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Melanic individuals in color polymorphic *Enallagma* damselflies result from phenotypic, not genetic, variation

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This paper is dedicated to Georg Ruppëll, in honor of his 75th birthday and with great appreciation for his dedication to capturing the stunning beauty of odonates on film.

Genetically determined color polymorphisms have a long history in the study of evolutionary change acting on populations. The Odonata exhibit relatively high levels of sex-specific color polymorphisms in mature adults. In *Ischnura* and *Coenagrion*, female-specific polymorphisms are known to be controlled by Mendelian genes. Nearly half of *Enallagma* species have polymorphic females, but the inheritance of any has yet to be determined. Our aims here were to determine: (1) the inheritance of the color polymorphism in *E. hageni*; and (2) inherent reproductive characteristics of blue female andromorphs and green heteromorphs reared under controlled conditions as tenerals. Maternal morphs, which developed normal coloration in field enclosures within a week, did not differ in copulation time or clutch size, and their offspring did not differ in sex ratio or survivorship to emergence. Surprisingly, no laboratory-reared offspring developed normal mature coloration. Rather, the initially pale parts of the thorax and abdomen, that normally would turn either blue or green, became melanized. Black novel phenotypes also developed in adults of *E. civile*, *E. anna*, *E. carunculatum*, and *E. annexum* that as larvae or teneral adults were reared to sexual maturity under greenhouse conditions that differed from the laboratory conditions used to rear *E. hageni*. We hypothesize that the phenotypic plasticity in body coloration documented in *Enallagma* results from the quality of UV radiation experienced as a sexually immature adult, which is known to affect melanization in other insects. These examples in *Enallagma* offer insights into the origin of color novelty in Odonata.

Keywords: Odonata; phenotypic novelty; larval rearing; color polymorphism; fitness characters

Introduction

Some of the best evidence for rapid evolution has come from quantifying population changes in genetic color polymorphisms (e.g. Linnen et al., 2013; Majerus, 1998; Roulin, 2004). Ford (1957) defined a genetic polymorphism as two or more distinct phenotypes that are genetically determined and coexist within a population at frequencies in which the least common morph occurs in numbers too large to result from recurrent mutation.

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Selection can act rapidly on a color morph if it is genetically determined, as found in the above examples of polymorphisms that occur in both sexes. Within the Odonata, color polymorphisms tend to occur only within a specific sex (e.g. Cordero & Andres, 1996; Fincke, Jödicke, Paulson, & Schultz, 2005). Color polymorphisms in male odonates are often associated with wing patterns and alternative mating strategies (e.g. Hooper, Tsubaki, & Siva-Jothy, 1999; Tsubaki, 2003), whereas those that occur only in females are typically associated with body coloration and pattern (but see Zhang and Cai, 2014). In non-territorial species, female color polymorphisms are thought to be a response to sexual conflict of interests, as a way for females to reduce male harassment (Johnson, 1975, reviewed by Van Gossum, Sherratt, & Cordero-Rivera, 2008). In female-specific polymorphism, the andromorph resembles the male in color (i.e. reflectance spectra), whereas other female phenotypes, called heteromorphs (Johnson, 1975) or alternatively, 'gynomorphs' (Cordero, 1990) differ from the males in coloration. Female-specific coloration polymorphisms are known to be genetically controlled at a single autosomal locus in six species of odonates: five *Ischnura* species (*I. elegans*, *I. graellsii*, *I. demorsa*, *I. damula*, and *I. senegalensis*) and *Ceriagrion tenellum* (Andres & Cordero, 1999; Cordero, 1990; Johnson, 1964a, 1966; Sanchez-Guillen, Van Gossum, & Cordero Rivera, 2005; Takahashi, Yoshimura, Morita, & Watanabe, 2010). Although 49% of *Enallagma*, the sister genus of *Ischnura*, exhibit female-specific color polymorphism, which is assumed to be genetically determined (e.g. Johnson, 1964b), that assumption has yet to be tested. Our aim here was to determine whether color variation of the two color morphs of *E. hageni* are under similar Mendelian inheritance as *Ischnura*. Our study species has been the focus of studies on reproductive fitness and sexual conflict of interest (e.g. Fincke, 1986; Fincke, Fargevieille, & Schultz, 2007; Schultz & Fincke, 2013; Xu & Fincke, 2011). Thus, because the determination of inheritance required rearing both morphs and their offspring under controlled conditions, we also asked if the two color morphs inherently differed in reproductive characteristics; specifically, copula time, number of newly hatched offspring (our surrogate of clutch size), and offspring survivorship.

We raised *Enallagma hageni* in the laboratory from controlled crosses of females reared to sexual maturity under controlled field conditions, and their offspring reared under controlled laboratory conditions. We found no inherent differences between the color morphs with respect to reproductive characteristics. To our surprise, however, offspring that survived to an age at which they would normally develop mature coloration in the field (i.e. blue andromorphic and green heteromorphic females), instead developed a novel black melanic phenotype. We obtained similar results from four other *Enallagma* species, even though the latter were reared under more favorable conditions in a greenhouse where they enjoyed adult survivorship comparable to that in the field. We propose that the excess melanization resulted from a lack of sufficient UV radiation in the laboratory and greenhouse, compared to field conditions. We discuss our findings in the context of phenotypic plasticity and the evolution of novel color morphs.

Material and methods

Color development in **E. hageni** *from controlled crosses*

Enallagma hageni (Coenagrionidae) is a female-specific color polymorphic species native to North America. In the field, newly emerged adults of both sexes are initially pale. The normally non-melanized areas of males quickly turn a pale blue, becoming a bright, saturated blue in about three to four days. By the end of the first day, the normally non-melanized areas of females turn tan on the thorax and abdomen and within a day or two, changing to pale blue in sexually immature females of both morphs. After 6–9 days, the pale blue of andromorphic

females becomes a saturated blue similar to that of the male, whereas the pale blue of hetero-morphic females becomes a saturated green or greenish brown (Fincke, 1982 and unpublished; Fincke et al., 2007). Both sexes have a black-striped thorax, but each female morph has a uni-formly black abdominal dorsum that is distinct from the male's blue-and-black striped abdominal dorsum (Xu, Cerreta, Schultz, & Fincke, 2014).

During July and August 2011, we performed controlled crosses among individuals from the *E. hageni* population of the Chase Osborn Preserve, Sugar Island, MI, USA (46°24′ N, 84°12′ W). We collected newly emerged females and reared them to sexual maturity in isolation from males in outdoor insectaries (1.8 m × 3.6 m × 1.8 m, Bioquip Inc., Rancho Dominguez, CA, USA). The insectaries were placed in forest light gaps and contained bushes and a water-filled plastic pool (1 m diameter × 0.1 m depth). We provided mosquitoes, moths and small flies as food and daily sprayed the insectaries with water to lower the air temperature and increase humidity.

Once females reached sexual maturity, noted by mature body coloration and thicker abdomens, we enclosed multiple virgin, individually marked females of single morph type in a mating cage (0.5 × 0.5 × 1 m) equipped with stems of emerging plants as perching sites. Two males that had been caught in tandem or copula and individually marked were released into each cage. We allowed a focal male to mate with each morph sequentially by moving a male to the appropriate cage after his first mating. Immediately after her first mating, each female was put individually in a plastic jar containing a piece of wet filter paper as an oviposition substrate, and was allowed to lay eggs for four days. The eggs in their water-filled containers were kept cool during transport to the laboratory in Oklahoma.

Once hatching of a clutch began, eggs were checked daily and newly hatched 'neonates' were transferred to rearing cups. We determined that the hatching of a clutch was completed when no more neonates appeared for three consecutive days, and all eggs were empty when checked under a dissecting microscope. Within an egg clutch, neonates that hatched on the same day were randomly assigned to groups of three, and each triad was put in a plastic, water-filled (120 ml) cup with strips of filter paper as perching sites. Only one larva ultimately survived per cup due to cannibalism (individual rearing of the > 5000 original neonates was not logistically feasible). Larvae were fed *Artemia* once daily and the water in the cups was changed every 2–4 weeks. To ensure a spring emergence, in December we transferred larvae to a room whose temperature was kept at 5°C. Feeding was reduced to once every 4–7 days. In mid-March, larvae were moved back to the 20°C laboratory, where feeding of brine shrimp and *Daphnia* to larger larvae was resumed until larval emergence.

Final instar larvae were moved to emergence tanks when they stopped feeding, an indication that they would soon emerge. Emergence enclosures were 19–38 liter glass aquariums with mesh tops, lined with fine window screening to provide perching sites for tenerals. A wooden dowel inserted into each cup served as an emergence site. Once its wings dried, a teneral adult was moved to a dry aquarium covered with clear cellophane on top and positioned adjacent to the laboratory windows. The tanks contained cups of water to increase humidity and aquatic plants or artificial ferns as perching sites. Teneral adults were fed *ad lib* fruit flies. Females shared a tank with up to four other females from the same clutch. We checked individuals daily, noting survivorship and color development.

Color development of other **Enallagma** *species*

We also noticed abnormal melanization exhibited by individuals from separate experiments that involved raising four additional *Enallagma* species in captivity: *E. civile*, *E. anna*, *E. carunculatum* and *E. annexum*. In June 2013, we obtained *E. civile* final instar larvae from an artificial outdoor pond at the Aquatic Research Facility (ARF) on the University of Oklahoma campus

and reared them in a greenhouse there. We kept the larvae in pond water in 500 ml containers that were kept in dark green mesh cages (30.5 cm^3, Bioquip, Inc.), and fed them one *Lumbriculus* worm per day until emergence. After emergence, adult tenerals were kept in the same cages in the greenhouse and fed *ad lib* fruit flies. Humidity in the cages was maintained above 50%.

In July and August 2013, we obtained eggs from newly mated females of *E. anna*, *E. carunculatum*, and *E. annexum* in Whitefish, MT, USA (48°24′ N, 114°18′ W); eggs were transported back to Oklahoma and reared in an ARF greenhouse. Larvae from each clutch were kept in communal 19-liter aquaria until they reached roughly 8 mm in length, at which point they were moved to individual cups. We fed them *ad lib*, a combination of *Artemia*, *Daphnia*, *Lumbriculus*, and chironomid larvae. After emergence, adult tenerals were kept in 30.5 cm^3 white mesh cages in the greenhouse and fed *ad lib* fruit flies. Humidity in the cages was maintained above 50%. Additionally, we caught teneral female *E. carunculatum* from the field in Montana, and kept them locally in a laboratory at the University of Montana's Flathead Lake Biological Station. Each female was housed individually in a small plastic cup with holes in the lid and a piece of moist filter paper to maintain humidity, and hand-fed *Drosophila* twice per day. The cups were kept near a window, and thus exposed to a natural photoperiod. Individuals that lived more than six days (sufficient to develop color beyond the teneral stage) were categorized either as having excess black or having normal pigmentation patterns.

Statistical analyses

We used a paired *t*-test to compare copulation duration of the andromorphs and heteromorphs that mated with the same focal male. Independent sample *t*-tests were used to compare neonate number, adult life span between the two maternal morphs, and a generalized linear model (GLM) with binomial error distribution was used to compare larval survivorship. To investigate whether larval survivorship between half-sib clutches were correlated, we excluded clutches sired by males that mated with two females of the same morph as well as the alternative morph. Including these half-sib cases by taking the mean survivorship of the clutches from the same morph mothers did not change the conclusion. We calculated larval survivorship in two ways. First, we calculated survivorship between egg hatch and successful emergence for 27 clutches for which we counted the number of neonates. Second, to exclude any effect of larval cannibalism, we also calculated larval survivorship between 30 January 2012, when cannibalism had reduced offspring to one per cup, and emergence. For the second calculation, we excluded clutches with fewer than three surviving larvae on 30 January. Because larval survivorship was not normally distributed, we used arcsine transformed survivorship for the *t*-tests. Throughout, means are given with standard errors unless noted otherwise.

Results

Inherent reproductive characteristics of female color morphs

In our outdoor insectaries in Michigan, virgin females developed mature coloration in 5.9 ± 0.9 days. For focal males that had copulated with females of both morphs, copulation duration did not differ between the two female morphs (andromorphs: 28.5 ± 1.8 min, heteromorphs: 29.5 ± 3.0 min, paired *t*-test, $t = 0.3$, df = 21, $p = 0.77$), nor did it differ between the first and the second copulation (first: 27.3 ± 2.5 min, second: 30.7 ± 2.4, paired *t*-test, $t = 1.17$, df = 21, $p = 0.25$). We obtained 53 egg clutches sired by a total of 32 males, of which 14 clutches had no
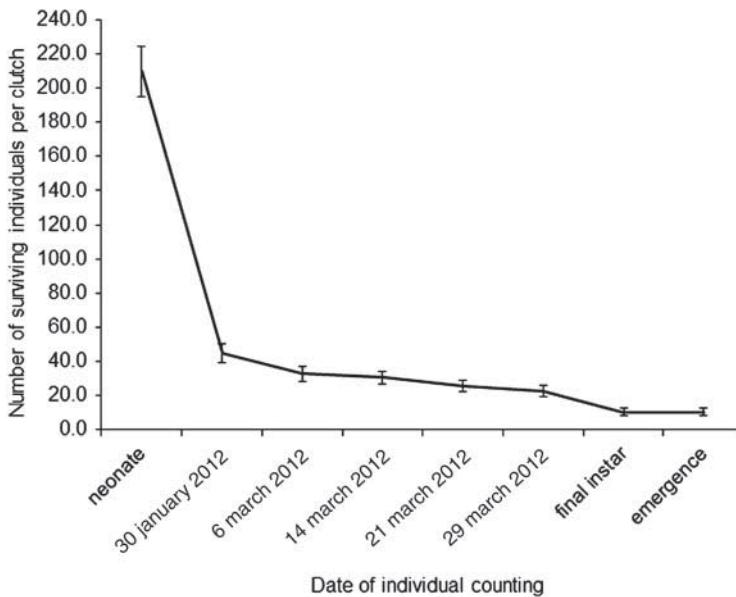
Figure 1. Mean number of *Enallagma hageni* individual neonates surviving to emergence, from the 27 clutches for which the number of neonates were counted in the controlled cross experiment. Error bars show standard error.

half-sib family, and 39 clutches comprised half-sib families from mothers of each color morph with the same sire.

Eggs began hatching on average 17.8 ± 0.6 days after being laid (range 14–29 days). Excluding one outlier clutch that hatched after 29 days, hatch time did not differ between maternal morphs (heteromorph: 17.3 ± 0.2, andromorph: 17.2 ± 0.5, $t = 0.34$, df $= 20$, $p = 0.74$). The average number of neonates was 209.7 ± 14.9 per clutch ($n = 27$ clutches). Neonate number did not differ between heteromorphic (212.8 ± 23.3) and andromorphic mothers (206.4 ± 19.0; $t = 0.21$, df $= 25$, $p = 0.83$).

Survivorship of morph offspring and family effects on survivorship

Nine of the 43 clutches had no offspring surviving to emergence. For the 27 clutches for which we counted the number of neonates, larval survivorship between egg hatch and successful emergence was $4.9\% \pm 1.0\%$ (median $= 3.6\%$, interquartile range $= 4.3\%$, Figure 1). This survivorship did not differ between offspring of andromorphs (median $= 4.0\%$, interquartile range $= 6.4\%$) and heteromorphs (median $= 3.3\%$, interquartile range $= 3.4\%$, GLM, $\chi^2_1 = 1.29$, $p = 0.26$, Figure 2). The survivorship between 30 January (after cannibalism had reduced offspring to one per cup) and emergence was $25.7\% \pm 3.9\%$ (median $= 18.8\%$, interquartile range $= 31.9\%$, $n = 41$). Among half-sib clutches with one andromorph mother and one heteromorph mother (and same sire), survivorship from hatching to emergence was not correlated ($r = 0.46$, $n = 10$ families, $p = 0.18$); nor was their survivorship between 30 January and emergence correlated ($r = 0.58$, $n = 10$, $p = 0.08$).

Adults began emerging 22 April 2012, 249 days after the first egg hatch. A total of 318 adults emerged, of which 86 (27%) failed to exit from the exuvia. The sex ratio was 1.1 ± 0.2 (male/female, $n = 14$ clutches), excluding clutches with fewer than three adults of either sex that had survived to the stage that we could distinguish sex. The number of adult male and female offspring did not differ (male: 7.9 ± 1.3, female: 8.0 ± 1.4, paired *t*-test, $t = 0.08$, df $= 13$, $p = 0.94$), suggesting a 1:1 sex ratio (male:female).
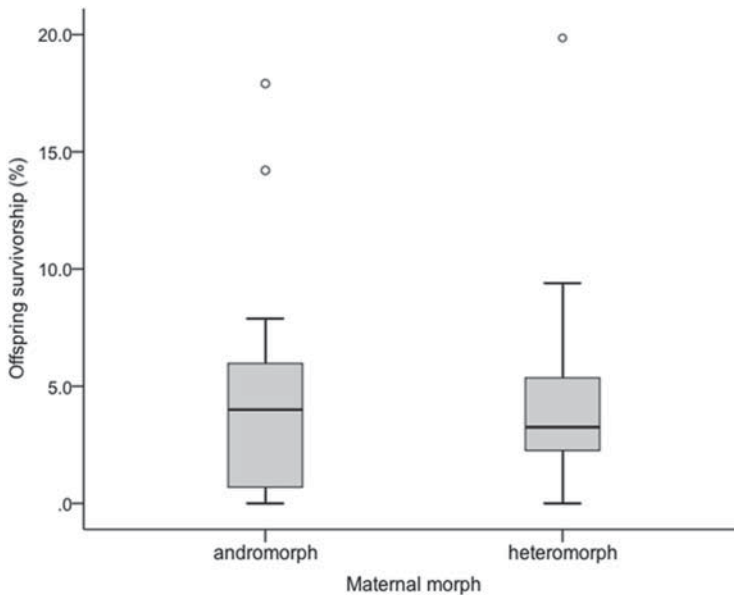
Figure 2.   Survivorship from the neonate stage to emergence of *Enallagma hageni* offspring of andromorphic and heteromorphic mothers from 27 clutches for which the number of neonates were counted.

Mean adult lifespan from 30 clutches was 2.8 ± 0.3 days ($n = 203$). Females lived 2.9 ± 0.5 days ($n = 107$); males lived for 2.2 ± 0.3 days ($n = 96$). Excluding one outlier clutch in which the only surviving female lived for 14 days, male and female offspring lifespan did not differ (paired *t*-test, $t = 1.01$, df $= 28$, $p = 0.32$). The average lifespan did not differ significantly between color morph of mothers (andromorph: 2.3 ± 0.3 days, heteromorph: 3.0 ± 0.3 days, $t = 1.38$, df $= 27$, $p = 0.18$).

Color development in **E. hageni** *offspring*

In the laboratory, all newly emerged tenerals were light tan. Among 188 adults that lived for at least one day, 62 individuals (33%) changed color. Forty-four males developed a pale blue thorax coloration, typical of older tenerals, in 2.3 ± 0.2 days. No male developed full coloration as seen in the field; at best, the thorax and/or abdomen turned a pale blue with a darker, but not completely saturated, blue tip. None of the males turned black. Eighteen females, which lived an average of 7.4 ± 0.7 days, exhibited some color change, with the tan parts turning pale blue after 4.8 ± 0.6 days. In nine of the 18 females, between days 3 and 10 post-emergence (mean $= 6.1 \pm 0.7$ days), melanization occurred in the normally green or blue parts of the thorax and sides of the abdomen (similar to the *E. carunculatum* females in Figure 3d). This was in addition to the normal black stripes on the thorax and the black abdominal dorsum. Six of the black offspring were from four andromorph clutches, and three came from two heteromorph clutches. The black melanization developed in otherwise normal, active females whose wings and bodies were fully sclerotized.

Abnormal color development in other **Enallagma** *species*

As shown in Table 1, under our greenhouse rearing conditions, many male and female *E. civile*, *E. carunculatum*, *E. anna*, and female *E. annexum* developed melanization in excess of that
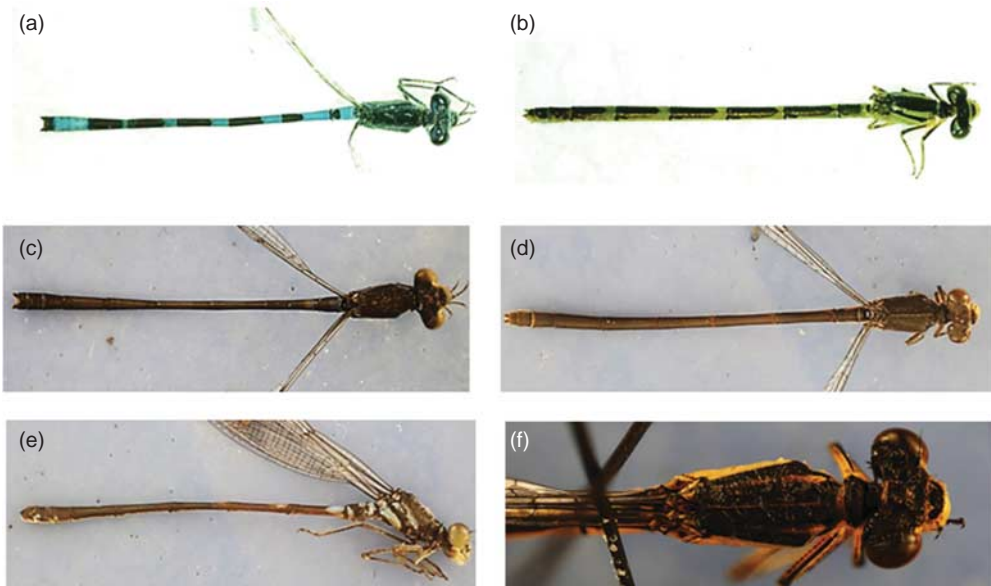
Figure 3. Color development in captive-reared *Enallagma carunculatum:* (a) normal color development in male; (b) normal color development in green female, showing normal black pattern; (c) melanized male, 16 days post-emergence; (d) melanized female, 17 days post-emergence; (e) partially melanized male, in which small areas of blue were visible only on anterior and posterior abdomen, 22 days post-emergence; (f) female thorax showing gradual darkening of antehumeral stripes, 10 days post-emergence.

Table 1. Individual *Enallagma* reared from eggs that survived > 6 days as adults (i.e. long enough to begin to develop adult color under normal conditions).

Species	Sex	Melanized	Normal	Proportion melanized	Proportion early[a]
E. anna	F	41	8	0.84	0.02
E. anna	M	31	38	0.45	0.03
E. carunculatum	F	25	10	0.71	0.26
E. carunculatum	M	16	7	0.70	0.19
E. annexum	F	3	0	1.00	0
E. annexum	M	0	1	0.00	0

[a]"Proportion early" refers to larvae that emerged before 1 April 2014 under shorter photoperiods.

which normally develops under natural conditions (e.g. Figure 3a, b). The normally blue or green areas turned black, similar to the melanization that occurred in *E. hageni* females. The extent of black increased over time for any given individual. In both sexes, the excess melanization tended to first obscure the posterior portion of the antehumeral stripes so that they ended in points mid-thorax (Figure 3f). Within a few days, the black coloration typically extended anteriorly until the pale shoulder stripes were no longer visible. The eyespots and entire dorsal abdomen was often blackened at the anterior portion of each segment (Figure 3c, d, f), a region that is typically pale. On males, S7, which is normally blue at the anterior end (Figure 3a), was commonly entirely black (Figure 3e).

For field-caught teneral *E. carunculatum* females for which time of melanization was noted ($n = 11$), females began to blacken at 6.5 ± 0.5 days. Eventually, 68% of the total females ($n = 22$) were melanized. *E. carunculatum* and *E. anna* adults reared from eggs showed even higher frequency of melanization. Of adults that survived more than six days, 70% of the 23 *E. carunculatum* males (Figure 3c) and 71% of the 35 females were melanized (Figure 3d),

compared with 45% of 69 *E. anna* males and 84% of the 49 *E. anna* females. *E. carunculatum* and *E. anna* males readily took black, conspecific females in tandem during mating trials, and black females readily accepted black males as mates.

Males without excess melanization developed the normal blue coloration and pattern (Figure 3a). However, most unmelanized *E. anna* and *E. carunculatum* females failed to develop any blue or green color on the lateral thorax or dorsal abdomen, and remained light brown in these areas, a color characteristic of 1–3-day-old teneral *Enallagma* females. Of adults surviving more than six days, only one of the 35 *E. carunculatum* females (3%), and eight of the 49 *E. anna* females (16%) developed the natural blue or green color. The frequency of melanization was lower for males that emerged later in the spring. Of the above males, 71% that emerged between 3 February and 31 March 2014 turned black, compared with only 45% of those that emerged between 1 April and 4 June 2014.

Additionally, from the single *E. annexum* clutch reared at the same time, a single male survived to maturity and developed normal coloration, but all three females were melanized. Finally, several female *E. civile* caught in the field as tenerals and raised in a greenhouse became melanized, although the frequency of melanization was not recorded. One additional teneral female *E. civile* was collected from the field in 2011; her wings were clipped and she was kept in a plastic vial used for rearing *Drosophila.* She survived in the vial for 17 days and became completely black.

Discussion

A unique result of our work was the finding that laboratory-reared female *Enallagma hageni*, *E. civile*, *E. carunculatum*, *E. anna*, and *E. annexum* all developed unnatural, excess melanization of body areas normally colored blue or green. The result was a black phenotype that, to our knowledge, has not been observed in *Enallagma* under natural conditions. Our results suggest that excess melanization is a phenotypically plastic trait (see also Michie, Mallard, Majerus, & Jiggins, 2010). The nine totally black female *E. hageni* from the inheritance experiment were offspring of both andromorphic and heteromorphic mothers. Melanization also occurred in male *E. carunculatum*, *E. anna* and *E. civile*, suggesting a plastic change in epidermal control over the formation of color pattern. Natural phenotypic plasticity is known to result in color polymorphism, such as brown migratory and green non-migratory phases of locusts (Pener & Yerushalmi, 1998). Nevertheless, despite extensive field research on multiple *Enallagma* species over the past 10 years, no melanized individuals were noted by O. M. Fincke or T. D. Schultz (personal communications). Females develop either blue or green coloration (presumed to be genetically determined) within a week or so of emergence, and coloration of marked individuals does not change from the mature, saturated hue over an individual's life (Fincke, 1982). Similarly, in his extensive field work with *Ischnura*, A. Cordero has noted only a single, naturally melanic individual (personal communication), a female *I. graellsii* with very dark violet coloration (Cordero, 1992).

Our second major result was that we did not find any inherent reproductive characters that differed between the female color morphs under controlled conditions. Copulation duration, and number of neonates did not differ significantly between blue and green mothers in our study. Nor did offspring survivorship or adult offspring lifespan differ. Our results were consistent with the conclusion that any differences found between these variables in the field are unlikely the result of morph-specific, genetic effects. Although we did not measure egg size of morphs, we found no difference in fecundity between morphs, consistent with findings for *Nehalennia irene* (Iserbyt, Bots, Van Gossum, & Sherratt, 2013) but in contrast with differences in egg size and number found in *Ischnura senegalensis* (Takahashi & Watanabe, 2010).

A major challenge in determining the inheritance of color polymorphic female *Enallagma* is to understand how to prevent abnormal melanization in a controlled environment. In our inheritance experiment, melanization made it impossible for us to determine the color of offspring that survived sufficiently long for normal color development under natural conditions. The high mortality of *E. hageni* larvae in our inheritance experiment could have been reduced by not limiting the prey to *Artemia*. When larvae were fed a variety of prey items, including *Lumbriculus* worms for larger instars, survivorship for *E. anna* and *E. carunculatum* reared in the greenhouse was relatively high and cases of failed emergence were rare (A. Barnard, personal observation), similar to that found in other studies when larvae were provided a variety of size-appropriate aquatic prey (e.g. Johansson, Stoks, Rowe, & De Block, 2001; Van Gossum, Sanchez, & Rivera, 2003).

Past problems of color development and melanization in laboratory-reared damselflies likely have a common cause. Cordero, Santolamazza-Carbone, and Utzeri (1995) found that female *Coenagrion scitulum* failed to develop blue coloration in the laboratory. In addition to our study, melanization of laboratory-reared males and females was also found in *Enallagma cyathigerum*, *Pyrrhosoma nymphula*, *Coenagrion scitulum*, and *Ischnura genei* (A. Cordero, personal communication). Inheritance was successfully determined for morph color in *Ischnura graellsii*, *I. demorsa*, *I. damula*, *I. senegalensis*, and *Ceriagrion tenellum* (Andres & Cordero, 1999; Cordero, 1990; Johnson, 1964a, 1966; Takahashi et al., 2010), in which laboratory-reared individuals developed coloration normally, without reports of any melanization (see also Takahashi & Watanabe, 2010). However, in those species, females exhibit a teneral color morph which could be determined within a few hours of emergence, well before melanization began in the *Enallagma* species we studied here.

Blue coloration of mature Odonates is produced by nanospheres within the endoplasmic reticulum of box-shaped subcuticular epidermal cells (Prum, Cole, & Torres, 2004). The arrangement of the nanospheres within the cells determines the body coloration. Blue coloration is produced when nanospheres are distal within the cell. Developing chromatophores in tenerals are irregular in size and shape and are distributed throughout the cytoplasm instead of distally (Charles & Robinson, 1981; Vernon, O'Farrell, & Dixon, 1974). Whereas disruption of this process might lead to unnatural coloration, it fails to explain the abnormal production of excess melanin.

The production and deposition of melanin primarily occurs during the young adult stage in insects. Several genes contribute to melanogensis, and in *Drosophila melanogaster*, the over-expression of certain genes causes ectopic melanin deposits that alter the coloration and patterning of individuals (True, Edwards, Yamamoto, & Carroll, 1999; Wittkopp, Vaccaro, & Carroll, 2002). Environmental variables that may affect the expression of genes involved in melanin production in insects include diet, temperature, and stress caused by parasites (e.g. Koch, Hasson, & Soto, 2012; Lee, Simpson, & Wilson, 2008; Schlein, 1976; Siva-Jothy, Tsubaki, Hooper, & Plaistow, 2001). Comparison of our results from the laboratory and outdoor insectaries suggested that none of these variables are implicated in the black phenotypes we found in our study. First, field-reared larvae that were brought into the laboratory and greenhouse as teneral adults also developed the black phenotype, suggesting that larval diet was not the cause of the melanization. Second, despite the fact that tenerals (not reared from eggs) in outdoor insectaries and tenerals in the laboratory (some reared from eggs and others not) were fed similar prey (i.e. mosquitoes and small dipterans versus *Drosophila*, respectively), all newly emerged teneral *E. hageni* females kept in outdoor insectaries under ambient temperature developed natural mature coloration. In contrast, laboratory-reared females and adults of both sexes reared in the greenhouse developed the black phenotype. Third, although stress to the immune system can result in melanin deposition in the cuticle (e.g. Abro, 1982; Nappi & Vass, 1993; Siva-Jothy et al., 2001), such melanization is localized (Schnitger, Kafatos, & Osta, 2007), rather than covering most of the body as occurred in our black individuals (Figure 3). Furthermore, teneral

females of *E. anna* and *E. carunculatum* reared in the greenhouse developed abnormal melanin patterns despite eating well, producing normal egg clutches, and surviving as long or longer than *Enallagma* females under natural conditions.

Light quality is one variable that differed in the treatment of adults in our study and corresponded to the differences we found in mature adult phenotypes. Immature females in our field cages on Sugar Island enjoyed close to natural illumination (for spectra, see Xu & Fincke, 2011), whereas the light in the laboratory and greenhouse was transmitted through glass and plastic, respectively. Ultraviolet radiation (UV), which comprises wavelengths of 100–400 nm, has long been known to affect melanization in organisms (e.g. Pathak, Riley, & Fitzpatrick, 1962; Schlein, 1976; reviewed in Lynch & Livington, 2001). For example, when Schlein (1976) exposed newly eclosed *Sarcophaga* flies to long wave, UV-A radiation (315–400 nm), melanization of the naturally black abdomen was suppressed, producing abnormally pale flies. Glass and plastic transmit only a portion of ambient solar UV radiation (Krizek, Clark, & Mirecki, 2005).

If *Enallagma* requires a certain amount of UV-A radiation during teneral maturation to suppress melanization of the normally blue or green parts of the thorax and abdomen, then partial blocking of UV-A by glass or plastic may be sufficient to result in the phenotypic differences in melanization that we found between adults that matured in field cages and those that matured in the laboratory or greenhouse (i.e. reared as larvae or only tenerals). Additional, indirect support for our 'UV hypothesis' comes from the fact that male *E. carunculatum* and *E. anna* exhibited lower frequency of the black phenotype if they emerged later in the year, when the longer days would provide more exposure to the UV radiation that was transmitted through the plastic covered greenhouse. Unnatural photoperiod is unlikely to explain our results because even *E. civile* tenerals that developed and emerged in the field under their natural photoperiod melanized after being brought into the laboratory.

However, we noted that the majority of *E. carunculatum* emerged before 1 April (i.e. developing under day lengths different from those of their natural population), whereas *E. anna* typically emerged later than that, under photoperiods more similar to those of their natural population. *E. carunculatum* may also be more susceptible to abnormal melanization than *E. anna*. Our UV hypothesis could be tested directly by the addition of ambient UV lights to the adult rearing environment in a laboratory or greenhouse.

The novel, melanized black phenotype we discovered is one that males have never encountered. To our knowledge, there are no entirely black *Enallagma* species. Nevertheless, male *E. carunculatum* and *E. anna* readily took melanized conspecific females in tandem during mating trials. Females accepted melanized male conspecifics as mates, although preference by either sex for melanized versus normal mates was not tested. This lack of mating bias against a novel, biologically produced phenotype is consistent with results generated from earlier field tests with novel pink and orange phenotypes that were produced by painting males and females with acrylic paint (Xu et al., 2014). That earlier study identified a male decision rule by which males recognize non-blue individuals as potential mates. Our current results offer a way to produce 'naturally novel' phenotypes, which can be used in studies of sexual conflict under otherwise natural conditions. Additionally, such novel phenotypes offer the opportunity to investigate the interaction of genes and environmental effects on gene expression in *Enallagma* and *Ischnura*, two non-model genera whose genomes are currently being sequenced.

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