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ABSTRACT

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Pigmentation patterns can be used as a communication signal in a variety of taxa, and can convey information relative to sexual selection, dominance, and species identification. Pigmentation is also sometimes used in mimicry to deceive the signal receiver into thinking the signaler is something other than itself. Mimicry can occur in several contexts, including sexual interactions, where one sex mimics another. There are relatively few examples of species with females that mimic males. Proposed hypotheses to explain female mimicry of males are that mimicry is used to reduce male harassment or that mimicry is used to display dominance over other females. In this study, we tested these two hypotheses using an experimental approach. Researchers have hypothesized that *Brachyrhaphis* fishes provide an example of sexual mimicry because females have pigmentation of the same coloration and shape, and in the same location as male genitalia. To test if female mimicry of males reduces male harassment, we designed an experiment to observe male preference for females with and without male-like pigmentation. To test the effect that female mimicry of males has on female dominance, we observed female behavior based on the pigmentation patterns of companion females. We found that neither of these hypotheses was supported by our data. We conclude that similarities in anal fin pigmentation between male and female *Brachyrhaphis* fishes cannot be explained as a way to reduce male harassment of females and is not a good predictor of female dominance interactions. Alternative explanations must exist for this pattern of anal fin coloration include the possibility that these similarities are simply non-adaptive.

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Introduction

Visual signals are used in many taxa as a way to communicate both between and among species (1; 2). Success of the visual signal is dependent upon the efficiency of the signal and the ability of the receiver to perceive and appropriately interpret the signal (3). In some systems, individuals have evolved traits that mimic the signals of other individuals. Mimicry function to deter predators (4; 5), to lure prey (6) or to deceive hosts to allow the mimic to act as a parasite (7). Mimicry can also occur in a sexual context. Sexual mimicry occurs when one sex mimics the other sex of the same species. Examples are found in a variety of taxa (*Drosophila melanogaster*, 8; cicadas, 9; poeciliid fish, 10; goby fish, 11; hyenas, 12; flycatchers, 13). In most cases of sexual mimicry, males mimic females. For example, in bluegill sunfish, small "sneaker" males deceive larger male bluegill sunfish into thinking the small males are in fact females. This allows for small males to enter into the nest and fertilize eggs that larger males are protecting (14). Examples similar to this are found in a variety of taxa (side-blotched lizards, 15; goby fish, 11; cuttlefish, 16).

In contrast, examples of females mimicking males are less common and not well understood. Despite the paucity of examples, two compelling hypotheses have been developed to explain why it may be beneficial for a female to mimic a male. First, male mimicry could be used to reduce male harassment of females. For example, female damselflies mimic males to avoid long, unwanted copulations (17; 18). Males are less attracted to male mimicking females than to typical females. By reducing male harassment, which leads to costly copulatory events, females are able to dedicate more time and energy to obtaining nutrients needed for survival (19). The second hypothesis is that male-mimicry could be used by females to signal dominance over other females. For example, in spotted hyenas females develop a pseudopenis that mimics a

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male penis. The larger the pseudopenis, the more dominant the female. Hence, the pseudopenis appears to help establish social structure by signaling dominance among females (20).

Fishes in the genus *Brachyrhaphis* appear to also show female sexual mimicry (21). These fishes are members of the family, Poeciliinae, a monophyletic group characterized by internal female fertilization and external male genitalia (gonopodia). Male fish of this family try to court females as well as force copulations when courtship is unsuccessful, or when a predator is near, or there are high light levels (22). Forced copulation is an opportunistic behavior in contrast to a specialized phenotype, which helps maintain the presence of the trait; being able to decipher when forced copulation is a better strategy than courtship (or vice versa) is a way to enhance reproductive success (22). In *Brachyrhaphis*, females typically prefer large males, while males typically pursue females close to their own size (23). What is most striking about *Brachyrhaphis* species is that females have anal fin pigmentation (melanin) similar in shape, size, and color to gonopodial pigmentation of their male counterpart (21). Although many studies on sexual selection of poeciliid fishes focus on sexually dimorphic studies species (21, 24, 25), *Brachyrhaphis* provide a unique opportunity to study a nearly sexually monomorphic genus. Gonopodial and anal fin pigmentation is found in all species except *Brachyrhaphis terrabensis* and *B. hartwegi*, which seem to be returning to an ancestral, less pigmented state (21). Furthermore, females display their anal fin to conspecifics in such a way as to clearly show their gonopodial-like anal fin pigmentation (21). Mojica (21) first proposed sexual mimicry in *Brachyrhaphis* fishes due to the nature of forced copulations by *Brachyrhaphis* males, as well as the way females seem to use their anal fins in communication. She used phylogenetic analyses as well as pigmentation and behavioral comparisons between males and females of several species in this genus to identify when pigmentation evolved and how similar pigmentation and behavior are between males and females of the same species. She proposed that females mimic

males to discourage unwanted courtship and that pigmentation is used to signal status to females. However, she did not explicitly test these hypotheses, leaving a need to evaluate social behaviors between males and females (to test for harassment) and between females (to evaluate social status).

In this study, we explored the two hypotheses to explain how, and if, female mimicry of males has evolved in *Brachyrhaphis* fishes: (1) females mimic males to reduce male attention and (2) females mimic males to signal dominance to other females. To evaluate the first hypothesis, we predicted that males would be more attracted to non-pigmented females than to pigmented females with the markings on their anal fin that mimicked the male gonopodium. To evaluate the second hypothesis, we predicted that females who interact with a pigmented female would show fewer aggressive behaviors than females who interact with a non-pigmented female. Surprisingly, we found that neither of these hypotheses was supported in *Brachyrhaphis* fishes, and we explore possible reasons for these unexpected results.

Methods

Study System

We used three species of *Brachyrhaphis* for this study: *B. terrabensis, B. roseni,* and *B. rhabdophora.* These species were chosen due to their variation in anal fin and gonopodial pigmentation (Figure 1) (21). *Brachyrhaphis terrabensis* males and females have little, and in some cases, no anal fin or gonopodial pigmentation; when pigmentation is present, it is expressed only as a small, circular area of pigment. *Brachyrhaphis roseni* males have pigmentation that covers most of their gonopodium, while most females have a distinct, dark, gonopodial shape along their anal fin. *Brachyrhaphis rhabdophora* males have a heavily pigmented gonopodia, while all females have a dark, inverted triangle shape along their anal fin.

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We tested two populations of *B. rhabdophora* because prior work shows that a variety of traits have diverged within this species coincident with the presence or absence of predators (26; 27). Although there was no *a priori* reason to predict that predation would affect mimic behavior, we evaluated two populations of this species separately: '*B. rhabdophora* Javilla' co-occurs with piscivorous predators and '*B. rhabdophora* Grande' occurs in the absence of predators (28).

Brachyrhaphis roseni and *B. terrabensis* were collected from two streams in the Rio Caño Seco drainage in Puntarenas, Costa Rica in April 2014. *Brachyrhaphis roseni* were collected from a low-elevation tributary (N 8.65427, W 82.93489; elevation 70 m) and *B. terrabensis* collected from a high-elevation tributary (N 8.81299 W 82.97408; elevation 962 m). *Brachyrhaphis rhabdophora* populations came from two streams in Guanacaste Province Costa Rica in April 2014. One population was collected from low-elevation Rio Javilla (N 10.40245, W 85.07610; elevation 99 m) and the other population was collected from high-elevation Quebrada Grande (N 10.44194, W 84.98804; elevation 363 m). After collection, fish were transported to Brigham Young University. Fish were held in the lab under the following conditions. Fish were kept on a normal light schedule of 12 h day and 12 h night and temperature was kept at 21 degrees C. Fish were fed daily with TetraMin® (Tetra, Melle, Germany). All fish utilized in this study were wild caught.

Experimental Design

Our first experiment was designed to determine if males are more likely to attend to non-pigmented females than to pigmented females with markings that mimic the male gonopodium. Our second experiment was designed to determine how a focal female behaves when placed with a female whose anal fin pigmentation mimics the male gonopodium compared to her behavior

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when she is with a female without any anal fin pigmentation. We predicted that the focal female would be more aggressive towards the female who mimics a male.

Male Attendance to Females

We used a dichotomous choice test to evaluate male attendance behavior. If males spend more time associating with non-pigmented females than with pigmented females, this would be consistent with the hypothesis that sexual mimicry is used to deter unwanted male attention. Association time has been shown to be a good indicator of attendance, therefore suggesting which type of female a male would attend to (29; 30; 31). In order to control for differences in pigmentation patterns among females, we used freeze branding to remove all pigment from each female prior to applying the pigment treatment (32). We then randomly marked half of the individuals with a temporary pigment. We painted female anal fins in the shape of a male gonopodium with Dr. Naylor's BLU-KOTE. This pigment was used in previous studies on swordtail (*Xiphophorus cortezi*) mate choice to manipulate coloration as an appropriate substitute for melanin (24; 33).

We then tested male association time when given a choice between marked versus unmarked females. We first grouped females into size-matched pairs, with the requirement that individuals in each pair had to be within 3 mm of each other. On the day of the trials, one female was anesthetized and had Dr. Naylor's BLU-KOTE added and the other was anesthetized and painted with a paintbrush wetted with water, as a control. We then placed these two females, one painted and one control, in separate small tanks attached to either end of a larger central tank. Smaller side tanks were 15 x 27 x 30.5 cm while the larger central tank was 56 x 28 x 30.5 cm. Clear glass separated the central tanks from the side tanks. The tanks were housed in a soundproof room with full spectrum overhead lighting. A male was placed in the center of the central tank and observed outside of the experimental room via webcam. The male was given 10

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minutes to swim to each side tank and back to the center. As soon as this task was performed, we began recording the position of the male for 10 minutes using a web cam. Data were recorded in the program, ObjectTracker (34), which marks the location of the focal fish throughout the trial time.

We visually divided the central tank into three sections: two association zones on the ends and a center zone. We scored time spent in the end zones as a measure of when the male was associating with the female; time spent in the center zone was not included in the analysis as this space was considered neutral, inferring that the male had not "chosen" a female to associate with. Time in the outer association zones was measured in seconds.

To ensure that Dr. Naylor's BLU-KOTE was an appropriate substitute for melanin pigmentation in female anal fins, we used a dichotomous choice test as a control, giving males the choice between unpainted and painted females. We included only *B. roseni* and *B. rhabdophora* populations in this control study, as there were no naturally pigmented female *B. terrabensis*. Time spent on each side of the tank was square root transformed to normalize data then analyzed by a t-test in the program JMP (SAS Institute). We found no statistical difference between time spent with natural females versus females marked with Dr. Naylor's BLU-KOTE (*B. roseni:* t-crit$_6$= 1.28, P= 0.25, n=8; *B. rhabdophora* Javilla*:* t-crit$_6$= 0.58, P= 0.58, n=8; *B. rhabdophora* Grande: t-crit$_6$= 0.01, P=0.99, n=8), suggesting that our pigment treatment is a valid approach.

Female Dominance

To determine if females mimic male traits to signal dominance over other *Brachyrhaphis* females, we set up two experiments. Both experiments evaluated dominance behavior when a female was with a pigmented female compared to when she was placed with a non- pigmented female. The first test was designed to evaluate the number of aggressive displays shown by a

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pair of females when both females were equally pigmented. Paired females were within 3mm standard length. For *B. roseni* and *B. rhabdophora* populations, we utilized naturally pigmented females. For *B. terrabensis,* Dr. Naylor's BLU-KOTE was applied in the shape of the anal fin pigmentation found on *B. rhaphdophora* females (See above for a description of pigmentation patterns in each species). Females were placed in a shoebox tank (26.7 x 15.25 x 17 cm) and observed via webcam from a separate room. Females were given up to 45 minutes to acclimate and start establishing dominance. Once either female showed an aggressive signal (nips, chases, or "S" displays) we recorded behavior for 15 minutes. Nips were defined as when an individual bit or tried to bite her partner. Chases were defined when a female quickly swam towards the other female. "S" displays were defined when a female would jerk tail back and forth several times (22). We performed the second test to compare total aggressive displays in un-matching pairs compared to the number of aggressive displays demonstrated by the pigment matched pairs. This test also allowed us to analyze the number of aggressive displays by a female while interacting with a pigmented or non-pigmented female. Again, pairs were size-matched within 3 mm. For these tests we controlled pigmentation by freeze-branding each female. Females were then placed in isolated pairs for twenty-four hours. After isolation, both females were anesthetized. We painted Dr. Naylor's BLU-KOTE along one female's anal fin in a gonopodial shape, while the other was painted with a paintbrush wetted with water. Females were placed in a shoebox (27 x 15 x 17 cm) tank and observed given up to 45 minutes to acclimate and display an aggressive behavior. Once an aggressive display was demonstrated, we recorded the behavior for fifteen minutes in another room via webcam. For each trial we counted the number of nips, chases and "S" displays. Females were then placed back in their isolated 10-gallon tanks for 24h to allow for recovery and to let pigmentation fade. After recovery, the other female was pigmented and the test was re-run in the same manner.

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Statistical Analyses

We square root transformed all time measurements to normalize data. To analyze male association times, we used a t-test to compare the amount of time males of each species spent with pigmented versus non-pigmented females. To analyze the female aggressive displays tests, we first compared total number of aggressive behaviors in a trial (sum of both females) between naturally pigmented pairs and non-matching pigmented/non-pigmented pairs. These count data were compared using a Kruskal-Wallis rank sum test. We also analyzed aggressive behaviors displayed by the focal female in non-matched pairs, based on the pigmentation type of female she was interacting with. The total number of displays was square root transformed to normalize the data. We used a t-test to compare the number of aggressive displays between a female interacting with a pigmented female versus a non-pigmented female. The type of female viewed was the independent variable and the number of aggressive displays was the dependent variable. All statistics were analyzed using JMP (SAS Institute).

Results

In each of the three *Brachyrhaphis* species, we found no difference in the amount of time males spent associating with either pigmented or non-pigmented females (See Table 1 and Figure 2). Moreover, males from both *B. rhabdophora* populations were almost equal in the amount of time spent with both pigmented and non-pigmented females (See table 1).

Table 1. T-test to analyze association time of males with females in a dichotomous choice test.

SPECIES	PIGMENTATION	MEAN	SD	DF	T-CRIT	P
B. roseni	Not Pigmented	14.9417	4.9160			
	Pigmented	15.7126	3.9740	36	0.5316	0.5983
***B. rhabdophora* Javilla**	Not Pigmented	14.8991	3.5124			
	Pigmented	14.0044	4.5857	32	-0.6386	0.5276
***B. rhabdophora* Grande**	Not Pigmented	14.7931	4.8938			
	Pigmented	14.4942	4.3556	28	-0.1767	0.8610
B. terrabensis	Not Pigmented	15.0897	3.1688			
	Pigmented	14.5207	4.8973	36	-0.4254	0.6732

Male fishes were given a choice between pigmented and non-pigmented females. Time was measured in seconds and square root transformed to normalize data.

In *Brachyrhaphis roseni, B. rhabdophora* Grande, and *B. rhabdophora* Javilla females showed no statistically significant difference in aggression levels between female pairs with matching pigmentation and female pairs with non-matching pigmentation. In *Brachyrhaphis terrabensis* females did show a higher number of aggressive displays in matching pigmented female pairs than in non-matching pigment female pairs (see Table 2). Finally, we found no statistical difference in aggression between pigmented and non-pigmented females (see Table 3 and Figure 3).

Table 2. Kruskal-Wallis analysis comparing aggression levels in paired females of of B. roseni, B. rhabdophora Javilla, B. rhabdophora Grande, and B. terrabensis.

SPECIES	PAIR TYPE	SCORE SUM	EXPECTED	MEAN	PROB > Z
B. roseni	Different	35.5	30	5.9	0.1948
	Same	9.5	15	3.2	
B. rhabdophora Javilla	Different	73	63	8.1	0.1421
	Same	18	28	4.5	
B. rhabdophora Grande	Different	60	52	7.5	0.2027
	Same	18	26	4.5	
B. terrabensis	Different	44.5	33	7.417	0.0183*
	Same	10.5	22	2.63	

*Females were either both naturally pigmented or one pigmented and one with pigment removed. In the case of B. terrabensis, females in "same" pairs were pigmented with BLU-KOTE. Significant values marked with an *.*

Table 3. T-tests to analyze number of female aggressive displays while interacting with a female with opposite pigmentation.

SPECIES	PIGMENTATION	MEAN	SD	DF	T-CRIT	P
B. roseni	Not Pigmented	2.9773	4.0203			
	Pigmented	2.0264	1.8639	10	-.5258	0.6105
***B. rhabdophora* Javilla**	Not Pigmented	2.6668	2.4526			
	Pigmented	3.0411	2.7259	14	0.2887	0.7770
***B. rhabdophora* Grande**	Not Pigmented	3.7342	2.5480			
	Pigmented	3.6300	1.7560	14	-0.0952	0.9255
B. terrabensis	Not Pigmented	5.0884	1.0108			
	Pigmented	5.0967	2.3145	10	0.0056	0.9956

Females were placed in pairs with one pigmented and one non-pigmented female. We compared aggression events for each individual, based on what was viewed by each female (pigmented or non-pigmented female). Aggression events were recorded and square root transformed to normalize data.

Discussion

Our results were consistent across species; male *Brachyrhaphis* fishes show no difference in attendance to females based on the presence or absence of gonopodial pigmentation on female anal fins. Although male-like pigmentation in females is hypothesized to reduce male harassment in other species (8; 17; 18), such does not seem to be the case in this system. Our findings suggest that the hypothesis that sexual mimicry is used to deter male interest may not be generally applicable. In capuchinbirds (*Perrisocephalus tricolor*), where male-mimicry has been explored, males and females are monomorphic. In some cases, females wait to reveal their sex to males until they have reached an alpha opportunity (35), allowing females to have more choice

in mating. In contrast, males can likely detect the sex of female *Brachyrhaphis* relatively quickly at a close distance, as it is more difficult for females to hide their sex due to the visibility of gonopodia and anal fins (see 36). This could be a question of mimicry at a different distance than was tested. As capuchinbird females get closer to a male, their true sex becomes more apparent (35). Could it be that in *Brachyrhaphis*, females appear as males from a further distance to reduce decisions for mate pursuits made at a further distance? Further exploration would need to be done to test this hypothesis.

We also found in pairs with mismatched pigmentation that females interacting with non-pigmented females did not show a higher level of aggressive displays than females interacting with pigmented females. By measuring the number of aggressive behaviors displayed by a female, based on what type of female she was interacting with, we were able to assess the success of the visual signal. Our results showed that there was not a significant difference in amount of aggressive displays, no matter the type of female that was interacted with. These findings demonstrate that pigmentation is likely not a factor in dominance signaling among females of the same species. This suggests that the hypothesis that sexual mimicry is used by females to establish dominance does not apply to this study. In a study on red-throat patches on three-spined sticklebacks (*Gasterosteus aculeatus*), Yong *et al* (37) similarly found no difference of aggression levels between patched and non-patched females. This study combined with other studies that found no statistically significant difference in aggression levels between ornamented and non-ornamented females (see 38 for a meta-analysis on the topic) point to the hypothesis that examples of male-like females, or female ornamentation, may be non-adaptive in some systems (37). In reference to the dark pigmentation present in *Brachyrhaphis,* we do know that melanic pigmentation is sometimes used as a dominance signal within other fish species (39). For example, in bluefin killifish, melanic anal fin pigmentation is used as a dominant status signal,

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and showed an increase in aggression when placed with other pigmented fish (40). But, again, in the case of anal fin pigmentation in *Brachyrhaphis* females, pigmentation appears to not significantly affect the behavior of receivers.

We evaluated females in pairs where both were naturally pigmented, and in mismatched pairs wherein we artificially removed pigment from both females and then reapplied to just one. We found that naturally pigmented females in pigment-matched pairs showed no statistical difference in number of aggressive displays from females in mismatched, manipulated pairs, except in *B. terrabensis*. *Brachyrhaphis terrabensis* females in non-matching pairs had lower amounts of aggressive displays pairs with matching, pigmented females, opposite of what our hypothesis predicted. *Brachyrhaphis terrabensis* females likely differed in behavior due to the natural lack of pigmentation in *B. terrabensis* females tested. Thus, females in pigmented pairs possibly thought they were interacting with unfamiliar females, likely causing higher amounts of aggressive behavior. Similar examples of organisms showing higher levels of aggression when interacting with an unfamiliar individual exist in other systems. For example, in cichlids, researchers found that if unfamiliar conspecifics were presented, the cichlid increased levels of aggression (41). Although these findings in *B. terrabensis* were unexpected, they could provide interesting direction for studies focusing on *Brachyrhaphis* behavior when interacting with unfamiliar females.

Neither of the hypotheses evaluated here to explain female anal fin pigmentation in *Brachyrhaphis* fishes is supported by our data. What other explanations exist? It is possible that anal fin and gonopodial pigmentation could be used for intra-species communication and identification, but more studies would need to be conducted to explore this hypothesis. It is likely that gonopodial anal fin pigmentation of males and females is a non-adaptive trait. Lande (42) suggested that the presence of male traits in females, in some systems, is non-adaptive and

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simply reflects cases where dimorphisms have not yet evolved. For example, in caribou, males typically have large antlers used in sexual displays. Females also have small antlers that are similarly structured to males. It is uncertain how antlers are utilized at all in females (43), yet they remain because shared alleles are expressed in both sexes (44).   Similarly, the evolutionary benefit of female anal fin pigmentation in *Brachyrhaphis* fishes currently lacks conclusive data to provide an adequate explanation, consistent with the possibility of the trait being non-adaptive.

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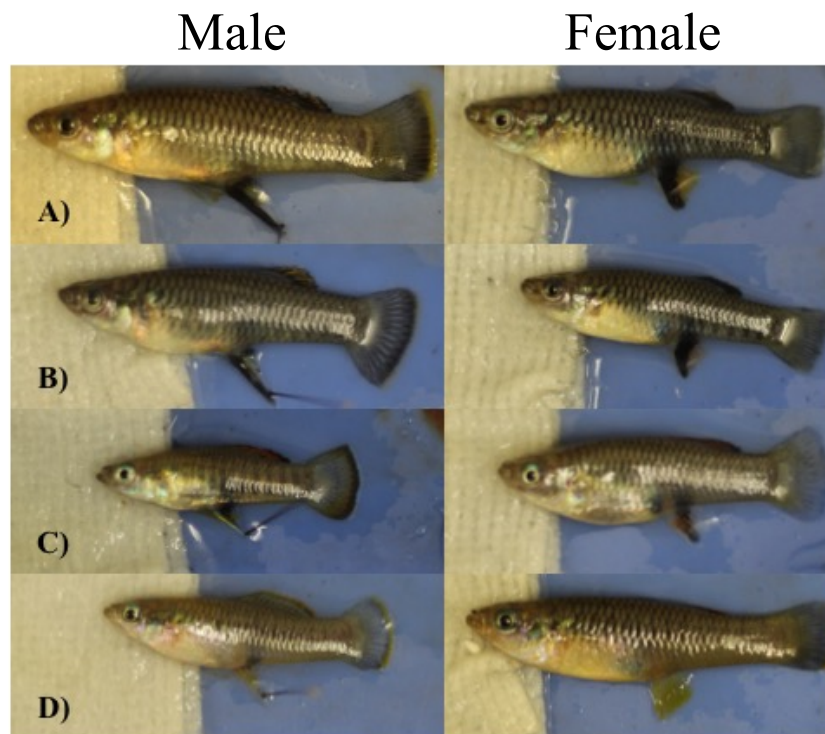43. Berglund A. Why are sexually selected weapons almost absent in females? Current Zoology. 2013;59(4):564-8.

Figure 1. Gonopodial and anal fin pigmentation in male (on left) and female (on right) Brachyrhaphis fishes: (A) B. rhabdophora *Javilla; (B)* B. rhabdophora *Grande; (C)* B. roseni*; and (D)* B. terrabensis.
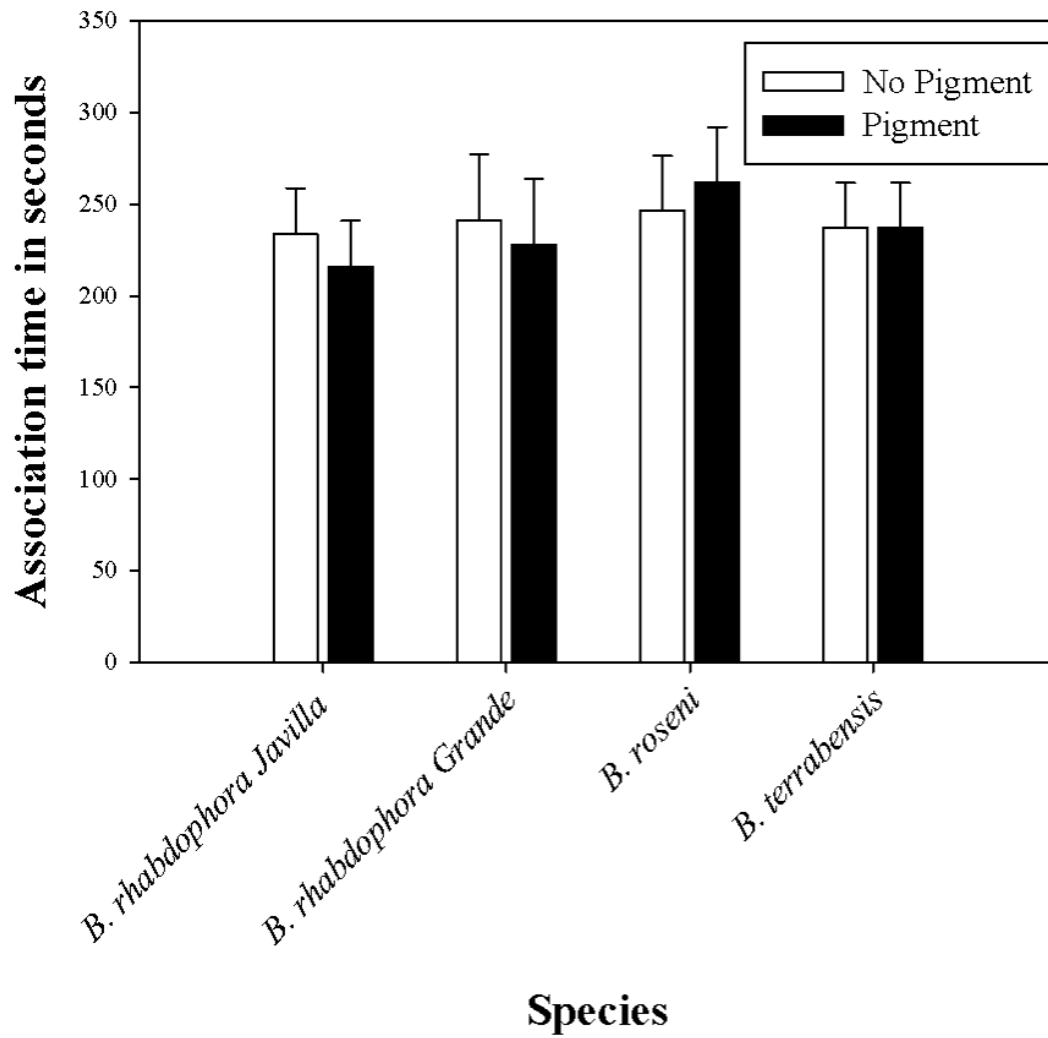
Male Dichotomous Choice Test

Figure 2. Amount of time a male spent with either a pigmented or non-pigmented female in a dichotomous choice test. Time was measured in seconds. For the statistical analysis, time was square root transformed to normalize data.
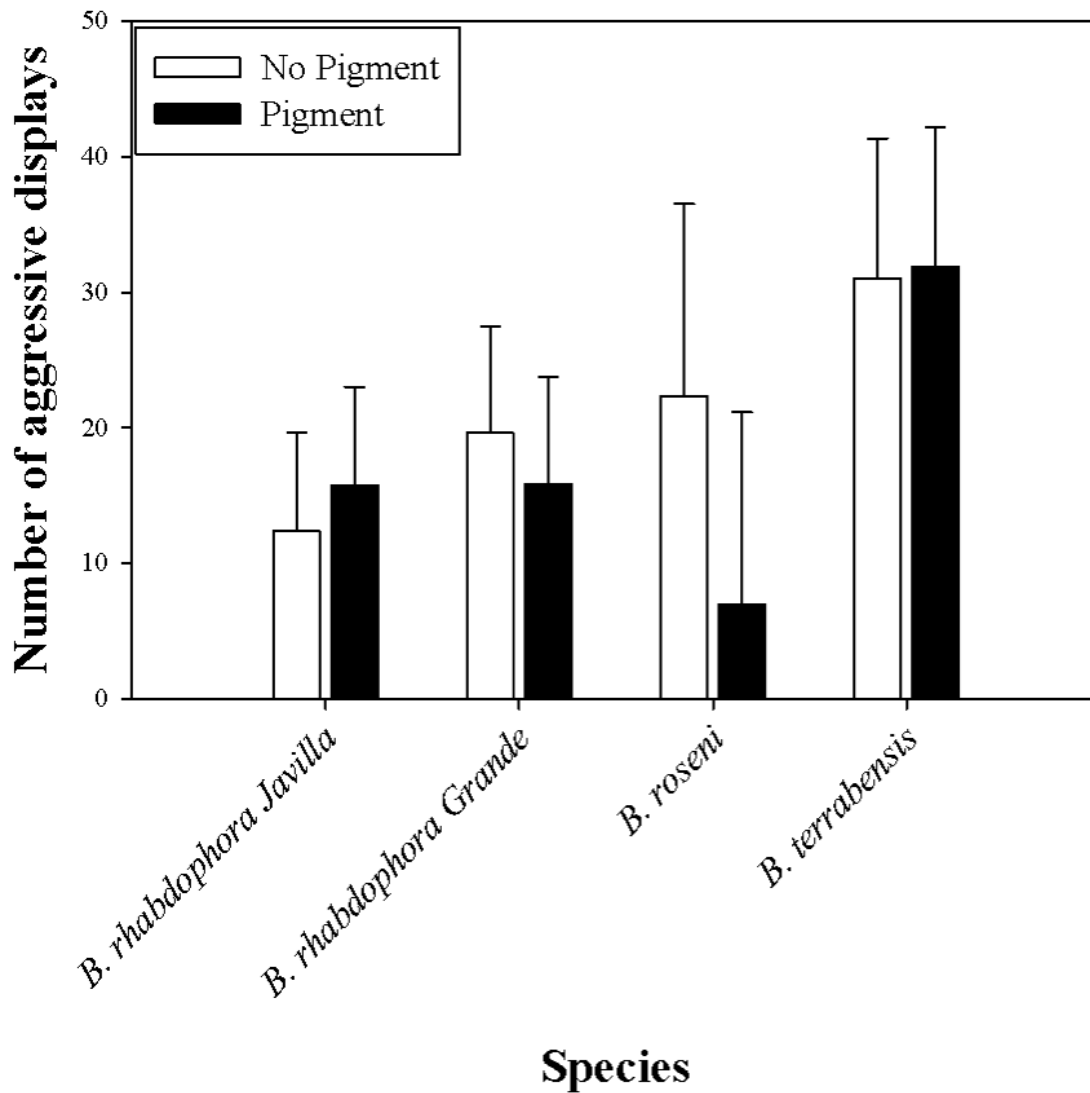
Female Aggression

Figure 3. Number of aggressive events by a focal female based on what type of female she interacted with (a pigmented or non-pigmented female). For the statistical analysis, aggression events were square root transformed to normalize data.