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Thermoregulatory Behaviours of *Anartia jatrophae* (Insecta: Lepidoptera: Nymphalidae): A Baseline study

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Abstract
Butterflies, a well-studied, easily tractable taxon, provide some of the most robust evidence for the ecological effects of recent climate change. In this study, we provide baseline data on how temporal ambient temperature at an equatorial site is related to butterfly behaviour. We observed the behaviours of, measured thoracic temperature and counted free-flying *Anartia jatrophae* butterflies. Further, we measured abiotic variables during four three-hour diurnal time blocks within their environments. Wild butterflies were also caught and observed in flight cages from June to July 2012 and 2013 at the CEIBA Biological Center, Guyana. We observed the highest species abundance during Time Block II (0900-1159 hrs). We also noted that time blocks significantly influenced all *A. jatrophae* observed activities except for fanning. The primary activities observed in each time block in addition to flight were as follows: TB I - Dorsal Basking, TB II – Territorial Defense, TB III - Diurnal Roosting and TB IV - Territorial Defense. Overall, we found that the thoracic temperatures of butterflies and ambient air temperatures were statistically similar. Furthermore, we detected statistically significant variations of these temperatures during activities, with significant correlations between thoracic and air temperatures for Diurnal Roosting, Fanning, Flight, and Nocturnal Roosting. Although this study does not show long-term behavioural patterns based on seasonality, it provides baseline line information on the relationship between ambient temperature and butterfly physiology and behaviour that can be expanded on as we design butterfly monitoring programmes to track how species respond behaviourally to climate change.

Keywords: *Anartia jatrophae*, butterflies, Guyana, thermoregulatory, behaviour

Introduction

Climate is changing at an unprecedented rate, and it is predicted to lead to substantial increases in global mean temperatures with a 2-6 °C increase by 2100 [1, 2]. With this changing climate, there are increased amplitude, frequency, and duration of extreme temperature events recorded [2, 3], which has led to dire consequences for species survival globally [4]. Organisms in tropical climates are even more vulnerable, given the comparative magnitude of change experienced relative to their temperate counterparts [5, 6]. Therefore, in warm climates, small changes in air temperatures will drive organisms in these climates to exceed their upper physiological temperature thresholds [3, 5]. The results are currently evident in declining biodiversity and deleterious effects on ecosystems, communities, and species [5, 7].

The effect of rising temperatures will specifically impose a significant constraint on ectothermic tropical species [8] as physiologically, these organisms have a restricted capacity to raise the upper limits of their body temperature and have to maintain their body temperature and have to regulate their temperatures [5, 9]. As such, ectotherms have evolved diverse adaptive strategies to buffer various shifts in their environment and understanding these strategies enables us to predict organisms' responses to changing climate [5, 6, 10]. These strategies include morphological, physiological, and behavioural adjustments that enable them to adapt/reduce to fluctuating conditions and maintain their physiological capabilities [5, 10]. Previous studies have mainly focused on endotherms and ectothermic vertebrates [5, 11, 12] rather than terrestrial arthropods, which are critical for biodiversity maintenance and ecosystem stability during global warming. Despite insects being the world's most species-rich taxa and recent progress in climate change-arthropod research [3, 13–16], there remains a sizable knowledge gap in the relationship between thermoregulation and population level ecology of these taxa [8, 17].

Insects can rapidly sense small fluctuations in their ambient temperatures, display physiological, molecular and symbiotic-mediated responses, exploit diverse thermal microhabitats, have morphological adaptations and exhibit various behaviours to regulate body temperature and reduce exposure to heat stress [3, 15, 18, 19]. Of all the insect groups, Lepidoptera has the most varied sets of physiological and behavioural processes for controlling their body temperature, as thermoregulation is directly related to their flight and, consequently, their survival, population biology and ecology [20], as such butterflies have been used extensively as a model study organism for thermoregulation [21]. Specifically, butterflies (genus *Anartia* Hübner; tribe Victorinini) are among the most common and conspicuous in the Neotropical world and, as such, have several species that have been used extensively for biological studies [22, 23]. This study focuses on thermoregulatory behaviours in tropical butterflies and aims to explore the behaviours of the white peacock butterfly (*Anartia jatrophae*, Linnaeus, 1763) as a model organism. Our study focuses on behavioural processes, as these enable an animal to adjust its body temperature, allowing for control of their energy budget and protection from the deleterious effects of heat stress.

Furthermore, although these processes are critical for butterflies' continued adaptation and survival as the earth continues to warm rapidly [3, 8], these behaviours and their uses remain unclear. Therefore, our objectives for this research were threefold: i) to observe the daily activities of our study species, ii) to examine relationships between their presence during varying daily time blocks and their activities, and, iii) to measure and calculate correlations between daily thermoregulatory activities, ambient air temperature, and butterfly thoracic temperatures. Our study is essential as it provides information that will lead to a better understanding of the ecological needs of the studied species and, overall, how the selective pressure of ambient temperature affects individuals. It will also enable us to make inferences about the relationship between air temperature and behaviour, and it will serve as a reference point for future studies monitoring butterfly populations as global temperatures rise.

Materials and Methods

Study Site

We conducted this study at CEIBA Biological Center (N 06/29.945//, W 058/ 13.106//), Madewini, Guyana. This white sand forested area comprises a low seasonal forest dominated by the fast-growing *Eperua falcata* and tall primary-growth flooded forests dominated by *Mora excelsa*. We observed butterflies on a fixed line transect that started in the forested area west of the biological station's buildings, extending towards the path to an abandoned farm located to the west of the biological station, northwest bound to the flooded forest, then north towards the spring of Younge Creek upwards in an easterly direction to the camp area and finally culminating northwards to the Splashmins Ecotourism Camp Grounds to an open field that leads to the modified Madewini River.

Study Species

***Anartia jatrophae* (Nymphalidae)**

This Neotropical butterfly is ubiquitous [23] and it is distinguished dorsally by its whitish-grey wings with marginal and sub-marginal golden or dull orange colouration which is lighter ventrally [23, 24]. They persist in open areas, disturbed environments and secondary forests [24–26]. They are conspicuous, abundant in multiple habitats [23, 25], and exhibit geographic and seasonal patterns [23, 26].

Data Collection

We conducted a pilot test five days before the experiment to select the appropriate study species and observation locations. Subsequently, based on population size and access to habitat locations, the *A. jatrophae* was selected. Its daily activities were observed and recorded for 18 d during the following periods in 2012: 1–6 June, 12–16 June, June 20–July 1, and concluded on July 12 and June 1 to July 31 2013 in open areas. We also caught, marked and observed twenty [20] butterflies in flight cages during four three-hour diurnal time blocks from June 1 to July 31 2013, at CEIBA Biological Center, Guyana.

We observed behaviours, measured thoracic temperature and counted the number of individuals within a 5 m radius using a modified Pollard walk and count method [27] along a fixed 2 km line transect utilising a combination of scan sample and focal animal sampling [28], as detailed below:

- Scan Sampling: Butterfly activities were recorded at pre-selected moments (i.e. every 10 seconds).
- Focal Animal Sampling: all occurrences of specified actions of one individual were recorded during a predetermined sample period.

A checklist table with the ethogram below and an additional comment column was prepared based on the following times

- 0600-0859 h TIME BLOCK I (TB I)
- 0900-01159 h TB II
- 1200-1459 h TB III
- 1500-1800 h TB IV

The ethogram was developed from (29) and pers. obs. G. Maharaj & G.R. Bourne

- Emergence from chrysalis – enclose from pupae by slitting pupal case
- Pumping wings – emerged butterfly hangs from pupal case as fluid is pumped in the veins of the wings
- Resting after emergence – butterfly with fully formed wings hangs from pupal case until wings harden and to gain strength before flight
- Flight – movement action in search of perch (P), food (F), mate (M), escape predators (EP), territorial patrol (TP), ovipositing (O), roost (R), undetermined (?)
- Courtship – display by male or female to attract potential mates
- Mating/Copulation – male and female with their abdominal tips in contact
- Ovipositing – female deposits egg/eggs on host plant.
- Feeding – extending proboscis onto food source
- Temperature regulation – warming – basking in full sun, lateral basking (LB) (closed wings held perpendicular to sun), dorsal basking (DB) (wings held open) or cooling, close wings parallel to sun rays or fly into shade (puddling behaviour separated)
- Lekking/swarming/groups gathering – aggregating behaviour of butterflies but not on moist soil or at the edges of puddles
- Puddling – aggregating on moistened patches of soil or at the edges of puddles

- Diurnal roosting – inactive periods where butterflies may hide on the underside of leaves or on dead flower heads, on the ground etc.
- Fans – opens and closes its wings repeatedly while resting
- Territorial defence – linear, circular or spiral chases
- Territorial display – periods during territorial defence when males remain perched in opposite directions flapping their wings at regular internals
- Nocturnal roosting – perch on vegetation

We photographed butterflies using a NIKON D 70 and NIKON Coolpix L120 camera and identified butterflies to species level using Butterflies of Costa Rica [24]. Abiotic variables which included air temperatures and relative humidity were measured using a RadioShack digital wired indoor/outdoor thermometer/hygrometer 6300334, thoracic and substrate temperature with the use of a Raytex Minitemp MT infra-red temperature gun and light intensity with a Extech Model 401025 light meter in each time block.

Statistical Analysis

We tested the assumptions for using a parametric approach using the Kolmogorov and Smirnov method. To measure the distribution of activities for *A. jatrophae* in the survey site, we extracted the time of capture for each individual and converted the diel data to radian. We treated the time data as random samples from a continuous distribution and applied kernel density estimation to estimate peak activity hours.

We applied the Kruskal Wallis Test, a non-parametric test based on mean ranks [31] using the 'kruskal. Test ()' function to determine if there were any significant differences ($p<0.05$) between the thoracic and ambient temperatures of *A. jatrophae* across different activities. We then conducted a Dunn's Test [32], a *Post hoc* multiple pair-wise comparison of *A. jatrophae* activities with Bonferroni correction. We also applied the Chi-squared Test to identify significant differences in the frequencies of *A. jatrophae* activities across different time blocks.

Finally, we prepared stacked bar graphs and summary tables to show the daily activities of each study species. The illustrations were made using the *R* packages 'Overlap' [33], 'ggpubr' [33] and 'ggstatsplot' [26] on R version 4.3.1 [35].

Results and Discussion

Behavioural thermoregulation is a complex interaction between morphology, behaviour and the ambient thermal environment that can be influenced by multiple factors [26, 36]. Further, as ectotherms, butterflies have strict thermal tolerance ranges and variation in temperature serves as a significant constraint to basic daily and metabolic activities, which also have profound repercussions on fitness outcomes, especially in cases of extremely high temperatures [15].

From Fig. 1 the daily activity patterns indicated that the *A. jatrophae* were most active between 0900-1000 hrs (Time Block II), followed by 1600-1800 hrs (Time Block IV) with periods of reduced activity between 0600-1000 hrs (Time Block III) and 1200 and 1500 hrs (Time Block III). This coincides with changes in average air temperature, where reduced activity periods were noted during the coolest and hottest periods of the day and high activity periods are during warmer periods. Similarly, Clench, Ma and colleagues indicated that as insects experience diurnal temperature cycles they have reduced activities during cooler hours of the day as they are unable to reach optimal thoracic temperatures for flight owing to their environment. In contrast, there is decreased activity during the hottest potions of the day to avoid heat injury from increased temperatures due to continued flight and solar irradiation [15, 37].

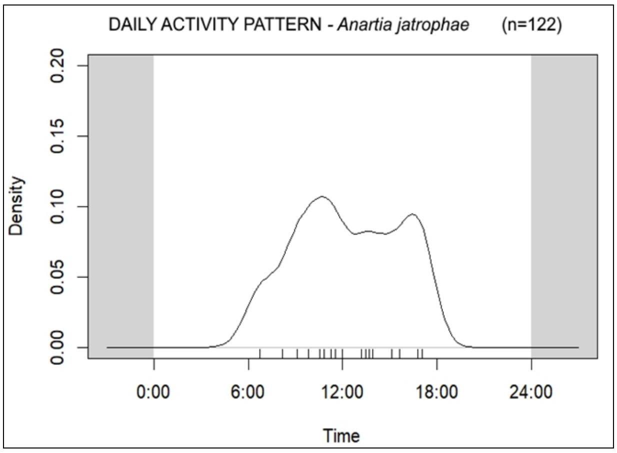
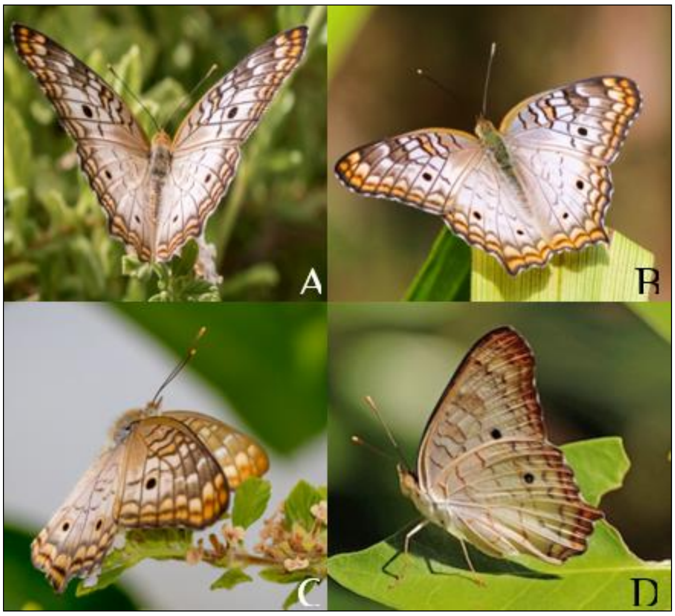


Fig 1: Daily activity patterns indicated that the *A. jatrophae.*

Table 1: *A. jatrophae* activities during each time block in an open area

Activities	Time Blocks				Species Richness per activity
	TB I	TB II	TB III	TB IV	
Diurnal Roosting	10	17	10	0	37
Dorsal Basking	32	17	5	0	54
Fanning	1	1	2	0	4
Flight	83	142	46	10	281
Territorial Defense	13	30	6	4	53
V Basking	1	11	0	1	13
Species Richness per TB	140	218	69	15	442



Plates 1-4: Wing positions of *A. jatrophae* observed in field

Observations, as seen in Table 1, showed the majority of butterflies were observed in Time Block II (0900-1159 hrs), followed by Time Block I, III and IV. The most frequently observed behaviours were flight followed by Dorsal Basking, territorial defence, and Diurnal roosting. It should be noted that the area in which these butterflies were observed was an open field [25, 35, 36] that served as both a flight and basking ground with very few feeding plants. Therefore, during all time blocks, butterflies were observed flying rapidly, landing on perch locations such as leaves for varying periods of time. While on their perch, we observed basking with wings in four positions, as seen in plates 2-5 or they frequently engaged in territorial defence (presumably of perch) in the form of upward spiral chases.

We found a significant relationship of *A. jatrophae* activities observed across the various Time Blocks (X2 [15] = 41.84, p-value = 2.4e-04, n =442). Specifically, we found that time blocks had significantly influenced all *A. jatrophae* activities except for fanning, where only 4 observations were recorded (2 observed in time block II). Aside from flight in each Time Block, the primary activity observed was TB I - Dorsal Basking, TB II - Territorial Defense, TB III - Diurnal Roosting and TB IV - Territorial Defense.

Similar to our findings, Kingsolver (1985) noted that butterflies regulate their internal body temperature to meet the elevated thermal requirements for flight and accomplish other essential activities such as travelling to a feeding patch [37] Therefore, it is not surprising that we observed most butterflies basking in the first time block to increase body temperature. Most other activities including seeking shade that we observed occurred during other time blocks, including shade seeking in Time Block III, which coincides with the warmest period of the day, as a heat loss device to prevent injury [15, 37].

Kemp and Krockenberger (2004) also noted as male butterflies seek potential mates they engage in two behaviours; 1) if a female enters their territory they engage in courtship behaviours, whereas, 2) if males come within their visual range they engage in territorial battles. However, as heliotherms, their ability to move in their environment and successfully compete for mating and territorial defense is constrained by their ability to regulate the temperature of their thoracic muscles which is linked to thoracic power output [25, 36, 38]. Similarly, females depend on air temperature/solar radiation as it directly correlates to daily egg production [39]. Limited flight activity time in females also reduces visits to oviposition sites, reducing fecundity [39]. As noted in our study, territorial defence and flight occurred during the warm periods of the day (TB II and IV) during which they can maintain higher thoracic temperatures. These observations were unsurprising as *A. jatrophae* is known for its territorial behaviour [35]. Furthermore, flight would have been noted throughout the day as it allows for the production of metabolic heat, which when coupled with other heating and cooling thermoregulatory activities and natural occurrences such solar irradiation and evaporative cooling enable the butterfly to maintain its body temperature for all daily activities [37].

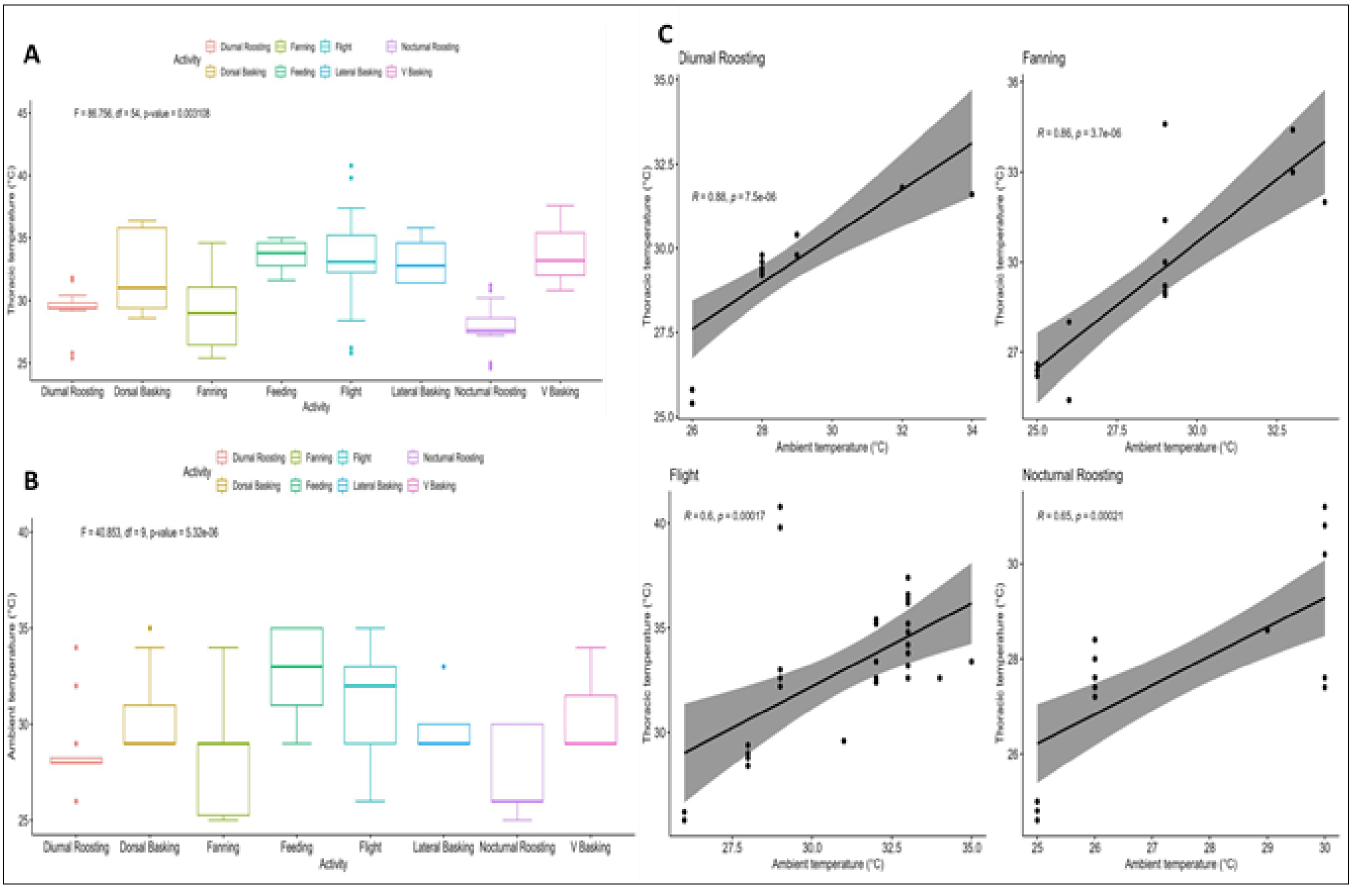


Fig 2 (A, B, C): Boxplots and correlation plots of the variations in thoracic temperatures of butterflies and ambient temperatures with daily activities

Boxplots and correlation plots (Fig. 2) comparing thoracic temperatures (Kruskal-Wallis chi-squared H= 86.756, df = 54, p-value = 0.003108) and ambient temperatures (Kruskal-Wallis chi-squared H= 40.853, df = 9, p-value = 5.32e-06) show a statistically significant difference of these temperatures during different activities. The regulation of heat gain and loss are the two methods for controlling a raised body temperature since body temperature is the outcome of a balance between the rates of heat gain and heat loss [39]. There are two levels at which this regulation of heat gain and loss can take place: the regulation of heat production and transfer within the body (known as physiological mechanisms) and the regulation of heat exchange between the body and its surroundings (known as behavioural mechanisms) [21, 38]. Therefore, as temperature changes, changes in the behaviour of ectotherms will be noted, as was seen in our study. Further, behavioural changes can reduce selection pressure on thermally sensitive characteristics and even lead to co-adaptation and significant selection on morphological features closely associated with the behavioural strategy; therefore, behaviour impacts how a species can respond to environmental changes [38].

Further analyses (Multiple pairwise comparisons using Dunn's Test), indicated that ambient temperatures during Feeding were significantly different from Diurnal roosting (p-value = 0.086), fanning (p-value = 0.0155), and Nocturnal Roosting (p-value = 0.0002). Similarly, ambient temperatures during flight were significantly different from Diurnal Roosting (p-value = 0.0095), fanning (p-value= 0.0179), and Nocturnal Roosting (p-value = 0.003). With thoracic temperatures, we noticed Nocturnal Roosting was significantly different from fanning (p-value = 0.0028), Dorsal Basking, (p-value = 0.0004), Feeding (p-value = 0.0002), Flight (p-value = 0.00) and Lateral Basking (p-value = 0.0191). Similarly, ambient temperatures during fanning were significantly different from flight (p-value= 0.0028). There are statistically significant positive correlations between ambient and thoracic temperatures for Diurnal Roosting, Fanning, Flight, and Nocturnal Roosting.

Results of the paired t-test indicated that there is no statically significant difference between External Temp/^{0}C (M = 31.1, SD = 1.3) and Internal Temp/^{0}C (M = 30.9, SD = 1.6), t (23) = 0.8, p = .410. The test statistic T equals -0.8388, which is in the 95% region of acceptance: [-2.0687, 2.0687]. The after minus before (-0.18), is in the 95% region of acceptance: [-0.4316, 0.4316]. The 95% confidence interval of after minus before is: [-0.6066, 0.2566]. The p-value equals 0.4102, ($P(x \leq -0.8388) = 0.2051$). It means that the chance of type I error, rejecting a correct H_0, is too high: 0.4102 (41.02%). The observed effect size d is very small, 0.17. These results indicate that the magnitude of the difference between the average of the differences and the expected average of the differences is minimal. Since the p-value > α, we cannot reject the H_0 i.e. The External (ambient) Temperature average is considered equal to the butterfly's internal Temperature average.

Analyses show that *A. jatrophae* butterflies changed their main activities during different times of the day and that their thoracic temperature is similar to air temperature. Clench (1966) described behavioural devices and patterns that are exhibited to ensure that they meet their daily needs without exceeding their physiological temperature threshold. As ectotherms, butterflies must raise the temperature of their thoracic muscles to within the small ranges necessary for effective flight and they have accomplished this by carefully choosing their microhabitats (such as perch sites) and using basking body positions and orientations [38]. Interestingly, although we noted positive correlations between thoracic and ambient temperature for activities such as Diurnal Roosting, Fanning, Flight, and Nocturnal Roosting, there was no statistically significant corollary relationship for the basking activities. In the absence of thermoregulatory activities the body temperature of insects is dependent on the external environment (as is seen in Fig. 4) as such they cannot maintain a constant body temperature independently of the temperature of their environment [40]. Therefore, thoracic temperature for activities such as fanning, flight and roosting will positively correlate with ambient temperatures. However, during basking where wing posture affects thoracic heat gain [21, 37]. Therefore, butterfly thoracic temperatures will not correlate to ambient temperatures as they actively try to increase their body temperatures.

Wings of butterflies are important structures in thermoregulation as when open and the butterfly orients it allows for maximum exposure to the sun or when closed the butterfly can significantly reduce solar irradiation [37]. When exposed to the sun the blood in the veins of the wings gains heat and then be transported around the body; similarly, when not exposed to heat the circulating blood can dissipate heat [37]. The various basking positions that we have observed may be a result of the butterflies' attempts to control the amount of surface area of the wings exposed to direct sunlight, thus regulating their body temperature [19–21]. Although different species have different basking methods, generally, heat is gained through radiation maximized and heat loss through convection is reduced [21, 35]. When basking butterflies are known to orient their bodies perpendicular to the plane of incident solar radiation so that the maximum area of the wings is exposed to the sun [21, 37]. The wings were then placed in specific positions, i.e. horizontal (dorsal basking), vertical (lateral basking), basking (angled) and downward V (appressed) and positions as also noted by Kemp and Krockenberger (2002) for *Hypolimnas bolina.* Although for *A. jatrophae* the downward V (appressed) position was rarely noted and therefore not included in the statistical analyses. Kemp and Krockenberger (2002) indicated in their experiments that the apression posture is adapted for maximum heat gain and it may have been possible that this was present in the first time block on higher perch sites (leaves of tall trees) and therefore, we may not have detected this behaviour. We did note that *A. jatrophae* butterflies spend long periods in their basking ground. We have postulated that because their wings are not dark colour and do not have large dark patches as their other open field loving counterparts *Junonia everte* (Nymphalidae) (also observed but not documented in this study) they are not efficient passive heat gatherers and as such must spend long and multiple times per days basking.

This study provided baseline information that will set the stage for longer-term studies to better understand the studied species' ecological needs and the selective pressures that affect the fitness outcomes of individuals. Although this study provides a snapshot of how temperature affects butterfly activities, rather than conclusive evidence of the effects of temperature on behaviours, it does elucidate how activities vary with daily temperature and the corollary relationship of thoracic temperature and ambient air temperature. Therefore, a long-term study building upon this study is needed as it will enable us to make inferences about how temperature, the major climatic factor associated with widespread local extinctions [4], affect butterfly populations as global air temperature rises. Additionally, an expanded behavioural study with a more detailed characterization of thermoregulatory devices and patterns and how they influence thoracic temperatures and vary with ambient air temperature is needed to elucidate how thermoregulatory behaviours can affect daily activities, reproduction and survivorship of these butterflies in a changing climate [19] as behaviour is a critical thermoregulatory mechanism for protection against overheating in the tropics [6].

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Conflict of 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.

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