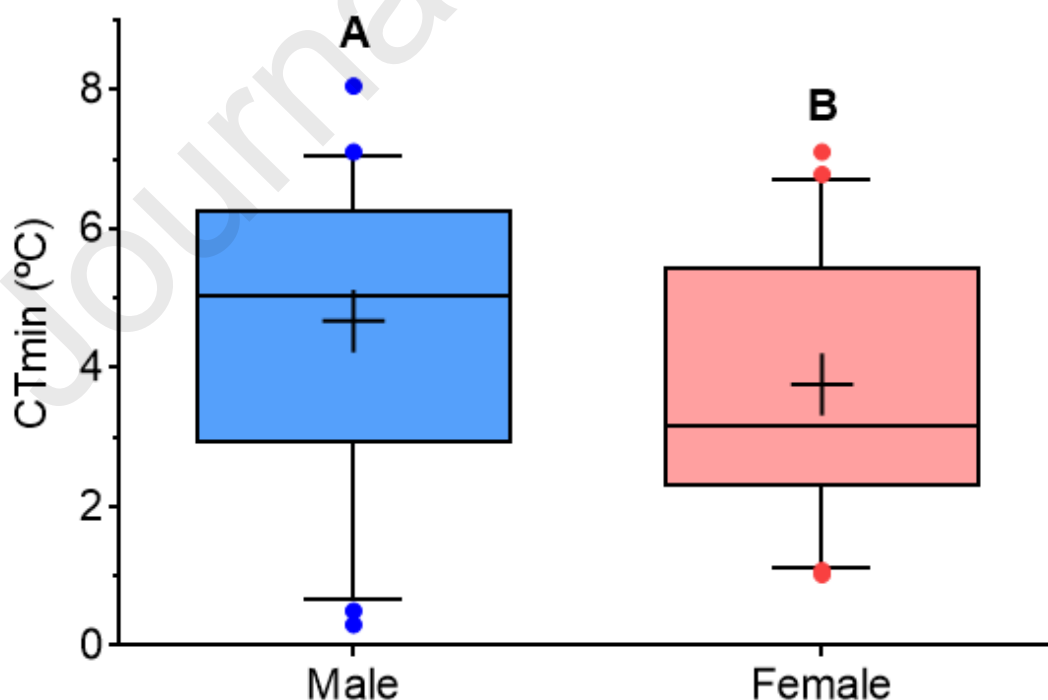
Figure 2. Boxplot of CTmin (°C) of males (blue, N=45), and females (pink, N=48) of *D. suzukii*. Cross denotes the mean value and points show extreme values. Different letters indicate significant differences between groups.

3.2. Effects of sex and anesthesia on heat tolerance (CTmax) of *D. suzukii*

The model showed significant differences for both main factors (Table S6). Overall, females showed significantly higher heat tolerance (CTmax of 37.87 ± 0.07ºC) than males (37.36 ± 0.09ºC; Figure 3). Likewise, differences across groups treated with different anesthesia were highly significant (Table S6). Tukey's tests revealed that cold anesthesia significantly decreases CTmax with respect to control and other anesthesia groups (Figure 4 and Table S7). No interaction between sex and anesthesia was found (Supplementary Table S6).
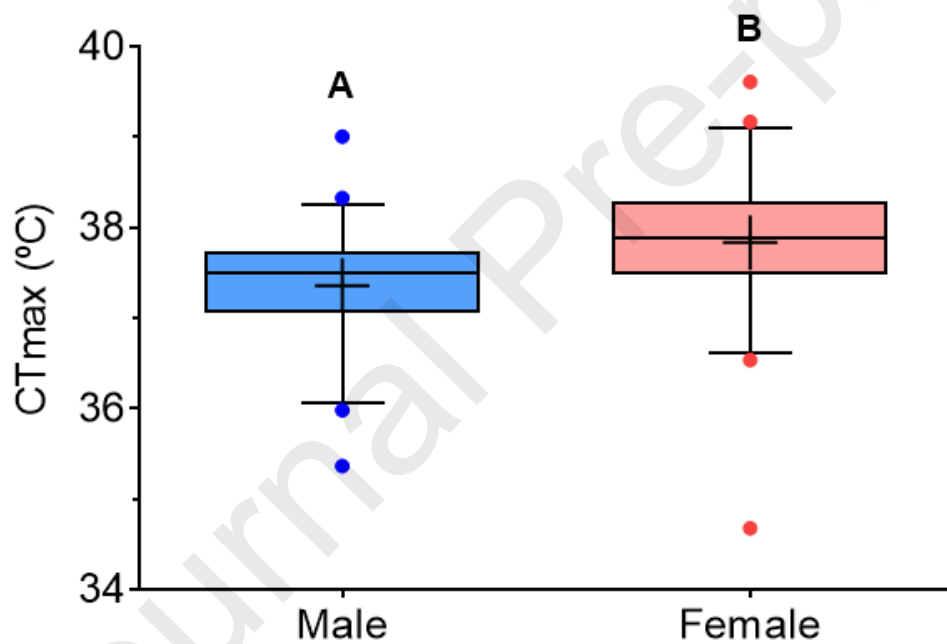

Figure 3. Boxplot of CTmax (°C) of males (blue, N=48), and females (pink, N=47) of *D. suzukii*. Cross denotes the mean value and points show extreme values. Different letters indicate significant differences between groups.
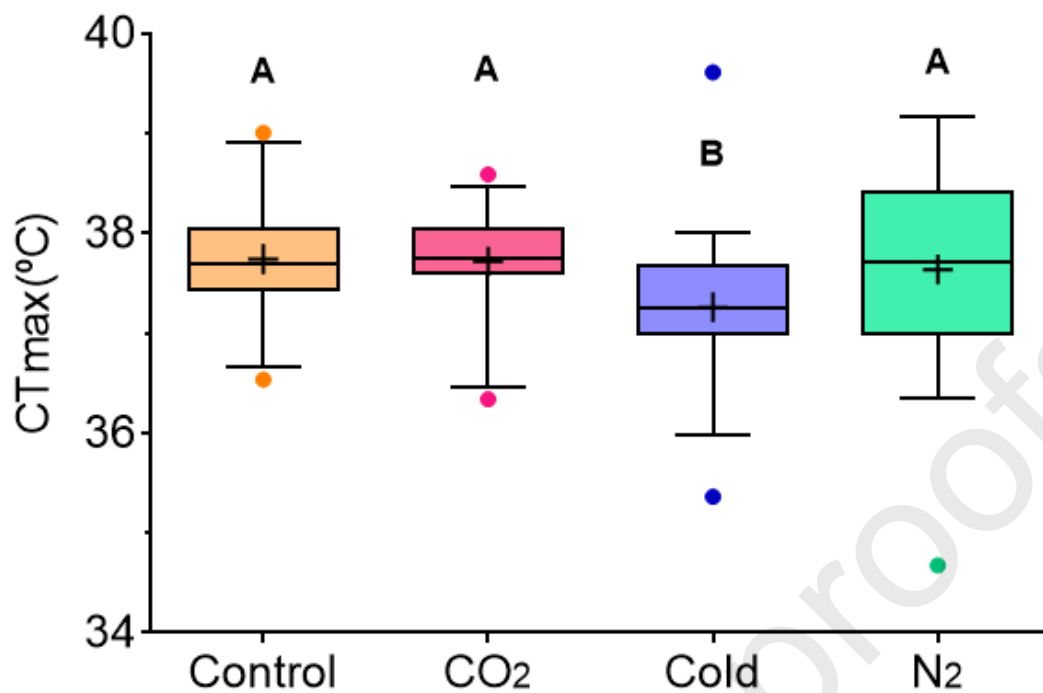
Figure 4. Boxplot of CTmax (°C) for different groups: Control group (orange, N=23), CO_2 anesthesia (red, N=24), Cold anesthesia (blue, N=24), and N_2 anesthesia (green, N=24). Cross denotes the mean value and points show extreme values. Different letters indicate significant differences across groups.

3.3. Effects of sex and anesthesia on metabolic rate of *D. suzukii*

We analyzed the response through time of CO_2 production while files were exposed to an increasing temperature profile (see Materials and methods for details). The temporal response we found was like the response previously described by Ligthon and Turner in 2004. Females showed higher MR values than males in both, inactive MR at 25°C and MMR (Table 1). This result is mainly explained by the differences in body mass. Female flies are bigger and heavier than males (Table 1 and S8). For this reason, MR values were corrected by body mass before analyzing the data. The analysis of the mass-independent inactive MR at 25ºC did not show interaction between fixed factors or effect of anesthesia treatments or differences between sex (see Table S8). The same result was found for mass-independent MMR (Table S8). It is important to note that the significant difference found in the MR without transformation of the data disappear not only when divided by live mass of the flies raised to the 0.856 power (Table S8), but also by 0.75 power ("Kleiber's exponent"), 0.67 power (which is related to the mass to surface-area relationship) or directly by mass (see Table S9 and S10).

	Units	Females	Males
Inactive MR at 25 ºC	**µl h^{-1}**	4.15 ± 0.20	3.04 ± 0.15
	µW	24.2 ± 1.17	17.7 ± 0.88
MMR	**µl h^{-1}**	8.55 ± 0.28	6.26 ± 0.28
	µW	49.87 ± 1.63	36.51 ± 1,63
Mass	**mg**	1.44 ± 0.22	1.06 ± 0.14
mass-independent inactive MR at 25 ºC	**µl h^{-1} mg$^{-0.856}$**	17.7 ± 0.85	16.8 ± 0.84
	µW mg$^{-0.856}$	103.25 ± 4.96	98.10 ± 4.90
mass-independent MMR	**µl h^{-1} mg$^{-0.856}$**	36.5 ± 1.63	34.7 ± 1.61
	µW mg$^{-0.856}$	212.9 ± 9.51	202.42 ± 9.39
Slope Ramping phase	**µl h^{-1} ºC^{-1}**	0.028 ± 0.001	0.037 ± 0.002
	µW ºC^{-1}	0.16 ± 0.005	0.21 ± 0,01
Q$_{10}$	**-**	1.98 ± 0.06	2.19 ± 0.07
Intercept Ramping phase	**(ºC)**	-0.171 ± 0.049	-0.341 ± 0.057

Table 1. Mean and SEM (standard error) values for females and males.

We did not find effects of anesthesia on ramping phase for both slope and intercept values, in this case no interaction between fixed factors were found (Table S8). As observed for values of thermotolerance differences between sexes were found in this phase with differences in both slope and intercept (Table S8). Males increase $\dot{V}CO_2$ in 0.037 µl h^{-1} for each degree of increasing temperature, which is faster than the rate of 0.028 µl hºC^{-1} exhibited by females (Table 1). This may indicate that the sensitivity of metabolic rate to temperature is higher in males than females. We confirmed this pattern by calculating the Q_{10} for each sex. As expected, males had a significantly higher Q_{10} (2.19) than females (1.98) (Table 1 and S5). This means that males had a more sensitive MR to temperature than females. We analyzed differences in locomotor activity across groups and sexes, using the activity index ADS activity min^{-1}. We found a significant effect of anesthesia on the locomotor activity of the flies (X^2=9.5279; P=0.023), but not between sexes (Table 1 and

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S8). In this case, the cold-anesthetized flies showed significantly lower activity than control flies (Tukey test, see Table S11 for mean values). Finally, we analyzed the relationship between $\bar{V}CO_2$ slope and activity of flies, and we did not find a significant correlation between these two variables (S=95606, P=0.08, rho=0.18; Spearman's rank correlation coefficient), suggesting that the significant increase of MR with temperature was not due to an increase in activity.

4. Discussion

The critical temperatures are the limits of the thermotolerance range of ectothermic organisms (Huey et al., 1992), and allow scientists to estimate their fundamental niche and potential geographic distribution changes due to global warming or anthropic disturbances (Addo-Bediako et al., 2000; de la Vega et al., 2015; de la Vega and Schilman, 2018; Coulin et al. 2019). This is particularly important for disease vectors, pests and invasive species, like *D. suzukii*. The use of anesthesia is a common practice for insect immobilization and handling. However, it has been shown that the use of anesthesia produces alterations in thermotolerance measurements (Nilson et al. 2006; MacMillan et al. 2017) that may lead to wrong estimations of current and future species distributions (de La Vega & Corley, 2019). In this study, we assessed, for the first time, the effects of brief anesthesia on thermal tolerance and metabolic rate in *D. suzukii*. All measurements were done right after anesthesia exposure. While cold exposure affects heat tolerance, but not cold tolerance or metabolic rate, anoxia does not affect any measurements. Interestingly, we found sexual dimorphism for thermotolerance traits, with females having a broader range of thermotolerance than males (*i.e.,* higher CTmax and lower CTmin) and lower sensitivity of MR to temperature.

4.1. Effects of anesthesia on thermotolerance and metabolic rate of *D. suzukii*

We exposed individual adults of both sexes of *D. suzukii* to cold anesthesia. This brief exposure to anesthesia did not produce any effects on aerobic metabolic rate (inactive MR and MMR) or cold tolerance (CTmin and CCRT), suggesting a lack of "rapid-cold hardening" effect (Czajka and Lee, 1990, Denlinger and Lee, 2010, Kelty and Lee, 2001; Lee et al., 1987). Also, cold exposure can alter metabolic homeostasis, for example, changing metabolic profile (Colinet and Renault, 2012; Overgaard et al., 2007) or changing transcription rate of genes as heat shock proteins to mitigate chill injury (Colinet et al., 2010; Enriquez and Colinet, 2019). However, we did not find any differences in the inactive or maximum metabolic rates. On the

other hand, cold anesthesia decreased CTmax compared to all the other groups. A brief exposure (1 minute) in a freezer at -22ºC was sufficient to decrease CTmax and motor activity, shown as significantly lower ADS activity in both sexes. These results could be due to chill injury caused by exposure to a low extreme temperature added to the stress produced by heat during CTmax assays. The drop in CTmax and activity due to anesthesia is not reflected on changes of the MR, suggesting an independence of aerobic metabolic pathways. Despite the costs observed in heat tolerance and activity, the flies did not reduce cold tolerance, which may be convenient to cope with the cold stress experienced.

Cold exposure affects locomotor activity in *D. suzukii* flies as seen in *D. melanogaster,* for example when flies experienced an exposure of 180 min to 0ºC (MacMillan et al., 2017) or when they are cold acclimated at 0ºC (Garcia and Teets, 2019). Locomotor activity is affected, probably because nerves and muscles suffer injury produced by the interruption of ion homeostasis in cells (Overgaard and MacMillan, 2017). It is important to mention that we did not find any evidence of an interacting effect between the anesthesia treatments and the sexes on thermotolerance or metabolic rate. Accordingly, in the case of heat tolerance, cold anesthesia negatively affects both sexes similarly. In summary, it is better a brief exposure to anoxia than use of cold anesthesia previously CTmax or motor activity measurements in *D. suzukii*.

Our results showed that a brief exposure to anoxia by CO_2 or N_2 did not affect either thermal tolerance traits nor activity and metabolic rate in *D. suzukii*. According to previous works, the reperfusion of oxygenated blood to ischemic organs after periods of anoxia happens in parallel with an overgeneration of reactive oxygen species, generally from mitochondria, and induction of lipid peroxidation, protein oxidation and DNA damage (Hermes-Lima and Zenteno-Savín 2002; Guzy et al. 2005). Similarly, repeated short pulses (1 minute) reperfusion followed by episodes of anoxia damaged the integrity of the spiracular control system in a dose-dependent fashion in *D. melanogaster* (Lighton and Schilman, 2007), with temperature, and thus indirectly of metabolic flux rates, strongly affecting both the rates of damage occurring in anoxia and the recovery processes in normoxia (Schilman et al., 2011). In *D. melanogaster*, anoxia affects thermotolerance detrimentally, with the effects of exposure to nitrogen being greater than those of carbon dioxide exposure (MacMillan et al., 2017; Milton and Partridge, 2008; Nilson et al., 2006). In addition, carbon dioxide exposure decreases the pH of hemolymph (Badre et al., 2005; Nicolas & Sillans, 1989), and leads to loss of metabolic homeostasis (Colinet & Renault, 2012). Thus, our results for *D. suzukii* did not show any effect of anoxia (100% N2) or anoxia + hypercapnia (100% CO2), suggesting that *D. suzukii* could be a more anoxia-tolerant species than *D. melanogaster* or our exposure time was not long enough to produce a detrimental effect. Overall, we can conclude, there is no evidence that a short anoxia

exposure affects neither thermotolerance nor metabolic rate in both sexes, for this reason the brief use of anoxia as anesthesia prior to these types of measurements could be employed. This finding is promising, as it indicates that subjecting the flies to one minute of anoxia results in an average anesthesia duration of three minutes (Table S3). This extended duration proves advantageous in situations that require prompt measurements following rapid manipulations, such as placing them in an experimental device or sex sorting.

4.2. Sexual dimorphism in thermotolerance and metabolic rate in *D. suzukii*

We found significant differences in thermotolerance between sexes in *D. suzukii*. Females showed a broader thermotolerance range than males by showing a higher CTmax and lower CTmin. Following the same tendency, results of CCRT showed that females recover faster from chill coma than males, although these differences were not statistically significant. Most of the previous studies of cold tolerance in *D. suzukii* analyzed survival after exposure to low temperatures. Some of them showed females having higher survival to cold than males (Dalton et al., 2011; Jakobs et al., 2015; Zerulla et al., 2015), while others found opposite results (Enriquez and Colinet, 2017), or no difference between sexes (Ryan et al., 2016). More recent studies assessed CTmin of *D. suzukii* using a dynamic method as we did in this work. For example, Enriquez and Colinet (2019) did not find differences between sexes, while Sato and Takahashi (2022) did, in accordance with our results. Although *D. suzukii* is a chill-susceptible species (Enriquez and Colinet, 2017), females could have physiological mechanisms to deal with events of low temperatures. Alternatively, but not mutually exclusive, we cannot rule out the possibility that *D. suzukii* flies survive the coldest events of winter using shelters as human constructions or leaf litter (Dalton et al., 2011; Enriquez and Colinet, 2017; Rossi-Stacconi et al., 2016; Zerulla et al., 2015). Recently, Panel, et al., (2020) suggest that *D. suzukii* females can store sperm over winter and start reproducing early in the following spring, as it was also shown in females of *Drosophila buzzati,* but not in its sibling species *Drosophila koepferae* (Kreiman et al., 2023). In addition, females of *D. suzukii* can enter into a state of reproductive arrest (Toxopeus et al., 2016; Wallingford et al., 2016) until spring arrives, and environmental temperatures are warm enough to lay eggs.

Up to now, heat tolerance *of D. suzukii* was assessed mainly on survival, development time and female fertility (Winkler et al., 2020). For example, Enriquez and Colinet (2017) showed better tolerances to high temperatures in females than males in terms of survival in the range of 34-37ºC. In accordance with our results, recent CTmax studies, using a dynamic method, showed higher values for females than for males (Sato and Takahashi, 2022; Xue and Ma,

2020), although CTmax values across studies were slightly different. Being 33 and 34ºC (Xue and Ma, 2020), 37.4 and 37.9 (present study) and 38.3 and 38.5 (Sato and Takahashi, 2022), for males and females, respectively. These differences could be explained by the different ramping rates used. As demonstrated for *D. melanogaster* by Chown et al. (2009) slower ramping rates lead to lower CTmax values. It should be noted that this is the first time that thermotolerance of *D. suzukii* was measured using lines from South America (Gandini et al., 2023). Despite the geographic variation across studies all these papers report sexual dimorphism in heat tolerance, being females more heat tolerant than males. These results suggest that such dimorphism is a species characteristic. This better heat tolerance could allow females to escape from heat stress and hide in cooler shelters. In addition, females have a better capacity to preserve fertility after heat stress than males (Kirk Green et al., 2019). It is important to note that the upper lethal temperature (ULT) is close to the CTmax. For example, in *D. melanogaster* applying a ramp of increasing temperature at a rate of 0.25ºC min^{-1}, the ULT is 1.35ºC above its CTmax (Rolandi et al., 2018). Together, the higher heat tolerance, survival and the lower fertility loss after thermal stress in females compared to males may allow population persistence following the frequent increase of extreme events, including heat waves predicted under global warming scenario (Easterling et al. 2000).

In relation to the metabolic rate, *D. suzukii* females showed higher inactive MR at 25ºC and maximum metabolic rates than males. These differences disappeared after mass correction, being the females bigger than males. Inactive MRs of *D. suzukii* are similar to the cosmopolitan species *D. melanogaster* (Lighton et al. 2004; Videlier et al. 2021; Brown et al. 2022). Our results showed a Q_{10} about 2, which is common for invertebrates, including *D. melanogaster* (Berrigan and Partrige, 1997), specifically a Q_{10} of 2.19 for males of *D. suzukii*, which is very close to the Q_{10} of 2.21 in males of *D.* melanogaster (Schilman et al., 2011). Interestingly, females showed lower Q_{10} values than males, consequently their MR are less sensitive to temperature change. In other words, female MR increases slower with temperature than MR of males. It is possible that temperature sensitivity could be related to the critical thermal limits. If that is the case, the slower increase of metabolic rate with temperature (lower Q_{10}), could maintain metabolic homeostasis up to higher temperatures preventing loss of motor control, thus evinced higher CTmax. However, further studies to test the relation between temperature sensitivity and critical thermal limits are required.

5. **Conclusions**

There is broad and rapidly growing interest in the use of *D. suzukii* as a model species in thermal biology. Up to now, the best practices after using anesthesia consisted of delaying thermo-tolerance measurements after 2-3 days of the application to wash away the carry-over

effects (Jakobs et al., 2015; Toxopeus et al., 2016). Our study, however, demonstrates that a very brief exposure to cold or anoxia anesthesia produces no deleterious effects in most of the thermal and metabolic measurements (except for CTmax after cold anesthesia), implying that our quick protocol can be a useful tool for the study of *D. suzukii*. This is particularly important when it is not possible to wait long enough after the application of anesthesia to perform the measurements. For example, when the insects are inside a device in which they cannot be left for many hours or days before the measurement, in the case that you want to take measurements of newly emerged flies as adults or for measuring thermo-tolerance traits in wild-caught flies during the collecting trips. Finally, our study revealed that females exhibit a broader thermotolerance range compared to males, characterized by higher CTmax and lower CTmin. This physiological advantage could contribute to their successful establishment in invaded temperate regions characterized by fluctuating environmental temperatures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are available on supplementary material.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Highlights

- *D. suzukii* females have broader thermotolerance range and lower Q_{10} than males
- Cold anesthesia detrimentally affects heat tolerance
- No effect of brief exposure to anesthesia on MR or cold tolerance
- Sex differences in MR is due to mass

St... /A...th...i...   M...   ...t   R...

Anesthe...
vs

Drosophila
suzuk

C

Co

Contro

CTmi → N eff

CTma → ↓ Co

CCR → N eff

M → N eff

→ N eff