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Life History Traits and Metabolic Pool Variation in Neotropical Species of *Drosophila* (Diptera, Drosophilidae)

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The differential exploration of natural resources by *Drosophila* species has effects on fitness, with changes in life history and metabolic traits. There is a lack of research on the variation in these characters in different environments in Neotropical species of *Drosophila*. The purpose of this study was to evaluate the profile of life history traits, including viability, development time, and dry weight (as a measure of size), as well as the metabolic pools of triglyceride, glycogen, and protein, in populations from the southern and southeastern regions of Brazil of four Neotropical *Drosophila* species: *D. willistoni*, of the *Sophophora* subgenus, and *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*, which belong to the *Drosophila* subgenus. Life history and metabolic traits showed interpopulational variation in at least one species. When significant differences in life history parameters occurred, species of the same subgenus presented similar profiles, *i.e.*, southern populations were larger, less viable, and showed longer development time. This was also observed for triglyceride. However, for the other two metabolic pools (glycogen and total proteins), *D. maculifrons* and *D. ornatifrons* presented inverse patterns to the other two species, with the highest values in southeastern populations and the lowest in southern populations. These populational variations indicate plasticity of the examined life history traits, which allows distinctive responses to different environmental conditions shared by species of the same subgenus. Nevertheless, interspecific comparisons did not reflect phylogenetic relationships, with the highest viability being found for *D. willistoni* and *D. mercatorum*, which is probably correlated to the ability of these species to explore a broader variety of habitats. On the other hand, the storage capability of metabolic pools seems to be species specific, determined by the adaptive history to the quality and availability of resources, with *D. mercatorum* (low) and *D. ornatifrons* (high) having opposing capacities to store metabolites from their diets.

Key words: Viability, Development time, Dry weight, Adaptive traits, Energy storage molecules

BACKGROUND

Life history traits, such as viability, development time and weight, and the concentration of metabolites in an organism, are all quantitative characteristics resulting from the interaction between the genotype and the environment. These traits are influenced by several environmental variables, as well as internal factors, such as the stress levels, immunological system activity, composition of the intestinal microbiome, and quality

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and caloric content of the diet, among others (Rose 1983; Hoffmann and Parsons 1989; Partridge and Sibly 1991; Rose and Bradley 1998; Matzkin et al. 2009 2011; Jumbo-Lucioni et al. 2010; Jehrke et al. 2018; Flatt 2020). The result of the joint action of all these parameters determines the fitness of the individuals. Thus, research on these relationships is essential to understand the adaptive process and evolution of life history traits of a species.

Matzkin et al. (2009) studied the variation in the metabolic pools of twelve ecologically divergent *Drosophila* species with sequenced genomes. Nine of these species belonged to the *Sophophora* subgenus, and three to the *Drosophila* subgenus, two of which are cactophilic. Therefore, there is a gap for non-cactophilic Neotropical models of the *Drosophila* subgenus, including the analysis of interpopulational variation in these characteristics in populations from different types of habitats for both subgenera of this region, where the process of adaptive divergence could be accentuated by the variety of resources and accessible habitats. One of the conclusions of Matzkin et al. (2009) indicated that although the control of the analyzed metabolites may be similar among species, it appears to be evolutionarily plastic, being able to reflect the response to nutritional necessities of populations.

In this context, in order to shed some light on this matter and to increase sampling for species in other regions, the current study analyzed metabolism variation and life history traits in four non-cactophilic *Drosophila* species native to South America, which were recently collected from different natural areas of the Brazilian Atlantic Forest. Three of the analyzed species belong to the *Drosophila* subgenus: *D. mercatorum* (*repleta* group), *D. maculifrons* (*guaramunu* group, Robe et al. 2010), and *D. ornatifrons* (*guarani* group, Robe et al. 2010). These species are more closely associated with natural areas of the Neotropical region, the first being found in a broader diversity of environments (open areas and forests, frequently containing enclaves of xerophytic vegetation), and the last two in forest fragments. We also analyzed one species from the *Sophophora* subgenus, *Drosophila willistoni*, which, in contrast to the other studied species, also inhabits anthropized environments.

The *Drosophila* populations of this work were collected from the Brazilian southeastern and southern regions. The first region is in the tropical zone and the second is in the subtropical zone. These regions have distinctive Atlantic Forest phytophysiognomies and climate conditions. In the Southeast, seasonal semideciduous forest (SSF) phytophysiognomy of Atlantic Forest is the main type of vegetation, characterized most of the year by higher temperatures and two annual distinctive seasons, wet and dry. In the South region, another Atlantic Forest phytophysiognomy can be detected, mixed ombrophilous forest (MOF), with a predominance of Araucaria pine (*Araucaria angustifolia*). Part of this forest is known for its high altitude, rigorous winters with frequent frost, high rainfall, and high relative humidity rates. However, in the southern region, the SSF can also be found as fragments within the Pampa Biome. In this case, it has intermediate climate conditions from the southeastern SSF and southern MOF (Instituto Brasileiro de Geografia e Estatística 1992; Backes 1999; Oliveira-Filho et al. 2015; Instituto Nacional de Meteorologia 2019).

Furthermore, the species selected for this study belong to groups of the most generalist Neotropical species, with high ecological versatility for breeding sites in different plant tissues, fungi, and even dung and carrion (Val et al. 1981; Pereira et al. 1983; Medeiros and Klaczko 2004; Mateus et al. 2006 2018; Gottschalk et al. 2007 2009; Döge et al. 2008; Hochmüller et al. 2010; Goñi et al. 2012; Cavasini et al. 2014; Coutinho-Silva et al. 2017; Mendes et al. 2017; Valadão et al. 2019; TaxoDros 2022). The applied approach allows the inference of which factors related to adaptation and historical evolution could be important to determine the extent of resource usage and habitat occupation of a species.

Considering the variety of resources, types of habitats and climate conditions available in the different regions of the Brazilian Atlantic Forest, the main purpose of this study was to evaluate intra and interspecific differences in classical life history traits (viability, development time, dry weight) and metabolic response (triglyceride, glycogen, and protein contents) in Neotropical species of *Drosophila*. Ultimately, all analyzed characteristics demonstrated different populational adaptive responses in at least one species. *Drosophila mercatorum*, *D. maculifrons*, and *D. ornatifrons*, species from the *Drosophila* subgenus and associated with natural environments, showed a similar populational pattern for most of the studied traits. However, the interspecific comparison did not result in the same pattern, *i.e.*, *D. willistoni* and *D. mercatorum* were similar regarding life history traits, and *D. willistoni* presented metabolic pools similar to *D. ornatifrons* and *D. maculifrons*. The possible role of the environment in the adaptive response of the examined characteristics and the capability of resource exploration demonstrated by the species are discussed.

MATERIALS AND METHODS

Collection areas

Drosophilids were sampled in five areas of the Atlantic Forest in the southern and southeastern regions of Brazil, with distinctive climatic and phytophysiognomic characteristics. The collections occurred in two sites of the southeastern region, Serrana and Cajuru, in the state of São Paulo, and in three southern sites, Guarapuava, in the state of Paraná, and Santiago and Porto Alegre in the state of Rio Grande do Sul (Fig. 1). The distance between the closest populations from the southeastern and southern regions (Serrana-SP and Guarapuava-PR) is approximately 600 km, in a straight line, and these areas are divided by the Tropic of Capricorn, which determines, besides different vegetation landscapes, distinct climate conditions: southeastern populations are in the Tropical zone and southern populations are in the Subtropical zone. Additional information about each area is described below:

1) Serrana-SP (SER) – fragment of the Seasonal Semideciduous Forest with xerophytic vegetation; average temperature (T°) = 22.7°C (minimum – MIN = 15.6°C; maximum – MAX = 30.4°C); average relative humidity (RH) = 67%. Summer is warm and humid and winter is characterized by long periods without precipitation;

2) Fazenda Santa Cecília, Cajuru-SP (CAJ) – 32 km away in a straight line from SER, fragment of the Seasonal Semideciduous Forest without xerophytic vegetation; T°, RH, and seasonality data are the same as described for SER;

3) Parque Municipal das Araucárias, Guarapuava-PR (PMA) – fragment of the Mixed Ombrophilous Forest (*Araucaria* Forest), without xerophytic vegetation; T° = 17.6°C (MIN = 13.4°C; MAX = 24.1°C); RH = 82.2%. Mild temperatures characterize summer, and frequent and severe frosts occur in autumn and winter;

4) Santiago-RS (SAN) – fragment of the Seasonal Semideciduous Forest with xerophytic vegetation, inside Pampa biome; T° = 18.9°C (MIN = 14.4°C; MAX = 24.9°C); RH = 74.8%. Region with humid subtropical climate, no defined dry season;

5) Morro Santana, *Campus* of Universidade Federal do Rio Grande do Sul, Porto Alegre-RS (POA) – 380 km away in a straight line from SAN, fragment of the Seasonal Semideciduous Forest without xerophytic vegetation, inside Pampa Biome; T° = 20.1°C (MIN = 16.1°C; MAX = 25.8°C); RH = 77.1%. Region with

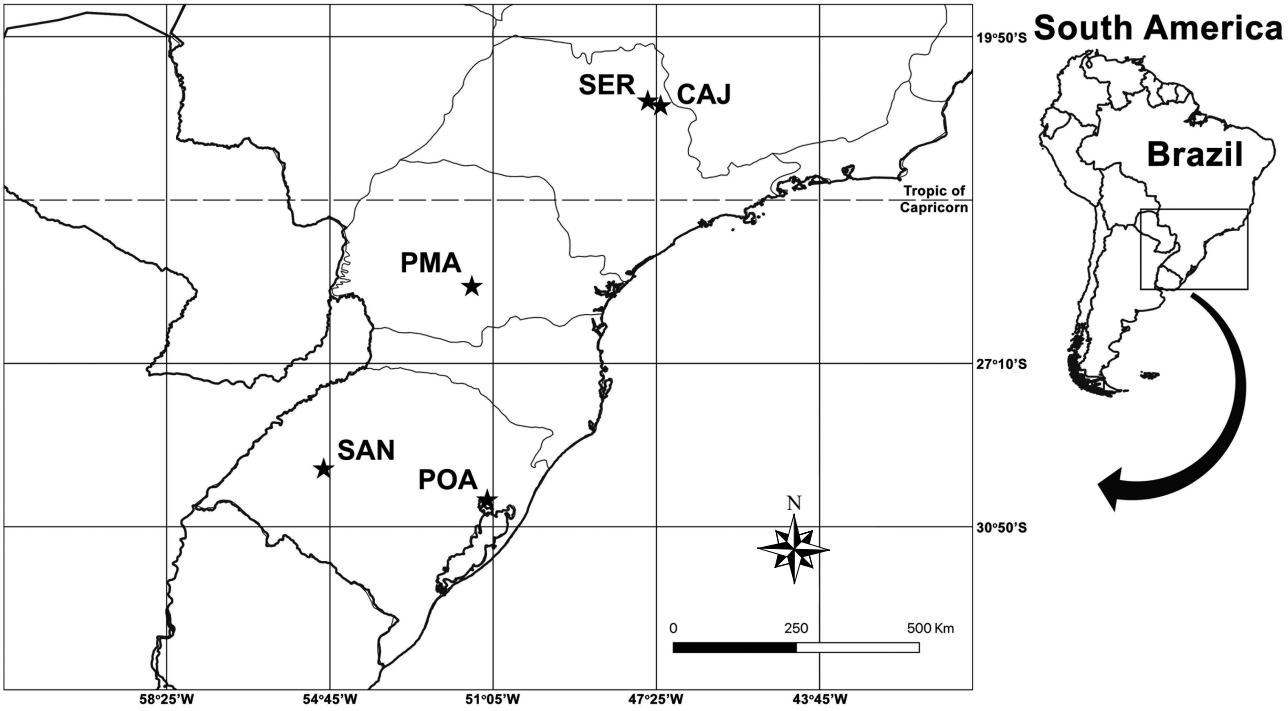

Fig. 1. Map of the collection areas of *Drosophila* populations from which isofemale lines were obtained and analyzed. The locations are described in the Material and Methods section. SER: 21°15'15.23"S, 47°34'34.95"W - Altitude 830 m; CAJ: 21°21'35.45"S, 47°17'32.89"W - Altitude 830 m; PMA: 25°21'3.23"S, 51°28'4.41"W - Altitude: 1,000 m; SAN: 29°23'0.54"S, 54°45'41.58"W - Altitude: 135 m; POA: 30°4'9.94"S, 51°7'36.34"W - Altitude: 115 m.

humid subtropical climate, no defined dry season.

The data for temperature and relative humidity refer to the average in the period between January 2009 and December 2018 (Instituto Nacional de Meteorologia 2019).

In the current study, *D. mercatorum* isofemale lines (referred to as populations throughout this paper) from all collection areas, except from PMA, were utilized. The analyzed *Drosophila willistoni* populations were from CAJ, PMA, and POA; and for *D. maculifrons* and *D. ornatifrons*, the populations were from CAJ and PMA. The collections were performed between February and April 2018 and the experiments were initiated in the same year, as soon as isofemale lines were established in laboratory conditions, around three generations.

Experimental design

Between 50 and 80 sexually mature female and male virgins of each population were put in embryonic chambers containing a Petri dish with agar 0.5% and enriched with *Saccharomyces cerevisiae* and sucrose to induce the larvae to hatch. Daily, dishes with agar were replaced and the removed dishes that contained eggs were stored. At 48h (*D. willistoni* and *D. mercatorum*), and 144h (*D. maculifrons* and *D. ornatifrons*) after mating, 2nd instar F1 larvae were transferred to vials with a standard banana diet. Ten replicates for each population of the four species were obtained, each containing 40 (*D. willistoni* and *D. mercatorum*) and 30 (*D. maculifrons* and *D. ornatifrons*) larvae. The experiments and *D. willistoni* and *D. mercatorum* isofemale line maintenance were performed at 25°C ± 1°C, and at 20°C ± 1°C for *D. maculifrons* and *D. ornatifrons*, all in a natural photoperiod. These experimental incubation temperatures followed previous knowledge about the best adaptation conditions for each species to laboratory conditions, which provides higher fitness for each species.

The *Drosophila* groups of the four species analyzed in this study exhibit a broader amplitude of resource usage among drosophilids captured with fruit baits (Valadão et al. 2019). Therefore, we assumed that the standard banana diet utilized in the experiments should not be a stressful factor for larval development; and the differences in the examined traits would reflect the adaptation to the collection environment, rather than a response to a resource different to those found in nature.

Analysis of life history traits

From larvae to emerged adults, viability (VI) and development time (DT) were estimated for each of the four populations of the four species. VI was expressed as the proportion of larvae that survived until the adult stage, and DT was measured as the average period elapsed (in hours) between the transference of second instar larvae to the culture medium and the appearance of adults (males and females). The observations were carried out every four hours after the emergence of the first adults. All the emerged adults were separated by sex and stored at -20°C for posterior analysis of the metabolic pools.

Dry weight (DW) was determined in 10 groups for each population and each sex of the four species. Each group contained five (for *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*) or 15 flies (for *D. willistoni*). The higher number for *D. willistoni* is due to its smaller size, requiring more individuals to be able to establish the weight. Each group of flies was incubated at 50°C in an oven for three days, and then weighed in a Shimadzu micro scale, model AY 220. The weight obtained for each group was then divided by the number of individuals in the group.

Analysis of metabolic pools

The metabolic pool analyses of the dried groups of flies were performed according to Matzkin et al. (2009). Each group of flies was homogenized in 1 mL of phosphate buffer (25 mM $KHPO_4$, pH 7.4) and centrifuged for 2 minutes at 12,000 rpm in order to remove particles that could interfere with the colorimetric tests. A total of 800 μL of the supernatant from the homogenized mixture was collected and stored at -20°C for later analysis of the metabolic pools.

The colorimetric examinations were performed for the quantification of glycogen (GL), triglyceride (TG), and total soluble protein (PR) contents. GL levels were measured using a Glucose Oxidase and Peroxidase enzyme kit (Sigma-Aldrich P7119), adding 0.1 units of Amyloglucosidase (Sigma-Aldrich) per mL of reaction buffer. The samples (40 μL of the homogenized mixture + 200 μL of the reaction buffer) were incubated at 37°C for three hours, and the absorbance was measured at 445 nm. TG content was determined using a Triglycerides kit (Gold Analisa REF. 459, MS 80022230062). The samples (40 μL of the homogenized mixture + 200 μL of reagent kit) were incubated at 37°C for 30 minutes, and the absorbance was measured at 500 nm. Only the triglyceride analysis was performed, and not the total lipids (which include cuticular lipids), due to the fact that the objective of this study was to evaluate the components involved in storing energy. The PR concentration was determined using the Bicinchoninic acid assay following the instructions of

the manufacturer (Sigma B9643), and the absorbance for quantifying the proteins was measured at 562 nm. These measurements were performed in the Spectramax 190 spectrophotometer from Molecular Devices. Each metabolic grouping was calculated by the average of triplicates and normalized by DW before the statistical analyses.

Statistical analyses

The VI, DT, and metabolic pool data were examined by the multifactorial analysis of variance (ANOVA) method, utilizing population and sex as factors. We also used temperature as a factor in the DT analysis because two culture temperatures were applied in different species. The comparison between the species was carried out using nested ANOVA of populations within species. Paired comparisons between fixed factors were carried out by applying Tukey *post-hoc* analysis. All data were transformed before analysis: VI – arc sine of square root; DT and DW – square root; and metabolic pools (TG, GL, and PR) – arc sine. All statistical examinations were performed in Statistica 7 (StatSoft, Inc.) software, using $\alpha = 0.05$ (Sokal and Rohlf 1995).

RESULTS

Viability (VI)

A significant difference in viability among populations within species was found only for *D. ornatifrons* (Fig. 2), with PMA being significantly less viable than CAJ ($F = 147.00$, $p \leq 0.001$). The interspecific comparison with nested populations within species showed a significant difference and similar viability for *D. willistoni* and *D. mercatorum*, with values significantly higher than the similarly viable *D. maculifrons* and *D. ornatifrons* (Table 1).

Development time (DT)

A significant difference in development time between males and females was found only in the PMA population of *D. willistoni*, in which the females presented faster DT than the males ($F = 5.454$, $p = 0.002$). Among populations within species, except for *D. willistoni*, the populations in lower latitudes (southeastern populations) tended to have shorter development time. There was significant variance in *D. mercatorum* ($F = 1{,}641$, $p = 0$): POA with the longest DT, followed by SAN, and SER with the shortest DT ($p < 0.001$ for all paired comparisons). In *D. maculifrons* and *D. ornatifrons*, PMA showed significantly higher DT than CAJ ($F = 292.15$, $p \leq 0.001$; $F = 451.53$, $p \leq 0.001$, respectively), which had similar DT in both species (Fig. 3).

The general comparative analysis revealed different DTs among species, populations nested within species, and temperature, between sexes, and in the interaction between sex and nested populations (Table 2). The paired comparison revealed that *D. willistoni* has a significantly faster development time, followed

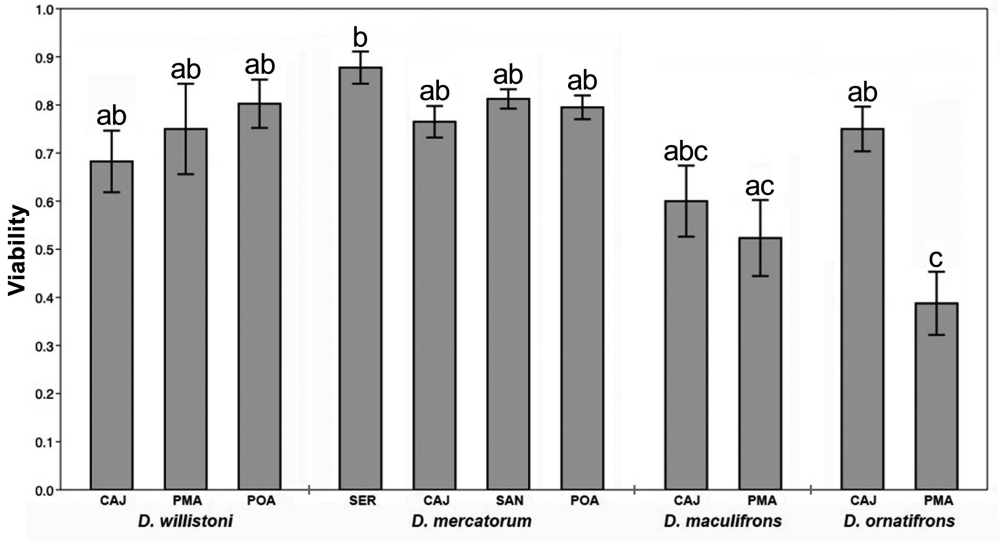

Fig. 2. Viability of second instar larva to adult for populations of *Drosophila willistoni* (CAJ, PMA and POA), *D. mercatorum* (SER, CAJ, SAN and POA), *D. maculifrons* (CAJ and PMA), and *D. ornatifrons* (CAJ and PMA). Viability was calculated as the emerged adults/total larvae ratio. Different letters above the bars indicate significant differences at a *p* value of 0.05 determined by the ANOVA followed by Tukey *post-hoc* test. Error bars represent standard error of the mean.

by *D. mercatorum*, while both *D. maculifrons* and *D. ornatifrons*, were similarly slower (Fig. 3, Table 2).

Dry weight (DW) of adults

Females were larger than males in some comparisons: combining the three populations of *D. willistoni* (F = 16.96, $p < 0.001$); SER (Tukey $p < 0.001$) and CAJ (Tukey $p < 0.05$) of *D. mercatorum*; and CAJ of *D. maculifrons* (Tukey $p < 0.05$). The population/sex interaction was significantly different only in *D. mercatorum* (F = 6.36, $p < 0.001$). Only *D. willistoni* did not show a significant difference in DW among populations (*D. mercatorum* - F = 13.64, $p = 0$; *D.*

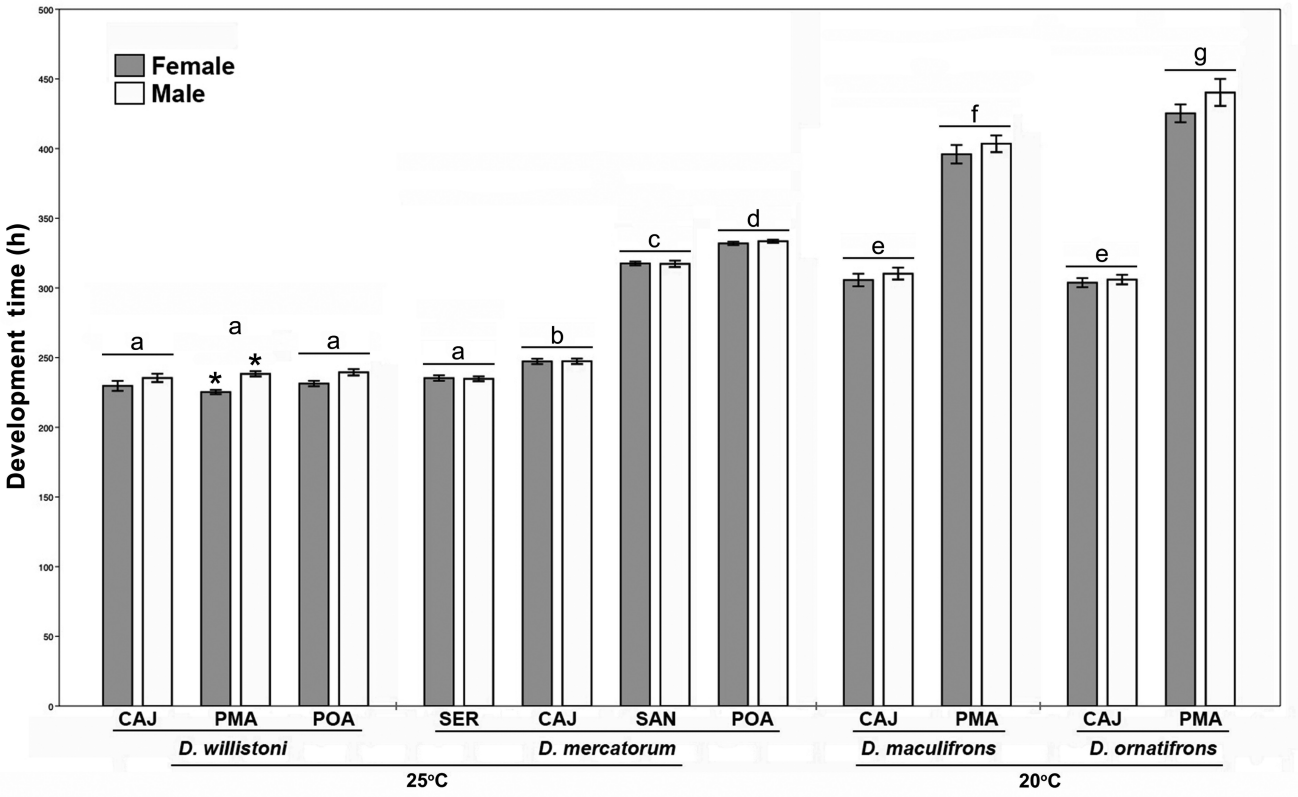

Fig. 3. Development time from second instar larva to emerged adults for populations of *Drosophila willistoni* (CAJ, PMA and POA), *D. mercatorum* (SER, CAJ, SAN and POA), *D. maculifrons* (CAJ and PMA), and *D. ornatifrons* (CAJ and PMA). The development times were measured in hours. Lines above bars group statistically similar values. Asterisks above bars and different letters above lines indicate significant differences (sex and populational, respectively) at a *p* value of 0.05, determined by the ANOVA followed by Tukey *post-hoc* test. Error bars represent standard error of the mean.

Table 1. Nested ANOVA (1) and *post-hoc* Tukey test (2) of viability for *Drosophila willistoni*, *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*

1	SS	MS	DF	F
Species	1.8209	0. 6070	3	10.298***
Population (Species)	1.1503	0.1643	7	2.788**
Error	5.8349	0.0589	99	
2	*D. willistoni*	*D. mercatorum*	*D. maculifrons*	
D. mercatorum	0.6733			
D. maculifrons	4.653**	5.499***		
D. ornatifrons	4.866**	5.723***	0.1943	

SS = sum of squares; MS = mean squares; DF = degrees of freedom. *$p \leq 0.05$; ***$p \leq 0.01$; ***$p \leq 0.001$.

maculifrons - F = 10.164, $p = 0.003$; *D. ornatifrons* - F = 30.256, $p < 0.001$), with southern populations being larger than the southeastern ones (Fig. 4).

Dry weight showed significant differences for species, populations nested in species, sex, the interaction of populations nested in species with sex, and in all paired species comparisons. *Drosophila mercatorum* was the largest species, followed in order of size by *D. ornatifrons*, *D. maculifrons*, and *D. willistoni* (Table 3, Fig. 4).

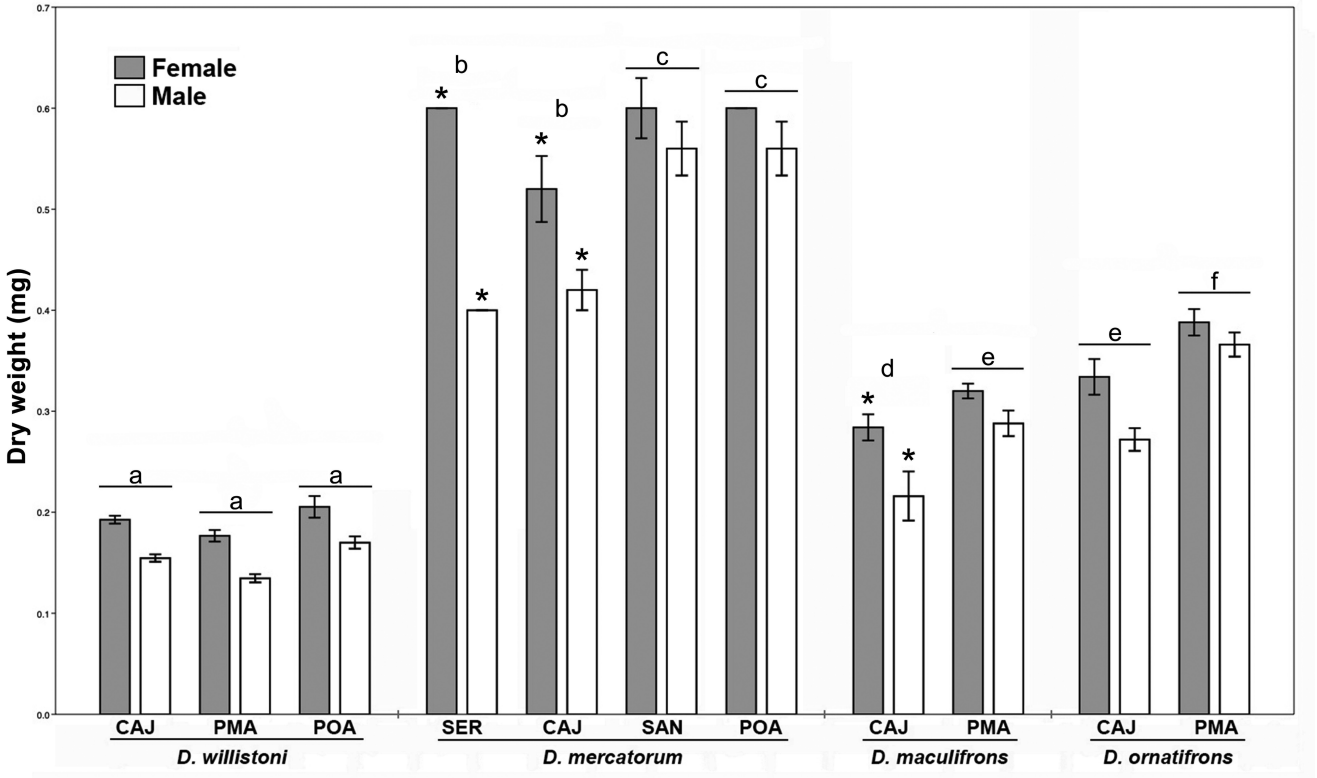

Fig. 4. Dry weights in milligrams of adult females and males from populations of *Drosophila willistoni* (CAJ, PMA and POA), *D. mercatorum* (SER, CAJ, SAN and POA), *D. maculifrons* (CAJ and PMA), and *D. ornatifrons* (CAJ and PMA). Individual dry weights were calculated from samples of 15 recently emerged flies of *D. willistoni*, and 5 recently emerged flies of *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*. Lines above bars group statistically similar values. Asterisks above bars and different letters above lines indicate significant differences (sex and populational, respectively) at a p value of 0.05, determined by the ANOVA followed by Tukey *post-hoc* test. Error bars represent standard error of the mean.

Table 2. Nested ANOVA (1) and *post-hoc* Tukey test (2) of development time for *Drosophila willistoni*, *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*

1	SS	MS	DF	F
Species (Temperature)	4963.1	1654.4	3	2061.6***
Population (Species (Temperature))	3433.1	490.4	7	611.2***
Sex	14.4	14.4	1	18.0***
Population (Species (Temperature)) x Sex	15.3	1.5	10	1.9*
Error	2290.2	0.8	2854	

2	*D. willistoni*	*D. mercatorum*	*D. maculifrons*
D. mercatorum	34.5***		
D. maculifrons	54.14***	31.84***	
D. ornatifrons	51.63***	29.39***	1.719

SS = sum of squares; MS = mean squares; DF = degrees of freedom. *$p \leq 0.05$; ***$p \leq 0.01$; ***$p \leq 0.001$.

Metabolic pools

Triglyceride (TG)

Differences between sexes were detected only in the PMA population of *D. maculifrons*; females showed significantly higher TG contents than males (Tukey $p < 0.01$). Differences among populations were observed for *D. mercatorum* ($F = 166.741$, $p = 0$), *D. maculifrons* ($F = 5.943$, $p < 0.01$), and *D. ornatifrons* ($F = 17.6531$, $p < 0.001$), evidently because of southern versus southeastern population comparisons. For these species, populations from the South of Brazil had higher TG content than populations in the southeastern region. Furthermore, in the case of *D. mercatorum*, no significant differences were detected between populations of the same region, *i.e.*, between SER and CAJ (from the southeastern region), and between SAN and POA populations (from the southern region) (Fig. 5A).

There was a significant difference in TG content among species, populations nested in species, and in the interaction of populations nested in species with sex. The paired comparison revealed that *D. maculifrons* presented, in average, the highest TG content, followed by *D. ornatifrons*, and by *D. willistoni* and *D. mercatorum*, which showed similarly lower values (Fig. 5A, Table 4).

Glycogen (GL)

In the comparisons between sexes within populations, only *D. maculifrons* from PMA showed females with significantly higher concentrations of GL than males (Fig. 5B). No other comparisons resulted in a significant difference in this metabolite content between sexes and in the population/sex interaction. There was a significant difference in GL content among populations in *D. willistoni* ($F = 35.636$, $p = 0$), *D. mercatorum* ($F = 27.6538$, $p = 0$), and *D. maculifrons* ($F = 18.9932$, $p < 0.001$). *Drosophila willistoni* and *D. mercatorum* presented the same populational pattern, with southern region populations (PMA and POA of *D. willistoni*, SAN and POA of *D. mercatorum*) presenting higher GL contents than southeastern region populations, with no difference between populations of the same region. On the other hand, *Drosophila maculifrons* displayed the opposite pattern compared to the first two species: The southeastern population (CAJ) showed higher GL content than the southern population (PMA). Though *D. ornatifrons* showed a similar pattern (Fig. 5B), the difference between its populations was not significant.

There were significant differences in GL content among species (Table 4); the paired comparison revealed that *D. mercatorum* has a significantly lower concentration of this metabolite in relation to the other species, mostly because of the southeastern populations, which were statistically different to all others (Fig. 5B). There was also significance in the comparison of populations nested in the species, between sexes, and in the interactions of populations nested in species with sex (Table 4).

Total proteins (PR)

The PR content was similar between sexes within populations of all species. Regarding populations within species, the same pattern observed for GL was partially detected for PR, *i.e.*, higher concentration in southern when compared to southeastern populations of *D. willistoni* and *D. mercatorum*, and the opposite for the other two species (Fig. 5C). For *D. willistoni*,

Table 3. Nested ANOVA (1) and *post-hoc* Tukey test (2) of dry weight for *Drosophila willistoni*, *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*

1	SS	MS	DF	F
Species	3.50758	1.16919	3	646.15***
Population (Species)	0.18371	0.02624	7	14.50***
Sex	0.15639	0.15639	1	86.43***
Population (Species) x Sex	0.06070	0.00607	10	3.35***
Error	0.35828	0.00181	198	
2	*D. willistoni*	*D. mercatorum*	*D. maculifrons*	
D. mercatorum	43.68***			
D. maculifrons	12.67***	25.17***		
D. ornatifrons	19.53***	17.94***	6.267***	

SS = sum of squares; MS = mean squares; DF = degrees of freedom. *$p \leq 0.05$; ***$p \leq 0.01$; ***$p \leq 0.001$.

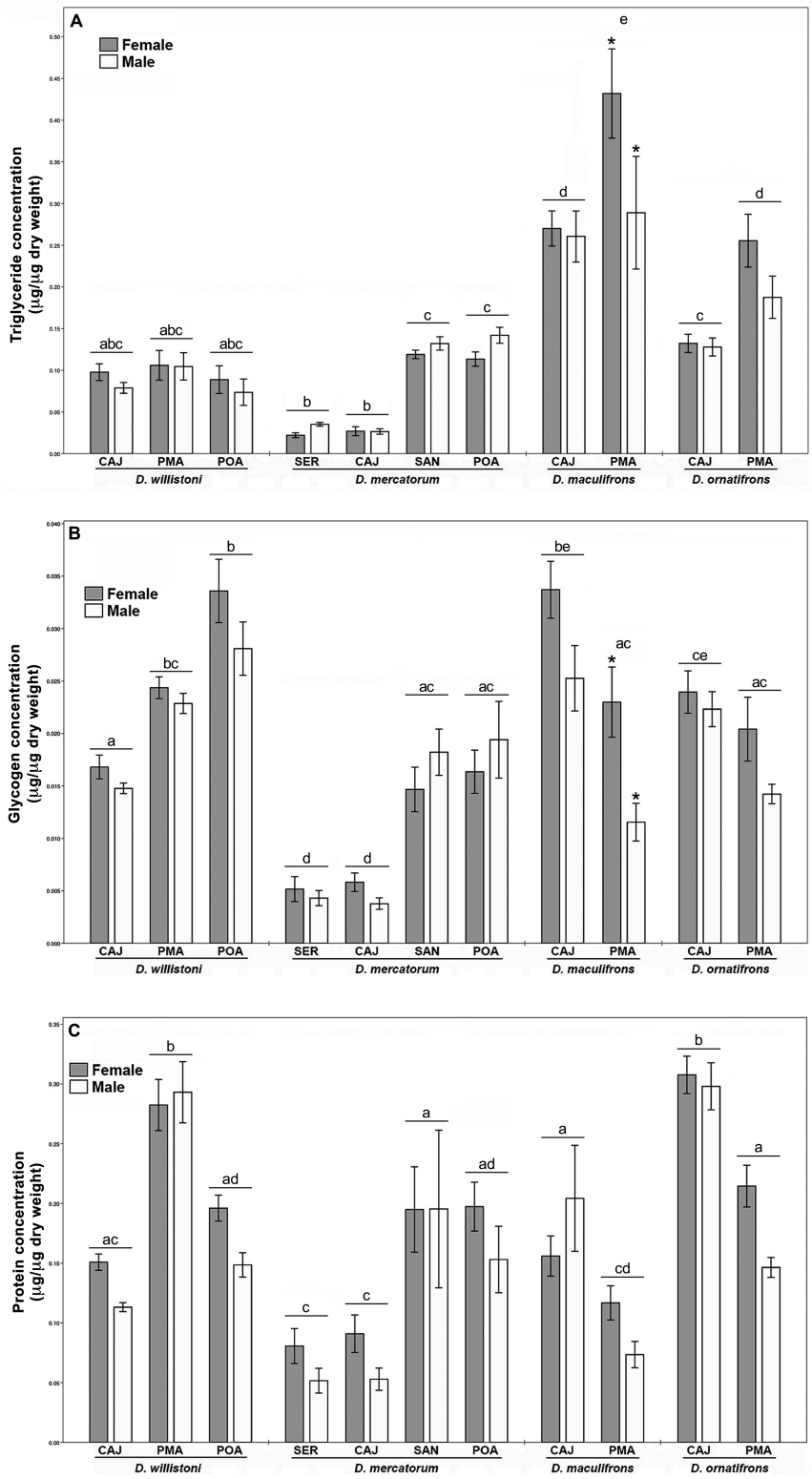

Fig. 5. Triglycerides (A), Glycogen (B), and Total Protein (C) concentrations per mg of dry weight of adult females and males from populations of *Drosophila willistoni* (CAJ, PMA, and POA), *D. mercatorum* (SER, CAJ, SAN, and POA), *D. maculifrons* (CAJ and PMA), and *D. ornatifrons* (CAJ and PMA). Values are means and SE for homogenates of 15 flies for *D. willistoni* and 5 flies for *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*. Lines above bars group statistically similar values. Asterisks above bars and different letters above lines indicate significant differences (sex and populational, respectively) at a *p* value of 0.05, determined by the ANOVA followed by Tukey *post-hoc* test. Error bars represent standard error of the mean.

only CAJ (southeastern population) and PMA (southern population) were different (F = 8.94, $p < 0.001$). The decreasing order of PR contents for the analyzed populations of this species was PMA, POA, both from southern region, and CAJ, from the southeastern region. For *D. mercatorum*, both southern populations presented higher PR concentration than southeastern ones (CAJ and POA: F = 5.707, $p < 0.01$; CAJ and SAN: F = 7.03, $p < 0.001$; SER and POA: F = 5.859, $p < 0.01$; SER and SAN: F = 7.144, $p < 0.001$), and the content of this metabolite was not different between southern populations (SAN and POA), or between southeastern populations, (CAJ and SER). For *D. maculifrons* and *D. ornatifrons*, the southeastern population (CAJ) showed higher PR content in relation to the southern (PMA) population (F = 11.0034, $p < 0.01$; F = 58.7521, $p = 0$, respectively).

There was a significant difference in average PR content among species. The paired comparison revealed a higher concentration of this metabolite in *D. ornatifrons*, followed by *D. willistoni*, whilst *D. mercatorum* and *D. maculifrons* showed similar PT contents. There was also a significant difference in the comparison of populations nested in species and between sexes (Table 4).

DISCUSSION

Among the life history traits evaluated, viability was the only one that did not show populational variation for most of the analyzed species (except for *D. ornatifrons*). On the other hand, development time and dry weight were similar among populations only for *D. willistoni*. Metabolic pools also presented consistent populational differentiation, except for triglyceride contents among populations of *D. willistoni*. When significant differences between populations occurred,

Table 4. Nested ANOVA (1) and *post-hoc* Tukey test (2) of Triglycerides, Glycogen, and Total Protein contents per mg of dry weight for *Drosophila willistoni*, *D. mercatorum*, *D. maculifrons*, and *D. ornatifrons*

1		SS	MS	DF	F
Triglyceride	Species	1.854886	0.618295	3	99.9980***
	Population (Species)	0.383478	0.054783	7	8.8601***
	Sex	0.018527	0.018527	1	2.9964
	Population (Species) x Sex	0.128304	0.012830	10	2.0751*
	Error	1.193334	0.006183	193	
Glycogen	Species	0.007034	0.002345	3	52.199***
	Population (Species)	0.007149	0.001021	7	22.737***
	Sex	0.000481	0.000481	1	10.699**
	Population (Species) x Sex	0.001025	0.000103	10	2.283*
	Error	0.008714	0.000045	194	
Total Protein	Species	0.442345	0.147448	3	23.254***
	Population (Species)	0.788636	0.112662	7	17.768***
	Sex	0.028351	0.028351	1	4.471*
	Population (Species) x Sex	0.059537	0.005954	10	0.939
	Error	1.230123	0.006341	194	

2		*D. willistoni*	*D. mercatorum*	*D. maculifrons*
Triglyceride	*D. mercatorum*	1.319		
	D. maculifrons	17.84***	20.01***	
	D. ornatifrons	6.295***	7.779***	10.29***
Glycogen	*D. mercatorum*	11.42***		
	D. maculifrons	0.0285	10.04***	
	D. ornatifrons	1.87	7.753***	1.691
Total Protein	*D. mercatorum*	5.504***		
	D. maculifrons	4.215***	0.4611	
	D. ornatifrons	3.162*	8.17***	6.734***

SS = sum of squares; MS = mean squares; DF = degrees of freedom. *$p \leq 0.05$; ***$p \leq 0.01$; ***$p \leq 0.001$.

D. maculifrons and *D. ornatifrons* presented the same variation profile for all analyzed characteristics, *i.e.*, the southern populations showed the longest development time, the highest dry weight and triglyceride, and the lowest glycogen and protein content. *Drosophila willistoni* and *D. mercatorum* were also similar for some of the analyzed traits (such as highest levels of viability and lowest development time and triglycerides). The species of the *Drosophila* subgenus, *D. maculifrons*, *D. ornatifrons*, and *D. mercatorum*, presented the same populational profile, except for glycogen and protein levels. For these metabolic molecules, *D. mercatorum* showed populational variation similar to *D. willistoni*, with higher contents in southern populations. Dry weight was the only parameter analyzed that was significantly different among all species. *Drosophila ornatifrons* was the species with the highest energetic molecule storage capacity, while *D. mercatorum* had the lowest.

Even though the analyzed species are generalist, some habitat features, such as abiotic factors and food and breeding sources, associated with vegetation type, can also influence the breadth of geographic distribution. The populational differences in all traits in *D. mercatorum* and *D. maculifrons* did not cause differential viability within these species, as they did in *D. ornatifrons*. Also, *D. mercatorum* and *D. willistoni* were similarly viable, suggesting more stable adaptive pathways for these species compared to *D. maculifrons* and *D. ornatifrons*. The lower viability of *D. maculifrons* and *D. ornatifrons* could be a result of rarer and/or non-usual preferential/ideal dietary requirements in nature, with nutritional content specificity distinct from *D. willistoni* and *D. mercatorum*. This fact is particularly evident in *D. ornatifrons*, which was the only analyzed species with populational variation in viability (lower in the southern region, PMA), and was also the only analyzed species that was captured in dung and carrion (Goñi et al. 2012).

According to the points highlighted above, *D. willistoni* showed populational variation only in glycogen and protein contents, with *D. mercatorum* exhibiting a similar pattern (higher values in the southern), and *D. malculifrons* and *D. ornatifrons* displaying an opposite one (higher values in the southeastern population). These different populational responses in areas where the species are sympatric can be explained by the differential resource preferences of each species in these locations. Matzkin et al. (2009) argued that the difference in metabolic pools between species of the *Sophophora* and *Drosophila* subgenera are a result of a tendency of the *Sophophora* subgenus species to consume decomposing fruits, while species of the *Drosophila* subgenus feed on yeast that develops not only in fruits, but also in other parts of decomposing plants. However, the Neotropical *D. willistoni* was also found in fungi, in addition to fruits (Gottschalk et al. 2009). Therefore, the species and the amount of yeast biomass that occurs in these substrates are probably different, and may be related to the variety of hosts that can be explored, as well as the different population responses of life history traits and metabolic pools discussed previously (Gottschalk et al. 2009; Anagnostou et al. 2010; Valadão et al. 2019; Koerte et al. 2020).

The development time is a plastic characteristic, sensitive to selective pressures like biotic factors, such as intra and interspecific competition, density, and desiccation, and also abiotic factors, such as temperature and humidity, even presenting a transgenerational effect (Bakker 1962 1969; Zwaan et al. 1995; Nunney 1996; Chippindale et al. 1997; Prasad et al. 2000; Valtonen et al. 2012; Ghosh et al. 2019; Shrader et al. 2020). *Drosophila willistoni* and *D. mercatorum* showed a faster development time than *D. maculifrons* and *D. ornatifrons*. The two faster species were cultured at a higher temperature (25°C) and the two slower species were cultured at a lower temperature (20°C), so we cannot exclude the effect of this culture temperature difference on these results. Nevertheless, they are naturally adapted to these temperatures, which even limit their laboratory maintenance in different temperatures. Our results showed that the Southern populations of *D. mercatorum* (cultured at 25°C) developed slower than southeastern populations of *D. maculifrons* and *D. ornatifrons* (cultured at 20°C). This can be evidence that differences in the development time detected among species can also be related to their adaptive history. A shorter period of larvae development, detected for *D. willistoni* and *D. mercatorum*, could allow for a greater ability to respond to desiccation and starvation as the larvae can explore relatively small fragments of resources that rapidly deploy, especially when densities are high. Thus, a decrease in the time until the emergence of imagos would bring advantages to individuals that passed faster through this unfavorable condition (Joshi and Mueller 1988 1996; Chippindale et al. 1998; Hoffmann and Harshman 1999; Krijger et al. 2001; Wertheim et al. 2000). A greater competitive ability, due to the reduction in development time, could also have favored the occupation of a broader amplitude of habitats by *D. willistoni* and *D. mercatorum*.

Nevertheless, besides a shorter development time favoring species in competition, adaptation to cold should lead to the opposite effect. A longer development time in low temperatures could be advantageous because it allows more time to increase body size and to accumulate energy reserve molecules, which

enables individuals to survive starvation periods and, for some *Drosophila* species, to pass through diapause during winter. In addition, the increase in body size, propitiated by a longer development time, frequently results in higher fecundity and longevity for females, and more successful mating for males (Tantawy and Vetukhiv 1960; Tantawy 1961; Zwaan et al. 1991; Hillesheim and Stearns 1992; Ohtsu et al. 1992 1998; Partridge and Fowler 1993; Chippindale et al. 1996; Betran et al. 1998; Harshman et al. 1999; Prasad et al. 2000; Michaud and Denlinger 2007; Angilletta 2009; Nelson and Cox 2014; Zonato et al. 2017; Kauranen et al. 2019; Flatt 2020). In this way, lower temperatures, such as those found in the southern region of Brazil, are probably the reason for the longer development time in POA and SAN for *D. mercatorum*, and in PMA for *D. maculifrons* and *D. ornatifrons*, with significant consequences for increasing body size in these three species in these populations, and also greater metabolic contents for *D. mercatorum* and triglyceride for *D. ornatifrons*. However, it seems that the adaptation of *D. ornatifrons* to cold produced a trade-off with viability in PMA.

Glycogen is the primary energetic source of carbohydrates in animal cells, and an excess of this metabolite could rapidly be converted into triglyceride. In insects, this metabolite has an important role in metamorphosis and in desiccation, since it not only represents an energy source that is quickly available, but is a reserve of metabolic water, stored in its bonds (Graves et al. 1992; Djawdan et al. 1998; Roach et al. 2012; Gáliková et al. 2015; Fernández-Elías et al. 2015; Matsuda et al. 2015; Yamada et al. 2018 2019). *Drosophila maculifrons* and *D. ornatifrons* showed higher concentrations not only of glycogen, but also protein, in CAJ, which could provide resistance to desiccation in periods of a hostile dryer environment for these species, whose abundance is positively related to high relative humidity (dos Santos et al. 2010; Cavasini et al. 2014; Gustani et al. 2015).

In *D. melanogaster*, larger accumulation of energetic molecules occurs in temperature conditions favorable to the adaptive history of this species, rather than in disadvantageous conditions, as described above. This species increases fat and glycogen reserves in mild temperatures, and lowers them when exposed to temperatures lower than 15°C or higher than 27°C (Klepsatel et al. 2019). For *D. willistoni* and *D. mercatorum*, the southeastern populations presented the lowest concentrations of glycogen and protein, and also of triglyceride in the case of *D. mercatorum*. Considering that temperature and humidity are two of the most important environmental factors for the adaptive evolution of insects (Nevo et al. 1998), these observations for these species suggest similarities with *D. melanogaster*: a higher concentration of energetic molecules occurs in populations with more satisfactory conditions for the species' survival, such as the southern populations, where annual temperatures are lower and relative humidity is higher than those of the southeastern populations (Instituto Nacional de Meteorologia 2019).

Matzkin et al. (2009) suggested that triglyceride contents would distinguish species of the *Sophophora* and *Drosophila* subgenera, and that the metabolite levels would be similar among species belonging to the same subgenus rather than to a different one. The results obtained here do not confirm these relationships, since none of the metabolites showed significant differences between *D. willistoni*, from the *Sophophora* subgenus, and the other three species from the *Drosophila* subgenus. *Drosophila willistoni* did not present a significant difference in triglyceride contents compared to *D. mercatorum* and the CAJ population of *D. ornatifrons*, as its glycogen and protein pools were different only from those of the southeastern populations of *D. mercatorum*. Moreover, triglyceride measurements for recently emerged adults in this study were similar to the zero-day adults of the *Sophophora* subgenus species, and glycogen and protein values were superior to species of both subgenera analyzed by Matzkin et al. (2009).

The differences in the results found here and by Matzkin et al. (2009) may have distinct, and not exclusive, causes: 1) the responses found for the lineages used by Matzkin et al. (2009) could be a consequence of adaptions after many generations in laboratory conditions (Tucson *Drosophila* Species Stock), while our results may more appropriately reflect natural conditions, since the populations were obtained from recently collected lineages (only about three generations in the laboratory); 2) differences in the nutritional components in the culture mediums used by Matzkin et al. (2009) (banana/*Opuntia*) and this research (banana) could have influenced some of the metabolic response, as considered by Ormerod et al. (2017); 3) the results in this paper demonstrate that the storage capacity of energetic molecules is determined by adaption to the environment, rather than by phylogenetic relationships. Thus, Neotropical populations and species can present distinctive responses of species belonging to other regions, probably due to their higher diversity of available habitats. This aspect highlights the importance of the current study in filling the gaps on the knowledge about the *Drosophila* species native to the Neotropical region, which could help to shed some light on the adaptive divergence in this region, and so become a model for other species of insects, and other animals.

CONCLUSIONS

The current work detected phenotypic plasticity in the studied characteristics, which is essential for adaptation to different environments. In general, species of the *Drosophila* subgenus showed the same populational profile, with southeastern region populations (region with higher temperature and lower relative humidity) presenting significantly smaller flies, with faster development and lower levels of triglyceride content. The interspecific comparison suggests that adaptive history plays a bigger role than phylogenetic relationships, since species for different subgenera, *D. willistoni* (*Sophophora*) and *D. mercatorum* (*Drosophila*), presented the highest viability values, and the lowest development time and triglyceride content. These results were probably related to their higher ability to explore resources and habitats when compared to the other two species. Populational differences in some characters analyzed did not influence the viability of *D. willistoni*, *D. mercatorum*, and *D. maculifrons*, revealing that they have an adaptive history distinct from *D. ornatifrons*. This aspect deserves better clarification, and we are currently performing a study that analyzes the same traits under different conditions of energy resources (experimental diets), aiming to verify the amplitude of the adaptive capacity of these species.

List of abbreviations

CAJ, Cajuru-SP.
SER, Serrana-SP.
PMA, Parque Municipal das Araucárias.
SAN, Santiago-RS.
POA, Porto Alegre-RS.
VI, Viability.
DT, Development time.
DW, Dry weight.
TG, Triglyceride.
GL, Glycogen.
PR, Protein.
RH, Relative humidity.
T°, Mean temperature.
MAX, Mean maximum temperature.
MIN, Mean minimum temperature.

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Authors' contributions: CHS and RPM collected the specimens; RPM and LPBM designed the research; CHS, KAVS, LPBM and RPM generated, analyzed and interpreted the results, and wrote the article. All authors contributed, read and approved the final version of this article.

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