SEASONAL PLASTICITY IN *JUNONIA COENIA* (NYMPHALIDAE): LINKING WING COLOR, TEMPERATURE DYNAMICS, AND BEHAVIOR

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ABSTRACT. The common buckeye, *Junonia coenia* (Hübner), is a North American nymphalid that shows seasonal wing color plasticity. Throughout much of its range the ventral hindwings are tan in the spring and summer and dark red in autumn. Although this species has long been used as a model to study the physiology and genetics of plasticity, the function of its seasonal color variation is still largely unknown. Here we investigate the effect of buckeye wing color on wing and body temperature and ask to what extent seasonal plasticity is associated with behavioral differences. By using real time infrared imaging of different seasonal morphs from a laboratory colony of North Carolina *J. coenia*, we observed that red autumnal butterflies warm up faster and reach higher final temperatures than tan summer butterflies. Furthermore, behavioral trials at field sites revealed that red butterflies are less physically active than tan butterflies. Based on our findings, we propose that the dark red wing coloration of autumn morphs could be useful for helping individuals reach higher body temperatures faster while basking on colder days. We also speculate that lower activity levels of autumnal butterflies may help conserve energy.

Additional key words: butterfly, polyphenism, wing patterns, thermoregulation, infrared imaging

The North American nymphalid *Junonia coenia* (Hübner), also known as the common buckeye, has long served as a model system for studies of phenotypic plasticity (Roundtree and Nijhout 1995a, 1995b, Nijhout 1997, Nijhout 2003, Daniels et al. 2012, Daniels et al. 2014). This species shows distinct seasonal wing color variation, where across most of its range adults have tan hindwings in the spring and summer and red hindwings in the autumn (Daniels et al. 2012). Ventral hindwing coloration is determined by environmental cues during larval and pupal development—the tan morph is induced by long day lengths and high temperatures, whereas the red morph is induced by short day lengths and low temperatures (Smith 1991, Daniels et al. 2012). Interestingly, however, in coastal Southern California populations plasticity is drastically reduced and individuals do not develop red wing coloration even under normally inductive conditions (Daniels et al. 2012). This evidence of local geographic variation in plasticity has led to the proposal wing color could play an ecological role in this species. Despite a long history of research on buckeyes, however, the functional significance of its wing color plasticity is still poorly understood.

Here we assess the hypothesis that buckeye wing color plasticity plays a role in temperature regulation. Indeed, similar cases of ventral wing color plasticity have been shown to have clear thermal consequences in other butterfly species (e.g. Watt 1968, Kingsolver 1987, Kingsolver 1995). We tested the influence of buckeye wing color on wing and body temperature by using real time infrared imaging to generate warming profiles of red versus tan individuals reared under controlled laboratory conditions. We also compared adult butterfly activity levels at field sites in North Carolina and Southern California to determine whether alternate seasonal morphs show differences in early morning behavior. We found that seasonal color variation has a strong effect on warming and that red autumnal morphs show reduced levels of activity.

Materials and Methods

Analysis of temperature dynamics. For temperature measurements we used laboratory-reared *Junonia coenia coenia* from a colony originating from Durham, North Carolina. Tan morphs were obtained by rearing larvae and pupae at 27°C with a 16:8 hour light:dark cycle. Red morphs were obtained by rearing larvae and pupae at 20°C with 8:16 hour light:dark cycle. Experiments were performed within three days of adult emergence. Adults were weighed before experiments, and digital images of wings were made afterwards. Wing color was scored as shown in Fig. 1A, and butterflies scored as a one or two were considered tan (equivalent to the *linea* and light intermediate forms of Smith, 1991), and butterflies scored as a three, four, or five were considered red (equivalent to the dark intermediate and *rosa* forms of Smith, 1991). Experiments were conducted in a cold chamber set to a constant temperature of 6 ± 2°C. Butterflies were immobilized on a cardboard sheet with string (Fig. 1B, C) and placed under a 150W incandescent light source (Philips 150A21/RS/VS/BR 120/130V) at 30 cm distance. As for natural daylight, this bulb has a full spectrum emission (see Philips bulb spectrum in Evstratov et al. 2006). Incandescent bulbs, including
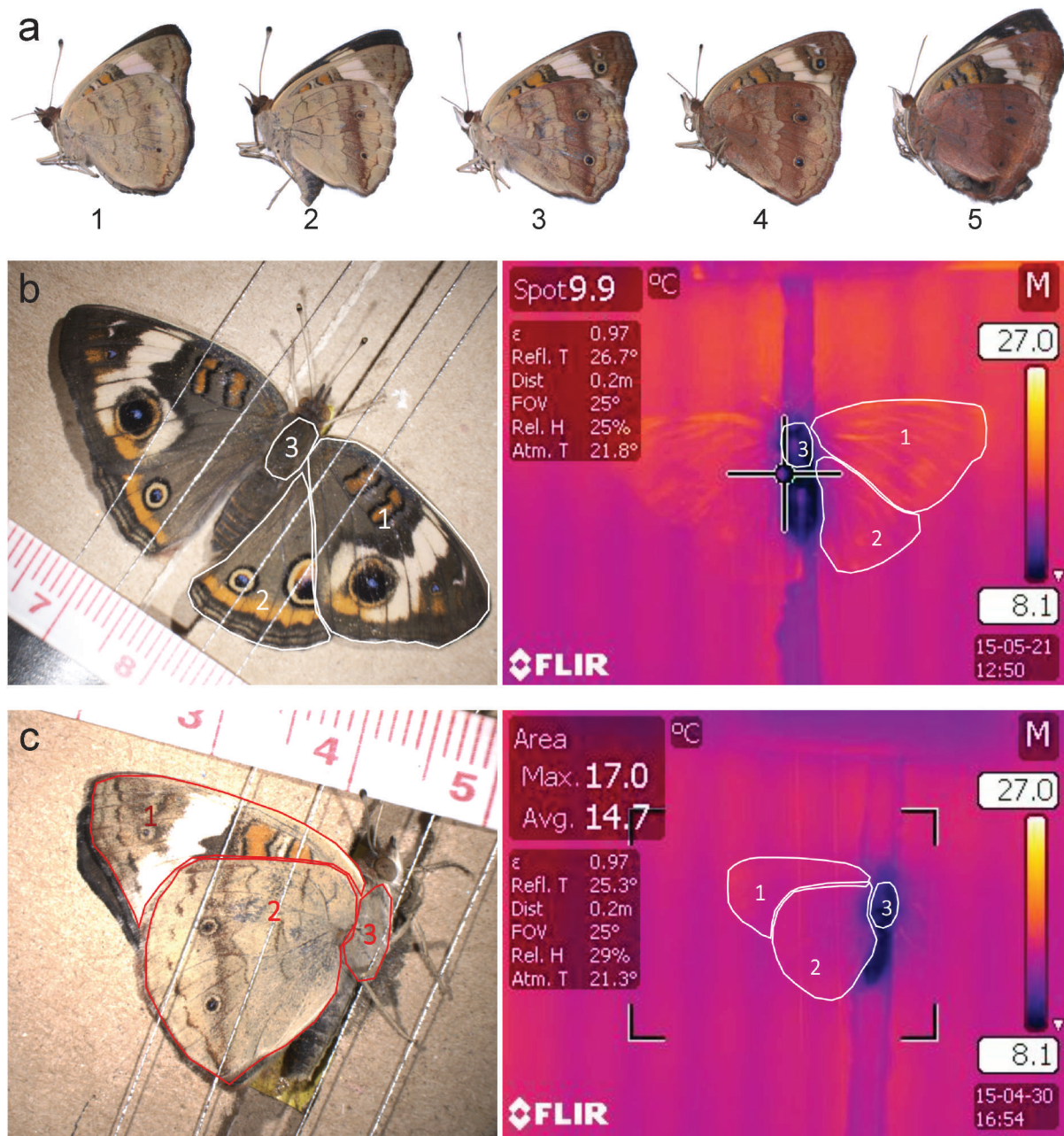
FIG. 1. Wing scoring and staging for thermal imaging. (**a**) Scale used to score butterfly wing color for temperature experiments. Butterflies scored as a one or two were considered tan, and butterflies scored as a three, four, or five were considered red. (**b**) Set up used to immobilize butterflies for dorsal measurements. (**c**) Set up used to immobilize butterflies for ventral measurements. **1**: forewing, **2**: hindwing, **3**: thorax.

this one, however, have relatively greater intensity in the red and infrared wavelengths compared to unfiltered sunlight, thus resulting in greater heating performance (Wurtman 1975, Prescott & Wathes 1999). A FLIR T400 infrared camera recorded images at ten second time intervals for ten minutes—sufficient time for each butterfly to reach a constant plateau temperature. This

was repeated for the dorsal (Fig. 1B) and ventral (Fig. 1C) wings, with a 10 min 6 ± 2°C cool down period between dorsal and ventral exposures. Three regions of interest were mapped onto each butterfly for both the dorsal and ventral sides—one covering all basking-visible regions of a forewing, one covering all basking-visible regions of a hindwing, and one covering the

thorax (Fig. 1B, C). A control region was also mapped onto the cardboard surface above each butterfly for both ventral and dorsal images. The ThermaCAM Researcher Pro 2.9 software package was used to calculate the average temperature for each region in each image, and these measurements were used to generate six temperature curves for each butterfly: dorsal forewing, dorsal hindwing, dorsal thorax, ventral forewing, ventral hindwing, and ventral thorax.

Heating curves were fitted to the equation $T_b(t) = T_{inf} + (T_i - T_{inf}) \exp[-t/\tau]$ as described (Kingsolver and Watt 1983), where T_b is body temperature, T_i is the initial steady state temperature, T_{inf} is the final steady state temperature, t is time, and τ is the time taken for T_b to reach 63.2% of the way from T_i to T_{inf}. We used R 3.3.2 (R Core Team (2016)) to fit curves using maximum likelihood. We tested whether T_{inf} values were significantly different for tan and red butterflies using a linear model for each region of interest using sex, mass, and control values as covariates. We fitted six separate models for T_{inf}, one for each combination of surface (dorsal vs. ventral) and region. We also tested whether the values of τ were significantly different for tan and red butterflies with a linear model for each combination of side and region using sex, mass, and the corresponding value of τ for the cardboard control regions as covariates. We also built a linear model to examine the effect of butterfly wing color as a continuous variable on T_{inf} for each butterfly region, using sex, mass, and T_{inf} of the control region as covariates. We calculated the slope of the regression line, the R-squared statistic of the line, and the p-value. All p-values in the above analyses were corrected for multiple comparisons using the Benjamini-Hochberg method. Finally, to estimate heating rates, we used the inverse of the heating equation to determine the time point at which each region of interest reached 6, 7, 8, 9, 10, 11, 12, and 13°C, respectively. To estimate heating rates over time we calculated the slope for each 1°C interval. To test whether heating rates differed significantly between red and tan butterflies, we used a linear model to determine the effect of butterfly color on heating rate for each temperature interval using sex, mass, and heating rate for the control region as covariates. We again fitted six separate models for each heating rate, one for each combination of side and region.

Behavioral observations. For behavioral assays, we used lab-reared NC morphs (subspecies *coenia*) and wild-caught butterflies (likely subspecies *grisea* in California). Lab-reared pupal and adult butterflies (raised under conditions described above) were transported to the field site and kept at room temperature until the day of testing. Wild butterflies were captured at field localities and kept at room temperature until testing. Before sunrise on the day of testing two mesh cages with dimensions of approximately $35 \times 80 \times 60$ cm, with three compartments each, were set up next to each other at the field site. Ambient temperature and humidity were recorded. One lab-reared tan, one wild-caught tan, and one lab-reared red butterfly of random sexes were placed in each compartment and two observers recorded behavioral data. Butterflies were observed for 90 min at the same time each morning at the onset of exposure to natural sunlight. Wild vs. lab-reared tan butterflies were easy to differentiate in trials thanks to minor individual differences in size, color pattern, hue, etc. A time-lapse camera was also placed in front of each cage and took pictures at 1 sec intervals. All butterflies were sexed and imaged after the behavioral trials. We conducted North Carolina work July 22–25, 2016, near Durham, NC (36°07′38.1″N 78°49′56.1″W), at the edge of a grassy field in a waterfowl impoundment. Temperatures when observations started ranged 21–24 °C, and cumulative time of sunlight on each cage ranged 43–67 min per session. North Carolina trials took place approximately 10 min after sunrise. We conducted California work August 4–8, 2016, at Audubon Starr Ranch, Trabuco Canyon, CA (33.6321° N, 117.5555° W) at the top of a hill near the entrance to the sanctuary, a site chosen because it receives sunlight earlier than sites in the valley. Temperatures when observations started ranged 19–24 °C, and cumulative time of sunlight on each cage ranged 0–42 min per session (on three days the cages had no direct sunlight because of cloud cover). California trials took place approximately one hour after sunrise — a bit later than the North Carolina trials in order to allow the marine layer to subside.

To analyze behavior, at the beginning of each observation minute each of four behaviors (open wings, fluttering, crawling, and flight) was scored 1 if present or 0 if absent. These scores were summed for each butterfly and behavior for three consecutive 30 min time windows. For each behavior, we fitted a full linear mixed effects model predicting behavior value sums using behavioral state ("state"), source population ("population"), time window ("time-window"), and time after sunrise ("time-after"), including all two-way interactions between these effects (Table 1). Three-way interactions of state:time-window:time-after and population:time-window:state as fixed effects, and cumulative minutes of sun on each cage in each time window and the sex of each butterfly were also included in the model. We also included random effects of day, cage, sector within day and cage, and individual
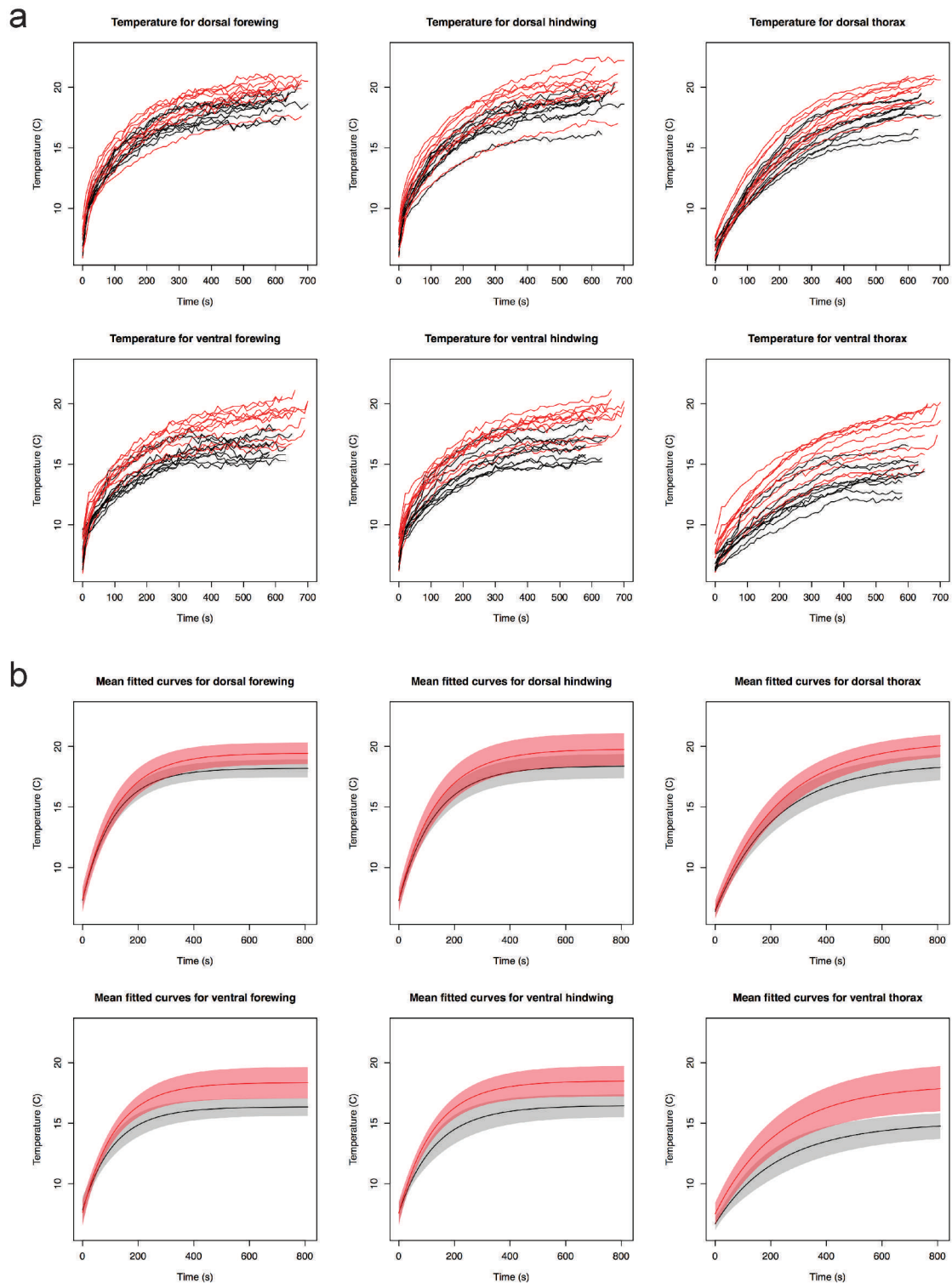
FIG. 2. Wing and thorax heating profiles. (**a**) Raw data used to generate heating curves. Graphs represent temperature of each butterfly for each anatomical region as butterflies were heated. Temperature values were collected every ten seconds. Red lines indicate red butterflies (n = 11), and black lines indicate tan butterflies (n = 10). (**b**) Heating curves differ between tan and red butterflies. Lines represents means of fitted curves for tan (black lines) and red (red lines) butterflies for each side and region. Shaded regions indicate a single standard deviation for each fitted curve.

TABLE 1. Significant p-values for factors and interactions in behavioral frequency models.

Variables and interactions	Open wings	Fluttering	Crawling	Flight	Mean temperature
population	1.50E-09	2.70E-05	0.0089		
time-after					
state					
cumulative sun		5.50E-05	2.10E-04	6.10E-05	2.20E-16
time window	3.90E-09	5.10E-04	0.0012	1.80E-04	2.80E-14
sex	0.043	0.032			
time-after:state					
population:state	1.20E-04			8.20E-04	
time-after:time-window	2.20E-05	4.90E-04	0.0017	0.0021	7.10E-04
state:time-window		0.0036	0.0019		0.0013
population:time-window	0.0016				
population:time-after		0.041	0.012		
time-after:state:time-window					0.024
population:state:time-window					

butterfly. Unfortunately, we could not include age as an effect because we could not determine the age of wild caught butterflies. Models were built using the R package lme4 (Bates et al. 2015). For flight and crawling behaviors, value sums were log(x+1) transformed to meet assumptions of the linear mixed effects model. We conducted backward selection to remove non-significant interactions to improve the model. For each behavior, we followed the linear mixed effects model analysis with multiple comparisons on the interaction of population and time window within each state with a Tukey correction for multiple comparisons, all using the lsmeans package in R (Lenth 2016). This was done to determine statistically significant differences between behavior levels of the three butterfly populations within each time window within each state.

RESULTS

Red autumnal buckeyes warm faster and to higher temperatures than tan summer buckeyes. To characterize the effects of wing color on adult temperature, we measured surface temperatures of summer morphs (tan ventral hindwing) and autumn morphs (red ventral hindwing) as they warmed under a heat radiating full spectrum light source in a cold room. The raw data (Fig. 2A) were fitted to the temperature change equation described above, and we calculated means and standard deviations of the fitted curves for tan and red butterflies for each butterfly surface (dorsal and ventral) and region (forewing, hindwing, and thorax) (Fig. 2B). The linear model that examined the effect of color on final temperature revealed higher final temperatures of red butterflies (p < 0.05) for all anatomical regions except for the dorsal hindwing. There was also an effect of butterfly sex on final temperature of the ventral thorax (p < 0.05), where males tended to be slightly warmer, and an effect of butterfly mass on final temperature of the dorsal thorax and ventral hindwing (p < 0.05), where heavier butterflies tended to have lower final temperatures. This result is perhaps unsurprising since *J. coenia* females tend to be larger and heavier than males. We used another linear model to determine the effect of graded wing color on final temperature for increasing temperature curves using sex, mass, and final temperature of the control region as covariates (Fig. 3). This analysis also revealed that butterfly color was a significant predictor of final temperature (p < 0.05), where darker and redder ventral hindwings predicted higher final temperatures. We also looked at whether different color morphs warm at different rates and found that red butterflies warmed significantly faster than tan butterflies, with the strongest effects for the hindwings and thorax (Fig. 4). In sum, our experiments
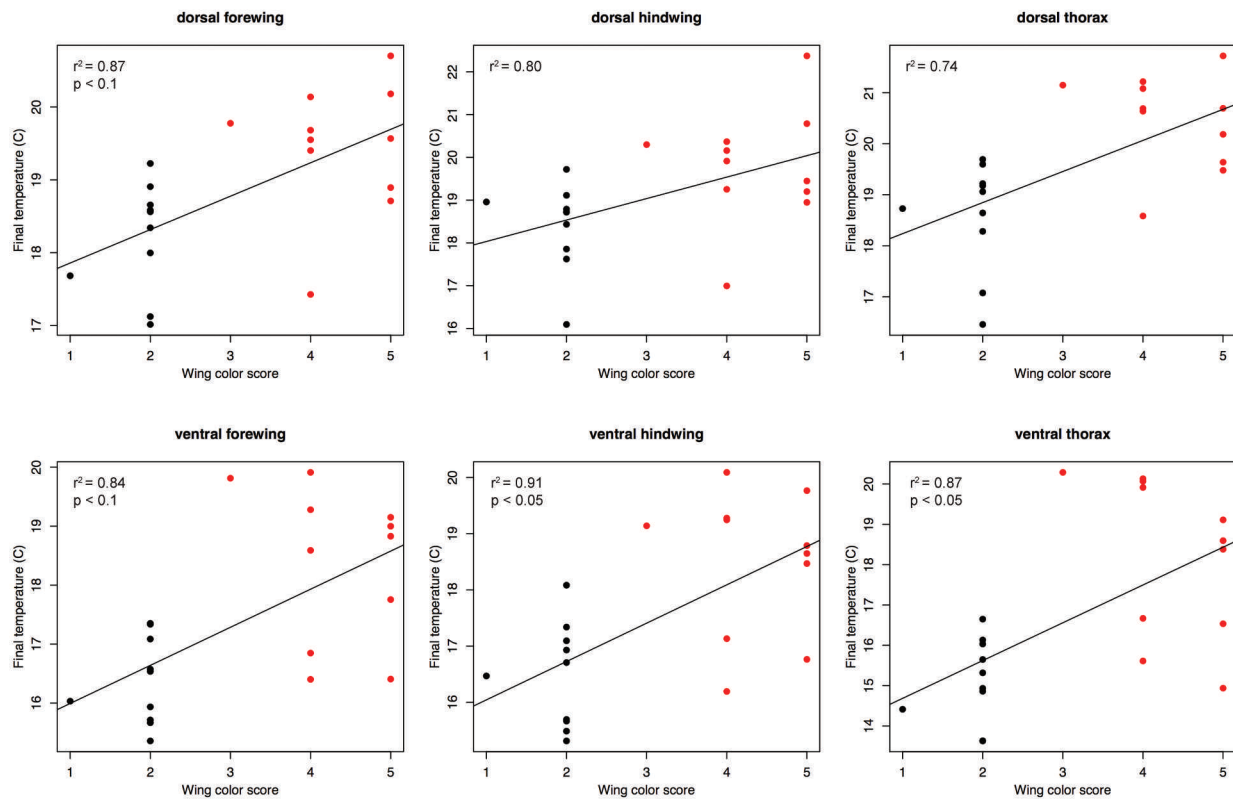
FIG. 3. Wing color predicts final temperature in heating experiments. Wing color score of each butterfly (see Fig. 1A) is plotted against final temperature for each anatomical region. R-squared values and significant Benjamini-Hochberg adjusted p-values for each regression line are shown for each plot. Red dots indicate red butterflies (n = 11), and black dots indicate tan butterflies (n = 10).

showed a strong pattern of red autumnal morphs warming faster and to higher final temperatures than tan seasonal morphs, with the strongest effects across analyses on the ventral hindwings, where color plasticity is most pronounced.

Red autumnal buckeyes are less physically active than tan summer buckeyes. To compare morning behaviors of the different butterfly populations observed (NC summer morphs (tan), NC autumn morphs (red), and wild-caught tan butterflies (NC or CA)), we analyzed numerical behavior data as described above (Table 1). Significant behavioral differences within each time window and state are shown in Fig. 5. The most consistent result across comparisons was that lab-reared tan morph butterflies showed much higher levels of open wings, fluttering, and crawling than the lab-reared red autumnal morphs, and, to a lesser extent, wild-caught tan butterflies. We thus conclude that tan morphs of *J. coenia* have a higher baseline activity level than red morphs, however activity levels may decrease in butterflies that have been living for a period under natural conditions (all lab-reared butterflies were tested within a few days of emergence, however we could not control for the age of the wild-caught butterflies).

DISCUSSION

Many butterfly species show seasonal plasticity in morphological and behavioral traits. The results we present here for *J. coenia* are consistent with previous work in other butterflies, where darker coloration is correlated with increased heating rates (Trullas et al. 2007; Zeuss et al. 2014). For butterflies, basking in the sun is essential for reaching temperatures necessary for behaviors such as flight, oviposition, and courtship, and wing pigmentation has been shown to impact body temperature in many species (Watt 1968, Douglas & Grula 1978, Wasserthal 1975, Kingsolver 1985, Kingsolver 1987, Heinrich 1993, Van Dyck & Matthysen 1998, Ellers & Boggs 2004, Xing et al. 2016). The findings we present here support a similar role for
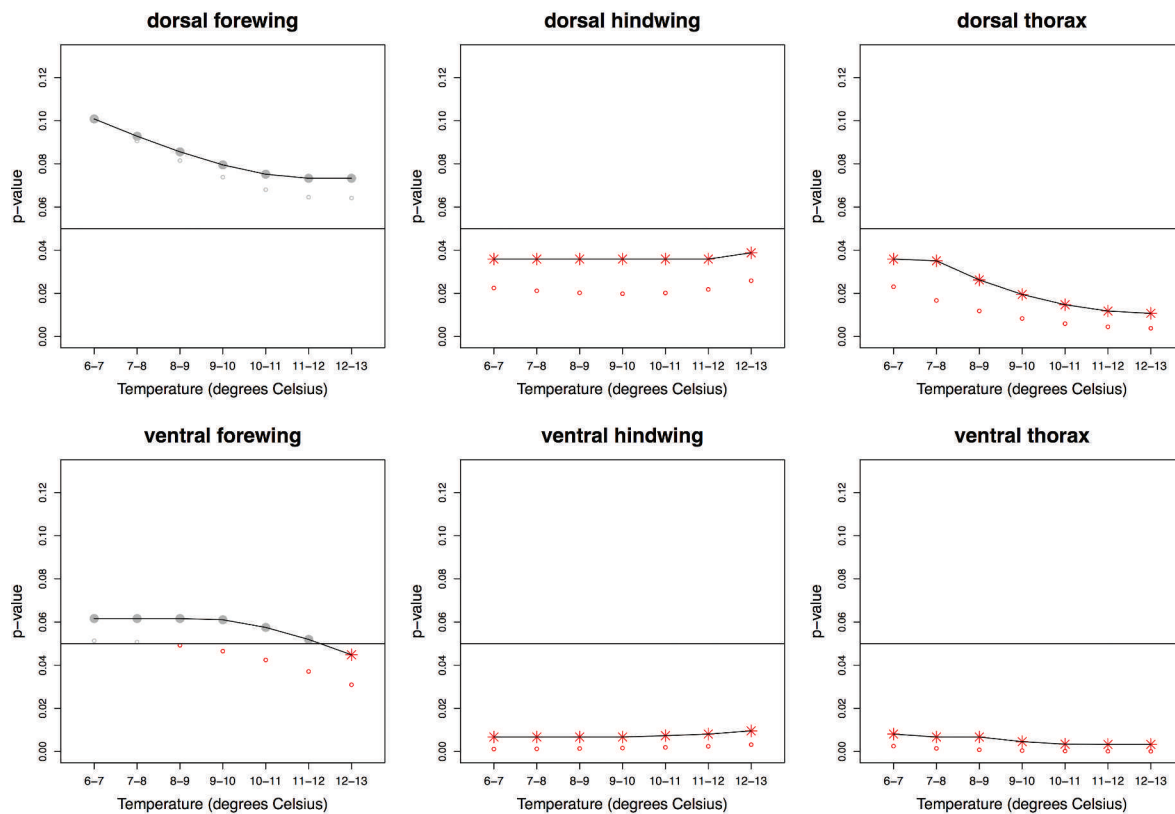
FIG. 4. Red butterflies heat up faster than tan butterflies. p-values demonstrate significant differences between heating rates of tan and red butterflies for seven different 1°C temperature intervals. Gray dots and red asterisks indicate Benjamini-Hochberg corrected p-values. Red asterisks additionally indicate significant difference in heating rate between tan and red butterflies for a temperature interval. Gray and red circles indicate unadjusted p-values. Black lines indicate p = 0.05 threshold.

wing pigmentation in *J. coenia*, where we found that seasonal wing color differences have significant effects on wing and body temperature dynamics. While it is true that this work shows a clear connection between wing color and warming, there may be other ecological functions of wing color plasticity, including predator avoidance, that our experiments cannot rule out (Clark & Sheppard 1960, Windig et al. 1994, Roskam & Brakefield 1996).

As with morphological traits, seasonal plasticity in behavioral traits has also been observed in many butterfly species. For example, seasonal environmental cues will determine *Bicyclus anynana*, *Danaus plexippus*, and *Polyognia c-album* activity levels, including reproductive activity (Tuskes & Brower 1978, Brakefield & Reitsma 1991, Karlsson et al. 2007, Prudic et al. 2011). Behavioral variation is linked with wing coloration in some species as well, including where darker pigmentation is associated with increased flight duration in *Colias nastes*, and greater activity and longer

patrol flights males in *Pararge aegeria* (Roland, 1982, 2006, Van Dyck et al. 1997, Van Dyck & Matthysen 1998). Based on these previous case studies we initially predicted that a similar pattern would be seen in *J. coenia*, where darker autumn forms would be more active. We observed the opposite trend, however, where darker buckeyes were distinctly less active. In this respect buckeyes show seasonal differences in behavior more similar to what is seen in *D. plexippus* and *P. c-album*, which show reduced activity levels while overwintering (Tuskes & Brower 1978, Karlsson et al. 2007). We speculate that this kind of behavioral seasonality may be advantageous in *J. coenia* where autumn morphs exhibit lower activity levels in order to conserve energy. These seasonal activity differences may also be related to migration and/or hibernation, although thus far these phenomena are not well understood in *J. coenia*. One unexpected trend in our data was that tan wild-caught butterflies tended to have reduced activity levels similar to lab-reared red
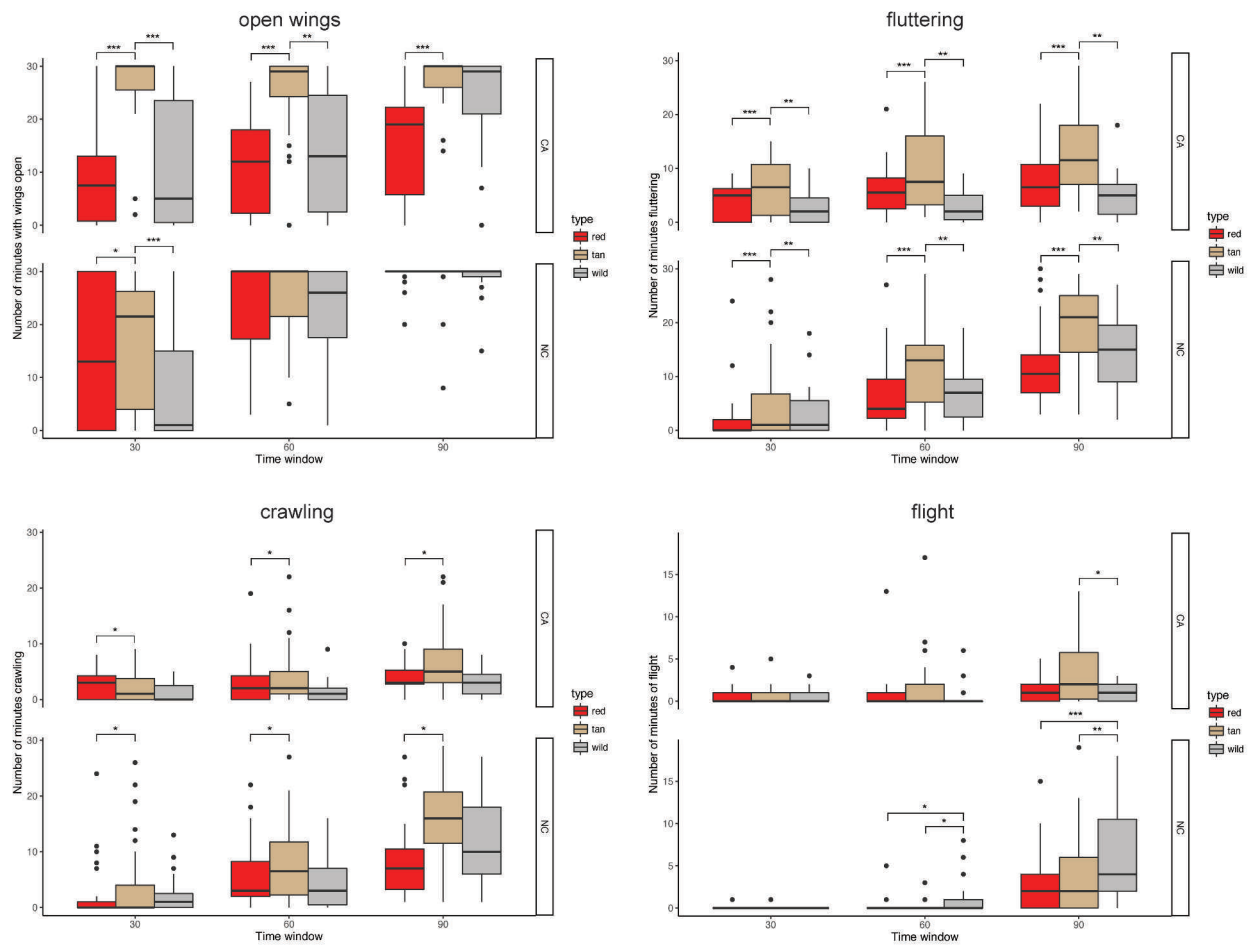
FIG. 5. Median number of minutes of each behavior observed in each state (NC and CA): open wings, fluttering, crawling, and flight. Box plot hinges indicate first and third quartiles, whiskers indicate 1.5 times the interquartile range, and dots indicate outliers. Brackets and asterisks indicate significant differences between butterfly populations. *: p < 0.05, **: p < 0.01, and ***: p < 0.001. CA sample sizes were n=20, n=30, and n=5 for red, tan, and wild-caught butterflies respectively. NC sample sizes were n=24, n = 24, and n=21 for red, tan, and wild-caught butterflies respectively.

butterflies, and unlike lab-reared tan butterflies. This suggests that other factors in the wild-caught butterflies' life history may be affecting their behavior. We speculate that the generally lower activity levels of wild-caught butterflies may be a result of the stress of capture and containment, old age, dietary differences, or other similar factors. Differences could also be attributable to other effects of captive breeding of our North Carolina stock (e.g. Lewis & Thomas 2001, Sorenson et al. 2012).

In conclusion, the work we present here demonstrates clear differences in warming dynamics between red and tan seasonal morphs of *J. coenia*. We also observed significant behavioral differences between these seasonal morphs under natural conditions, where red autumnal butterflies show significantly decreased activity levels compared to tan summer butterflies. This work serves as a foundation for future studies on seasonal plasticity in the model species *J. coenia*.

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