



E-ISSN: 2320-7078

P-ISSN: 2349-6800

www.entomoljournal.com

JEZS 2020; 8(6): 23-31

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Received: 10-09-2020

Accepted: 16-10-2020

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Egg laying patterns and egg development of the butterfly *Actinote brylla* (Heliconiinae: Acraeini)

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Abstract

The increase in global temperature will directly affect the phenology of insects and other ectothermic organisms. Understanding how temperature will affect these organisms is necessary to make conservation decisions. Butterflies are indicated as biological models to assess abiotic pressures. This paper describes the influence of temperature on egg development of the *Actinote brylla* butterfly. Egg-clusters were searched on seven sites in the study area. During immature rearing temperature was continuously monitored by an Arduino Uno-based datalogger with a digital temperature sensor programmed to record environmental temperature at each minute. Statistical analyzes were performed using R software packages. Mean accumulated degree-days during development of 92 recent eggs clusters collected during winter in six sites in the study area ranged from 362.5°C to 385.6°C in a mean temperature interval that ranged from 21.8°C to 22.3°C. The development of *Actinote brylla* eggs responded positively to temperature increase. Female egg-laying concentrated in the week from July, 7 to July, 13, responding to the increase of day length and the fall of mean temperature.

Keywords: Degree-day, global warming, egg-clusters

Introduction

The latest report by the Intergovernmental Panel on Climate Change ^[1] points to an average increase of 1.5°C in the planet's temperature over the next 100 years. Therefore, it is necessary to know their impacts on organisms. Global warming changes development and the phenology of organisms and is becoming increasingly common ^[2-9]. Such changes can influence all organisms, affecting, for example, the laying of eggs in birds ^[10, 12] or accelerating the development of insects, such as butterflies ^[3].

There is an inverse relationship between the increase in temperature and the development of ectothermic organisms ^[12]. For example, the fruiting and flowering of plants and the development of insects are influenced by the accumulation of temperature, known as degree-days or thermal tolerance for development ^[9, 13-22].

Insects are ubiquitous organisms, with many generations per unit of time and, because they are ectothermic, they can provide information for understanding how climate change is affecting biological systems ^[23]. Insects also show rapid responses to environmental changes, especially regarding temperature ^[20, 24]. Phytophagous insects can also be affected in different ways by the changes undergone by their host plants ^[25]. Such changes can be diverse and eventually change the pattern of distribution and abundance ^[26-35].

For some species, the increase in temperature can be considered beneficial ^[36] or be disastrous for monophagous species ^[19, 25, 37]. The metabolism of ectothermic animals is considerably complicated because various temperature ranges are thought to be associated with different systemic states ^[19, 25, 37-38].

To understand how climate change can affect individuals of different species, it is necessary to know how such changes can affect biological communities. It is necessary to carry out research with a higher level of detail in a specific taxon making it possible to generate hypotheses for possible impacts on other organisms ^[39]. Butterflies are indicated for such forecasting models due to the wide knowledge about the group in relation to the pressure of abiotic factors on their life history ^[40-43].

In the Neotropical region, species richness in assemblages of the butterfly tribe Acraeini (Nymphalidae) are low in hotter and/or dryer areas and practically absent in Amazon watershed were its sister group, Heliconiini are highly diverse. High Acraeini richness was observed in subtropical or/and mountain areas where mean temperatures were lower ^[44-47].

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This work describes the dynamics of the egg cluster process and the development of eggs in populations of *Actinote brylla* sampled in sites in Southeastern Brazil where they are present [45-47] together with its larval food plant, *Mikania lundiana* [45-46].

Materials and Methods

Study area

Egg-clusters were searched on seven sites in the study area (Table 1). They were chosen because they had enough larval food plants or because many *A. brylla* adults were observed there.

Climate

At geographical coordinates -23.94° and -46.35°, the mean annual temperature is 21.2°C and mean total annual rainfall is 2,667 mm with more than 37% of the rains concentrated on summer [50]. According to Köppen's classification, the climate of study area is type Af [51, 52]. On 2019 the mean annual temperature was 24.1°C, being 2.9°C above the normal. Rainfall was 3301.2mm or 23.8% bigger than normal. On six months the rainfall was bigger than normal with February and May two times bigger. Daylength was calculated based on sunrise/sunset tables [53].

Table 1: Sites where samples were done, all in the coast of Sao Paulo, Brazil (see [49] for more details)

SITE CODE	GEOGRAPHICAL COORDINATES	SITE NAME	COUNTY, STATE	DETAILS
VRQUI	-23.839959° -046.323585°	Quilombo River valley	Santos, Sao Paulo	Dirt road (9 km) on the right bank of the Quilombo River.
TRAPU	-23.874535° -046.347534°	Quilombo River valley	Santos, Sao Paulo	Dirt road (2 km) on the left bank of the Quilombo River.
TTREM	-23.874535° -046.347534°	Quilombo River valley	Santos, Sao Paulo	Dirt road (2 km) on the left bank of the Quilombo River.
JURUB	-23.876566° -046.315060°	Jurubatuba River valley	Santos, Sao Paulo	Dirt road at right bank of the Jurubatuba River.
LIXAO	-23.882063° -046.324002°	Jurubatuba River valley	Santos, Sao Paulo	Dirt road at right bank of the Jurubatuba River.
NCINT	-23.943675° -046.362566°	Nova Cintra Hill	Santos, São Paulo	Urban area of Santos.
PORCH	-23.980043° -046.371675°	Porchat Island	São Vicente, Sao Paulo	Urban area of Sao Vicente.

Weather data

Averages of hourly solar radiation ($J/m^2/s$), temperature at shade (°C), wind velocity (m/s) and direction (quadrant) were obtained in CETESB site [54]. Temperature at shade (°C) was measured using an Hikari HT-450 (precision = 0.1°C) infrared thermometer and solar radiation using a solarimeter Instrutherm MES-100 (precision = 10 W/m^2). Rainfall data was obtained from Cemaden site [55].

Field protocol

In all sites, a careful search was done on all accessible leaves of the larval food plants, up to four meters high, and all visible egg-clusters were collected.

During 2018-2019, a total of 211 leaves with 236 egg clusters were collected including those of females recorded laying eggs. The winter and spring egg clusters of 2018 were used only to estimate the duration of stages after hatching and qualitative observation of these larvae. Comparisons between the number of eggs per egg cluster were made only between the fall and winter generations of TRAPU, JURUB and VRQUI sites of the 2019.

In the winter generation of year 2019, 134 leaves containing 140 egg clusters were collected. To estimate the dates of each found egg cluster, we subtracted 17 days from the date of collection using the median found for egg development of the 12 collected egg laying females. We used these estimates to verify the weeks where there were most females laying eggs.

Egg development and temperature monitoring

Eggs were reared in laboratory with no temperature control receiving only indirect daylight. Temperature variation was continuously monitored by an Arduino Uno-based datalogger

with a digital temperature sensor DS18B20 programmed to record environmental temperature at each minute. The average of daily 1,440 recorded temperatures was used in the analysis. Temperatures of egg clusters from collection to transport to the laboratory (for 3 hours) were also considered.

Data analysis

Normality of data was checked using shapiro-wilk test. Regression of averages of mean daily temperature and duration of egg-clusters of each site were analyzed using a Generalized Linear Model (GLM). Differences of accumulated degree-days between sites were estimated by a one-way anova. PCA analysis used package FactoMineR, v. 2.3 [56, 57], factoextra, v. 1.0.7 [58] and ggplot2 [59]. All tests were done using R software packages [60].

Results and Discussion

Distribution and characteristics of larval food plant

The larval food plant *Mikania lundiana* used by *A. brylla* is relatively common in isolated hills and at foothills of Serra do Mar to elevations near 500 m. on the coast. It grows as a vine and could reach to the canopy of high trees. It is particularly abundant near rocky shores (Figure 1 A) and in low elevation hills (Figure 1 B-C) in coastal area of Sao Paulo state, from Cardoso Island to Picinguaba, in the Rio de Janeiro board. Despite this, it grows mainly in soils where clay component is higher than the sand component which are common in hill slopes. The consequence is that is not common in "restinga" forests on the coastal plains due to their high sand content. The effect promoted by the impact of *A. brylla* larvae when larvae reach last instar, in plants which were colonized by this species, is strong. When almost all leaves are eaten at least six

months are necessary by leaf reposition ($n = 9$) and in some case the plant is killed. In areas where the number of available plants is small this implicates that after mating, female adult butterflies when emerging need to search plants in other sites.



Fig 1: (A) Some aspects of the bushes of *M. lundiana* in the Porchat Island south rocky shore (PORCH) on October 14, 2019. (B) TRAPU December 2, 2018. (C) NCINT August 11, 2019

Leaf characteristics and microclimate environment

Leaves of *M. lundiana* can present different leaf morphology in the same individual. Depending if the leaf is in a sunnier or shadier spot which determines the microclimates' moisture. Spots not exposed to direct sun have bigger old leaves than plants in sunny areas ($n = 6$). The complexity of the arrangement of the leaves means that there are sunnier and darker spots on the same plant. During the day, with the variation of the height of the sun, each leaf receives different amounts of solar radiation and all those exposed directly to the sun have higher temperatures. The surface temperature of a leaf partially exposed to the sun may be lower at the point where it is shaded (Figure 2 A-D). Variation in leaf shape in the same individual plant due to different environmental conditions, as in a sun and shade spot, is known in several angiosperms' species [60]. Other *Mikania* species, used as larval food plant also present variation in leaf shape (e.g. *M. lanuginosa*, *M. scabrida*, *M. micrantha*, and *M. cordifolia*).

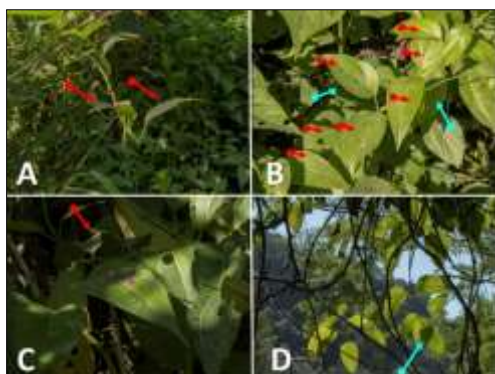


Fig 2: Differences on direct solar radiation reaching the leaf surface. Red arrow indicates sun incidence in relation to leaf surface and blue arrow a shaded leaf. All pictures from LIXAO on August 30 between 09:50 and 10:00h when solar radiation ranged from 650 to 700 W/m^2 . (A) Leaves at shade and (B) exposed to direct solar radiation. Recorded surface temperature on leaf (C) showed a difference of 2.3°C between point a, b, and c. (D) Group of leaves totally exposed to direct solar radiation with leaf indicated by blue arrow partially shaded by other above it

Leaf area from VRQUI, LIXAO and PORCH ranged from 1696.5 mm^2 to 9000.0 mm^2 ($n = 90$) and were significant different ($F_{2,87} = 74.62$; $p < 0.0001$). Tukey multiple comparisons of means showed significant differences between VRQUI and LIXAO ($p < 0.0001$) and VRQUI and PORCH ($p < 0.0001$) (Figure 3 A).

A total of 40 leaves measured at field on August 30, have significant differences in the surface temperature between the sun and shade leaves ($F_{5,33} = 25.19$; $p < 0.0001$; Figure 3 B).

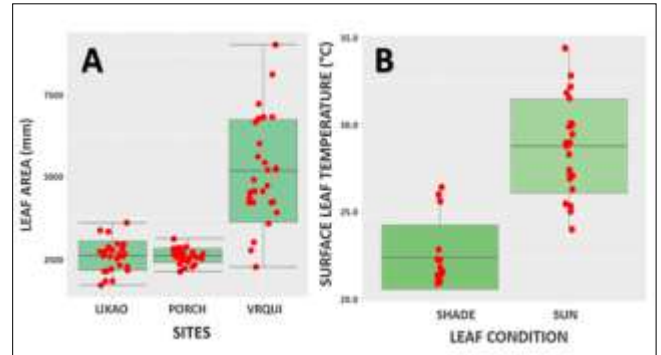


Fig 3: (A) Variation of leaf area (mm) in three sites in the study area showing the significant differences between leaves from VRQUI to leaves from LIXAO and PORCH. (B) Differences on the surface leaf temperature on August 30, between 09:50 and 11:10h. In LIXAO, JURUB, and VRQUI ($n = 40$) when solar radiation ranged from 650 to 700 W/m^2 . Difference between means are significant ($F_{5,33} = 25.19$; $p < 0.0001$)

Laboratory leaf heating experiments

Leaves heated in controlled laboratory environment showed an increase of 10.54°C (SD = 0.86°C; $n = 5$; Figure 4) after 10 minutes of exposition to an incandescent lamp which emits 800 W/m^2 of radiation at working distance. Therefore, actual degree-minutes accumulation in leaves in shade and in sun are significant different because exposed leaves accumulate more relative heat than those in shade. Since egg-clusters are immobile, those that are placed by the females in leaves in the sun should develop faster.

On the other hand, as the larvae can move their thermoregulation is done behaviorally.

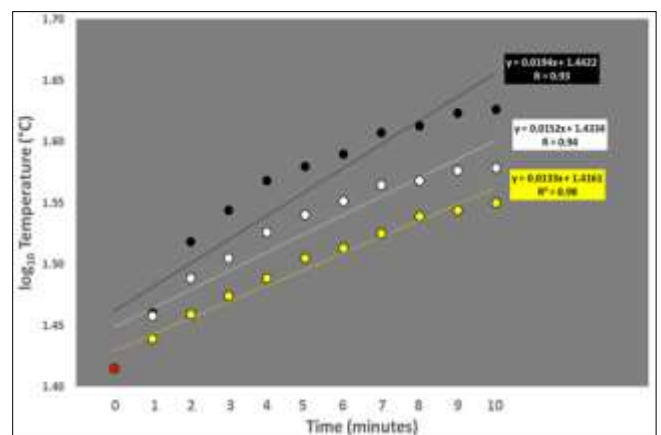


Fig 4: Temperature increase of five leaves and two foam surfaces, one black and other white (see methodology) exposed during 10 minutes to an incandescent lamp which emits 800 W/m^2 at working distance. Data transformed (Log_{10}) to fit a linear distribution. Initial temperature (red point) was 26°C. Black surface had an increase of 16.3°C, and white surface of 11.9°C. Leaves had an average increase of 9.5°C ($n = 5$)

Temporal dynamics of egg laying by females

During the period of June 17 (Autumn) July 22 (Winter), the estimates of dates of female ovipositions' showed that 71.3% egg clusters were laid were from 6 to 18 July; Figure 5 A). The Principal Component Analysis of environmental conditions for the period of 36 days when ovipositions occurred (Figure 5 B) showed that the first two dimensions of analyses expressed 55.34% of the total dataset's inertia which is high enough representing both first planes of data variability. The cluster in the top right quadrant grouped

mostly days when egg laying was high and has a positive significant correlation with the photoperiod (PHOTO) and a strong negative correlation with mean temperature (TMED). At the end of autumn and beginning of winter, the decrease in the photoperiod (daylength) is expected and the average temperature should decrease. However, what happened is that it remained high (above 25°C), dropping only in the week of July 7-13 when egg laying was high. Most of egg-clusters were laid when daylength ranged 644 to 647 minutes and mean temperature from 14.6°C to 18.6°C.

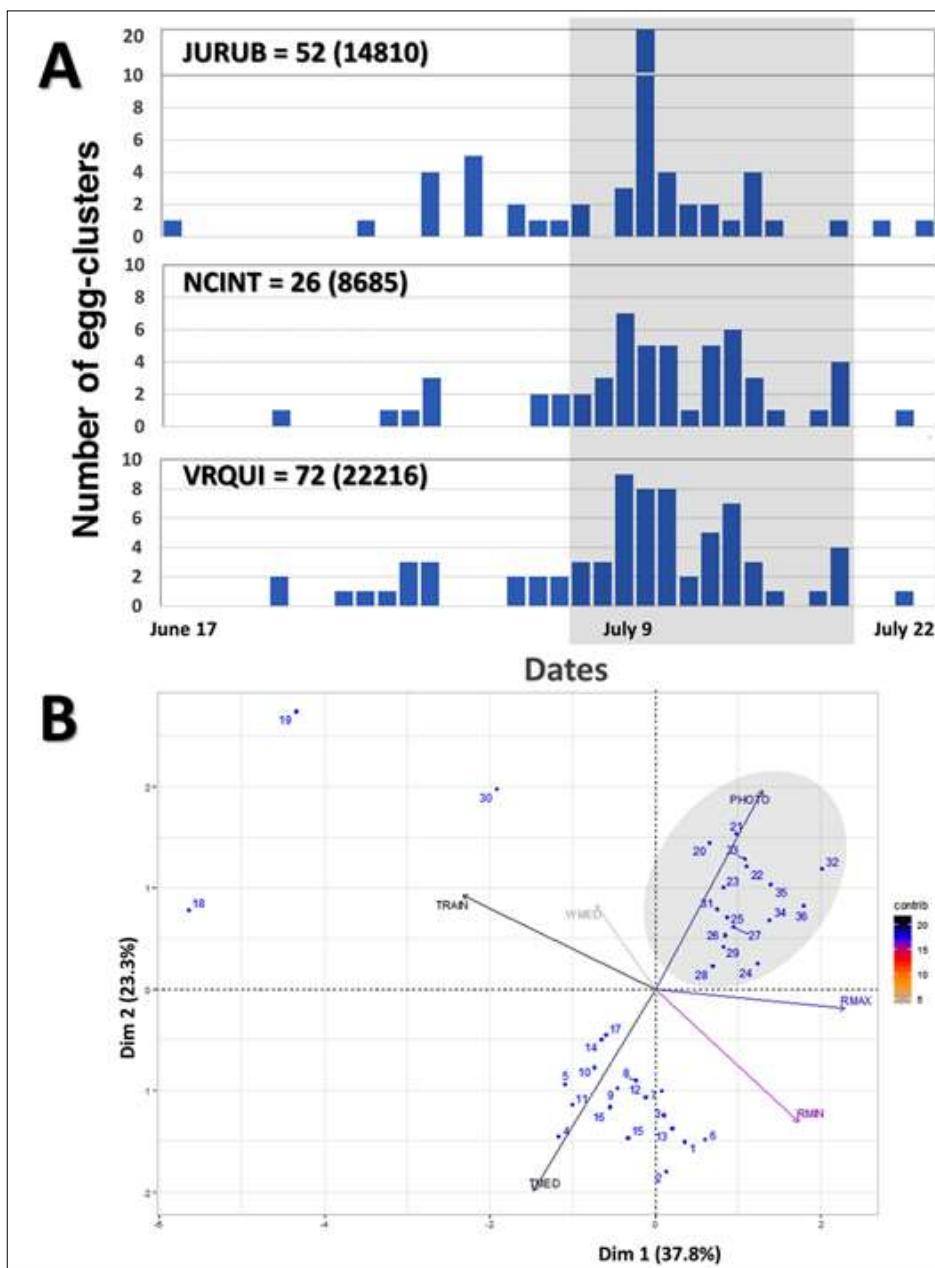


Fig 5: (A) Number of egg clusters from June 17 (Autumn) to July 22 (Winter) in three sites of the study area. Numbers after site name are the total egg clusters and inside parenthesis, total number of eggs. Egg laying was concentrated in the period from July 6 to 18 (gray shaded area). (B) Principal Component Analysis of environmental conditions during the period of winter ovipositions in 2019 in the study area, from June 17 to July 22. The gray area indicates the days when the higher egg laying number occurred. This cluster has a positive significant correlation with the photoperiod (PHOTO) and a strong negative correlation with mean temperature (TMED). Vector color indicates the variable contribution of the variable according with the color scale at right. Mean wind velocity (WMED), rainfall (TRAIN), minimum and maximum integrated solar radiation (RMIN and RMAX) were variables that had low contribution to variance

Result of the General Additive Model analysis of total number of egg clusters (TOTAL) shows the significant additive role of photoperiod (PHOTO) and mean temperature (TMED) (Table 2). The egg clusters in VRQUI (n = 71) were

distributed in eight *M. lundiana* patches (mean = 8.9 egg clusters/patch); in JURUB (n = 52) in five patches (10.4 egg clusters/patch) and NCINT (n = 26) in one patch of the larval foodplant.

Table 2: Results of the General Additive Model analysis of total number of egg clusters (TOTAL) as a function of the photoperiod (PHOTO) and mean temperature (TMED). Significance codes: 0 (***); 0.001(**); 0.01(*)

Family: gaussian					
Link function: identity					
Formula: TOTAL ~ s(PHOTO) + s(TMED)					
(Intercept)	Estimate	SE	t value	Pr(> t)	
4.1667	0.4695		8.875	***	
Smooth terms	edf	Ref. df	F	p	
s(PHOTO)	3.601	4.384	2.94	*	
s(TMED)	7.959	8.640	2.64	*	
Adjusted R ²	Deviance explained	GCV	Scale est	n	
0.69	79.3%	12.188	7.9357	36	

Number of eggs in clusters

The average number of eggs in clusters of *A. brylla* follow the same pattern of other neotropical *Actinote* species whose life-cycle is known (Table 3) and Acraeini species from Afrotropical region^[69-71].

Three virgin females that were dissected emerged with an average of $354 \pm$ ovules ready to be fertilized and an additional 637 ± 42 ovules to develop, the same pattern observed for *A. pellenea pellenea*^[46]. So, if a female can

potentially lay an average of three eggs clusters along its brief life of few days, we can infer that female number in these three sites in the winter of 2019 was 17, 9 and 24, respectively. These numbers are not very different from the number of females that were observed in the field during the study period. The number of eggs per egg cluster and egg density per egg cluster between the three study sites was not significant when analyzed by one-way anova.

Table 3: Number of eggs in egg-cluster of some *Actinote* species

Species	Mean	sd	n	Range	duration (days)	Source
<i>Actinote brylla</i>	309.1	125.9	232	12-836	14-19	submitted
<i>Actinote brylla</i>	361.2	113.8	13	173-538	14-18	[46]
<i>Actinote canutia</i>	412.5	130.8	2	320-505	15	[46]
<i>Actinote carycina</i>	294	99.1	16	110-470	14-19	[47]
<i>Actinote conspicua</i>	485.3	88	3	406-580	18	[62]
<i>Actinote dalmeidai</i>	186.3	161.3	7	30-413	19-22	[46]
<i>Actinote genitrix</i>	295	63.6	2	250-340	16	[46]
<i>Actinote melanisans</i>	351.6	180.8	8	100-723	15-17	[47]
<i>Actinote paraphelus</i>	366.8	99.8	5	271-510	12-18	[46]
<i>Actinote pellenea</i>	361.2	132.8	291	34-650	11-15	[46]
<i>Actinote pellenea</i>	287.0	111.0	NA	15-450	NA	[63]
<i>Actinote pellenea</i>	NA	NA	NA	130-500	NA	[63]
<i>Actinote pyrrha</i>	316.4	240.5	18	240-300	14-17	[46]
<i>Actinote quadra</i>	190.3	71.8	33	63-357	14	[64]
<i>Actinote rhodope</i>	350.8	68.8	4	300-410	14	[46]
<i>Actinote rufina</i>	NA	NA	1	89	13	[65]
<i>Actinote surima</i>	NA	NA	2	129-254	13	[46]
<i>Actinote zikani</i>	354	143.8	3	269-520	13	[66]
<i>Altinote ozomene</i>	NA	NA	39	100-380	21-22	[67]
<i>Altinote trinacria cf. unicolor</i>	NA	NA	14	38-214	24	[68]

Temperature and egg development

A total of 92 egg-clusters from 2019 winter generation were reared from June 28, 2019 to August 7. During this period, the average laboratory temperatures ranged from 20.4°C to 23.7°C (mean = 21.6°C; Fig. 6 LABOR) and environmental temperature recorded by CETESB automatic station for the

same period ranged from 14.6°C to 26.4°C (mean = 20.3°C; Fig. 6 CETESB). The difference between laboratory temperatures and environmental temperatures ranged from -4.4°C to +8.0°C being significant different (Wilcoxon rank sum test with continuity correction $w = 1,137.5$; $p = 0.006$).

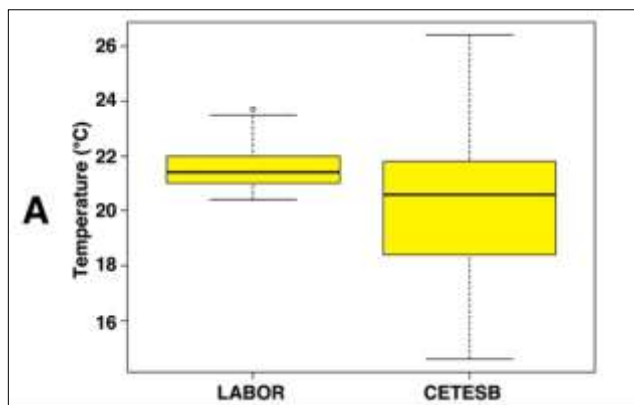


Fig 6: (A) Variation of daily mean temperature in the laboratory (LABOR) and in Santos environmental automatic station (CETESB) showing the difference between medians (Wilcoxon rank sum test with continuity correction $w = 1137.5$; $p = 0.006$)

Mean accumulated degree-days during development of 92 recent eggs clusters collected during winter in six sites in the study area ranged from 362.5°C to 385.6°C in a mean temperature interval that ranged from 21.8°C to 22.3°C (Fig. 7 A). Mean duration of egg stage ranged from 15.6 to 16.3 days (absolute values ranged from 14 to 19 days), diminishing with the increase of mean temperature (Fig. 7 B) but egg

duration between these sites were no significant (Fig. 8 A). Extrapolating the regression equation: duration (days) = $-1.3571 * x^{\circ}\text{C} + 45.824$ we can predict egg mean durations of 12, 9, 8, 6, and 5 days at mean temperatures of 25, 27, 28, 29, and 30°C, respectively. This will be checked in a next step of this project.

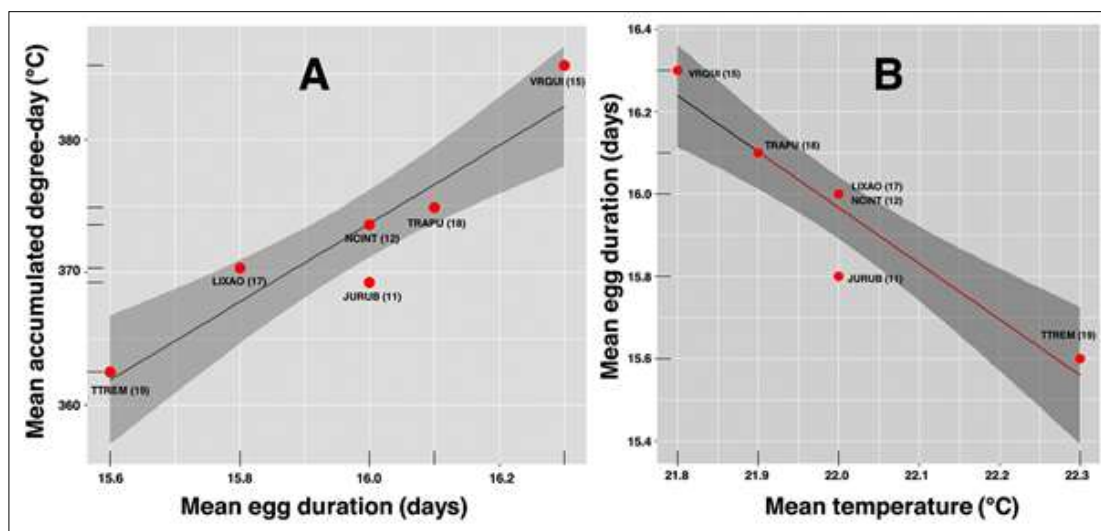


Fig 7: (A) Accumulated degree-day (°C) of egg-clusters of *Actinote brylla* in six different sites in the study area in function of development duration (days) (Adjusted R-squared = 0.83; $F_{1,4} = 25.99$; $p = 0.007$). (B) Mean egg duration (days) in function of mean temperature (°C) of egg-clusters of *Actinote brylla* collected in six different sites in the study area showing the decrease of egg stage duration with the temperature increase. Numbers inside parenthesis indicate the number of sampled egg-clusters (Adjusted R-squared = 0.85; $F_{1,4} = 29.07$; $p = 0.006$). Gray area indicates the 95 % confidence interval

Egg eclosion

The duration of eclosion of eggs of 11 egg-clusters ranged from 30 to 210 minutes (Fig. 8 B). The regression analysis between both variables showed no significant correlation

between egg number in the cluster and total time spent for eclosion (Fig. 8 C). This analysis ignored non-fecundated eggs, ovules, which keep the original yellow color while the others become reddish over time.

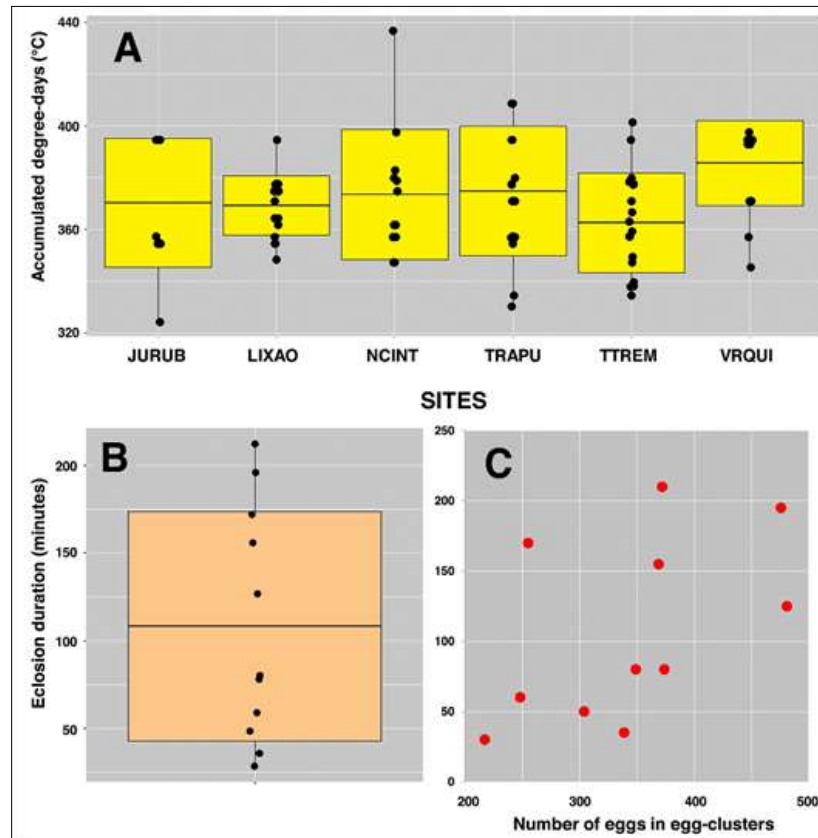


Fig 8: (A) Mean accumulated degree-days of egg-cluster of *Actinote brylla* collected in six sites of the study area. There were no significant differences between them ($F = 2.281$; $df = 5$; $p > 0.05$). (B) Duration (minutes) of egg eclosion in 11 egg-clusters of *Actinote brylla*. (C) There is no significant correlation between the number of eggs in the egg-clusters and the total time necessary for the eclosion of all eggs

The published records of egg duration for other 18 Neotropical montane Acraeini species shows values ranging from 12 to 24 days (Table 2). However, they do not have information for the temperature range during egg development of these species.

Our methodology maintaining the egg-clusters at environmental laboratory conditions (temperature and daylength) was effective since that quantity of egg-clusters was enough to work with averages. We need to consider that also the genetical information in the sampled eggs of each site could be relevant in development response to temperature.

Even so, for a next job, we intend to use the degree-hour as a metric to reduce any errors.

Being mainly subtropical, assemblages of *Actinote* species are richer in mountainous areas (above 1,000 m in our latitudes [48]) or more southern areas in Brazil (Paraná, Santa Catarina and Rio Grande do Sul) and living in climates where mean annual temperatures are under 20°C [55]. Therefore, optimal developmental temperature should be around this value.

Conclusion

Our data show that the development of *Actinote brylla* eggs reared in laboratory environment responded positively to temperature increase. Daylength and mean daily temperature had a role in the female egg laying pattern.

Acknowledgements

EFSF thanks to FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) for Scientific Initiation Grant (# 2018/20544-9). RBF thanks to Universidade Católica de Santos for logistic support. We thanks to one anonymous referee for his comments.

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