



Age and Appearance Shape Behavioral Responses of Phasmids in a Dynamic Environment

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Although morphological adaptations leading to crypsis or mimicry have been studied extensively, their interaction with particular behaviors to avoid detection or recognition is understudied. Yet animal behaviors interact with morphology to reduce detection risk, and the level of protection conferred likely changes according to the surrounding environment. Apart from providing a locational cue for predators, prey motion can also serve as concealing behavior in a dynamic environment to prevent detection by potential predators or prey. Phasmids are conventionally known to rely on their adaptive resemblance to plant parts for protection, and this resemblance may vary across life stages and species. However, little is known about how their behaviors interact with their appearance and their environment. We investigated two species of phasmids with varying morphology and color patterns at different ontogenetic stages and examined their behavioral responses to a wind stimulus as a proxy for a dynamic environment. While adult behaviors were mostly species-specific, behavioral responses of nymphs varied with appearance and environmental condition. Display of different behaviors classified as revealing was positively correlated, while the display of concealing behaviors, except for swaying, was mostly negatively correlated with other behaviors. Exhibition of specific behaviors varied with appearance and environmental condition, suggesting that these behavioral responses could help reduce detection or recognition cues. We discuss the differences in behavioral responses in the context of how the behaviors could reveal or conceal the phasmids from potential predators. Our results provide a novel investigation into adaptive resemblance strategies of phasmids through the interaction of behavior and morphology, and highlight the importance of considering the effects of dynamic environments on sending and receiving cues.

Keywords: adaptive resemblance, *Calvisia flavopennis*, crypsis, *Lonchodes brevipes*, motion masquerade, Phasmatodea

INTRODUCTION

Predator–prey relationships are fundamental in shaping animal morphology and animal behavior. In the constant evolutionary arms race (Dawkins and Krebs, 1979), predators need to be capable of detecting and catching prey, while potential prey need to avoid detection or escape capture through morphological or behavioral adaptations (Ruxton et al., 2018). The chances of survival

for prey can increase through minimizing cues that predators could exploit. Adaptive resemblance allows the prey to dupe a potential predator by pretending to be an inanimate object (Starrett, 1993). Concealing behaviors, such as specific postures or resting habits, can interact with morphological adaptations and decrease the risk of detection or recognition, thus improving protection from predators (Stevens and Ruxton, 2019). However, the undetectability of an inanimate object can also be broken by motion (Regan and Beverley, 1984; Ioannou and Krause, 2009; Hall et al., 2013; Cuthill, 2019). Revealing behaviors, such as locomotion, can provide information about the presence and location of an animal and function as a cue for predators (Ioannou and Krause, 2009). Although avoiding motion is clearly an effective way of reducing the risk of detection, other strategies have evolved to minimize cues that reveal the presence, location, or identity of an animal (Tan and Elgar, 2021). Motion can even decrease the probability of detection or recognition through motion camouflage or motion masquerade (Stevens and Merilaita, 2009; Hall et al., 2017; Cuthill, 2019).

Depending on the prevailing environmental conditions, animals can show different behavioral responses to improve their protection. Cryptically colored animals often modify their flight responses depending on their level of exposure (Cuadrado et al., 2001; Maritz, 2012). Behavioral modifications of resemblance strategies are especially important if the environment of the animal is not static but changes dynamically, e.g., through moving backgrounds or changes in illumination (Cuthill et al., 2019). Wind can be the underlying cause of a dynamic environment, e.g., by agitating leaves or creating water caustics (Cuthill et al., 2019). If the anti-predator behavior of an animal relies on blending in with its environment, it is necessary to take this dynamically changing background into account. For instance, several species of phasmids are known to sway in response to a wind stimulus (e.g., Rupperecht, 1971; Bian et al., 2016), which could potentially enhance the resemblance of phasmids to plants when seen against a backdrop of moving vegetation, as the swaying behavior resembles the movement patterns of the plants (Bian et al., 2016). Since wind can dynamically alter the background of an animal, its presence can be used as a proxy for a dynamic environment. If an animal can perceive changes in air currents and adjust its behavior as a response, it allows for maintaining the benefits of adaptive resemblance without the need to integrate detailed information about concrete background changes.

Animals can possess life-stage specific behaviors and color patterns to avoid detection by predators. Ontogenetic changes in color patterns can reduce detection or increase the warning signal as the animal increases in size (Grant, 2007; Tan et al., 2016), while behavioral responses can vary depending on individual size (Cuadrado et al., 2001). Ontogenetic changes in foraging behavior and habitat used are widespread across animal taxa and have profound impacts on individual survival and ecological interactions (Werner and Gilliam, 1984; Hughes et al., 1992; Lind and Welsh, 1994; Hochuli, 2001; Arthur et al., 2008; Nakazawa, 2015; Ohba and Tatsuta, 2016). However, organisms at the same life stage living in a similar habitat are expected to face similar challenges (Fairclough, 2016; Tan et al., 2017).

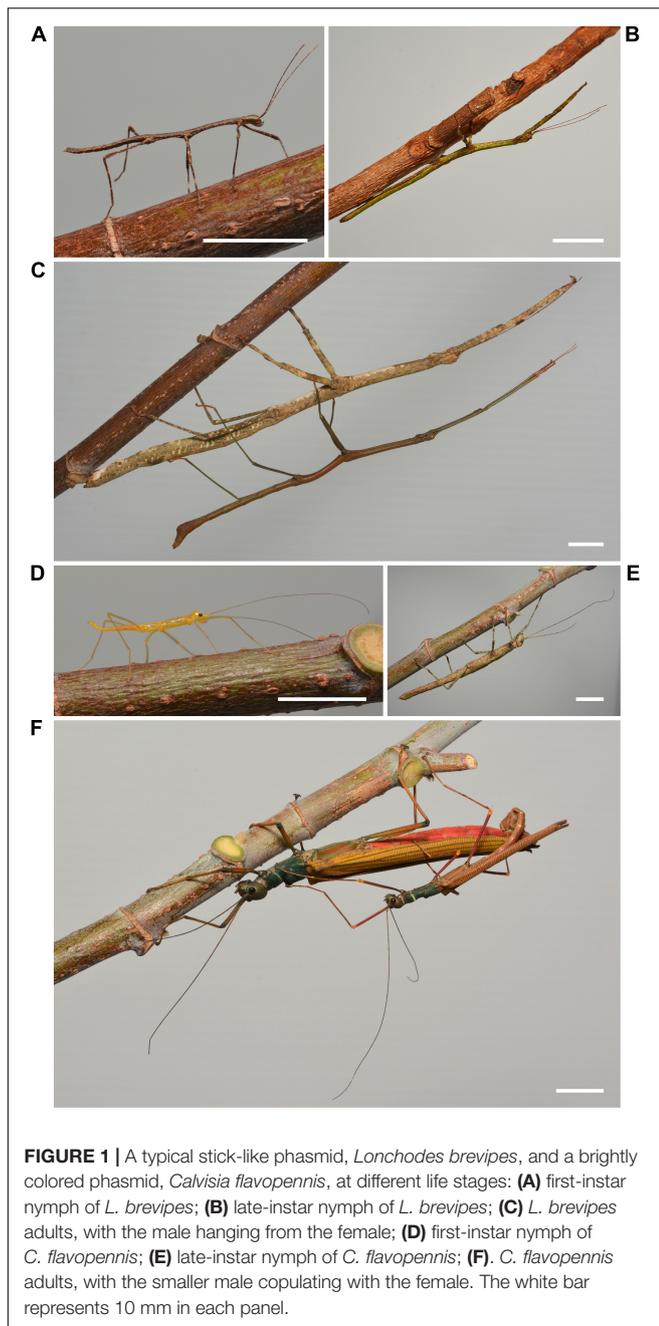
A textbook example of adaptive resemblance, phasmids are predominantly nocturnally foraging herbivores that are known for their remarkable resemblance to sticks or leaves. These plant-resembling phenotypes have existed for at least 47 million years (Wedmann et al., 2007), attesting to their evolutionary success. Phasmid coloration varies from cryptic green and brown colors (e.g., *Lonchodes brevipes*, **Figures 1A–C**) to more “conspicuous” red, blue, and yellow colors (e.g., *Calvisia flavopennis*, **Figures 1D,F**). As hemimetabolous insects, phasmids go through several nymphal stages before emerging as adults, which often differ to varying degrees in their appearance from the adult forms (e.g., *L. brevipes* and *C. flavopennis*, **Figure 1**). With different phenotypes and ecological requirements at different life stages, we can expect the behaviors across these life stages to differ as well. Several predator avoidance behaviors in phasmids have been described, yet their adaptive advantages have not been thoroughly studied. Upon disturbance, some phasmid species may escape by dropping from the substrates to the ground or jump off the substrate (Steiniger, 1933; Robinson, 1968a; Zeng et al., 2020), while other species of phasmids may remain immobile (Strong, 1975). In the case of detection by a predator, some phasmid species show a secondary line of defense behaviors, such as abdomen rearing, stridulation, deimatic displays, or spraying of defensive secretions (Eisner, 1965; Bedford and Chinnick, 1966; Löser and Schulten, 1981; Dräger, 2011; Hennemann et al., 2016).

To examine the interactions between behavior, life stage, and phenotype, we chose two species of phasmids that live in forest habitats in Southeast Asia. The first species, Gray’s Malayan stick insect (*L. brevipes*), is a cryptically colored phasmid with a typical stick-like morphology (Seow-Choen, 2017). The second species, *C. flavopennis*, is a brightly colored phasmid that is fully winged at adulthood and capable of flight (Seow-Choen, 2016). Both species look distinctly different at the various life stages of first-instar nymph (**Figures 1A,D**), late-instar nymph (**Figures 1B,E**), and as adult males and females (**Figures 1C,F**). We examined how the behaviors of these phasmids differ according to their appearance, as determined by species and life stage, using the presence or absence of a wind stimulus as a proxy for a dynamic environment. We predict that phasmids of different appearances exhibit corresponding differences in their behavior and selectively modify their behavioral responses in the presence of a wind stimulus. We envisage that phasmids show specific behaviors that provide them with protection from predators, according to their appearance. Specifically, we expect phasmids with cryptic coloration to adopt behaviors that enhance their adaptive resemblance to inanimate objects and thus reduce their likelihood of detection or recognition, and more conspicuously colored phasmids to adopt behaviors that lower their risk of detection or capture, such as hiding or startle displays.

MATERIALS AND METHODS

Experimental Subjects

To ensure that adaptations to domestication have not been introduced, none of the experimental subjects were from hobbyist



cultures. We used laboratory-reared offspring from field-caught phasmids in this study. We collected *L. brevipes* on Pulau Ubin, Singapore (1.409°N, 103.955°E), and *C. flavopennis* near the Kuala Belalong Field Studies Centre, Temburong District, Brunei Darussalam (4.547°N, 115.158°E). We carried out collections between 19:00 h and midnight while walking through the forests. Individuals of *L. brevipes* were collected from shrubs and trees between 0.5 and 2.5 m above the ground, while *C. flavopennis* was found above 3.5 m height. Phasmids were housed in a temperature-controlled laboratory at 24°C with a 12 h:12 h light:dark cycle. All individuals were provided food

plants *ad libitum* and monitored to be feeding and behaving normally before the behavioral trials. Individuals with missing or misshapen legs or wings were not used in our experiments, as these may impede their natural responses. No individual was subjected to more than one trial at each life stage. We performed 20 successful trials for each combination of species and life stage—first instar, late instar, adult male, adult female—to a total of 80 trials with *L. brevipes* and 80 trials with *C. flavopennis*. “Late instar” denotes nymphs of both species in the later stages of their development, which look dissimilar from both first instar nymphs and adults. In particular, we did not use the final instar of *C. flavopennis* as they started to develop the adult color patterns. Correspondingly, we did not use the final instar of *L. brevipes*.

Experimental Setup

Trials were performed in the same laboratory at 24°C during daylight hours under constant artificial light conditions (170–190 lx), in an enclosure (60 cm × 60 cm × 60 cm) with mesh and plastic sides to contain the phasmids during the trials. To simulate a branch for phasmids to rest on, a wooden dowel (300 mm length and 85 mm diameter) was elevated horizontally 145 mm from the bottom of the enclosure (**Supplementary Figure 1**). We used a portable fan, directed at the phasmid, to simulate a wind stimulus. The fan was moved along a fixed track parallel to the position of the phasmid on the dowel to ensure that the distance of the fan to the phasmid, and hence the wind speed, was constant during and across experiments. Before each trial, the wind velocity was measured to ensure that it was 2.0 ± 0.2 m/s from the track to the dowel. The trials were video-recorded for subsequent behavioral scoring.

Acclimatization

Before each observation, phasmids were introduced to the dowel and allowed to acclimatize for 120 s. Acclimatization was defined as successful when the phasmid adopted a stationary behavior (cf. **Table 1**) on the dowel for at least the last 30 s of the acclimatization period, and the observation commenced by exposing the phasmid to the wind or control treatment. If the phasmid moved off from the dowel, it was replaced onto the dowel and the acclimatization period would be repeated once, following the procedure above. If the phasmid did not adopt a stationary behavior within 90 s after the start of the acclimatization, the trial was aborted for the day and the phasmid was returned to its housing, until a new trial on a subsequent day.

Treatment

Each trial consisted of two separate observations, one per treatment (*control* or *wind*). Each individual was subjected to both the control and wind treatment. The order of the observations was determined by the flip of a coin, with a 180 s time-out between the two observations. During the time-out, the phasmid was placed in an interim cage with fresh leaves.

For the *wind treatment*, the fan was switched on while it was covered with a cardboard box, to allow the fan to pick up its final wind speed of 2.0 ± 0.2 m/s (see Bian et al., 2016). The box was then removed to expose the phasmid to the wind stimulus, marking the beginning of the observation. After 5 s, the cover was

TABLE 1 | List of scored behaviors and their definitions.

Behavior	Description	References
Active escape	Where the individual leaves the substrate through one of these: (i) Takes flight from the substrate (ii) Walks off from the substrate	–
Bounce	Insect body repeatedly moves toward and away from the substrate, while the insect remains in stance in contact with the substrate	–
Curl	The posterior end of the abdomen curls toward the anterior of the insect	Bedford and Chinnick, 1966
Evade	Insect orients away from the source of disturbance	–
Explore	Insect uses antennae and first pair of legs to probe gap spaces with the other four legs on the substrate	Blaesing and Cruse, 2004
<i>Extend</i>	Insect adopts a stationary position with the anterior limbs extended in line with the body axis	Robinson, 1968b
<i>Flatten</i>	Entire body is in contact with the substrate, with all legs around the substrate	–
<i>Hang</i>	Insect is hanging on the underside of the substrate	–
<i>Hug</i>	Insect brings its body closer to the stick by bending its legs, without <i>flattening</i> against the substrate	–
Raise	Abdomen raised from a position parallel to the substrate	–
<i>Stand</i>	Insect is stationary, with all legs partially or entirely straightened (compared to <i>hug</i> or <i>flatten</i>)	–
Stretch forward	Insect extends the anterior legs until they are in line with the body axis	–
Sway	Lateral rocking of the insect body, while the insect remains in stance, in contact with the substrate	Bian et al., 2016
Sway and Walk	Lateral rocking of the insect body, while the insect moves forward	–
Uncurl	Abdomen uncurls from curled position	Bedford and Chinnick, 1966
Walk	Forward movement along the substrate without any lateral movement of the body	–

All behaviors were scored as state events except for active escape, which was scored as a point event that ended the observation early. Stationary behaviors are marked in italics. Please refer to **Supplementary Materials** for videos and images of additionally described behaviours.

replaced to end exposure of the phasmid to the wind stimulus. For the *control treatment*, the procedure was identical, except that the fan was not switched on.

Behavioral Scoring

Video recordings of the observations were played back, and all behaviors were scored using the software BORIS (Friard and Gamba, 2016). The scored behaviors and their definitions are listed in **Table 1**. We referred to the literature for previously described behaviors, and list and describe additional behaviors (please refer to **Supplementary Materials** for videos and images of additionally described behaviours). An observation started with the beginning of the treatment and ended when the phasmid was stationary for at least 5 s after the end of the treatment. An observation could end prematurely if the insect displayed any of the active escape behaviors (e.g., flying away, **Table 1**). Behaviors were scored as *state* events, and the duration of the behavior was recorded. Two stationary behaviors (cf. **Table 1**) were mutually exclusive, while non-stationary behaviors could co-occur with stationary behaviors as well as with non-stationary behaviors. Active escape behaviors that ended an observation were scored as *point* events, i.e., they were either observed or not, and no duration was recorded.

Statistical Analyses

Statistical analyses were performed using the *R* base package (R Core Team, 2019) and PAST 4.03 (Hammer et al., 2001). To adjust for the different total duration of each observation, we calculated, separately for each observation and behavior, the behavioral response as the proportion of time (*p*) that the insect

displayed that behavior during an observation, by dividing the time an individual showed that specific behavior (*b*) by the total duration of the observation (*o*): $p = b/o$. We then performed a permutational multivariate analysis of variance (PERMANOVA) using the function *adonis2* from the *vegan* package (Oksanen et al., 2019) to test the differences in these behavioral responses, with Bray–Curtis distances and 9,999 permutations. The full model included morphological type (specified as the combination of species and life stage), treatment (wind or control), and an interaction term between morphological type and treatment, with the behavioral responses as response variables. We tested for pairwise differences between groups using the function *pairwise.adonis* from the *pairwiseAdonis* package (Martinez Arbizu, 2020), with Bray–Curtis distances, 9,999 permutations, and Benjamini–Hochberg adjusted *p*-values.

Using the *metaMDS* function from the *vegan* package (Oksanen et al., 2019), we performed non-metric multidimensional scaling (NMDS) with Bray–Curtis distances, 999 random starts, and *autotransform* set to *FALSE* to analyze the differences in behavioral responses between individuals. We used the *envfit* function from the *vegan* package with 9,999 permutations to determine the behaviors (i.e., the intrinsic variables) that shape the distribution pattern of the ordination. We visualized the results in NMDS ordination plots and added arrows to visualize the direction of behavioral responses that significantly contributed to the distribution pattern.

Integrating data from all individuals, separately for the control and the wind treatment, we created correlation matrices for the significantly contributing behaviors using the function *cor* from the *R* base package with Spearman correlation coefficients and

the packages *reshape2* (Wickham, 2007) and *ggplot2* (Wickham, 2016) to examine if any of these behaviors are correlated in the control or the wind treatment. We tested correlations for significance using the function *rcorr* from the package *Hmisc* (Harrell, 2021).

We performed Kruskal–Wallis and Dunn’s *post hoc* tests using PAST to determine differences in the proportion of total duration of these behaviors between the different groups (characterized by morphological type and treatment), as indicated by Bonferroni corrected *p*-values. We visualized the proportion of total duration of these behaviors in the different groups with radar plots using the package *fmsb* (Nakazawa, 2021).

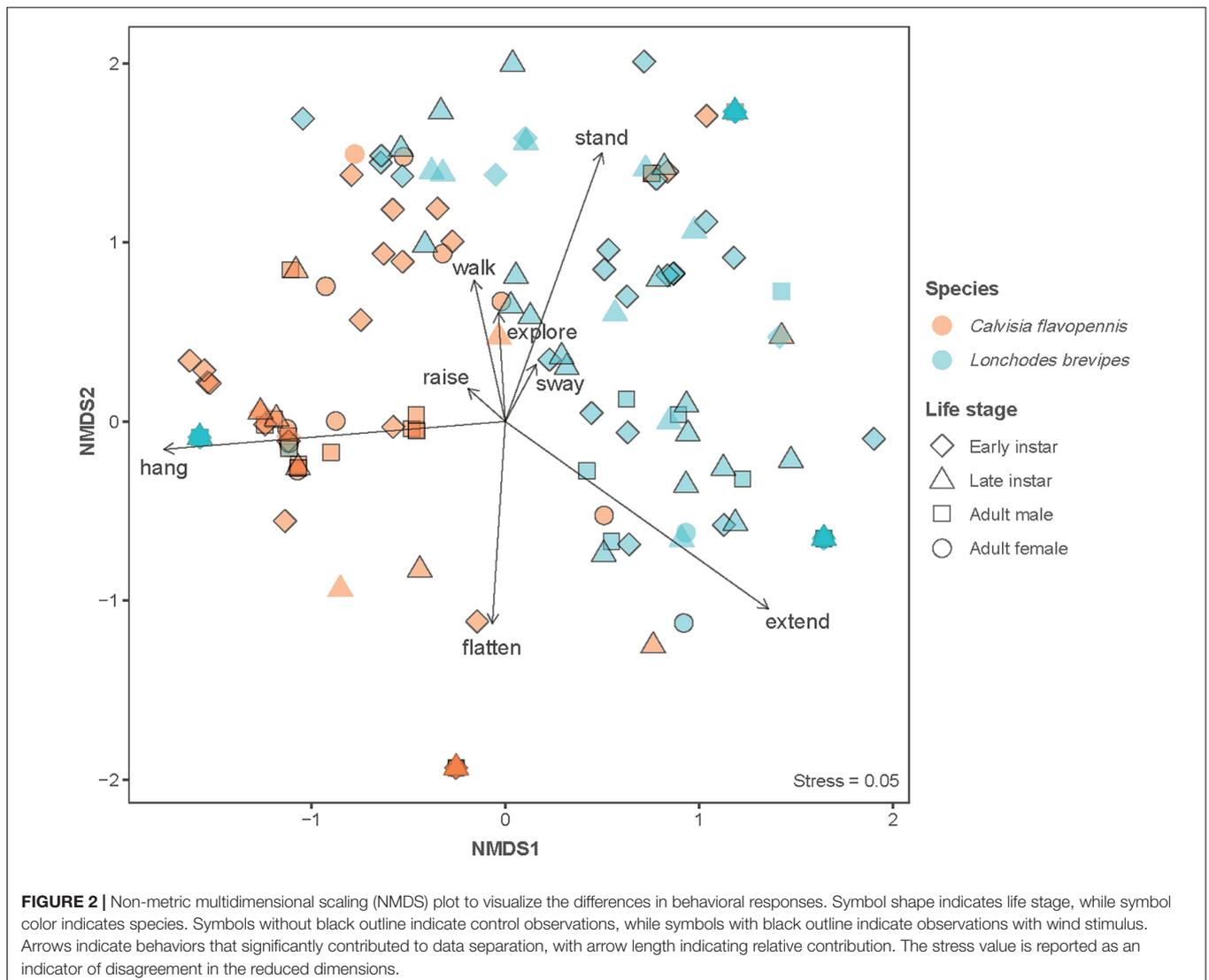
RESULTS

In our experiments, phasmids exhibited a range of different behaviors, in both the control and the wind treatment. We found differences in the behavioral responses between species as well

as across life stages of the same species. Morphological type and treatment affected the proportion of time a specific behavior was displayed. Revealing behaviors were positively correlated while concealing behaviors, except for *swaying*, were mostly negatively correlated.

Comparison of Overall Behavioral Responses Across Groups

Phasmid behavior differs with appearance and environmental condition. We found differences in the overall behavioral responses between individuals depending on morphological type (PERMANOVA, $F_{7,319} = 28.55$, $p = 0.0001$) and treatment (PERMANOVA, $F_{1,319} = 9.44$, $p = 0.0001$), as well as an interaction term between morphological type and treatment (PERMANOVA, $F_{7,312} = 1.77$, $p = 0.0134$). Differences in the overall behavioral responses between groups are visualized in an NMDS plot in **Figure 2**. Of the 15 recorded state behaviors, eight behaviors contributed significantly to the NMDS data



separation: explore, extend, flatten, hang, raise, stand, sway, and walk (all $p \leq 0.041$; **Table 2**). Pairwise comparisons revealed differences in the overall behavioral responses between specific groups (**Supplementary Table 1** and **Supplementary Figure 2**). In the control treatment, all morphological types differed in their overall behavioral responses from each other, except for two sets: (1) adult male *C. flavopennis*, adult female *C. flavopennis*, and first instars of *C. flavopennis*, and (2) adult male *L. brevipipes* and adult female *L. brevipipes* (**Table 3A** and **Figure 3A**). In the wind treatment, all morphological types show differences in their overall behavioral responses from each other, except for the following three sets: (1) all *C. flavopennis*, (2) adult male *L. brevipipes* and adult female *L. brevipipes*, and (3) first instar *L. brevipipes* and late instar *L. brevipipes* (**Table 3B** and **Figure 3B**). Comparing the overall behavioral responses of individuals of the same morphological type between the control and wind treatments, we found differences only for first instars of *C. flavopennis* ($F = 3.82, p = 0.0188$) and first instars of *L. brevipipes* ($F = 6.92, p = 0.0002$; **Table 3C**).

Comparison of Specific Behaviors Across Groups

For each of the eight behaviors that contributed significantly to the NMDS data separation, we found differences in the proportion of total duration of the behavior between the sixteen combinations of two species, four life stages, and two treatments (Kruskal–Wallis tests, all $p < 0.001$, **Table 4** and **Figure 4**). We highlight notable patterns of differences between combinations of morphological type and treatment for these behaviors, as indicated by Dunn's *post hoc* tests (all p -values are Bonferroni corrected; for full results, please refer to **Supplementary Table 2**). We group the behaviors according to their potential to conceal or reveal the phasmids.

TABLE 2 | The contribution of individual behaviors to the non-metric multidimensional scaling (NMDS) data separation.

Behavioral response	NMDS1	NMDS2	R^2	p -value
Bounce	0.429	−0.903	0.001	0.940
Curl	0.534	0.846	0.012	0.131
Evade	0.251	0.968	0.005	0.481
Explore	−0.059	0.998	0.092	<0.001
Extend	0.792	−0.610	0.734	<0.001
Flatten	−0.059	−0.998	0.319	<0.001
Hang	−0.996	−0.088	0.781	<0.001
Hug	−0.332	−0.943	0.002	0.726
Raise	−0.724	0.690	0.018	0.041
Stand	0.316	0.949	0.624	<0.001
Stretch forward	0.801	−0.598	0.010	0.189
Sway	0.447	0.894	0.032	0.006
Sway and walk	0.502	0.865	0.001	0.889
Uncurl	−0.718	−0.696	0.004	0.812
Walk	−0.199	0.980	0.161	<0.001

Behaviors with significant p -values at the 0.05 level are marked in bold.

Concealing Behaviors

Displays of concealing behaviors varied depending on species, life stage, and treatment. Adult *L. brevipipes* spent a higher proportion of time with their anterior limbs *extended* than any *C. flavopennis*, regardless of treatment (all $p < 0.001$). Within *L. brevipipes*, adults in the wind treatment spent a higher proportion of time with their anterior limbs *extended* than both first and late instars in either treatment (all $p < 0.016$). Adults in the control treatment spent a higher proportion of time with their anterior limbs *extended* than first instars in either treatment (all $p < 0.002$), and adult males in the control treatment spent a higher proportion of time with their anterior limbs *extended* than late instars in the control treatment ($p = 0.024$). Late instar *C. flavopennis* in the control treatment spent a higher proportion of time *flattened* than all other morphological types in either treatment (all $p < 0.001$), except for late instar *C. flavopennis* in the wind treatment ($p = 1$). First instar *C. flavopennis*, adult male *C. flavopennis*, and adult female *C. flavopennis* in the control treatment spent a higher proportion of time *hanging* than all life stages of *L. brevipipes* in either treatment (all $p < 0.008$). In the wind treatment, these three morphological types spent a higher proportion of time *hanging* than all life stages of *L. brevipipes* in the wind treatment (all $p < 0.036$). First and late instar *L. brevipipes* in the wind treatment spent a higher proportion of time *swaying* than all other morphological types in either treatment (all $p < 0.017$), except for adult female *C. flavopennis* in the wind treatment (all $p > 0.21$). We recorded a *raised* abdomen only occasionally ($N = 7$ observations) and only in the wind treatment. Except for one first instar *L. brevipipes* individual, only first instar *C. flavopennis* showed this behavior; the latter spent a higher proportion of time raising their abdomens than all other morphological types in either treatment (all $p < 0.001$).

Revealing Behaviors

Revealing behaviors were observed across the two species and varied depending on species, life stage, and treatment. Late instar *L. brevipipes* in the wind treatment spent a higher proportion of time *exploring* than all other morphological types in either treatment (all $p < 0.024$), except for first instar *L. brevipipes*, first instar *C. flavopennis*, and adult female *C. flavopennis* in the wind treatment (all $p > 0.57$). First instar *L. brevipipes* under wind conditions spent a higher proportion of time *exploring* than adult male *C. flavopennis* and adult female *L. brevipipes* in either treatment, than late instar *C. flavopennis* in the wind treatment, as well as than first instar *C. flavopennis* and adult male *L. brevipipes* in the control treatment (all $p < 0.013$). First instar *L. brevipipes* in the control treatment spent a higher proportion of time *standing* than all other morphological types in either treatment (all $p < 0.004$), except for first instar *L. brevipipes* in the wind treatment and late instar *L. brevipipes* in either treatment (all $p > 0.37$). First instar *L. brevipipes* in the wind treatment and late instar *L. brevipipes* in both treatments spent a higher proportion of time *standing* than adult female *L. brevipipes* in either treatment and adult male *L. brevipipes* in the wind treatment (all $p < 0.032$). First instar *C. flavopennis* in the wind treatment spent a higher proportion of time *walking* than adult female *L. brevipipes* in either treatment,

TABLE 3 | Pairwise comparisons of the overall behavioral responses between **(A)** all morphological types in the control treatment and **(B)** all morphological types in the wind treatment, and **(C)** comparisons of the overall behavioral responses between the wind and control treatments for individuals of the same morphological type.

Group 1	Group 2	df	Sums of squares	F model	R ²	Adjusted p-value	Sig.
(A)							
<i>C. flavopennis</i> first instar	<i>C. flavopennis</i> late instar	1	1.20	4.91	0.114	0.024	*
<i>C. flavopennis</i> first instar	<i>C. flavopennis</i> adult male	1	0.18	1.29	0.033	0.296	n.s.
<i>C. flavopennis</i> first instar	<i>C. flavopennis</i> adult female	1	0.17	1.28	0.033	0.323	n.s.
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> first instar	1	5.07	25.63	0.403	0.000	***
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> late instar	1	3.43	12.60	0.249	0.000	***
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> adult male	1	5.95	35.26	0.481	0.000	***
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> adult female	1	5.11	29.57	0.438	0.000	***
<i>C. flavopennis</i> late instar	<i>C. flavopennis</i> adult male	1	2.18	10.73	0.220	0.001	**
<i>C. flavopennis</i> late instar	<i>C. flavopennis</i> adult female	1	2.19	10.70	0.220	0.000	***
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> first instar	1	3.66	13.79	0.266	0.000	***
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> late instar	1	2.42	7.13	0.158	0.000	***
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> adult male	1	4.98	21.09	0.357	0.000	***
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> adult female	1	4.58	19.04	0.334	0.000	***
<i>C. flavopennis</i> adult male	<i>C. flavopennis</i> adult female	1	0.03	0.26	0.007	1.000	n.s.
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> first instar	1	5.38	34.25	0.474	0.000	***
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> late instar	1	3.88	16.76	0.306	0.000	***
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> adult male	1	6.58	51.45	0.575	0.000	***
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> adult female	1	5.69	43.10	0.531	0.000	***
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> first instar	1	5.68	35.84	0.485	0.000	***
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> late instar	1	3.97	17.02	0.309	0.000	***
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> adult male	1	6.60	51.05	0.573	0.000	***
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> adult female	1	5.66	42.44	0.528	0.000	***
<i>L. brevipes</i> first instar	<i>L. brevipes</i> late instar	1	0.98	3.35	0.081	0.033	*
<i>L. brevipes</i> first instar	<i>L. brevipes</i> adult male	1	4.46	23.47	0.382	0.000	***
<i>L. brevipes</i> first instar	<i>L. brevipes</i> adult female	1	5.01	25.76	0.404	0.000	***
<i>L. brevipes</i> late instar	<i>L. brevipes</i> adult male	1	1.76	6.64	0.149	0.002	**
<i>L. brevipes</i> late instar	<i>L. brevipes</i> adult female	1	1.95	7.25	0.160	0.002	**
<i>L. brevipes</i> adult male	<i>L. brevipes</i> adult female	1	0.10	0.59	0.015	0.547	n.s.
(B)							
<i>C. flavopennis</i> first instar	<i>C. flavopennis</i> late instar	1	0.58	1.82	0.046	0.143	n.s.
<i>C. flavopennis</i> first instar	<i>C. flavopennis</i> adult male	1	0.60	2.31	0.057	0.075	n.s.
<i>C. flavopennis</i> first instar	<i>C. flavopennis</i> adult female	1	0.32	1.17	0.030	0.325	n.s.
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> first instar	1	2.46	7.87	0.172	0.000	***
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> late instar	1	2.52	7.96	0.173	0.000	***
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> adult male	1	5.44	25.58	0.402	0.000	***
<i>C. flavopennis</i> first instar	<i>L. brevipes</i> adult female	1	6.22	33.90	0.471	0.000	***
<i>C. flavopennis</i> late instar	<i>C. flavopennis</i> adult male	1	0.60	2.16	0.054	0.114	n.s.
<i>C. flavopennis</i> late instar	<i>C. flavopennis</i> adult female	1	0.53	1.82	0.046	0.144	n.s.
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> first instar	1	2.95	9.01	0.192	0.000	***
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> late instar	1	2.82	8.52	0.183	0.000	***
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> adult male	1	4.27	18.79	0.331	0.000	***
<i>C. flavopennis</i> late instar	<i>L. brevipes</i> adult female	1	4.77	24.08	0.388	0.000	***
<i>C. flavopennis</i> adult male	<i>C. flavopennis</i> adult female	1	0.18	0.77	0.020	0.534	n.s.
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> first instar	1	3.66	13.65	0.264	0.000	***
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> late instar	1	3.79	13.95	0.269	0.000	***
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> adult male	1	6.25	37.30	0.495	0.000	***
<i>C. flavopennis</i> adult male	<i>L. brevipes</i> adult female	1	6.97	50.39	0.570	0.000	***
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> first instar	1	3.78	13.38	0.260	0.000	***
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> late instar	1	3.54	12.39	0.246	0.000	***

(Continued)

TABLE 3 | (Continued)

Group 1	Group 2	df	Sums of squares	F model	R ²	Adjusted p-value	Sig.
(B)							
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> adult male	1	5.57	30.61	0.446	0.000	***
<i>C. flavopennis</i> adult female	<i>L. brevipes</i> adult female	1	6.24	40.81	0.518	0.000	***
<i>L. brevipes</i> first instar	<i>L. brevipes</i> late instar	1	0.30	0.93	0.024	0.466	n.s.
<i>L. brevipes</i> first instar	<i>L. brevipes</i> adult male	1	3.78	17.24	0.312	0.000	***
<i>L. brevipes</i> first instar	<i>L. brevipes</i> adult female	1	4.76	25.03	0.397	0.000	***
<i>L. brevipes</i> late instar	<i>L. brevipes</i> adult male	1	2.26	10.15	0.211	0.000	***
<i>L. brevipes</i> late instar	<i>L. brevipes</i> adult female	1	3.06	15.82	0.294	0.000	***
<i>L. brevipes</i> adult male	<i>L. brevipes</i> adult female	1	0.07	0.77	0.020	0.558	n.s.
(C)							
<i>C. flavopennis</i> first instar	<i>C. flavopennis</i> first instar	1	0.92	3.82	0.091	0.019	*
<i>C. flavopennis</i> late instar	<i>C. flavopennis</i> late instar	1	0.29	0.90	0.023	0.433	n.s.
<i>C. flavopennis</i> adult male	<i>C. flavopennis</i> adult male	1	0.36	2.30	0.057	0.110	n.s.
<i>C. flavopennis</i> adult female	<i>C. flavopennis</i> adult female	1	0.36	2.12	0.053	0.118	n.s.
<i>L. brevipes</i> first instar	<i>L. brevipes</i> first instar	1	1.87	6.92	0.154	0.000	***
<i>L. brevipes</i> late instar	<i>L. brevipes</i> late instar	1	0.68	1.96	0.049	0.118	n.s.
<i>L. brevipes</i> adult male	<i>L. brevipes</i> adult male	1	0.11	0.79	0.020	0.474	n.s.
<i>L. brevipes</i> adult female	<i>L. brevipes</i> adult female	1	0.22	1.88	0.047	0.294	n.s.

Sig., significance level of Benjamini–Hochberg adjusted p-value. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; n.s., not significant.

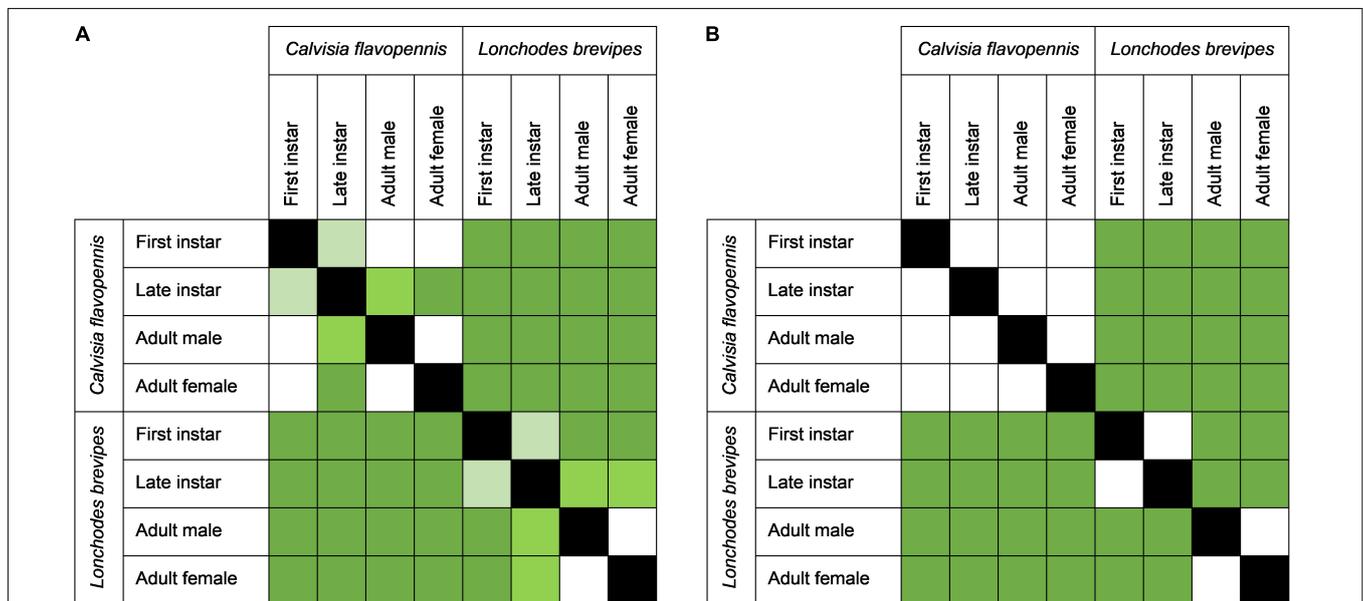


FIGURE 3 | Heat maps of the pairwise comparisons of overall behavioral responses across species and life stages in (A) the control treatment and (B) the wind treatment. The intensity of green in the squares indicates the significance levels of Benjamini–Hochberg adjusted p-values of comparisons between morphological types, dark green, $p < 0.001$; medium green, $p < 0.01$; light green, $p < 0.05$, while white squares represent comparisons that were not statistically significant. Black boxes indicate no comparison (same morphological type).

as well as than adult male *L. brevipes* and first instar and adult male *C. flavopennis* in the control treatment (all $p < 0.021$).

Correlation of Behaviors in Control and Wind Treatments

We found correlations between several behaviors in both the control treatment (Table 5A and Figure 5A) and the wind treatment (Table 5B and Figure 5B), integrating data from all

individuals in a treatment. Except for *swaying*, we found positive correlations only between revealing behaviors, while we found negative correlations between concealing behaviors and both other concealing behaviors and revealing behaviors alike. In the control treatment, *hang* was negatively correlated with *explore*, *extend*, *flatten*, *stand*, and *walk* (all $p < 0.043$). *Extend* was negatively correlated with *flatten* and *stand* (all $p < 0.024$). *Walk* was positively correlated with *explore* and *stand* (all $p < 0.002$),

TABLE 4 | Differences in individual behaviors between experimental groups, for behaviors that significantly contributed to the distribution pattern during NMDS.

Behavioral response	<i>H</i> (<i>ch</i> ²)	<i>H</i> _c (tie corrected)	<i>p</i> -value
Extend	134.9	205	<0.001
Explore	18.20	69.08	<0.001
Flatten	17.02	70.95	<0.001
Hang	119.3	153.6	<0.001
Raise	5.084	79.19	<0.001
Stand	55.04	105.4	<0.001
Sway	31.22	103.7	<0.001
Walk	12.29	45.34	<0.001

Behaviors with significant *p*-values at the 0.05 level are marked in bold.

and *explore* was positively correlated with *stand* ($p = 0.019$). In the wind treatment, *hang* was negatively correlated with *explore*, *extend*, *flatten*, *stand*, and *sway* (all $p < 0.024$). *Extend* was negatively correlated with *explore*, *stand*, *walk*, and *sway* (all $p < 0.024$). *Flatten* was negatively correlated with *stand* and *sway* (all $p < 0.041$). *Explore* was positively correlated with *stand*, *sway*, and *walk* (all $p < 0.001$). *Stand* was positively correlated with *sway* and *walk* (all $p < 0.011$), and *raise* was positively correlated with *hang* ($p = 0.019$).

DISCUSSION

Our study provides a first insight into the variability of behavioral responses of different phasmid species and life stages in response to a variable environmental factor. The results for both the control and the wind treatment indicate that the behaviors of phasmids varied through ontogeny and between species. The proportion of time spent on specific behaviors differed between groups of different morphological types and treatments. Within the same morphological type, only first instars showed significant changes in their overall behavior as a response to different environmental conditions. Through the interaction of behavior and morphology, phasmids may be able to adjust their adaptive resemblance strategies to specific situations. We discuss the differences in behavioral responses in the context of how the behaviors could reveal the phasmids or conceal them from potential predators. Investigating the behavioral components of predator avoidance strategies is a further step toward a more complete understanding of the complex interactions between morphology, behavior, sensory ecology, and environmental factors. Ultimately, this work will allow us to understand the selective pressures and evolutionary dynamics that shape evolutionary strategies of phasmids.

Overall Behavioral Differences

Adaptive strategies to evade predation can be shaped by extrinsic factors, such as the predator community, and intrinsic factors, such as current life history requirements (Mappes et al., 2014; Valkonen et al., 2014). Individuals at the same life stage leading a similar lifestyle in a similar habitat might be expected to face similar challenges and thus exhibit similar behaviors and color patterns, even across species (Tan et al., 2017). Life stage

influences phasmid behavior, particularly in the absence of wind, yet species is a better predictor of phasmid behavior across environmental conditions. The two species in this study differ in their overall behavior, regardless of life stage and treatment. Additionally, ontogenetic changes could be accompanied by changes in behavior. For example, the shield bug *Graphosoma lineatum* changes from a cryptic appearance pre-hibernation to an aposematic appearance post-hibernation, with aposematic forms showing higher mobility than cryptic forms (Johansen et al., 2011). The overall behavioral responses of *C. flavopennis* to wind do not differ across life stages, whereas *L. brevipipes* in the wind treatment show differences in the overall behavior between adults and nymphs, but not between adult sexes or nymphs of different ages. Intraspecific behavioral differences depending on the life stage are also observed in other phasmid species, with smaller and younger individuals of *Timema cristinae* stick insects more likely to exhibit thanatosis (Farkas, 2016). While our study directly compares the behavior of different life stages of two phasmid species, identification of broad evolutionary trends would require comparative analyses to adjust for phylogenetic relationships (Harvey and Pagel, 1991).

Group-Specific Behaviors

By disguising their shape, prey can become unrecognizable to predators (Merilaita et al., 2017). Behaviors such as extend and flatten have the potential to conceal the presence of an individual and hence reduce the likelihood of detection or recognition by potential predators. The *extension* of anterior limbs aligned with the antennae masks the typical morphology of an insect, as phasmids lose the distinctive shape of six legs and antennae (Robinson, 1968b). Unlike *C. flavopennis*, *L. brevipipes* individuals have legs that fit closely to the head when extended with the antennae, and all life stages of the cryptically colored species *L. brevipipes* resemble twigs and branches of plants. Extension of anterior limbs was more common in the adults of *L. brevipipes*. Possibly due to their larger size, and thus detectability, it might be more important for larger insects to adopt this behavior, which alters their appearance.

Plant resemblance could also play a role during the *flattening* behavior. By flattening themselves against the substrate, phasmids could lose the distinct morphology of an insect, and thus reduce detection. Flattening can additionally enhance protection by eliminating shadow casting, as seen in frogs (Ferreira et al., 2019). Combined with cryptic coloration, a phasmid flattened against its substrate would be concealed from predators. In fact, late instar *C. flavopennis*, which have a mottled brown, bark-like appearance (Figure 1E), behaved distinctly different from the other life stages: in the control treatment, they spent a higher proportion of time flattened than all other morphological types. As the wooden dowel used in the experimental setup resembles a plant branch, flattening against a branch could be a behavior that reduces the detection of the insect. The combination of flattened resting positions with a dorsoventrally flattened morphology has been demonstrated to reduce detection by predators in the spider mite *Aponychus corpuzae* (Chittenden and Saito, 2006).

Prey can reduce their exposure to predators by selecting specific microhabitats. For instance, prey location and foliage

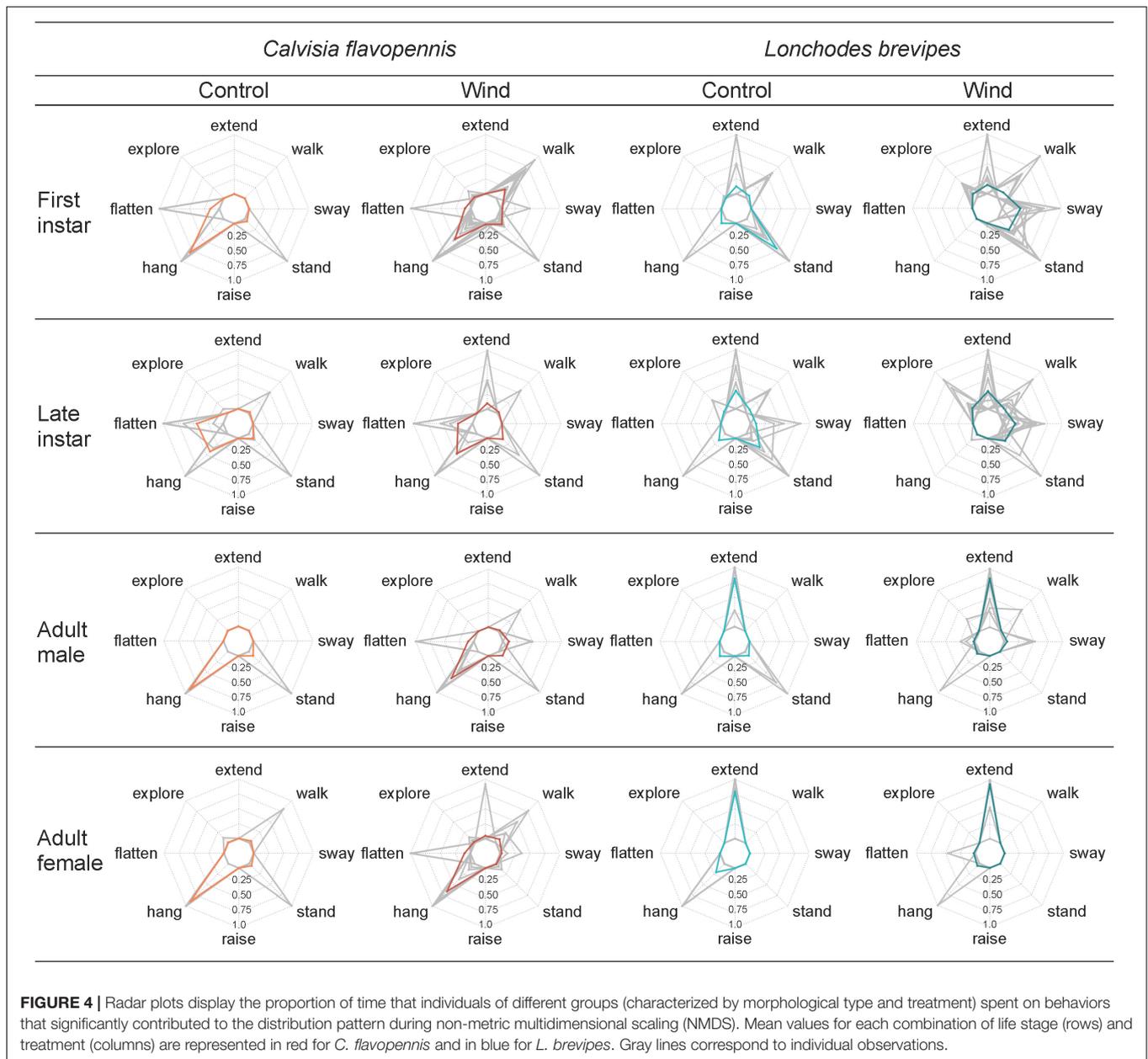


FIGURE 4 | Radar plots display the proportion of time that individuals of different groups (characterized by morphological type and treatment) spent on behaviors that significantly contributed to the distribution pattern during non-metric multidimensional scaling (NMDS). Mean values for each combination of life stage (rows) and treatment (columns) are represented in red for *C. flavopennis* and in blue for *L. brevipes*. Gray lines correspond to individual observations.

structure influence prey capture rates, with lower leaf surfaces experiencing less predation overall (Whelan, 2001; Johnson et al., 2007). By *hanging* under the substrate, the phasmid could be concealed from predators above. Contrary to our expectations, hanging was more often observed in brightly colored individuals of *C. flavopennis* than in *L. brevipes*. First instar *C. flavopennis*, adult male *C. flavopennis*, and adult female *C. flavopennis* spent a higher proportion of time hanging than all life stages of *L. brevipes* in the same treatment. Instead of performing evasive behaviors, hanging under a substrate could be an adaptation by the brightly colored life stages of *C. flavopennis* to reduce the risk of predation. Although the main predators of *C. flavopennis* are currently unknown, a field study of insectivorous bird species in a similar tropical forest in Malaysia found that

these insectivores rarely foraged from the underside of branches (Mansor and Mohd Sah, 2012).

While movement may alert a predator to the presence of prey, it does not necessarily compromise camouflage, as there are several ways in which prey may specifically incorporate motion into their arsenal of defense strategies to compromise the cues used by their natural enemies (reviewed by Stevens and Ruxton, 2019; Caro and Koneru, 2021). In some cases, specific motion can enhance the effectiveness of camouflage (Fleishman, 1985; Huffard et al., 2005; Cuthill et al., 2019). Under dynamic background conditions, such as those caused by wind, *swaying* could improve concealment by reducing the signal-to-noise ratio (Cuthill et al., 2019). Swaying has previously been reported as a behavior that enhances camouflage

TABLE 5 | Pairwise correlations between the proportions of time spent on behaviors that significantly contributed to the distribution pattern during NMDS, for **(A)** the control treatment and **(B)** the wind treatment, integrated for all individuals in a treatment.

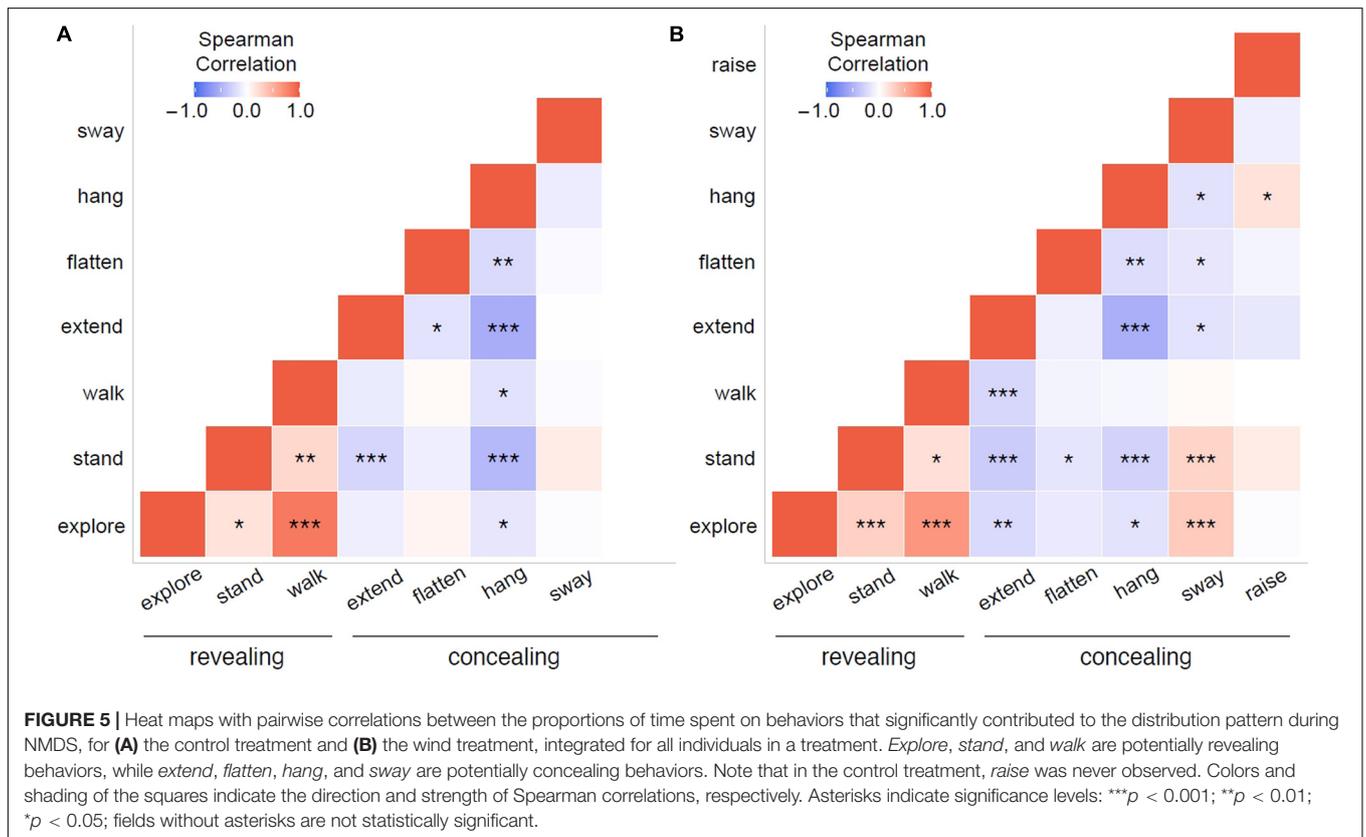
(A)

Control	Explore	Stand	Walk	Extend	Flatten	Hang	Sway
Sway	0.755	0.141	0.711	0.942	0.606	0.119	
Hang	0.042	3.32E-10	0.015	1.64E-13	0.002		-0.12
Flatten	0.402	0.148	0.640	0.023		-0.24	-0.04
Extend	0.173	4.99E-04	0.104		-0.18	-0.54	-0.01
Walk	0.000	0.002		-0.13	0.04	-0.19	-0.03
Stand	0.019		0.25	-0.27	-0.11	-0.47	0.12
Explore		0.18	0.83	-0.11	0.07	-0.16	-0.02

(B)

Wind	Explore	Stand	Walk	Extend	Flatten	Hang	Sway	Raise
Raise	0.774	0.146	0.962	0.053	0.388	0.019	0.174	
Sway	1.55E-05	6.76E-04	0.637	0.023	0.040	0.014		-0.11
Hang	0.024	2.28E-04	0.474	5.97E-13	0.006		-0.19	0.18
Flatten	0.076	0.033	0.295	0.200		-0.22	-0.16	-0.07
Extend	0.002	2.19E-05	9.14E-04		-0.10	-0.53	-0.18	-0.15
Walk	0.000	0.010		-0.26	-0.08	-0.06	0.04	0.00
Stand	1.20E-04		0.20	-0.33	-0.17	-0.29	0.27	0.12
Explore		0.30	0.66	-0.24	-0.14	-0.18	0.33	-0.02

Explore, stand, and walk are potentially revealing behaviors, while extend, flatten, hang, and sway are potentially concealing behaviors. Note that in the control treatment, raise was never observed. The upper-left triangle shows p-values, while the lower-right triangle shows Spearman correlations. Correlations with significant p-values at the 0.05 level and associated Spearman correlations are marked in bold.



in other cryptically colored animals, such as snakes, mantids, and spiders (e.g., Fleishman, 1985; Jackson, 1985; Watanabe and Yano, 2009, 2012, 2013). In the presence of wind, *L. brevipes* first and late instars generally spent more time swaying than the other morphological types. Swaying could enhance the resemblance of phasmids to an uninteresting object through motion masquerade (Hall et al., 2017; Cuthill, 2019). As prey animals will risk predation to continue with other profitable activities if the risk is low (Ydenberg and Dill, 1986), swaying could reduce the detection risk of phasmids during activities such as foraging, which is crucial for developing nymphs. Swaying in the phasmid *Extatosoma tiaratum* was quantitatively similar to that of moving plants, consistent with motion masquerade where animals may use motion as a concealing behavior against a moving background (Bian et al., 2016).

Unlike concealing behaviors, behaviors such as standing, exploring, and walking could reveal the identity and/or location of an individual. When insects are *standing* with their body elevated from the substrate, they exhibit a distinct form, with their body, six legs, and antennae visible. The cryptically colored first instar *L. brevipes*, particularly in the control treatment, spent more time standing than *C. flavopennis* and adult *L. brevipes*. It is possible that the combination of cryptic coloration and smaller size makes them less detectable and, hence, standing disrupts their cryptic appearance less. *Explore*, where the insect probes a gap with its antennae and first pair of legs (Blaesing and Cruse, 2004), reveals the presence of the insect and is similarly risky. In the field, one of the authors (ET) observed a phasmid being preyed on during exploration. Wind is a cue to inform jumping spiders whether it is appropriate to jump (Aguilar-Arguello et al., 2021) and might similarly serve as a cue for exploration in phasmids. *Walking*, which is the forward movement along the substrate, is risky as it can break the crypsis of the insect. In the presence of predators, the caridean shrimp *Tozeuma carolinense* decreases the time spent walking on their seagrass blade substrate and shows an increase in clinging behavior that resembles flattening in phasmids (Main, 1987). In the wind treatment, exploration behavior was increased in both *L. brevipes* instars, while first instar *C. flavopennis* spent a higher proportion of time walking than other groups. The risk of detection during these behaviors could be lower for nymphs due to their smaller size.

Interaction of Concealing and Revealing Behaviors

Once phasmids adopt a concealing behavior, a change of behavior could break their concealment. However, if phasmids already adopted a revealing behavior, there could be fewer costs to switching to a different revealing behavior. In fact, they could then benefit from a combination of different behaviors – for example, a combination of exploring and walking could lead them to a better-suited spot for resting or feeding. The phasmid *Haplopus scabricollis* was observed to intersperse walking with exploration behaviors often during locomotion (Stockard, 1908). We found that *sway* was the only concealing behavior that correlated positively with any revealing behaviors (*stand* and *explore*, in the wind treatment). Swaying at

intervals could thus provide phasmids some form of concealment between revealing behaviors through motion masquerade in a dynamic environment.

CONCLUSION

Future studies to test the predictions about the role of particular behaviors as antipredator strategies will need to determine how likely predators are to detect phasmids. Using prey models that systematically vary in size, coloration, posture, and location (e.g., Cuthill and Székely, 2009; Webster et al., 2009; Tan et al., 2016, 2020), it will be possible to elucidate the importance of these characteristics for concealment and predator avoidance in phasmids. Field observations can provide information about the range of natural predators of phasmids and indicate the preferred foraging microhabitats of the predators. Anecdotal reports describe spiders, mantids, and birds (Paine, 1968; Robinson and Robinson, 1973; Bragg, 1992; Suetsugu et al., 2018) as natural predators of phasmids in the wild. In captivity, phasmids are readily accepted by lizards and amphibians (Bragg, 1992), suggesting that these animals could be potential predators of phasmids in the natural environment. Specific knowledge about phasmid predators will also provide further insight into the role of coloration, as color vision differs between different predator groups (Jacobs, 1993; Briscoe and Chittka, 2001; Hart, 2001; Théry and Gomez, 2010; Fabricant and Herberstein, 2015). Species-specific attributes, such as the presence or absence of wings, and the resulting different niches could provide additional explanations for differences in the behaviors between species. Behavioral experiments would be required to investigate the effect and function of active behaviors. For example, to investigate whether swaying behavior in the species tested in this study is a form of motion masquerade as suggested for *E. tiaratum* (Bian et al., 2016), it is necessary to examine the effect of swaying on potential predators and to quantify the swaying behavior in response to wind. Future studies, such as a comparative analysis of morphology and behaviors across phasmid species that considers the phylogenetic relationships, would allow us to elucidate the evolution of predator avoidance strategies. By using an integrated approach that combines behavioral experiments with biophysical measurements and field observations, it will be possible to gain further insights into processes of adaptive resemblance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The research reported in this article, which involved insects only, was exempt from ethics approval procedures by the National University of Singapore. We collected *L. brevipes* in Singapore

(permit number NP/RP18-062) and *C. flavopennis* in Brunei Darussalam (export permit number BioRIC/HoB/TAD/51-131).

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AUTHOR CONTRIBUTIONS

ET and SP conceptualized the project. ET, SP, and YN-R acquired funding. RW coordinated the access to Bruneian phasmids. ET, SP, MS, and KL collected the specimens. KL, HB, MS, and YP performed data collection. ET and SP performed data analyses and wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.767940/full#supplementary-material>

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