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Male mate recognition and neighbor–stranger discrimination in rubyspot (*Hetaerina* spp.)
damselflies

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in
Biology

by

Maria Beatriz Cabezas Castillo

2017

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ABSTRACT OF THE THESIS

Male mate recognition and neighbor–stranger discrimination in rubyspot (*Hetaerina* spp.)
damsel­flies

by

Maria Beatriz Cabezas Castillo

Master of Science in Biology

University of California, Los Angeles, 2017

Professor Gregory F. Grether, Chair

Mating and territorial behavior have important fitness consequences. In this thesis, I investigate some processes that affect mating and territoriality in damselflies of the genus *Hetaerina*. In the first chapter, I carried out an experiment to mimic the initial stage of female color polymorphism, which is hypothesized to be maintained by negative frequency-dependent selection caused by male mating harassment. I tested how males respond to novel female color in a non-polymorphic species, *Hetaerina capitalis*, and the results supported the mating harassment-based hypotheses. In the second chapter, I experimentally evaluate the ability of rubyspot damselflies to discriminate between neighbors and non-neighbors. Specifically, I tested whether the dear enemy phenomenon (DEP) or a nasty neighbor effect (NNE) governs how territorial males respond to intruders. Two of the three species tested exhibited DEP, but one uses location cues to discriminate intruders while the other uses individual recognition.

The thesis of Maria Beatriz Cabezas Castillo is approved.

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2017

To Daniel, my life partner and best friend, I couldn't have done this without you.
Thanks for all your unconditional love and support along this way.

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Chapter 1. Effects of novel female color morphs on male mate recognition and aggression in territorial damselflies

Abstract

Female color polymorphisms in damselflies, which often include "andromorphs" that resemble males, are hypothesized to be maintained by negative frequency-dependent selection caused by male mating harassment. The frequency dependence is thought to arise from learned male mate recognition, with or without male mimicry. To test whether the conditions hypothesized to give rare female morphs an advantage exist in non-polymorphic, territorial damselflies, we presented male *Hetaerina capitalis* territory holders with conspecific females of two novel body color morphs: green and red. While neither color morph specifically resembles males, only males naturally have red coloration. As predicted by the learned mate recognition hypothesis, females of both novel color morphs elicited fewer sexual responses than unmanipulated females. As predicted by the male mimicry hypothesis, the red morph elicited fewer sexual responses than the green morph. However, some males attacked red females as though they were male territory intruders. We hypothesize that, in territorial species, the costs of such misdirected male aggression could override the benefits of reduced male mating harassment. Thus, the results of our field experiment support both leading hypotheses for the evolution of female color polymorphisms in damselflies, while also helping explain the rarity of andromorphs in territorial species.

Keywords

color polymorphism, learned mate recognition, male mimicry, mating harassment, misdirected aggression, sexual harassment

Introduction

The traditional explanation for color polymorphisms in prey species is frequency-dependent predation, where common prey types are attacked more frequently than rare prey types (Allen, 1988; Stamps & Gon, 1983; Reznick *et al.*, 2001; Bond & Kamil, 2002). Predators are hypothesized to focus on conspicuous features of the most frequently encountered prey type and to switch prey types when the relative frequency of prey types changes. This frequency-dependent prey switching prevents the elimination of rare morphs while regulating the abundance of common morphs (Bond & Kamil, 2002). Female-limited color polymorphisms, in which females occur in multiple morphs but males are monomorphic, have also been attributed to frequency dependent predation in combination with genetic or selective factors that limit the polymorphism to females (e.g., heterogamy, sexual selection; Stamps & Gon 1983; Herrell & Hazel 1995; Kunte 2008; Calsbeek & Cox 2012).

An entirely different selective mechanism has emerged as the leading hypothesis for female-limited color polymorphisms in damselflies (Odonata: Zygoptera): frequency dependent male mating harassment. In damselfly species that exhibit female color polymorphisms, usually one morph resembles the brightly colored males (andromorph) while the other morph or morphs are cryptic in coloration (gynomorphs; Cordero & Andrés 1996; Van Gossum *et al.*, 2005; Hammers & Van Gossum, 2008). The general hypothesis is that males predominately attempt to mate with the most common female morph, which reduces harassment of rarer morphs, and the resulting negative frequency-dependent selection maintains the polymorphism (Gosden & Svensson 2007, 2009; Takahashi *et al.*, 2014; Hammers & Van Gossum, 2008; Van Gossum *et al.*, 2001; Svensson *et al.*, 2005, 2009; Fincke 2015; Le Rouzic *et al.* 2015). Multiple versions of this hypothesis have been proposed. Under the learned mate recognition (LMR) hypothesis,

males are most likely to respond sexually to females of the morph they encounter most frequently, and the morph ratio drives frequency-dependent selection (Hammers & Van Gossun, 2008). Most versions of the male mimicry (MM) hypothesis also assume that male mate recognition is learned, but under this model, andromorphs benefit from their resemblance to males and the ratio of andromorphs to males drives frequency-dependent selection (Ting *et al.*, 2009). The LMR and MM hypotheses are not mutually exclusive, and some species appear to exhibit both types of frequency dependence (e.g., Ting *et al.* 2009).

While many studies have tested assumptions and predictions of the LMR and MM hypotheses in polymorphic damselflies, studies of monomorphic species could shed light on the conditions that initially favor the evolution of polymorphisms. Would novel female color morphs experience reduced male mating harassment in monomorphic species? If so, what has prevented female color polymorphisms from evolving in those species? Genetic constraints seem unlikely because female color polymorphisms in damselflies have been shown to involve single autosomal alleles (Johnson, 1966; Sánchez-Guillen, *et al.*, 2005). One selective factor that could explain the absence of andromorphs in some species is male territoriality. If territorial males mistook andromorphs for males, the costs of misdirected aggression could override the benefits of reduced male mating harassment. Most species of damselflies with polymorphic females are non-territorial, which suggests that this misdirected male aggression hypothesis warrants testing.

We carried out a simple field experiment to determine whether the conditions hypothesized to give rare female color morphs a fitness advantage exist in a monomorphic, territorial species of damselfly, *Hetaerina capitalis* (Odonata: Zygoptera: Calopterygidae). The experiment consisted of recording the responses of territorial males to tethered conspecific females of three different color treatments: (1) natural morph, unmanipulated (Fig. 1b); (2) red

morph, simulating a novel andromorph (Fig. 1c); and (3) green morph, simulating a novel gynomorph (Fig. 1d). The LMR hypothesis predicts that both novel morphs should be less likely than the natural morph to elicit a sexual (clasping) response. The MM hypothesis predicts that the red morph should be less likely than either of the other morphs to elicit a sexual response. Finally, the misdirected male aggression hypothesis predicts that the red morph should be more likely than the other morphs to elicit an aggressive response.

Methods

Study Sites

We replicated the experiment in two different river drainages on the Pacific slope of Costa Rica. The first replicate was carried out at Quebrada Socorro, near San Luis, Puntarenas (10.2783, -84.81894, elev. 780 m) from March 31 to April 8, 2016. The second replicate was carried out at Rio Tempisquito near Maritza in the Guanacaste Conservation Area, Guanacaste (10.9283, -85.5297, elev. 290 m) from May 12 to May 25, 2016. Quebrada Socorro and Rio Tempisquito are tributaries of Rio Guacimal and Rio Tempisque, respectively, which enter the Pacific Ocean separately in the Gulf of Nicoya.

Study Species

Male *H. capitalis* have reddish-brown body coloration and red wing patches (Fig. 1a), while females have greenish-brown body coloration and amber wing patches (Fig. 1b). As in most other species in this genus, mature adult males compete for small mating territories close to the surface of rivers in areas with flowing water and submerged vegetation, where females come to oviposit (Grether 1996; Cordoba-Aguilar et al. 2009). Females store sperm and may oviposit

without re-mating if circumstances allow, but generally they are pursued by one or more males and clasped as soon as they arrive to oviposit (there is no pre-clasping courtship). Clasping is usually followed by copulation, and then the pair flies in tandem while the female searches for a site to submerge and oviposit (Grether 1996; Cordoba-Aguilar et al. 2009).

Behavioral Observations

At each site, we captured most of the adult male *H. capitalis* along a 300 m transect using aerial nets and marked them with unique IDs using a previously described method (Anderson *et al.*, 2011). Throughout the period of the day that males defended territories (approximately 08:00 to 17:00 h), observers surveyed the transects, recording territorial fights and the locations of males to 0.1 m in three dimensions (distance along the transect, distance from the nearest bank, and height above the water) using hand-held computers. We considered males that consistently perched close to the water surface (≤ 0.5 m) near the same location (± 1.5 m) during two or more consecutive days to be territory holders eligible for testing (Anderson & Grether 2010). These damselflies habituate rapidly to the presence of humans, and most territory holders could be approached closely without visibly affecting their behavior, by the time we began the experiment.

Experimental Design and Procedure

Territorial males were presented sequentially with (1) an unmanipulated female (natural morph, Fig. 1b), (2) a female with red bands on her abdomen (red morph, Fig. 1c), and (3) a female with green bands on her abdomen (green morph, Fig. 1d). The colored bands were added using paint pens (200-S Fine Point, Marvy Decocolor Paint Marker, Uchida of America,

Torrance, CA, USA). The order of presentation was random in the first test at each study site and then varied systematically to achieve a balanced design. One person presented the tethered female while a second person recorded the male's responses. Females were tethered with transparent thread (0.076 mm diameter) to a thin fiberglass pole and directed to fly within 1 m of the male for 5 seconds or until the male stopped interacting with her, whichever happened last (as in Drury et al. 2015). Females were used in maximum of seven tests (range 1-7; median 3, $n = 101$) and there was no difference between treatments in the number of times females were used (Kruskal-Wallis test, $\chi^2 = 0.115$, $df = 2$, $p = 0.94$, $n = 34$ natural, $n = 33$ red, $n = 34$ green). Tests were carried out on non-rainy days between 09:00–17:00 h.

The possible responses of males were: no response (remained perched), approach (flew toward the female), clasp (clasped the female with his cerci), attempted clasp (approached the female with abdomen curled into a clasping position but did not clasp the female), slam (rammed into the female), grab (grasped the female with his legs) and perch (returned to perch). Slams and grabs are aggressive responses, frequently seen in male territorial fights, while clasps and attempted clasps are sexual responses (Drury et al. 2015). Approaches are a necessary prelude to both kinds of physical interactions and were not classified as aggressive or sexual.

Following previous studies, a “successful” test was defined a priori as one in which the male responded sexually to at least one of the three females presented, regardless of the female’s treatment group (Drury et al. 2015). At Quebrada Socorro, 31 of 50 tests were successful; at Rio Tempisquito 39 of 53 tests were successful. Males were retested if their first test was unsuccessful (range 1-5, median 2 repeat tests, $n = 24$ males), and eleven males responded sexually when retested. Aggressive responses were recorded in four unsuccessful tests: three

toward the red morph; one toward the green morph; and zero toward the natural morph. Only successful tests were included in the analysis.

Data analysis

To examine how the female color treatments affected male sexual responses, we used logistic regression with sexual response (0/1) as the dependent binary outcome variable, female color treatment, study site and trial order (i.e., the order in which females were presented) as categorical predictor variables, and all possible interactions. To compare levels of the categorical variables, we used the Bonferroni multiple pairwise comparisons procedure (which reduces the probability of type I errors). We were not able to use logistic regression to analyze male aggression because there were too few aggressive responses. In this case, we used Fisher's exact test to compare the frequency of aggressive responses between treatments, both with the data pooled and separately by study site. All statistical analyses were carried out in STATA 14.1 (StataCorp, College Station, Texas USA).

Results

Sexual Responses

The probability of males responding sexually to the tethered female was highest for the natural morph, intermediate for the green morph, and lowest for the red morph (Fig. 2; logistic regression, treatment $\chi^2 = 34.93$, $df = 2$, $p < 0.001$; pairwise comparisons with Bonferroni corrections: natural vs. green $\chi^2 = -4.07$, $p < 0.001$; natural vs. red $\chi^2 = -5.91$, $p < 0.001$; green vs. red $\chi^2 = -2.64$, $p = 0.025$; Supplementary Table 1). There was no difference in overall sexual responsiveness between study sites ($\chi^2 = 0.01$, $df = 1$, $p = 0.93$) and no interaction between color

treatment and study site ($\chi^2 = 1.65$, $df = 2$, $p = 0.44$). There was a trial-order effect ($\chi^2 = 6.47$, $df = 2$, $p = 0.04$), but no interaction between treatment and trial ($\chi^2 = 2.99$, $df = 4$, $p = 0.56$). The direction of the trial-order effect is that males were less likely to respond sexually to the third female than to the first or second female (pairwise comparisons: first vs. second $\chi^2 = 0.17$, $p = 1.00$; first vs. third $\chi^2 = -2.27$, $p = 0.07$; second vs. third $\chi^2 = -1.99$, $p = 0.14$).

Aggressive Responses

Territory holders were more likely to respond aggressively to the red morph than to the natural morph (Fisher's exact test, $p = 0.001$) and green morph ($p = 0.004$), but they did not respond differently to the green and natural morphs ($p = 1.0$). Overall, 11 of 70 males were aggressive to the red morph, one was aggressive to the green morph, and none was aggressive to the natural morph (Supplementary Table 3). Separate analyses by study site yielded similar results. At Quebrada Socorro, 7 of 31 males were aggressive to the red morph, one was aggressive to the green morph, and none was aggressive to the natural morph (Fisher's exact test, red vs. natural, $p = 0.011$; red vs. green, $p = 0.053$; green vs. natural, $p = 1.00$). At Rio Tempisquito, 4 of 39 males were aggressive to the red morph and none was aggressive to the other morphs (red vs. natural, $p = 0.115$; green vs. red, $p = 0.115$).

Discussion

The purpose of this experiment was to evaluate how males in a non-polymorphic, territorial species would respond to novel female color phenotypes. We found that putting either red or green bands on the abdomens of female *Hetaerina capitalis* reduced male sexual

responses, and the red markings caused a larger reduction in sexual responses than did the green markings. Aggressive responses were elicited almost exclusively by females with red markings.

Our findings support both leading hypotheses for the evolution of female color polymorphisms in damselflies. Under the learned mate recognition (LMR) hypothesis, females of novel (or rare) color morphs should elicit fewer sexual responses from males than do females of common color morphs. Our results indicate that a female *H. capitalis* with novel body coloration would indeed attract fewer mating attempts than do females of the natural morph. Our results also support the male mimicry (MM) hypothesis in that females with red markings received fewer mating attempts than did females with green markings. Thus, a mutant female *H. capitalis* with novel body coloration of either type would be expected to benefit from reduced male mating harassment but a mutant with red, male-like coloration would benefit more in this regard than a female with novel green coloration. Neither of our color manipulations eliminated male sexual responses – even females with red markings elicited sexual responses from 24% of the males tested. Thus, as is evidently the case in species with female color polymorphisms, a female *H. capitalis* with a rare color phenotype would probably mate frequently enough to store sperm and fertilize her eggs.

While our experiment was designed to test general predictions about the initial stage of the evolution of a color polymorphism, namely the appearance of novel female color morphs, it is logical to ask why such a polymorphism has not evolved in *H. capitalis*. Genetic constraints seem unlikely. Female color polymorphisms in damselflies have been shown to have a simple genetic basis: allelic variation at a single autosomal locus (Johnson, 1966; Sánchez-Guillen, *et al.*, 2005). Since females likely have unexpressed genes for developing male body coloration, a regulatory gene mutation might be all that is required for the appearance of red coloration in

female *Hetaerina*. On the other hand, the aggressive responses of males to the red morph in our experiment indicate that andromorphic female *H. capitalis* would often be attacked by territorial males. We hypothesize that in territorial species, the benefits of andromorphic coloration in terms of reduced male mating harassment would be more than offset by the costs of misdirected male aggression. An alternative explanation for the rarity of andromorphs in territorial damselflies is that females are subject to lower levels of mating harassment in territorial species than in non-territorial species (Cooper *et al.* 2016). These hypotheses are not mutually exclusive and they could be tested with field experiments in which the fitness of free-flying color-manipulated females is compared to that of sham-manipulated control females in both territorial and non-territorial monomorphic species.

As our experiment was only designed to mimic the initial appearance of a color polymorphism, the results have no bearing on the learning mechanism that is hypothesized to result in negative frequency dependence and maintenance of polymorphisms. However, future experiments in which color-manipulated females are presented repeatedly to the same male could be used to assess whether males can learn to recognize novel female morphs, and if so, whether this interferes with their ability to recognize females of the natural morph, as predicted by the LMR hypothesis, and whether repeated exposure to andromorphs causes males to respond sexually to males, as predicted by the MM hypothesis (Ting *et al.*, 2009; Gering 2017).

In summary, we simulated the initial appearance of a color polymorphism to examine how males of a non-polymorphic species would respond to new female color morphs. The results show that novel color morphs would receive less sexual harassment than color morphs that already exist in the population. This is consistent with LMR hypothesis for the maintenance of female color polymorphisms. The results are also consistent with the MM hypothesis because

females with novel red marks (simulating andromorphs) elicited fewer sexual responses than did females with novel green marks (simulating gynomorphs). Finally, our study provides the first experimental evidence that andromorphic coloration might be selected against in territorial species by the costs of misdirected male aggression.

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Fig 1. Photos of *Hetaerina capitalis* showing a) mature male, b) natural female, c) experimental red female morph, d) experimental green female morph.

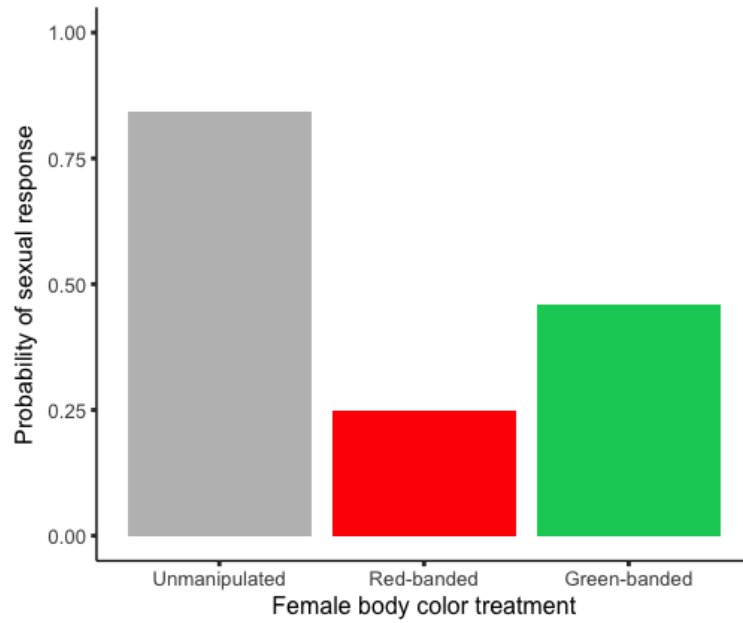


Fig 2. Predicted margins of sexual response on treatment. Probability of a sexual response to be categorized under control, green or red treatment according to the regression model. Marginal probabilities were calculated in STATA (14.1) with the command margins.

Supplementary Information

Table 1. Number of sexual and aggressive response under each female treatment: natural, green, and red.

Type of responses	Behaviors	Natural	Green	Red
No sexual/aggressive responses	No response	4	13	18
	Approach	8	23	24
Sexual responses	Clasp	30	14	10
	Attempted clasp	28	19	7
Aggressive responses	Slam	0	0	3
	Grab	0	1	8
	Total	70	70	70

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Chapter 2. Experimental test for neighbor–stranger discrimination in rubyspot damselflies (*Hetaerina* spp.): evidence for dear enemy phenomenon

Abstract

Many territorial animals have been reported to be less aggressive to neighboring territory holders than to strangers (floaters), a pattern referred to as the dear enemy phenomenon (DEP). The opposite pattern has also been reported and is known as the nasty neighbor effect (NNE). Observational studies are of limited use for documenting such patterns, however. Experiments are required to eliminate potentially confounding variables, including the behavioral responses of neighbors and floaters and differential encounter rates. To determine whether rubyspot damselflies (*Hetaerina* spp.) exhibit DEP or NNE, and if so, to identify the underlying mechanisms, we carried out a field experiment in which one neighbor and one stranger were flown into an owner's territory simultaneously from opposite directions. To test for discrimination based on location cues, we systematically varied the direction of intrusion in relation to the neighbor's territory. Two of the three species tested exhibited DEP, but the mechanism of discrimination differed between species. *H. cruentata* territory holders responded less aggressively to whichever male intruded from the neighbor's territory, which implies DEP based on location cues. *H. occisa* territory holders were less aggressive to neighbors than strangers regardless of the direction of intrusion, which implies DEP based on individual recognition. The third species, *H. capitalis*, showed neither type of discrimination. In both species that exhibited DEP, the level of aggression toward intruders increased with the length of time the focal male held a territory contiguous with the neighbor's territory. Whether this is a residency time or familiarity effect cannot be resolved with our data but could be addressed in

future experiments. Further research is needed, but we tentatively conclude that DEP occurs in *Hetaerina* and that, surprisingly, the mechanism of discrimination differs between species.

Keywords

dear enemy phenomenon, nasty neighbor effect, territoriality, individual recognition, location recognition, familiarity effect.

Introduction

Territoriality is a common behavior in animals. However, defending a territory is generally costly, owners must protect their spaces against individuals with lower quality territories, or against individuals without territories (Alonzo, 2004; Lenda *et al.*, 2012). Thus, territorial animals invest time and energy defending their territory, which in turn may jeopardize their survival due to injuries from physical attacks and exposure to predation (Bradbury & Vehrencamp, 1998). For this reason, the way territorial animals confront competitors is important for survival.

In many territorial animals, the identity of the opponent is important, since their level of aggression differs toward neighbors and non-neighbors (also called strangers or floaters). Some territorial holders reduce their aggression level to intrusions when established neighbors are involved. In contrast, the level of aggression increases when the intruders are unfamiliar individuals. This is called the dear enemy phenomenon (DEP; Fisher, 1954), and has been shown in different taxa (Langen *et al.*, 2000; Husak & Fox, 2003; Boulay *et al.*, 2007, Dimarco *et al.*, 2010; Wei *et al.*, 2011; Monclús *et al.*, 2014; Aires *et al.*, 2015). The hypotheses behind this phenomenon are: (1) the relative threat hypothesis, in which neighbors represent a lower threat than strangers. This difference could be due to the fact that neighbors already have an established

territory, while strangers may be looking for one (Wilson, 1980), and (2) the familiarity hypothesis which argues that when neighbors have an established relationship, they reduce the level of aggression to each other, whereas the relationship with strangers is temporally and spatially unpredictable with possible outcomes displaying high levels of aggression (Wilson, 1975; Temeles, 1994).

The opposite behavior, when owners react more aggressively toward neighbors than strangers, has also been observed in many animals (Sanada-Morimura *et al.*, 2003; Müller & Manser, 2007; Newey *et al.*, 2010; Schradin *et al.*, 2010). This is called the nasty neighbor effect (Temeles, 1994; Müller & Manser 2007). Like the DEP, territorial individuals learn to recognize their neighbors, but this time the hypothesis state that they may represent a greater threat, since the encounters may result in the loss of territory, while strangers could be a minor threat because they are just passing through (Temeles, 1994). On the other hand, many territorial animals do not show differences in the level of aggression toward neighbors and strangers, which would suggest that they are equally threatened by these two classes of individuals (Bee, 2003; Lachish & Goldizen, 2004; Battiston *et al.*, 2015; Christensen *et al.*, 2016).

In his review Temeles (1994) found that the territory purpose could be driving both the nasty neighbor and dear enemy phenomenon. The dear enemy tends to occur principally on multi-purpose territories (mating and breeding), principally because in this kind of territories, territorial males are more likely to be challenged by strangers, which may look for suitable places for mating; while neighbors would already have their own territory, thus representing a minor threat (REF?). Nasty neighbors, on the other hand, may occur in feeding or nesting territories. In this case neighbors represent a constant threat when defending resources, while

strangers are only a temporary threat. Together, these ideas suggest that the purpose of defending a territory could give clues about the phenomena driving individual relationships.

Rubyspot damselflies (*Hetaerina* genus) are territorial non-social insects that defend mating territories (Grether, 1996). Even though these characteristics make them good candidates to display a dear enemy phenomenon, a recent observational study performed on *Hetaerina vulnerata* found the opposite result, a nasty neighbor relationship (Munguía-Steyer *et al.*, 2016). Observational data can be subject to potential observer and sample biases. Also, the outcome can be influenced by uncontrolled variables altering animal behavior that could result in some events (e.g., aggression) being recorded more often than others (Salvia & Meisel, 1980; Balph & Balph, 1983; Mann, 1999). Experiments, on the other hand, allow investigators to evaluate the outcome influenced principally by the tested variable while controlling for other factors that might be influential (Martin *et al.*, 2010). The present study is a field experiment that has the potential to corroborate previous findings or consider new unexplored explanations, about the behavior of *Hetaerina* genus.

To explore if rubyspot damselflies show discrimination between neighbors and non-neighbors when defending territories, we evaluated the aggressive response of territorial males against neighbors and strangers. We conducted experiments with conspecific intruders, and separate experiment with heterospecific intruders, with territory holders of three species in the genus *Hetaerina*: *H. cruentata*, *H. capitalis*, and *H. occisa*. Some studies have demonstrated that competitor recognition in these species is based principally on visual recognition of spots that males display on their wings (Anderson & Grether 2010a, Anderson & Grether 2010b, Anderson & Grether 2011, Grether *et al.*, 2015, Drury *et al.*, 2015). Moreover, the study performed by Grether *et al* (2015) demonstrated that wing spots allowed discrimination of gender and age.

Some animals have the ability to discriminate intruders individually (Carazo *et al.*, 2008; Baird *et al.*, 2015; Chuang *et al.*, 2017), but others use location cues to distinguish neighbor from strangers (Falls & Brooks, 1975; Husak & Fox, 2003). To test if damselflies show one or both of these discrimination abilities resulting in the dear enemy phenomenon, we presented two intruders (neighbor and stranger) simultaneously to territory holders. To determine whether recognition was based on individual recognition or location cues, the intrusions were repeated with the entering locations of the intruders switched. We predicted, under a dear enemy phenomenon, that: 1) if damselflies recognize individuals then territorial males would be less aggressive to neighbors regardless of the side where they come from, and 2) if is based on location then, territorial males would show less aggression toward any intruder that comes from the neighbor's side. Finally, if territorial males would not show a decrease, instead show an increase in the level of aggression to neighbors (using either of these two mechanisms), then these species would be displaying a nasty neighbor effect.

Methods

Study Sites

The field experiment was carried out at two sites in Costa Rica: Quebrada Socorro, Monteverde (10.27826, -84.818937, elev. 780 m), from March 23 to April 19, 2016 and Río Tempisquito, Guanacaste (10.92830, -85.529677, elev. 290 m), from May 2 – 25, 2016.

Data Collection and Behavioral Observations

In both sites, we established a river transect of 300m. The transect lines were marked every meter with numbered flags, which allowed us to record males' precise locations. Using

aerial nets, we captured most of the adult males inside the transect and drew a unique color code on the abdomen of each captured individual (Anderson *et al.*, 2011). In order to identify territorial males, behavioral observations were carried out along each transect, between 8:00 to 17:00 hours, which is the time when territorial males defend territories. We considered individuals that consistently perched above 0.5 m of the water and near the same location on the river (± 1.5 m) during two or more consecutive days to be territorial males eligible for testing (Anderson & Grether, 2010a). The experiment was carried out on non-rainy days, between 9:00–17:00 hours (air temperature range, 22.0-28.5°C), which is when males usually defend their territories.

Experimental Design and Procedure

The experiment consisted of presenting known territory holders ("focal males") simultaneously with two tethered conspecific male intruders, the nearest territory holder ("neighbor") and another mature male captured outside the study transect ("stranger"). To make it possible, in principle, for focal males to identify neighbors using location cues, we selected focal males with only one close neighbor. Specifically, the criterion for designating a male as a focal male in the experiment was that he had one neighbor perching within 2 m and no other territory holders perching within 5 m of his usual perch. This criterion was applied to most tests. The exceptions were one male at Quebrada Socorro and two males at Rio Tempisquito that had neighbors perching approximately 3 m away.

The experiment was conducted as follow: each morning before the experiments, a stranger was captured and marked using the same individual marking system as was used for males on the study transect, and the designated neighbor was captured from his territory. The

two captured males were then tethered to fishing poles using ~ 0.2 m lengths of transparent thread and flown into the focal male's territory from opposite directions. Each presentation lasted 60 seconds, and was performed by flying males within the owner's territory. Two people performed the trials, one presented the neighbor and the another one the stranger. During intruders' presentation one of these two people recorded the focal male behavior on a continuously running audio recorder.

To clarify whether a possible DEP is associated with individual recognition or location recognition, two treatments were conducted. In treatment 1, neighbors were presented from the side where their territory was located and the stranger was presented from the opposite direction. In treatment 2, the presentation direction was switched. Thus, neighbors entered from the side opposite their territory and strangers entered from the neighbor's territory side. The treatments were randomly assign during the first test at each study site, then they were systematically alternated between tests. There are three possible outcomes that would be consistent with a form of DEP: (1) If the territory holder is more aggressive to the stranger than the neighbor, regardless of the presentation direction, this would suggest DEP based on individual recognition. (2) If the territory holder is more aggressive to the male entering from the side opposite the neighbor's territory, regardless of whether the intruder is the neighbor or stranger, this would suggest DEP based on location cues without individual recognition. (3) If the territory holder is less aggressive to the neighbor than the stranger only when the neighbor enters from the neighbor's side and responds equally to the stranger regardless of the direction of entry, this would suggest DEP based on both individual recognition and awareness of the neighbor's usual location.

To examine whether familiarity with a neighbor affected the response of focal males, we categorized focal males according to the length of time they held a territory contiguous with the

neighbor's territory prior to the experiment: (a) just 2 days or (b) 3 or more days. We established the category of two days, based on previous studies (Anderson & Grether, 2010a), showing that this is the minimal amount of time to define territorial holders; thus, following this finding, if two territorial males are tested when they only have two days living together, they are named “new establish neighbors”. In case of individuals living together, three or more days they are named “established neighbors”.

Our primary goal was to test for an intraspecific DEP but a secondary goal was to determine whether males are able to distinguish between neighbors and strangers of other *Hetaerina* species. Therefore, in cases where the nearest neighbor was a *Hetaerina* male of a different species from the focal male, we paired the neighbor with a stranger of the same species as the neighbor (i.e., so that both intruders were either conspecifics or heterospecifics of the focal male). We analyzed data from tests with conspecific and heterospecific intruders separately.

The behaviors recorded during the experimental were as follows: no response (the focal male never left its territory during the test), approach (the focal male leaves its perch and flies toward one of the intruders), chase (the focal male pursues one of the intruders), slam (the focal male rams into one of the intruders), grab (the focal male grasps one of the intruders with its legs), and perch (the focal male returns to his perch).

We performed a total of 17 tests at Quebrada Socorro and 26 at Río Tempisquito. At Quebrada Socorro we carried out 7 tests with *H. cruentata* territory holders and conspecific intruders, 3 tests with *H. cruentata* territory holders and heterospecific (*H. capitalis*) intruders, 6 tests with *H. capitalis* territory holders and conspecific intruders, and 1 test with *H. capitalis* territory holders and heterospecific (*H. cruentata*) intruders. At Río Tempisquito, we carried out 7 tests with *H. occisa* territory holders and conspecific intruders, 4 tests with *H. occisa* territory

holders and heterospecific (*H. capitalis*) intruders, 15 tests with *H. capitalis* territory holders and conspecific intruders, there were no tests with *H. capitalis* territory holders and *H. occisa* intruders.

Statistical Analysis

We analyzed the length of time focal males chased the tethered intruders using multilevel mixed-effects linear regression (xtmixed in STATA 14.1), with individual focal male entered as a random-effect grouping variable. To improve the fit to normality, we used the arc-sine-square-root transformation of the proportion of time spent chasing as the dependent variable. We carried out one analysis for cases in which the intruders were conspecifics of the focal male and a separate analysis for cases in which the intruders were heterospecifics of the focal male. The full model for conspecific intruders included the intruder's category (neighbor or stranger), the location treatment (neighbor entering from neighbor's side or stranger entering from neighbor's side), the length of familiarity category (2 days or ≥ 3 days), the focal male's species (*H. occisa*, *H. capitalis* or *H. cruentata*), and all possible interactions. We also fitted a separate regression model, with the aforementioned variables and all possible interactions, for each focal species separately.

As mentioned in the introduction, a secondary goal was to determine whether males are able to distinguish neighbors and strangers from other *Hetaerina* species. However, the number of test with heterospecific intruders was too small for analysis. Likewise, the number of physical attacks shown by territorial males, were too few to be analyzed.

Results

The full conspecific model showed main effects of species ($\chi^2 = 41.79$, $df = 2$, $p < 0.01$) and length of familiarity ($\chi^2 = 12.01$, $df = 1$, $p = 0.0005$), but there was an interaction between length of familiarity and species ($\chi^2 = 9.54$, $df = 2$, $p = 0.0085$) and a 3-way interaction between intruder category, location treatment and species ($\chi^2 = 6.66$, $df = 2$, $p = 0.03$; Table S1). We therefore analyzed the data separately by species. In *H. cruentata*, territory holders were less aggressive to intruders entering from the neighbors' side than to intruders entering from the opposite site (Fig 1; location treatment by intruder category interaction $\chi^2 = 15.14$, $df = 1$, $p < 0.01$); there was no main effect of intruder category ($\chi^2 = 0.05$, $df = 1$, $p = 0.82$) or location treatment ($\chi^2 = 0.79$, $df = 1$, $p = 0.37$; Table S2). In *H. capitalis*, territory holders did not respond differently to intruders based on intruder category ($\chi^2 = 0.19$, $df = 1$, $p = 0.66$) or location treatment ($\chi^2 = 0.67$, $df = 1$, $p = 0.41$), and there was no location treatment by intruder category interaction ($\chi^2 = 0.30$, $df = 1$, $p = 0.58$; Table S3). In *H. occisa*, territory holders were more aggressive toward strangers than neighbors (Fig 2; intruder category $\chi^2 = 7.31$, $df = 1$, $p < 0.01$); there was no main effect of location treatment ($\chi^2 = 0.22$, $df = 1$, $p = 0.63$) and no interaction between location treatment and intruder category ($\chi^2 = 0.09$, $df = 1$, $p = 0.76$; Table S4). Regarding the interaction between length of familiarity and species, the level of aggression was higher toward both intruders (neighbor and stranger) when the focal male had a established neighbor, this means that they had held contiguous territories for longer (≥ 3 days versus 2 days) in *H. occisa* (Fig. 3; $\chi^2 = 12.86$, $df = 1$, $p < 0.01$; Table S4) and *H. cruentata* ($\chi^2 = 10.20$, $df = 1$, $p < 0.01$; Table S2), but this was not the case for *H. capitalis* ($\chi^2 = 0.120$, $df = 1$, $p = 0.75$; Table S3).

The results also showed, among all species, a small number of tests where territorials males performed physical attacks. Specifically, *Hetaerina cruentata* showed 8 tests with physical attacks, *H. capitalis* 11 tests, and *H. occisa* 4 tests; the number of physical attacks toward neighbor and strangers differ among the aforementioned tests and are shown in Table S5.

Discussion

We found evidence for the dear enemy phenomenon (DEP) in two of the three species of *Hetaerina* included in this study, but surprisingly, the mechanism of neighbor-stranger discrimination appears to differ between the two species. *H. cruentata* territory holders were more aggressive to the intruder that came from the side opposite the neighbor's territory, regardless of the identity of the intruder, which suggests a DEP based on location cues. By contrast, territorial male *H. occisa* were more aggressive to strangers than neighbors, regardless of whether the neighbor intruded from the neighbor's territory or from the opposite side of the focal male's territory, which suggests a DEP based on individual recognition. We found no evidence for DEP or NNE in *H. capitalis*, despite having the largest sample size for this species.

Taken at face value, these results suggest that the existence and mechanisms underlying DEP can vary even among closely related species. Combined with the results of another recent study on *Hetaerina* (Munguía-Steyer *et al.* 2016), it would appear that species in this genus display the full range of territory neighbor relations, from “dear enemy” to “nasty neighbor”, and that even the mechanisms underlying these phenomena vary among species. We are not aware of any a priori reasons to expect this diversity of territory neighbor relations in *Hetaerina*, however, and we think some healthy skepticism, and further research, is warranted.

Munguía-Steyer *et al.* (2016) reported finding evidence for the nasty neighbor effect in *H. vulnerata* based on observations of naturally occurring fights. Their results could be explained simply by the proximity of neighbors and non-neighbors territories. Also, floaters are many times either juveniles or old individuals that are not strong enough to engage in long-lasting fights, or simply individuals that are just passing by. Thus, the lack of response could be a consequence of individuals' physical condition or lack of interest in the focal territory, rather than a nasty neighbor relationship. Our study was protected against these problems, since using an experimental approach, the behavior of both intruders can be controlled, and in this way, a more effective measure in the level of recognition and aggressive response of territorial males toward neighbors and strangers was performed.

How might male *H. occisa* distinguish between neighbors and strangers individually and why would *H. cruentata* use location cues instead of individual recognition? Individual recognition has only rarely been reported in insects. *Polistes fuscatus* wasps with individual recognition have individually distinctive face color patterns; experiments have demonstrated that these wasps learn to distinguish between conspecific faces in individually and that another species in the same genus, *P. metricus*, lacks individually distinct faces and the specialized ability to learn to recognize faces (Sheenan & Tibbets 2011). The hypothesized reason for the difference in individual recognition between the two wasp species is that *P. fuscatus* nests in groups with dominance hierarchies while *P. metricus* nests solitarily (Sheenan & Tibbets 2011). We are not aware of any differences between *H. occisa* and *H. cruentata* in social behavior – both are solitary, territorial species. Whether *H. occisa* have individually distinctive face color patterns remains to be investigated, but unlike the wasps, rubyspot damselflies generally do not have the opportunity to view each other at very close range. It seems very unlikely that the

compound eyes of these insects could resolve distinctive facial color patterns on flying conspecifics. The wing and body coloration of *H. occisa* is not individually distinctive; in fact, *H. occisa* is one of the least variable species of *Hetaerina* (Garisson 1990; Anderson & Grether 2010b). In short, we have no working hypothesis for how *H. occisa* distinguishes between neighbors and strangers or for why *H. cruentata* appears to lack this ability.

While chase duration is a good estimate to measure level of aggression in some species of *Hetaerina* genus, males from other species chase similarly different intruders, thus making difficult to measure aggression. Therefore, previous studies have also used the frequency of physical attacks in order to evaluate, the aggression level that territorial males showed toward intruders (Grether *et al.*, 2015; Drury *et al.*, 2015). The present study tried to take the number of physical attacks in form of slams and grabs into account, and further evaluate if the additional information could give more insights about aggressive response. However, due to the reduced number of physical attacks recorded, there was not strong evidence about neighbors and strangers discrimination by the territorial owner. Thus, future studies with larger sample sizes should be performed to increase the power to detect differences in physical attack responses.

Two factors could probably explain the lack of physical attacks observed in this experiment. First, since the territorial holder must confront two intruders, it is possible that they get confused resulting on an increase of chase and decrease of physical attacks. An increment on the duration of circling flights as result of confusion of residency has been observed in butterflies (Wickman & Wiklund, 1983; Kemp & Wiklund, 2001). Second, the lack of physical attacks could be an adaptive nonaggressive strategy. Specifically, in this case when two territorial males engage in fights against intruders, one of them could prefer to perch in order to save energy for future fights where it will have to repel intruders by itself. This action of leaving the other male

(neighbor) chasing the intruder, could be a modified strategy to save energy as a consequence of reducing the number of physical attacks. Previous studies have shown that animals are able to acquire information about other males' fighting abilities and use this information in their favor. This prior evaluation would allow territorial holders to save energy for future contests (Johnsson & Akerman, 1998). This energy saving hypothesis could be tested in future studies by evaluating if the time territorial males spend chasing the intruder is longer when two individuals are involved (focal male and stranger), or when three individuals are fighting (focal male, neighbor and stranger).

In addition to the treatment effects, we found that time of familiarity affected the results in *H. cruentata* and *H. occisa*. Male *H. cruentata* and *H. occisa* that were “established neighbors” were more aggressive to intrusions of both neighbor and strangers than were males with only 2 days of familiarity “new established neighbors”. We are aware of three plausible explanations for this result: (1) Two males that have held contiguous territories for a longer period are less likely to accidentally intrude on each other's territories. Therefore, if an intrusion occurs, it is more likely to be a take-over attempt. Increases in the level of aggression toward neighbors after a previous simulated intrusion has been interpreted as a tit-for-tat response in hooded warblers (Godard, 1993). (2) The longer a male has held a territory in a particular location, the higher the value of the territory to that male. This is called the resource value asymmetry hypothesis, and posits that the knowledge acquired from territories is a potential factor favoring territorial holders during fighting contests (Tobias, 1997). (3) The longer a male has a territory, the higher his fighting ability (resource holding power, RHP) and therefore lower the risk of losing a territorial fight. Length of residency was confounded with length of familiarity in our study, so we are unable to distinguish among these hypotheses, but the third

hypothesis seems unlikely to apply to *Hetaerina* and other species with mating territories because males rarely feed while on their territories and therefore males with territories are not expected to increase in condition (and therefore RHP) relative to males without territories. Future experiments could separate the effects of familiarity from residency through removal experiments, as has been done in birds (e.g., Tobias 1997).

In summary, this is the first experimental study to provide evidence that territorial damselflies have the ability to discriminate between neighbors and strangers. Even though evidence of dear enemy phenomenon was shown on two of the three species analyzed, the mechanism of neighbor-stranger discrimination appeared to be different. While *H. occisa* showed, a DEP based in individual recognition, *H. cruentata* displayed a DEP based in location recognition. Therefore, the result shown in this study coupled with previous findings suggest that species of *Hetaerina* displayed a wide range of territory neighbor relations. Furthermore, this study is the first, to the best of our knowledge, to suggest that the territory aggression level in rubyspot damselflies, can increase with the number of days that individuals remain in their territories. Finally, we suggest future studies to evaluate territorial relations among other species of *Hetaerina*, and in this form to have better understanding of the role of neighbor–stranger discrimination in territorial defense.

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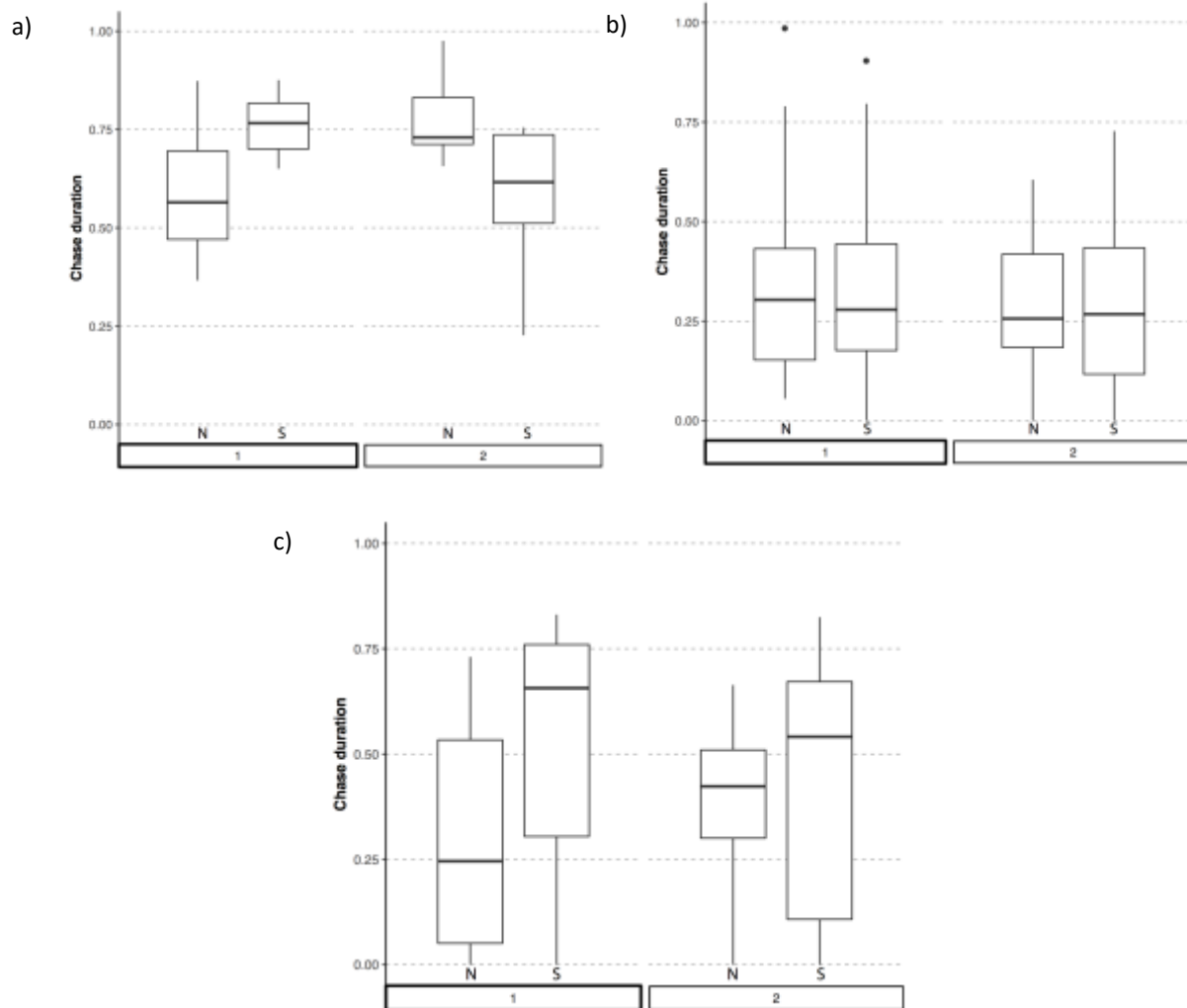


Figure 1: Chase response of territory holders based in individual: neighbors (N) and stranger (S), and location recognition neighbor entered from neighbor's side (1) and stranger entered from neighbor's side (2). Chase duration is the proportion of time spent chasing, after an arc-sine-square-root transformation. a) *H. cruentata*, b) *H. capitalis*, and c) *H. occisa*. Box plots depict the median (horizontal line within the box), interquartile range (box), lower and upper adjacent values (whiskers), and outside values (dots).

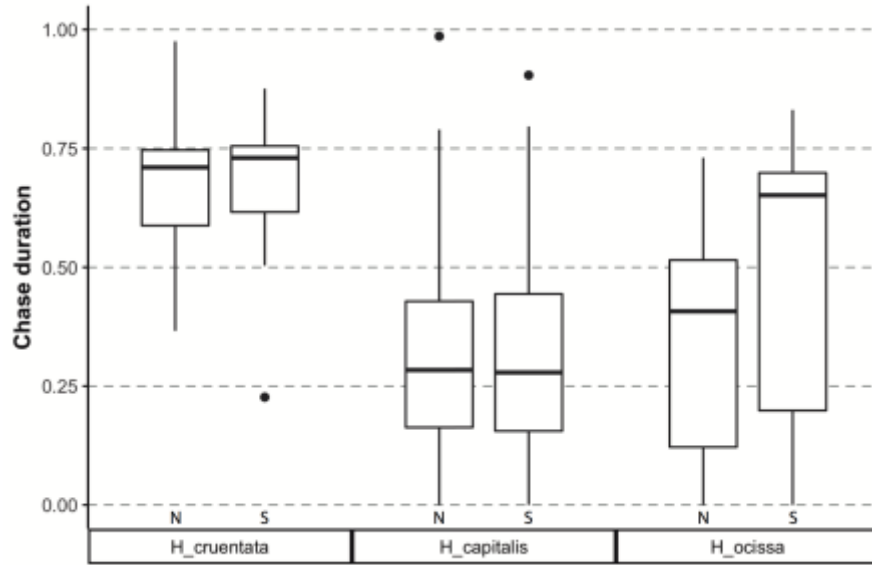


Figure 2: Chase response of territory holders based in individual recognition: neighbors (N) and stranger (S). Chase duration is the proportion of time spent chasing, after an arc-sine-square-root transformation. Box plots depict the median (horizontal line within the box), interquartile range (box), lower and upper adjacent values (whiskers), and outside values (dots).

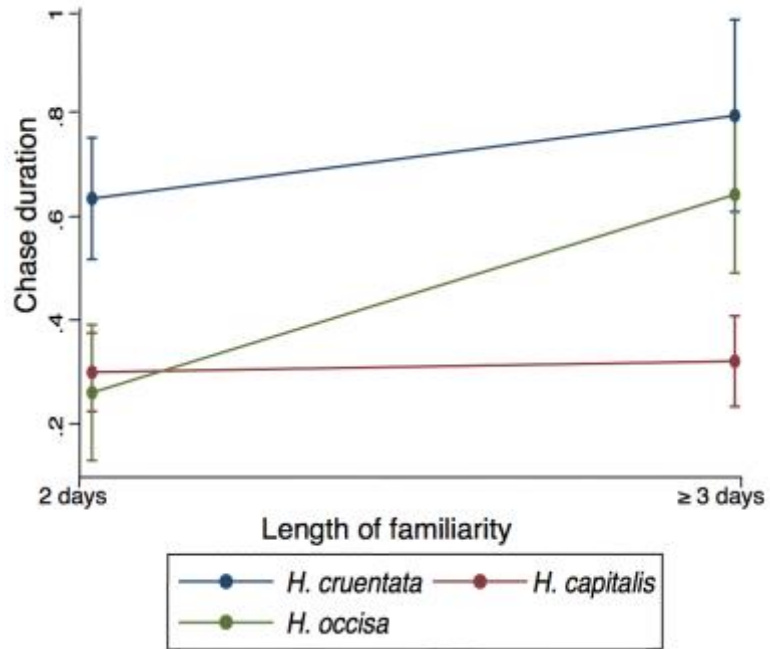


Figure 3: Chase response of territory holders based on length of familiarity (2 days or ≥ 3 days). Chase duration is the proportion of time spent chasing, after an arc-sine-square-root transformation. Species tested are indicated by different colors.

Supplementary information

Table 1: Full model with conspecific intruders and chase duration as the dependent variable (multilevel mixed-effects linear regression). This analysis is based on 140 observations (trials), clustered in 35 groups (territory holders)

Variables	df	Chi²	P>Chi²
Model		85.45	0.0000
Intruder category	1	2.93	0.0870
Location treatment	1	0.20	0.6534
Intruder category X location treatment	1	3.09	0.0786
Species	2	41.79	0.0000
Intruder category X species	2	4.83	0.0892
Location treatment X species	2	0.80	0.6690
Intruder category X location treatment X species	2	6.66	0.0357
Length of familiarity	1	12.01	0.0005
Intruder category X length of familiarity	1	0.13	0.7196
Location treatment X length of familiarity	1	1.88	0.1698
Intruder category X location treatment X length of familiarity	1	2.33	0.1268
Species X length of familiarity	2	9.54	0.0085
Intruder category X species X length of familiarity	2	3.36	0.1859
Location treatment X species X length of familiarity	2	0.42	0.8098
Intruder category X location treatment X species X length of familiarity	2	3.38	0.1847

Table 2: *H. cruentata* model with chase duration as the dependent variable (multilevel mixed-effects linear regression). This analysis is based on 28 observations (trials), clustered in 7 groups (territory holders).

Variables	df	Chi ²	P>Chi ²
Model		32.88	0.0000
Intruder category	1	0.05	0.8231
Location treatment	1	0.79	0.3732
Intruder category X location treatment	1	15.14	0.0001
Length of familiarity	1	10.20	0.0014
Intruder category X length of familiarity	1	0.78	0.3776
Location treatment X length of familiarity	1	2.08	0.1492
Intruder category X location treatment X length of familiarity	1	0.07	0.7953

Table 3: *H. capitalis* model with chase duration as the dependent variable (multilevel mixed-effects linear regression). This analysis is based on 84 observations (trials), clustered in 21 groups (territory holders).

Variables	df	Chi ²	P>Chi ²
Model		4.30	0.7445
Intruder category	1	0.19	0.6652
Location treatment	1	0.67	0.4133
Intruder category X location treatment	1	0.30	0.5849
Length of familiarity	1	0.10	0.7493
Intruder category X length of familiarity	1	2.61	0.1060
Location treatment X length of familiarity	1	0.27	0.6012
Intruder category X location treatment X length of familiarity	1	0.01	0.9027

Table 4: *H. occisa* model with chase duration as the dependent variable (multilevel mixed-effects linear regression). This analysis is based on 28 observations (trials), clustered in 7 groups (territory holders).

Variables	df	Chi ²	P>Chi ²
Model		27.17	0.0003
Intruder category	1	7.31	0.0069
Location treatment	1	0.22	0.6390
Intruder category X location treatment	1	0.09	0.7620
Length of familiarity	1	12.86	0.0003
Intruder category X length of familiarity	1	1.28	0.2580
Location treatment X length of familiarity	1	0.68	0.4087
Intruder category X location treatment X length of familiarity	1	5.00	0.0253

Table 5: Summary table with the number of tests with physical attacks that territorial holders showed toward neighbors and strangers, and the total number of physical attacks to neighbors and strangers.

Species	Number of tests	Number of physical attacks Neighbors	Number of physical attacks Strangers
<i>Hetaerina cruentata</i>	8	12	11
<i>Hetaerina capitalis</i>	11	4	12
<i>Hetaerina occisa</i>	4	3	6

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