Quantitative variation in female sensory structures supports species recognition and intraspecific mate choice functions in damselflies

Alexandra A. Barnard[1,2] and John P. Masly[1]

[1]Ecology & Evolutionary Biology Program, Department of Biology, University of Oklahoma, Norman, OK, USA
[2]Email: alex.barnard@ou.edu

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Abstract

Males and females exchange signals prior to mating that convey information such as sex, species identity, or individual condition. Tactile signals relayed during physical contact between males and females before and during mating appear to be important for mate choice and reproductive isolation in some animals. However, compared to our understanding of visual, auditory, and chemical signals, we know little about the importance of tactile signals in mating decisions. Among North American damselflies in the genus *Enallagma* (Odonata: Coenagrionidae) species-specific tactile stimulation contributes to reproductive isolation between species and may also be important for intraspecific mate choice. We quantified several mechanosensory sensilla phenotypes on the female thorax among multiple sympatric and allopatric populations of two

Enallagma species that occasionally interbreed in nature. Although each species differed in features of sensilla distribution within the thoracic plates, we found no strong evidence of reproductive character displacement among the sensilla traits we measured in regions of sympatry. However, substantial variation of sensilla traits was observed within populations of both species. Our results suggest that species-specific placement of female mechanoreceptors appears sufficient for species recognition, but mechanosensor variation among females within species may be important for mate choice.

For sexual species, maintenance of species boundaries relies on reproductive isolation (RI) between recently diverged species (Mayr 1942). Premating reproductive isolating barriers, including behavioral or sexual isolation, often evolve earlier in the speciation process than postmating barriers in a variety of animal taxa (*e.g.*, McMillan et al. 1997; Price and Bouvier 2002; Mendelson and Wallis 2003; Dopman et al. 2010; Sánchez-Guillén et al. 2012; Williams and Mendelson 2014; Castillo et al. 2015; Barnard et al. 2017). Behavioral isolation requires that mate recognition signals and/or preferences diverge between populations, which ultimately results in the ability for individuals to discriminate conspecifics from heterospecifics. Species recognition signals may rely on a variety of sensory modalities such as color (Wiernasz and Kingsolver 1992; Sætre et al. 1997; Jiggins et al. 2001; Boughman et al. 2005; Kronforst et al. 2006; Williams and Mendelson 2014), courtship behavior (Stratton and Uetz 1986), sound/vibration (Ewing and Bennet-Clark 1968; Wells and Henry 1998; Shaw 2000; Gerhardt and Huber 2002; Arthur et al. 2013), and volatile chemicals (Coyne et al. 1994; Noor and Coyne 1996; Trabalon et al. 1997; Rafferty and Boughman 2006). Often, multiple signals act in concert to affect species recognition (*e.g.*, Costanzo and Monteiro 2007; Girard et al. 2015).

Although much is known about the importance of visual, chemical, and auditory signals and responses in sexual communication and species recognition, we know relatively little about other sensory modalities that may have strong effects on individual mating decisions. Tactile signals have been hypothesized as a likely contributor to mating decisions (Mendelson and Shaw 2012), but it is unclear whether touch could represent a primary species recognition signal, given that visual and auditory cues usually act earlier during the mating sequence. Research on the prevalence of tactile signals in mating decisions is limited (Coleman 2008) because of the experimental challenge it poses: whereas other sensory modalities present male signals to a focal

59 female from a distance, studying female preference for tactile cues requires contact between
60 males and females, which is not always easily achieved or quantified under controlled
61 conditions.

62 Despite this challenge, the role of tactile signals along the continuum between
63 intraspecific mate choice and interspecific RI deserves attention because it broadens our
64 understanding of the causes and consequences of a common pattern in nature— the rapid
65 divergence of male genital morphology between species. It has been suggested that rapid genital
66 differentiation can cause RI (Dufour 1844), although mechanical incompatibilities between
67 heterospecific male and female genitalia do not appear to be a common cause of RI (Shapiro and
68 Porter 1989; Masly 2012; Simmons 2014). However, observations both within (Eberhard 1994;
69 Edvardsson and Göran 2000; Briceño and Eberhard 2009a; Briceño and Eberhard 2009b; Frazee
70 and Masly 2015) and between species (Patterson and Thaeler Jr 1982; Robertson and Paterson
71 1982; Eberhard 1992; Coyne 1993) suggest that male genitalia may convey tactile information to
72 females that affects their subsequent behavior and/or physiology. Although female genital
73 structures often appear invariant among closely related species (Shapiro and Porter 1989), subtle
74 morphological differences (*e.g.*, Kamimura and Mitsumoto 2011; Yassin and Orgogozo 2013)
75 could enable females to detect variation among males' genital morphology. This variation could
76 occur in signal processing at the level of neurons and neural networks and/or in the distribution
77 and morphology of sensory structures that receive male tactile signals.

78 Female sensory structures that reside in body regions that contact species-specific male
79 structures during mating have been documented in several arthropods, including flies (Eberhard
80 2001; Ingram et al. 2008) and damselflies (Cordoba-Aguilar 2005; Robertson and Paterson
81 1982). Other studies have demonstrated that tactile cues from male grasping organs influence

female mating responses, either via experimental manipulation of male structures and/or desensitization of females (Eberhard 2002; Briceño et al. 2007; Briceño and Eberhard 2009a; Eberhard 2010; Myers et al. 2016) or comparison of female behavior when grasped by males with varying genital morphologies (Sánchez-Guillén et al. 2012; Sánchez-Guillén et al. 2014; Barnard et al. 2017). Premating tactile isolation may also be important in vision-limited vertebrates. For example, contact cues via the lateral line system may influence female mate choice in a cavefish (Plath et al. 2004; but see Rüschenbaum and Schlupp 2013).

Tactile signals appear to be a significant cause of RI in Zygoptera, the damselfly suborder of Odonata (Krieger and Krieger-Loibl 1958; Loibl 1958; Robertson and Paterson 1982; Corbet 1999). Concentrations of cuticular mechanoreceptors (sensilla) on the female thorax have been described in several coenagrionid damselfly genera. These sensilla reside in areas where male grasping appendages contact the female thorax before and during mating, which has led to speculation that they allow females to evaluate male morphologies and identify conspecifics (Jurzitza 1974, 1975; Tennessen 1975; Robertson and Paterson 1982; Battin 1993a,b). Each mechanoreceptor is associated with a single sensory neuron (McIver 1975; Kiel 1997). The thoracic sensilla thus represent a spatial matrix that can transmit signals to the female central nervous system based on the pattern in which the sensilla are stimulated. Greater numbers of these receptors enhance a female's sensory resolution by increasing the combinatorial complexity of tactile signals that a female can perceive. For example, if a female possesses 25 sensilla, and each sensillum has two response states ("on" if contacted and "off" if not contacted), then the number of unique tactile patterns that the female could distinguish is $2^{25} = 3.4 \times 10^7$. A female that possesses just one additional sensillum would be able to distinguish among roughly twice as many patterns ($2^{26} = 6.7 \times 10^7$). Should individual sensilla respond to

105 quantitative variation in touch (rather than a binary response), this would dramatically increase
106 the number of response states and therefore further enhance tactile acuity (Gaffin and Brayfield
107 2017). Female damselfly thoracic sensilla thus present an external, quantifiable phenotype to
108 investigate the mechanistic basis of tactile stimuli and female mating decisions.

109 The North American damselfly genus *Enallagma* includes several recently diverged
110 species that often co-occur in the same habitats (Johnson and Crowley 1980; McPeek 1998), and
111 do not engage in premating courtship (Fincke et al. 2007; Barnard et al. 2017) or use chemical
112 cues for mate selection (Rebora et al. 2018). A female's first opportunity to assess a potential
113 mate occurs when the male uses his terminal appendages to grasp the mesostigmal plates on the
114 female's thorax to form tandem, the premating position. The males' superior appendages (cerci)
115 have species-specific morphologies, and differences in genital morphology are the primary cause
116 of RI in this genus (Paulson 1974; Barnard et al. 2017). Two species, *E. anna* and *E.*
117 *carunculatum*, occasionally hybridize in nature to produce males and females with morphologies
118 that are intermediate to each of the pure species (Donnelly 2008; Johnson 2009; Barnard et al.
119 2017). Females of both pure species discriminate strongly against both heterospecific and
120 interspecific hybrid males that take them in tandem, which shows that not only can *E. anna* and
121 *E. carunculatum* females detect large differences in male cercus morphologies, but also more
122 subtle differences such as those between conspecific and hybrid males (Barnard et al. 2017).

123 Because it appears that mesostigmal sensilla are used to mediate species recognition, they
124 might be expected to show signs of reproductive character displacement (RCD) in regions where
125 species co-occur (Brown and Wilson 1956; Howard 1993; Pfennig and Pfennig 2009). RCD can
126 evolve via direct selection on adult prezygotic phenotypes or via reinforcement, in which direct
127 selection against interspecific hybrids gives rise to selection for enhanced premating isolation

128 between species (Dobzhansky 1937). *Enallagma anna* and *E. carunculatum* can interbreed, but
129 these species produce hybrids with significantly reduced fitness (Barnard et al. 2017). This
130 suggests that species-specific sensilla phenotypes might show patterns consistent with RCD in
131 regions of sympatry, where females are known to experience frequent mating attempts from
132 heterospecific males (Paulson 1974; Fincke et al. 2007; Barnard et al. 2017). Here, we test the
133 hypothesis that variation in female sensilla phenotypes supports a function in species
134 recognition. We test this hypothesis by quantifying sensilla number, density, and location
135 phenotypes on the mesostigmal plates of a large set of *E. anna* and *E. carunculatum* females
136 from multiple populations across the western United States and comparing phenotypes of each
137 pure species from sympatric and allopatric populations to identify patterns consistent with RCD.
138

139 **Methods**

140 **Population sampling**

141 We measured the sensilla traits of 29 *E. anna* females across 13 populations, and 74 *E.*
142 *carunculatum* females across 19 populations (Figure 1, Table 1). We classified each population
143 as either allopatric, locally allopatric, or sympatric. Sympatric populations are those where *E.*
144 *anna* and *E. carunculatum* co-occur temporally as well as spatially. Because *E. anna*'s
145 geographic range falls completely within *E. carunculatum*'s range, only *E. carunculatum* has
146 completely allopatric populations. We designated populations that exist at sites within the area
147 of range overlap, but where only one species is known to occur, as locally allopatric. Some
148 specimens were collected as early as 1945, but the majority of samples (82 of 103) we studied
149 were collected between 2012 and 2016.
150

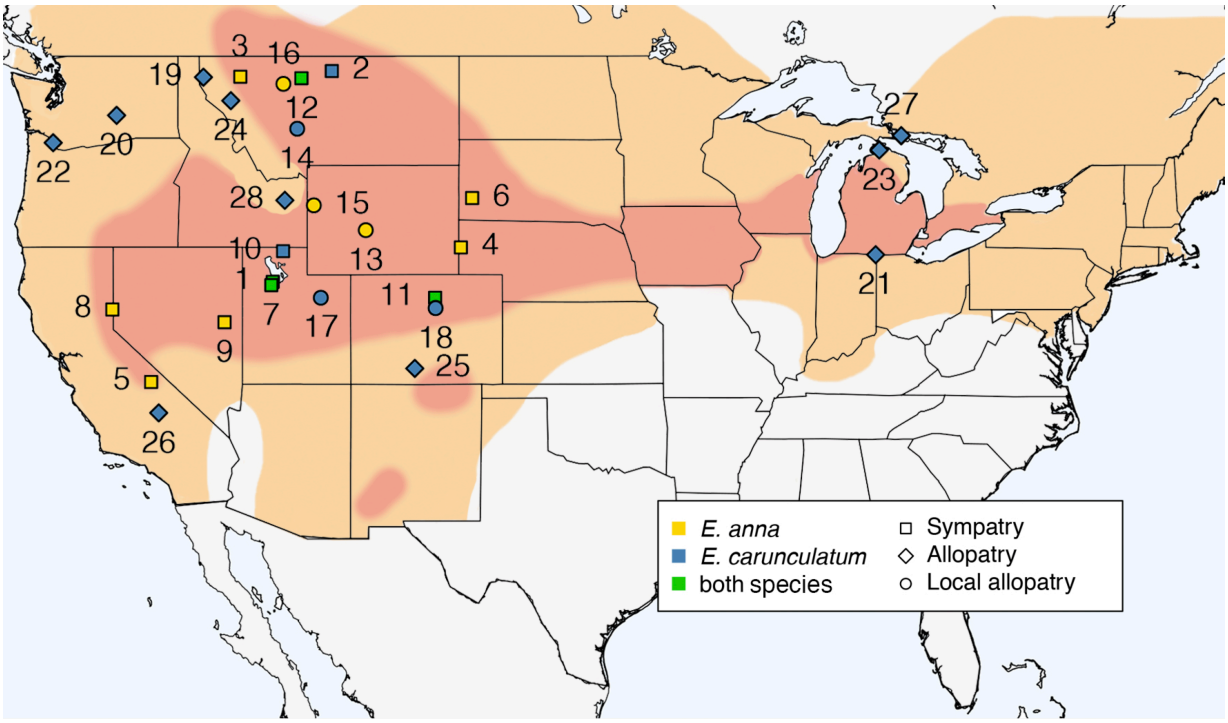

Figure 1. Sampling sites and species ranges. *Enallagma anna*'s geographic range (red) occurs within *E. carunculatum*'s geographic range (orange). Names of sites associated with each number are described in Table 1. Symbol color indicates the species sampled and symbol shape indicates the population type. (Species ranges adapted from Johnson 2009; Paulson 2009, 2011).

Trait imaging and quantification

We photographed each damselfly using a Nikon D5100 camera (16.2 MP; Nikon Corporation, Tokyo, Japan). We dissected the ventral thoracic cuticle from each female using forceps and imaged the mesostigmal plates using scanning electron microscopy. Specimens were mounted on aluminum stubs with carbon tape, sputter-coated with gold-palladium, and imaged at 200X magnification and 3kV using a Zeiss NEON scanning electron microscope.

To avoid any potential bias during measurements, we blind-coded image files before measuring traits. We measured abdomen length (abdominal segments 1-10, excluding terminal appendages) on the full-body photos as a proxy for body size using the segmented line tool in ImageJ (Abramoff et al. 2004). We quantified sensilla traits on the right mesostigmal plate of

each female damselfly unless the right plate was dirty or damaged, in which case we quantified the left plate. Sensilla counts on a subset of 57 females showed that left plate and right plate sensilla counts are highly correlated ($r = 0.85$). In cases where we quantified the left plate, we flipped the image horizontally, so it was in the same orientation as a right plate. We standardized the position of the mesostigmal plate in each image by cropping and rotating so that the lower medial corner of the plate was in line with the lower left corner of each image. We counted sensilla and obtained their *x* and *y* coordinates in ImageJ using the multi-point selection tool. We traced an outline around the plate image, excluding the lateral carina (Figure S1), using a Wacom Cintiq 12WX tablet and stylus (Wacom, Saitama, Japan) and the freehand selection tool in ImageJ. This procedure provided *x* and *y* coordinates that describe the plate outline. We performed all measurements twice for each specimen. Measurements across the two technical replicates were highly correlated ($r_{\text{abdomen}} = 0.96$, n = 155; $r_{\text{count}} = 0.97$, n = 183; $r_{\text{plate area}} = 0.98$, n = 157), so we used the mean trait values of the two replicates in subsequent analyses.

Sensilla trait analyses

We conducted all morphometric and statistical analyses using R v. 3.4.1 (R Core Team 2015). We used the plate outline coordinates to calculate each plate's two-dimensional area. To calculate the area of the sensilla-covered region of each plate, we generated a polygon connecting the coordinates of the outermost sensilla and calculated the area within this outline. We determined the proportion of each plate that is covered by sensilla by dividing the sensilla area by total plate area. We calculated sensilla density in two ways. First, we divided sensilla number by the area of the sensilla-covered region. This measures the number of sensilla that occur in a particular area but does not capture the relative arrangement of sensilla within that

area. Second, we computed the nearest neighbor distances among all sensilla within each plate based on their *x* and *y* coordinates and then calculated the mean and median nearest neighbor distances between the sensilla for each female. Nearest neighbor mean and median distances were highly correlated ($r_{E.\ carunculatum} = 0.83$; $r_{E.\ anna} = 0.88$), so we report only the analyses using the mean values.

To determine whether larger females possess more sensilla, we regressed sensilla number against abdomen length. We found no significant relationship between these traits in either species (*E. anna*: $R^2_{adj} = -0.02$, $F_{1,43} = 0.13$, $P = 0.73$; *E. carunculatum*: $R^2_{adj} = 0.005$, $F_{1,65} = 1.35$, $P = 0.25$). We thus present the results that compare sensilla counts without correcting for differences in body size.

Sensilla spatial analyses

To quantify sensilla distributions within each plate, we generated kernel density estimates (KDEs) for populations with at least four sampled individuals (two *E. anna* and six *E. carunculatum* populations) using the R package ks (Duong 2016). First, we randomly selected one of the two replicate sets of sensilla and plate outline coordinates for each female. To prepare the coordinate data for KDE analyses, we concatenated the sensilla and plate coordinates for each female and adjusted all plate outlines to have an area of one. This standardized each set of sensilla coordinates for size while maintaining their relative positions within each plate. Next, we translated each set of coordinates to place the origin of the coordinate system at the plate outline's centroid. We concatenated sensilla coordinates for all females sampled within each population to compute a representative KDE for each population.

212 Within each species, we conducted pairwise tests to compare each population's KDE
213 against every other population using the function kde.test() with the default settings. This test
214 returns a *P*-value that reflects the probability of generating the two sets of from the same
215 distribution of points. Because we performed multiple pairwise tests among *E. carunculatum*
216 populations, we adjusted the resulting *P*-values using the false discovery rate (Benjamini &
217 Hochberg 1995).
218 We generated an average plate outline for each population on which to visualize the
219 KDEs. The total number of coordinates that describe each plate outline varied among females,
220 ranging from 647-1078 for *E. anna* and 688-1028 for *E. carunculatum*. We standardized the
221 number of coordinates representing each plate by retaining the points for each of the upper and
222 lower medial corners and randomly sampled 198 points in between. We then treated each of
223 these 200 points as landmarks (the corners represented fixed landmarks and the remaining points
224 were designated as sliding semilandmarks) and used the R package geomorph (Adams and
225 Otarola-Castillo 2013) to perform general Procrustes analysis (Rohlf 1999) and obtain an
226 average two-dimensional plate shape for each population.
227
228 **Statistical analyses**
229 Some populations were well-sampled and other populations were represented by a single
230 female (Table 1). To avoid psedoreplication, for each population with N > 1, our analyses used
231 population means of trait values, so that each population was represented by a single
232 measurement. We arcsin transformed proportion data prior to analysis. We pooled data for
233 locally allopatric and fully allopatric *E. carunculatum* after *t*-tests showed that these groups did
234 not significantly differ with respect to sensilla number ($t_{2.7} = 0.80$, $P = 0.49$), sensilla density ($t_{9.2}$

235 = -1.62, $P = 0.13$), or the proportion of the plate that contained sensilla ($t_{10} = 0.06$, $P = 0.95$). To
236 compare traits between sympatric and allopatric populations of each species, we used *t*-tests or
237 Wilcoxon rank sum tests. We combined data for the two locally allopatric *E. carunculatum*
238 populations with the data from completely allopatric populations, after determining that these
239 data were similar enough to pool (sensilla number: $t_{2.1} = -0.91$, $P = 0.46$; proportion plate with
240 sensilla: $t_{11} = -1.24$, $P = 0.24$; sensilla density: $t_{5.8} = 0.51$, $P = 0.63$). To understand the
241 relationships between sensilla number, sensilla density, and the area of the plate occupied by
242 sensilla, we performed linear regressions between pairs of these traits.
243

244 **Results**

245 ***E. anna* and *E. carunculatum* females possess distinct sensilla traits**

246 *Enallagma anna* females possessed significantly more sensilla per plate ($\bar{x} = 46.2 \pm 1.4$)
247 than *E. carunculatum* females ($\bar{x} = 28.7 \pm 0.6$; $t_{19.4} = 7.37$, $P = 4.9 \times 10^{-7}$; Figure 2A).
248 *Enallagma anna* females also had sensilla distributed over a larger proportion of each plate ($W =$
249 240, $P = 2.6 \times 10^{-7}$; Figure 2B), and larger mean distances between sensilla ($t_{30} = 5.2$, $P = 1.3 \times$
250 10^{-5}; Figure 2C), which made *E. anna*'s overall sensilla distributions less dense than *E.*
251 *carunculatum*'s ($W = 239.5$, $P = 9.2. \times 10^{-6}$; Figure 2D). The sensilla occurred in different
252 locations on the mesostigmal plates of each species: they were more medial in *E. anna* and more
253 lateral in *E. carunculatum* (Figures 3, S2).

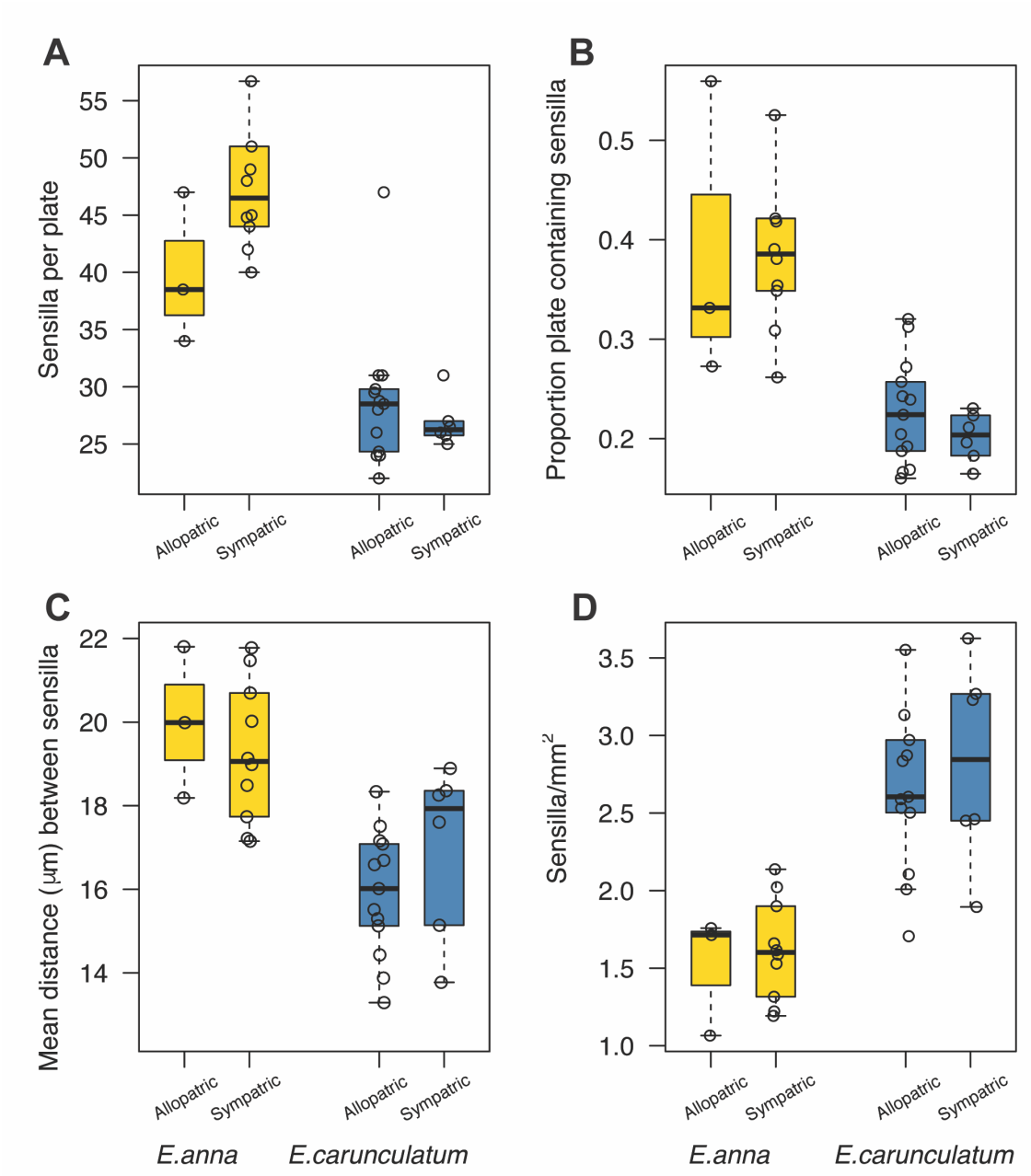

Figure 2. *E. anna* and *E. carunculatum* sensilla traits by population type. (A) The number of sensilla on one mesostigmal plate. **(B)** Proportion of the plate that contains sensilla. **(C)** Mean nearest neighbor distances between sensilla. **(D)** Sensilla density in the region of the plate that contains sensilla. Within each panel, each open circle represents the mean of one population. Boxplots show the interquartile range. The line within the box shows the median and whiskers extend to the most extreme observation within 1.5 times the interquartile range.

Both species showed a strong positive relationship between sensilla number and the absolute area of the plate occupied by sensilla (*E. anna*: $R^2_{adj} = 0.33$, $F_{1,27} = 14.71$, $P < 0.0007$;

264 *E. carunculatum*: $R^2_{adj} = 0.33$, $F_{1,72} = 37.68$, $P = 4.1 \times 10^{-8}$). Consistent with this result, linear
265 regressions also revealed that females with more sensilla also had a larger proportion of the plate
266 occupied by sensilla (*E. anna*: $R^2_{adj} = 0.26$, $F_{1, 27} = 10.65$, $P = 0.003$; *E. carunculatum:* $R^2_{adj} =$
267 0.20, $F_{1, 65} = 18.93$, $P = 4.4 \times 10^{-5}$). Females with more sensilla had smaller mean distances
268 between neighboring sensilla (*E. anna*: $R^2_{adj} = 0.11$, $F_{1, 27}$ 4.34, $P = 0.046$; *E. carunculatum*: R^2_{adj}
269 $= 0.09$, $F_{1,72} = 3.80$, $P = 0.01$). Overall, these results showed that a greater number of sensilla
270 was more strongly associated with a sensilla distribution that covers a larger area of the
271 mesostigmal plate rather a greater concentration sensilla within in a smaller area.
272

273 ***E. carunculatum* sensilla traits do not show a strong pattern of reproductive character**
274 **displacement**

275 We made several non-mutually exclusive predictions expected under RCD for sensilla
276 traits in sympatric populations relative to allopatric populations. In particular, we predicted to
277 observe at least one of the following phenotypic differences in sympatric females relative to
278 allopatric females: (1) more numerous sensilla (2) denser sensilla, (3) sensilla concentrated in
279 different regions of the mesostigmal plates. We did not find significant differences in any of
280 these traits between sympatric and locally allopatric *E. anna* females (Table S1). However,
281 because our *E. anna* samples included only four females from three locally allopatric
282 populations, we could not perform a robust comparison of *E. anna* sensilla traits between
283 populations that do, or do not encounter *E. carunculatum*. We thus focus our analysis on
284 comparisons between sympatric and allopatric *E. carunculatum* populations, for which we had
285 larger sample sizes.

286 Sympatric *E. carunculatum* populations did not differ significantly from allopatric
287 populations in sensilla number ($t_{16.3} = 0.98$, $P = 0.35$), proportion of the mesostigmal plate
288 covered by sensilla ($t_{16.8} = 1.33$, $P = 0.20$), or sensilla density (overall density: $t_{9.7} = -0.26$, $P =$
289 0.80; mean distance between sensilla: $t_{18} = -1.31$, $P = 0.21$). In addition to divergence of mean
290 trait values, RCD can also result in reduced trait variance in sympatry without affecting the mean
291 (Pfennig and Pfennig 2009). Sympatric *E. carunculatum* populations displayed less variance in
292 both sensilla number (Figure 2A) and the proportion of the plate covered by sensilla (Figure 2B).
293 However, these trends were not statistically significant (sensilla number with locally allopatric
294 outlier removed: Bartlett's $K^2_1 = 0.75$, $P = 0.39$; proportion of plate covered by sensilla: Bartlett's
295 $K^2_1 = 2.5$, $P = 0.11$). KDE comparisons also did not reveal significant differences in sensilla
296 distributions between sympatric and allopatric *E. carunculatum* populations (Table S2).
297 However, the analysis revealed significant differences in sensilla distributions between several
298 pairs of populations that are not sympatric with *E. anna* (Figure 3E). This result is consistent
299 with those described above that indicated higher variance in sensilla traits among allopatric
300 populations compared to sympatric populations.

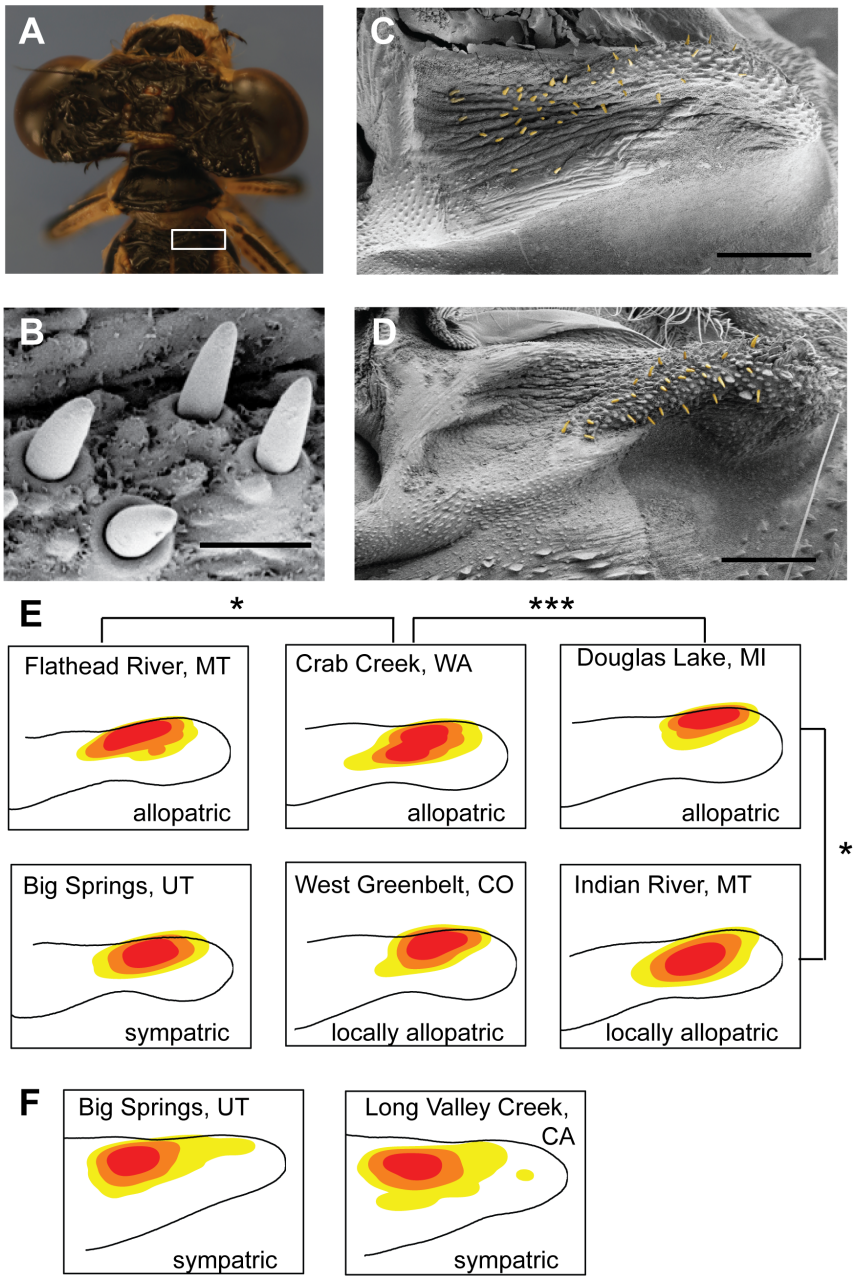

Figure 3. Sensilla locations. **(A)** White box indicates the location of right mesostigmal plate on the thorax. **(B)** Ultrastructural details of individual sensilla. Scale bar represents 10 µm. **(C, D)** Scanning electron micrographs show the locations of sensilla (yellow) on the mesostigmal plates of *E. anna* (C) and *E. carunculatum* (D). Scale bars represent 100 µm. **(E, F)** Population kernel density estimates for *E. carunculatum* (E) and *E. anna* (F) sensilla. The shading indicates different regions of sensilla density: red represents the 75-99th percentile, orange represents the 50-74th percentile of sensilla density, and yellow represents the 25th-49th percentile. Each outline represents the average mesostigmal plate shape for the population. Asterisks indicate *E. carunculatum* populations whose KDEs were determined to be significantly different (* indicates $P < 0.05$, *** $P < 0.001$).

312 Interestingly, although mean trait values did not differ significantly between sympatric
313 and allopatric populations, sensilla traits displayed considerable variation within the populations
314 we sampled. For example, within a single population, a particular female might have twice as
315 many sensilla than another female (Figure S2). This pattern was also observed in the *E. anna*
316 populations we studied.
317

318 **Discussion**

319 *Enallagma anna* and *E. carunculatum* females possessed different numbers of sensilla in
320 species-specific distributions on their mesostigmal plates. This result supports the idea that
321 receptors that receive male stimuli will occur in patterns that correspond to the male organs
322 during contact (Eberhard 2010). An association between male morphology and female sensilla
323 has been described for African *Enallagma* species (Robertson and Paterson 1982), and our
324 results show a similar pattern. *Enallagma anna* male cerci are considerably larger than *E.*
325 *carunculatum* cerci, and the observation that *E. anna* females had a larger number of sensilla
326 compared to *E. carunculatum* females is consistent with the difference in species-specific male
327 genital morphology.

328 When species make secondary contact after initial divergence in allopatry, the possible
329 outcomes are increased species divergence (*e.g.*, Sætre et al. 1997; Noor 2000; Naisbit et al.
330 2001; Yukilevich 2012; Dyer et al. 2014), decreased divergence (*e.g.*, Ritchie et al. 1989;
331 Shurtliff et al. 2013; Yang et al. 2016), one species goes locally extinct due to reproductive
332 exclusion (Hochkirch et al. 2007, Groning and Hochkirch 2008), or no change in either direction
333 (Abbott et al. 2013). Because *E. anna* and *E. carunculatum* produce reproductively
334 disadvantaged hybrids (Barnard et al. 2017), selection is expected to favor increased premating

335 isolation. Within each species, we predicted that female sensilla traits would show character
336 displacement in sympatric populations, which could indicate a shift in female preferences to
337 avoid mating with heterospecifics. Contrary to this prediction, *E. carunculatum* sympatric and
338 allopatric populations were not significantly different in mean sensilla trait values (Figure 2) or
339 sensilla density distributions (Figure 3E). Although we observed a trend toward more sensilla in
340 sympatric *E. anna* populations relative to allopatric populations (Figure 2A), it is difficult to
341 conduct a robust comparison for this species because (1) *E. anna*'s entire geographic range
342 overlaps with *E. carunculatum*'s range and (2) *E. anna* are often relatively rare (Acorn 2004; A.
343 Barnard, personal obs.). It was therefore difficult to collect sufficient *E. anna* samples from
344 populations that do not co-occur with *E. carunculatum*. We do, however, expect a stronger
345 pattern of RCD in sympatric *E. anna* females because *E. carunculatum* males can take them in
346 tandem relatively easily, whereas *E. anna* males are typically unsuccessful at taking *E.*
347 *carunculatum* females in tandem (Barnard et al. 2017). This means that *E. anna* females may
348 have more opportunities for mating mistakes than *E. carunculatum* females, which can result in
349 stronger asymmetric RCD (Lemmon 2009; Pfennig and Pfennig 2009).
350 There are at least four potential explanations for the absence of RCD in the form of
351 significant differences in the sensilla traits we measured between sympatric and allopatric
352 populations of *E. carunculatum*. First, species-specific sensilla distributions may be sufficiently
353 different to allow females to recognize when they are taken in tandem by heterospecific or
354 conspecific males. If this is true, small degrees of variation within the overall species pattern
355 among females might not affect females' species-recognition abilities. RCD is most easily
356 facilitated when the trait under selection already differs between species (Pfennig and Pfennig

357 2009). However, these traits may have already diverged enough sufficiently to preclude strong
358 selection for further divergence.

359 Second, it is possible that the external sensilla phenotypes we measured are not
360 representative of proximate female sensory traits, and the important variation lies deeper within
361 the female nervous system. For example, individual sensilla might differ in response rate or
362 ability to distinguish different levels of pressure applied by the cerci, and grasping pressure
363 might differ between males of each species. The direction of mechanosensor deflection is also
364 important for stimulus detection (Keil 1997), and different species' cercus morphologies may
365 contact sensilla from different angles. Female mate preferences may also be influenced by male
366 exposure and sexual experience (Svensson et al. 2014).

367 Third, the thoracic sensilla may not be a target of strong selection. For example, earlier
368 acting forms of RI may prevent most heterospecific interactions in the sympatric populations we
369 sampled. In one region where *E. anna* and *E. carunculatum* co-occur, habitat and temporal
370 isolation were close to zero (Barnard et al. 2017), but the strength of these isolating barriers may
371 vary geographically.

372 Finally, although we did not detect a statistically significant difference between group
373 means, the small differences we observed may still have biological relevance. If gaining just one
374 additional mechanosensor can (at least) double a female's tactile discriminatory power (Gaffin
375 and Brayfield 2017), then females in a population with a seemingly minor upward shift in
376 sensilla number could gain a remarkable increase in their ability to detect and avoid mating with
377 heterospecifics. Similarly, it is difficult to determine the features of sensilla density distributions
378 that may influence female preference solely by conducting statistical tests between KDEs. The
379 human eye can visually detect differences in the KDE plots shown in Figure 3, and it is thus

380 possible that these spatial differences reflect salient variation in the way females might receive
381 tactile stimuli.

382 These possible explanations highlight the interesting avenues that female damselfly
383 sensilla provide for investigating how females evaluate male tactile signals to make mating
384 decisions. The ability to quantify the number and locations of female mechanoreceptors in a
385 region contacted by male reproductive structures complements our understanding of patterns of
386 variation in male morphologies (McPeek et al. 2008; McPeek et al. 2009; McPeek et al. 2011;
387 Barnard et al. 2017). Females of both species display substantial intrapopulation variation in
388 sensilla traits (Figure S2) and this variation may play a role in sexual selection and female
389 preferences within species. Behavioral studies will be crucial to link mechanoreceptor
390 phenotypes to female mating decisions and clarify how sensilla traits influence both species
391 recognition and sexual selection. For example, do females with more sensilla make fewer
392 mating mistakes than females with fewer sensilla (Lemmon 2009)? Another outstanding
393 question of this system is how the cerci stimulate individual sensilla during tandem. This might
394 be determined by flash-freezing male-female tandem pairs and using micro-CT scanning to
395 understand how the male and female structures interact, similar to a recent approach used in seed
396 beetles (Dougherty and Simmons 2017). Once we understand how cerci contact the sensilla,
397 functional tests of sensilla electrophysiology could reveal how individual sensilla respond to
398 stimulation and indicate whether certain sensilla make greater contributions to reproductive
399 decision-making than others.

400 Female preference can drive sexual selection, promote trait divergence, and cause RI
401 between species (Ritchie 1996). A longstanding presumption in the literature on genital
402 evolution and speciation has been that female reproductive morphologies are less variant or

species-specific than male genitalia (Shapiro and Porter 1989). However, recent studies of variation in female reproductive structures suggest that variation does exist among individuals and species (Ah-King et al. 2014), and our data support the need to look beyond the visible external morphologies. When male genital morphologies are obviously divergent, but female morphologies are not, females may possess important variation at neurophysiological levels that affects how they evaluate male tactile signals, similar to the way females evaluate signals in other sensory modalities.

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Table 1. Sampling sites for *E. anna* and *E. carunculatum* populations. N refers to the number of females that were imaged and measured for this study. Sources: A. Barnard(AB), Ola Fincke (OF), Bill Mauffray (BM), and Dennis Paulson (DP).

Type	Site (site number)	Species	Year collected	N	Source
Sympatric	Big Spring, UT (1)	*E. anna*	2016	10	AB
		E. carunculatum		4	
	Big Sandy Creek, MT (2)	*E. carunculatum*	2015	1	AB
	Creston, MT (3)	*E. anna*	1972	1	BM
	Dry Sheep Creek, NE (4)	*E. anna*	2012	1	BM
	Fish Springs Run, CA (5)	*E. anna*	1998	2	BM
	Grace Coolidge Creek, SD (6)	*E. anna*	1969	1	BM
	Horseshoe Springs, UT (7)	*E. anna*	2016	1	AB
		E. carunculatum		1	
	Long Valley Creek, CA (8)	*E. anna*	1973	5	DP
	Murray Creek, NV (9)	*E. anna*	2001	1	
	Malad River, UT (10)	*E. carunculatum*	1983	2	BM
	Niwot Ditch, CO (11)	*E. anna*	2015	2	AB
		E. carunculatum		1	
	Pondera Coulee, MT (12)	*E. anna*	2015	1	AB
		E. carunculatum		1	
Locally allopatric	Beaver Creek, WY (13)	*E. anna*	2015	1	AB
	Indian Road Camp, MT (14)	*E. carunculatum*	2015	4	AB
	Jackson, WY (15)	*E. anna*	1971	2	BM
	Muddy Creek, MT (16)	*E. anna*	2015	1	AB
	Strawberry River, UT (17)	*E. carunculatum*	2016	1	AB
	West Greenbelt, CO (18)	*E. carunculatum*	2014	9	AB
Allopatric	Bull Lake, MT (19)	*E. carunculatum*	2015	1	AB
	Crab Creek, WA (20)	*E. carunculatum*	2016	20	DP
	Clear Lake, IN (21)	*E. carunculatum*	1945	1	BM
	Columbia River, WA (22)	*E. carunculatum*	1952	2	BM
	Douglas Lake, MI (23)	*E. carunculatum*	2016	17	OF
	Flathead River, MT (24)	*E. carunculatum*	2015	4	AB
	Home Lake, CO (25)	*E. carunculatum*	2015	1	AB
	Little Lake, CA (26)	*E. carunculatum*	1967	1	DP
	Drumond Island, MI (27)	*E. carunculatum*	2002	1	BM
	Snake River, ID (28)	*E. carunculatum*	1983	2	BM