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Altitudinal distribution of two sibling species of the *Drosophila tripunctata* group in a preserved tropical forest and their male sterility thermal thresholds

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ABSTRACT

Variation of ecophysiological traits may help to explain geographic distribution patterns of *Drosophila* sibling species. Many traits in ectotherms have optimal performance within specific temperature ranges. Altitudinal gradients are potentially informative for characterizing differences of sibling species distributions. We collected two sibling species of the *tripunctata* group – *Drosophila mediopunctata* (MPT) and *D. unipunctata* (UNI) – at eight altitudes (ranging from 593 to 1185 m above sea level) located at a continuous Atlantic Rainforest reserve in consecutive years (2009–2011), with two collections at the hot-rainy season and two at the cold-dry season. Mean altitude was significantly different between species and seasons. Their distributions showed a consistent pattern with MPT always occurring at higher altitudes than UNI. A significant correlation was found between altitude and species relative abundance. We characterized the thermal range of fertility, an important fitness component, for each species and found evidence for differential thermal adaptation. Our results suggest that the two species altitudinal distributions and seasonal relative abundances are consistent with their differential thermal adaptations: MPT seems to be adapted to lower temperatures, occupies higher altitudes and occurs at higher relative abundances in the cold-dry season; while UNI tolerates higher temperatures and occurs at lower altitudes and higher relative abundances in the hot-rainy season. However, their thermal ranges overlap at most temperatures, suggesting that additional variables (e.g. habitat choice, competition, differential survival *etc.*) may also play a role to determine their distribution in the field.

1. Introduction

Several evolutionary and physical processes affect distribution patterns of species-rich groups across a broad continuum of temporal and spatial scales (Pyrcz et al., 2009; Laiolo et al., 2017). In the long-term, environmental variation may shape differences in species physiological traits as contributing factors allowing their coexistence and maintaining local biodiversity (Aiken and Navarrete, 2014; Dell et al., 2014; Bozinovic and Pörtner, 2015; Ørsted et al., 2017). Among *Drosophila* species, variation in reaction norms of fitness components, such as viability and fertility, may be interpreted as climatic adaptations, which may be helpful to explain differences in their geographic distributions (Andrewartha and Birch, 1954; David et al., 2004; Kellermann et al., 2012; Mensch et al., 2017).

Male sterility is influenced by several genes (Buckley and Huey, 2016; Bundgaard and Barker, 2017) and is often observed with cytological abnormalities in spermatogenesis when exposed to heat or cold stress during development (Rohmer et al., 2004). Cold and heat temperature limits for male sterility define a sterility thermal range that is usually narrower than the viability range (David et al., 2005).

Several studies have shown that male sterility is a significant fitness component and a target of natural selection (Araripe et al., 2004; David et al., 2005; Buckley and Huey, 2016), which can be affected when a species is exposed to stressing temperatures in a variable environment (Vollmer et al., 2004). *Drosophila* species become sterile at distinctive upper and lower temperatures (David et al., 2005). Interspecific comparisons may show differences in thermal range they tolerate, reflecting thermal adaptation to local climatic variation (Chakir et al., 2002; David et al., 2005). Furthermore, thermal tolerance may be an important limiting factor for *Drosophila* species preventing their population expansion (Hoffmann, 2010).

Drosophila tripunctata species group encompasses 84 species (TaxoDros, 2017), mostly found within forests in Neotropical region (Throckmorton, 1975). Among them, *D. mediopunctata* and *D.*

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unipunctata are closely related (Yotoko et al., 2003; Hatadani et al., 2009; Robe et al., 2010) with a time of divergence estimated between 1.2 and 8.7 million years (Brianti et al., 2013). They are commonly found in coexistence among drosophilids collected in the Brazilian Atlantic Forest (Vilela and Mori, 1999; Toni et al., 2007; Döge et al., 2008; Medeiros and Klaczko, 2004; Batista and Klaczko, 2013; Batista et al., 2016, 2017).

The Brazilian Atlantic Forest biome is highly diverse with several types of forest dispersed over plateaus and mountainous areas (Ross, 2013; Joly et al., 2014). Altitudinal gradients are potentially informative for characterizing differences of sibling species distributions (Sexton et al., 2009). Currently, the Brazilian Atlantic Forest landscape is extremely fragmented (Ribeiro et al., 2009) and its biodiversity is suffering harmful effects of climate changes (Batista et al., 2012; Scarano and Ceotto, 2015). Nevertheless, there are still large areas of continuous preserved forest (Bergamin et al., 2017), especially in national or state reserves.

In this work, we examined the altitudinal distribution and variation in seasonal abundance of *D. mediopunctata* and *D. unipunctata* from a reserve with a large area of well-preserved continuous Brazilian Atlantic Forest. In addition, we analyzed the thermal range of male sterility as an indicator of differences in ecophysiological traits, taking into account Araripe et al.'s (2004) conclusion that "male sterility thresholds should also be considered and might be (…) informative for understanding the biogeographical distribution of a species".

2. Material and methods

2.1. Altitudinal distribution and seasonal abundance variation

We carried out four collection trips to *Parque Nacional do Itatiaia*, Rio de Janeiro, Brazil (22° 26' S, 44° 37' W), between May 2009 and March 2011 (Table 1). Banana baits were prepared using mashed bananas seeded with lyophilized commercial baker's yeast, which were left fermenting overnight. Roughly 200–250 ml of the baits were poured on plastic plates which were placed on the forest ground floor. We used about fifteen banana baits randomly distributed per site (altitude), which remained constant throughout the seasons (the same trails at each altitude). We sampled flies in up to eight altitudes ranging from 593 to 1185 m above sea level (m). We collected in altitudes above 590 m in the cold-dry season (**C-D**) and above 750 m in the hot-rainy season (**H-R**). Flies were collected by sweeping an entomological net over these banana baits. At the end of each collecting trip, the baits with plastic plates were removed and properly discarded to avoid polluting and to minimize any environmental change. Then, we brought the flies to our laboratory where they were sorted according to descriptions of their external morphology (Freire-Maia and Pavan, 1949; Frota-Pessoa, 1954). We established isofemale lines and used F1 male genitalia to compare with reference drawings for species identification (Frota-Pessoa, 1954; Vilela, 1992 for *D. mediopunctata* and *D. unipunctata*, respectively).

Table 1
Drosophila mediopunctata (**MPT**) and *D. unipunctata* (**UNI**) isofemale lines established from specimens collected in different altitudes (m) at *P. Nacional do Itatiaia* (22° 26' S, 44° 37' W). **Seasons**: Cold-Dry season = **C-D**; Hot-Rainy season = **H-R**.

Collecting date	Season	Alt. (m)	MPT	UNI	Total
May 30, 2009	C-D	1185	3	0	3
to June 5	C-D	1128	11	0	11
	C-D	1073	28	0	28
	C-D	955	22	0	22
	C-D	900	19	0	19
	C-D	808	40	2	42
	C-D	745	29	7	36
	C-D	593	27	4	31
Total			179	13	192
March 03, 2010	H-R	1128	9	0	9
to March 05	H-R	1073	9	1	10
	H-R	955	2	4	6
	H-R	900	3	3	6
	H-R	745	4	7	11
Total			27	15	42
August 23, 2010	C-D	1073	25	1	26
to August 27	C-D	955	50	0	50
	C-D	745	16	0	16
	C-D	593	6	2	8
Total			97	3	100
March 21, 2011	H-R	1073	23	33	56
to March 24	H-R	955	4	115	119
	H-R	808	1	35	36
	H-R	745	3	72	75
Total			31	255	286
Total			**334**	**286**	**620**

We found a great biodiversity with a total of 55 species (8 unpublished) from 15 *Drosophila* groups including *willistoni*, *guarani*, *cardini*, *calloptera*, and *tripunctata* (see: Batista and Klaczko, 2013; and Batista et al., 2016). However, we focused upon the two sibling species from the *tripunctata* group, since our objective was to characterize altitudinal and seasonal variation of their relative abundances, given they are the model organisms we work with (Klaczko, 2006). We performed an analysis of variance (ANOVA; Zar, 2010) to test whether the altitude of collection across the four occasions from 2009 to 2011 was affected by the species (UNI vs. MPT), season (hot-rainy vs. cold-dry) and the interaction between these variables. We estimated the mean altitude of collection for each species using the number of isofemale lines established from collected specimens in each altitude in each collection trip.

2.2. Fertility assay

In August 2012 and in March 2013 we collected flies especially to perform the fertility assay (a total of 161 *D. mediopunctata* and 17 *D. unipunctata*). Mass culture lines were established from isofemale lines (protocol adapted from Chakir et al., 2002; Araripe et al., 2004). Twenty couples per bottle were maintained with culture medium routinely used in our laboratory (Carvalho et al., 1989). Flies were let to oviposit overnight at 18 °C and transferred to new vials. Then, vials with eggs were left to develop in B.O.D. incubators with constant temperatures. Both species were exposed to different temperatures (*D. mediopunctata*: 12 °C, 14 °C, 16 °C, 18 °C, 23 °C, 24 °C, 25 °C, and 26 °C; *D. unipunctata*: 12 °C, 14 °C, 16 °C, 18 °C, 24 °C, 26 °C, and 28 °C). After emergence, flies were transferred to new vials. Then, for temperatures higher than 18 °C, they were kept in the same temperature for a week; and for temperatures lower than or equal to 18 °C, they were kept for 10 days.

After this period, sperm motility was checked. First, we dissected anesthetized flies in *Drosophila* Ringer's solution (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris·HCl) with forceps. Then, we separated testis and seminal vesicles from each male and examined sperm motility under a microscope. We dissected at least 28 males per temperature and counted the number of males with sperm motility. Finally, we compared graphically the two species countings (with standard errors) (Araripe et al., 2004).

3. Results

3.1. Altitudinal distribution and seasonal relative abundance variation

We analyzed the relative abundance of 620 isofemale lines established from specimens collected in different altitude and different seasons (Table 1). In three out of four occasions, *D. mediopunctata* was the most collected species (Fig. 1), but in March 2011, *D. unipunctata* was collected in higher relative abundance in all altitudes. In 2009, a significant correlation was found between altitude and species relative abundance after angular transformation (for *D. mediopunctata* – $r = 0.84$; $d.f. = 6$; $p = 0.009$).