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TECHNICAL NOTE

An assessment of marking techniques for odonates in the family Calopterygidae

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Introduction

Marking effects (e.g., changes to behavior, survival, or reproduction after manipulation) are likely to be common, as researchers have found effects in a variety of taxa, including mammals (Moorhouse & Macdonald, 2005), birds (Burley et al., 1982; Hunt et al., 1997; Gauthier-Clerc et al., 2004), and amphibians (McCarthy & Parris, 2004). Prior to the advent of the felt-tipped marker, marking techniques for odonates ranged from India ink to various types of paint carefully applied to the wings or abdomen (Borror, 1934; Bick & Bick, 1961; Cordero Rivera & Stoks, 2008). In recent years, most odonate biologists mark individual odonates by writing a unique identification code with permanent marker on the wings (Cordero Rivera & Stoks, 2008). Despite the recommendation to carefully test marking techniques (Hagler & Jackson, 2001), few researchers describe more than a qualitative assessment of potential marking effects. Furthermore, few researchers have compared alternative marking techniques for odonates (but see Bennett & Mill, 1995).

Natural variation in the extent of odonate wing coloration may influence inter- and intra-sexual interactions (Tynkkynen et al., 2004; Anderson & Grether, 2010), as well as foraging success (Grether & Grey, 1996) and the likelihood of predation by avian predators (Svensson & Friberg, 2007). Prior studies in the calopterygid damselfly genus *Hetaerina* used markers to experimentally increase the extent of red (Grether, 1996a,b; Grether & Grey, 1996) and black (Anderson & Grether, 2010, 2011) wing coloration. These manipulations significantly influenced mating success (Grether, 1996a,b), foraging success (Grether & Grey, 1996), survival (Grether, 1997), and territorial aggression against conspecifics and, in some instances, against heterospecifics (Anderson & Grether, 2010, 2011). Therefore, using wing marking to identify individuals may be problematic for investigations of reproductive, territorial, and foraging behavior of these species and their relatives.

Herein, we describe an alternative, simple, and effective marking technique for calopterygid damselflies and evaluate potential effects on biology and behavior. Briefly, abdominal sections 3–6 are marked with unique combinations of paint marks. We report on the use of this marking technique from two calopterygid populations: a population of *Calopteryx haemorrhoidalis* (Vander Linden) and a population of *Hetaerina titia* (Drury) (both Odonata: Calopterygidae). For each population, we investigate whether specific colors painted on the abdomen affect the probability of resighting. Furthermore, in a subsequent study, the *C. haemorrhoidalis* population was marked using the common technique of writing a number on the wing with a marker. This allowed us to directly compare the two aforementioned odonate marking techniques. To investigate the effects of abdominal coloring on territorial aggression, we utilized an experimental approach to compare aggression toward manipulated and unmanipulated territorial intruders in the *H. titia* population.

Materials and methods

Localities and behavioral observations

Calopteryx haemorrhoidalis observations were carried out along a 200-m transect near a small stream near Pontevedra, Spain (42.406°N, 8.684°W) in May 1996. In the first

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investigation conducted from May 2 to 6, 80 *C. haemorrhoidalis* individuals were captured with aerial nets and held carefully by the wing while marked on the abdomen with a unique combination of four colored marks. Marked individuals were resighted for four subsequent days after marking. The four colored marks were a unique combination of four Humbrol Pinturas acrylic paint colors (white: #22, orange: #18, yellow: #69, and lime: #38; Humbrol LTD, Kingston-upon-Hull, UK). Paint was applied by immersing the tip of a sharpened pencil into the paint and gently applying a dot (ca. 1.2 mm diameter) upon consecutive abdominal segments. Colors were applied in a predetermined random order. Randomization was completed by listing all possible color combinations and then applying the RAND function in Microsoft Excel in an adjacent column. We then sorted both columns by the random column. In a subsequent investigation conducted from May 4 to 8 at the same site, 80 *C. haemorrhoidalis* individuals were marked using a white Sharpie Brand marker (Extra fine point; Sanford, Oak Brook, IL, USA). The left hindwing of each captured individual was marked with a unique three-digit numbered code selected randomly between 001 and 300. As with the first investigation, resighting continued for 4 days after marking.

Hetaerina titia observations were carried out in July–August 2010 along the Otapa River southeast of La Tinaja, Mexico (18.687°N, 96.394°W). Two study transects, (each 200 m) ca. 1 km apart were established along riverbanks. Damselflies were captured with aerial nets, photographed, and individually marked with a unique combination of four colored marks on the abdomen using paint pens (200-S Fine Point, Marvy Decocolor Paint Marker; Uchida of America, Torrance, CA, USA).The colored marks encircled abdominal segments 3–6 and typically were as thick as the marker tip (ca. 1.2 mm diameter). The four colored marks were randomly selected from a set of seven colors (white: #0, yellow: #5, orange: #7, light blue: #10, light green: #11, rosemarie: #59, and hot purple: #79. A predetermined random order was obtained as above. In total, 416 *H. titia* received unique colored marks.

During a behavioral census, we recorded each individual's perch location relative to the transect line to the nearest 0.1 m. A male was classified as a territory holder if it was consistently seen at the same location (+1.5 m) on at least two consecutive days. Following marking, the *H. titia* population was censused for eight consecutive days. All individuals, territorial or not, were used for the investigation of color effects on the probability of resighting. Individuals identified as territorial were subject to paired intrusions to investigate the effects of marking on territorial behavior (see below).

Territorial intrusion tests with *Hetaerina titia*

To determine whether abdominal marking per se (as opposed to any individual color) influenced territorial behavior, we carried out experimental territorial intrusions. During a territorial intrusion test, an experimental or control tethered male was presented using 0.3 m of fine transparent thread and a modified fishing pole to a territory holder. During the 2-min test, an observer recorded observations on a continuously running audio recorder. A total of 14 *H. titia* territory holders were tested consecutively against control and experimental tethered intruders within a 20–30 min window (so that, each territory holder was tested twice). Experimental individuals were given three colored marks on the abdomen, whereas control individuals received similar handling, but remained uncolored. During a tethering test, movement of the modified fishing pole ensured continuous flight from the tethered male. A minimum of 5 min elapsed between consecutive tests on a territorial male, and the order of treatments was varied systematically (trial order has not significantly influenced the degree of aggressive response in similar prior investigations; Anderson & Grether, 2010). Tethered males were obtained from stream sections adjacent to the study transect and used for at most two tests before release outside the transect. Following the tests, both control and experimental males were uniquely marked to prevent use in future tests and returned to their site of origin. All simulated intruder tests were carried out between 10:00 and 18:00 hours under sunny or lightly cloudy conditions (territory defense wanes under heavy cloud cover).

Statistical analysis

We used contingency table analyses (χ^2 tests) to compare the probability of resighting for individuals within a population marked with a specific color to the probability of resighting for all other individuals in the population (for a similar approach, see Hagan & Reed, 1988). A contingency table approach was also used to compare between marking methods (abdomen coloring vs. wing numbering). We used the Wilcoxon matched pairs signed-rank test to compare the response of territory holders (rate of aggressive attacks) to intruders with colored abdomens vs. unmanipulated intruders. Statistical analyses were conducted in Stata 10 (StataCorp, College Station, TX, USA).

Results

For *H. titia*, probabilities of resighting, given a specific color ranged from 46.4 to 51.6%. No single color significantly affected the probability of resighting (Table 1). In

Table 1 The probabilities of resighting for white, pink, orange, yellow, green, blue, and violet-colored *Hetaerina titia* damselflies

	n	Probability of resighting	P
White	198	0.510	0.84
Non-white	218	0.500	
Pink	183	0.464	0.15
Non-pink	233	0.536	
Orange	179	0.497	0.79
Non-orange	237	0.511	
Yellow	201	0.493	0.63
Non-yellow	215	0.516	
Green	192	0.516	0.68
Non-green	224	0.496	
Blue	178	0.500	0.87
Non-blue	238	0.508	
Violet	184	0.516	0.68
Non-violet	232	0.496	

Individuals with one or more abdominal mark(s) of the focal color are compared with all other individuals without the focal color; n, number of damselflies. P-values are from χ^2 contingency tests.

C. haemorrhoidalis, probabilities of resighting given a specific color ranged from 70.9 to 77.4%. As with *H. titia*, no color significantly affected the probability of resighting in the *C. haemorrhoidalis* study (Table 2). A significantly higher proportion of *C. haemorrhoidalis* was resighted in the census following abdomen marking (73.8%) than in the census following wing marking (55.0%; $\chi^2 = 6.13$, d.f. = 1, P = 0.013).

In the *H. titia* territory intrusion tests, the average rate of attack toward abdomen marked conspecific intruders was not significantly different from the rate of attack toward unmanipulated intruders (signed-rank test: z = 1.193, P = 0.23; n = 14).

Table 2 The probability of resighting for white, yellow, orange, and green-colored *Calopteryx haemorrhoidalis* damselflies

	n	Probability of resighting	P
White	53	0.774	0.30
Non-white	27	0.667	
Yellow	53	0.755	0.62
Non-yellow	27	0.704	
Orange	55	0.709	0.39
Non-orange	25	0.800	
Green	56	0.732	0.87
Non-green	24	0.750	

Individuals with one or more abdominal mark of the focal color are compared with all other individuals without the focal color; n, number of damselflies. P-values are from χ^2 contingency tests.

Discussion

Our results suggest that marking the abdomen with opaque paint is a viable alternative to wing numbering for behavioral investigations of calopterygid damselflies. Our results do not concur with prior claims that calopterygids show negligible effects when populations are uniquely marked by wing numbering (Cordero Rivera & Stoks, 2008). A direct comparison of these two techniques showed that abdominal painting was associated with higher resighting rates in the *C. haemorrhoidalis* population. We failed to detect any significant effect of any particular color on the probability of resighting – despite having robust sample sizes (*H. titia*, n = 417; *C. haemorrhoidalis*, n = 80).

Several reasons may account for why painting the abdomen appears to be associated with an increased resighting rate over marking the wings. First, wing marking may affect conspecific or interspecific aggression and thus affect territory occupancy time. Wing pigmentation is an indicator of fighting ability in several calopterygid species (Cordoba-Aguilar, 2002; reviewed by Suhonen et al., 2008) so that a change in the wing pigmented area may change other males' perception of the marked male's fighting ability. Second, wing marking may increase detection by predators, as natural variation in odonate wing color may affect predation (Svensson & Friberg, 2007). Abdomen painting could also affect how odonates are perceived by potential competitors and predators; however, and this needs to be investigated.

In the *H. titia* population, we compared rates of aggression by territory holders against tethered intruders with painted and unmanipulated abdomens. No significant differences were found in measurements of aggression against the two types of intruders. In contrast, prior studies using an identical tethering protocol found effects of wing spot size manipulations on the extent of territorial aggression elicited (Anderson & Grether, 2010). Abdominal painting appears to be a preferable method of individually marking *H. titia*, at least when the scope of the study includes an investigation of territorial behavior.

We conclude that painting abdomens of individual calopterygid damselflies with unique paint color combinations is a simple, effective method of individual identification. Abdominal painting yielded resighting proportions of close to 50% for *H. titia* damselflies, and close to 75% for *C. haemorrhoidalis* damselflies. One explanation for these different resighting rates is a difference in site fidelity

between the two species. Damselflies in the family Calopterygidae (also known as 'broad-winged damselflies') are among the most robust odonates in the suborder Zygoptera (Corbet, 1999). Rings of opaque paint may not be appropriate for damselflies in other families (e.g., Coenagrionidae), as the added weight to the abdomen may affect the efficacy of flight. Furthermore, while we did not find any effect of any individual color for calopterygids, researchers using the method for other odonates could consider whether the colors used should preferably be 'camouflage colors' (e.g., green, brown) instead of 'warning colors' (e.g., bright yellow, red). We concur with the recommendation of Hagler & Jackson (2001) to carefully test marking techniques prior to behavioral study.

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