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

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

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

Female sensory structures that reside in body regions that contact species-specific male
structures during mating have been documented in several arthropods, including flies (Eberhard
2001; Ingram et al. 2008) and damselflies (Cordoba-Aguilar 2005; Robertson and Paterson
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
quantitative variation in touch (rather than a binary response), this would dramatically increase
the number of response states and therefore further enhance tactile acuity (Gaffin and Brayfield
2017). Female damselfly thoracic sensilla thus present an external, quantifiable phenotype to
investigate the mechanistic basis of tactile stimuli and female mating decisions.

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

Because it appears that mesostigmal sensilla are used to mediate species recognition, they
might be expected to show signs of reproductive character displacement (RCD) in regions where
species co-occur (Brown and Wilson 1956; Howard 1993; Pfennig and Pfennig 2009). RCD can
evolve via direct selection on adult prezygotic phenotypes or via reinforcement, in which direct
selection against interspecific hybrids gives rise to selection for enhanced premating isolation.
between species (Dobzhansky 1937). *Enallagma anna* and *E. carunculatum* can interbreed, but these species produce hybrids with significantly reduced fitness (Barnard et al. 2017). This suggests that species-specific sensilla phenotypes might show patterns consistent with RCD in regions of sympatry, where females are known to experience frequent mating attempts from heterospecific males (Paulson 1974; Fincke et al. 2007; Barnard et al. 2017). Here, we test the hypothesis that variation in female sensilla phenotypes supports a function in species recognition. We test this hypothesis by quantifying sensilla number, density, and location phenotypes on the mesostigmal plates of a large set of *E. anna* and *E. carunculatum* females from multiple populations across the western United States and comparing phenotypes of each pure species from sympatric and allopatric populations to identify patterns consistent with RCD.

**Methods**

**Population sampling**

We measured the sensilla traits of 29 *E. anna* females across 13 populations, and 74 *E. carunculatum* females across 19 populations (Figure 1, Table 1). We classified each population as either allopatric, locally allopatric, or sympatric. Sympatric populations are those where *E. anna* and *E. carunculatum* co-occur temporally as well as spatially. Because *E. anna*’s geographic range falls completely within *E. carunculatum*’s range, only *E. carunculatum* has completely allopatric populations. We designated populations that exist at sites within the area of range overlap, but where only one species is known to occur, as locally allopatric. Some specimens were collected as early as 1945, but the majority of samples (82 of 103) we studied were collected between 2012 and 2016.
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.
Within each species, we conducted pairwise tests to compare each population’s KDE against every other population using the function kde.test() with the default settings. This test returns a $P$-value that reflects the probability of generating the two sets of from the same distribution of points. Because we performed multiple pairwise tests among *E. carunculatum* populations, we adjusted the resulting $P$-values using the false discovery rate (Benjamini & Hochberg 1995).

We generated an average plate outline for each population on which to visualize the KDEs. The total number of coordinates that describe each plate outline varied among females, ranging from 647-1078 for *E. anna* and 688-1028 for *E. carunculatum*. We standardized the number of coordinates representing each plate by retaining the points for each of the upper and lower medial corners and randomly sampled 198 points in between. We then treated each of these 200 points as landmarks (the corners represented fixed landmarks and the remaining points were designated as sliding semilandmarks) and used the R package geomorph (Adams and Otarola-Castillo 2013) to perform general Procrustes analysis (Rohlf 1999) and obtain an average two-dimensional plate shape for each population.

### Statistical analyses

Some populations were well-sampled and other populations were represented by a single female (Table 1). To avoid pseudoreplication, for each population with $N > 1$, our analyses used population means of trait values, so that each population was represented by a single measurement. We arcsin transformed proportion data prior to analysis. We pooled data for locally allopatric and fully allopatric *E. carunculatum* after $t$-tests showed that these groups did not significantly differ with respect to sensilla number ($t_{2.7} = 0.80, P = 0.49$), sensilla density ($t_{9.2}$
= -1.62, \( P = 0.13 \)), or the proportion of the plate that contained sensilla (\( t_{10} = 0.06, P = 0.95 \)). To compare traits between sympatric and allopatric populations of each species, we used \( t \)-tests or Wilcoxon rank sum tests. We combined data for the two locally allopatric \textit{E. carunculatum} populations with the data from completely allopatric populations, after determining that these data were similar enough to pool (sensilla number: \( t_{2.1} = -0.91, P = 0.46 \); proportion plate with sensilla: \( t_{11} = -1.24, P = 0.24 \); sensilla density: \( t_{5.8} = 0.51, P = 0.63 \)). To understand the relationships between sensilla number, sensilla density, and the area of the plate occupied by sensilla, we performed linear regressions between pairs of these traits.

\textbf{Results}

\textit{E. anna} and \textit{E. carunculatum} females possess distinct sensilla traits

\textit{Enallagma anna} females possessed significantly more sensilla per plate (\( \bar{x} = 46.2 \pm 1.4 \)) than \textit{E. carunculatum} females (\( \bar{x} = 28.7 \pm 0.6; t_{19.4} = 7.37, P = 4.9 \times 10^{-7} \); Figure 2A). \textit{Enallagma anna} females also had sensilla distributed over a larger proportion of each plate (\( W = 2.6 \times 10^{-7} \); Figure 2B), and larger mean distances between sensilla (\( t_{30} = 5.2, P = 1.3 \times 10^{-5} \); Figure 2C), which made \textit{E. anna}’s overall sensilla distributions less dense than \textit{E. carunculatum}’s (\( W = 239.5, P = 9.2 \times 10^{-6} \); Figure 2D). The sensilla occurred in different locations on the mesostigmal plates of each species: they were more medial in \textit{E. anna} and more lateral in \textit{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*: $R^2_{adj} = 0.33$, $F_{1,27} = 14.71$, $P < 0.0007$;
E. carunculatum: $R^2_{adj} = 0.33, F_{1,72} = 37.68, P = 4.1 \times 10^{-8}$). Consistent with this result, linear regressions also revealed that females with more sensilla also had a larger proportion of the plate occupied by sensilla (E. anna: $R^2_{adj} = 0.26, F_{1,27} = 10.65, P = 0.003$; E. carunculatum: $R^2_{adj} = 0.20, F_{1,65} = 18.93, P = 4.4 \times 10^{-5}$). Females with more sensilla had smaller mean distances between neighboring sensilla (E. anna: $R^2_{adj} = 0.11, F_{1,27} = 4.34, P = 0.046$; E. carunculatum: $R^2_{adj} = 0.09, F_{1,72} = 3.80, P = 0.01$). Overall, these results showed that a greater number of sensilla was more strongly associated with a sensilla distribution that covers a larger area of the mesostigmal plate rather a greater concentration sensilla within in a smaller area.

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

We made several non-mutually exclusive predictions expected under RCD for sensilla traits in sympatric populations relative to allopatric populations. In particular, we predicted to observe at least one of the following phenotypic differences in sympatric females relative to allopatric females: (1) more numerous sensilla (2) denser sensilla, (3) sensilla concentrated in different regions of the mesostigmal plates. We did not find significant differences in any of these traits between sympatric and locally allopatric E. anna females (Table S1). However, because our E. anna samples included only four females from three locally allopatric populations, we could not perform a robust comparison of E. anna sensilla traits between populations that do, or do not encounter E. carunculatum. We thus focus our analysis on comparisons between sympatric and allopatric E. carunculatum populations, for which we had larger sample sizes.
Sympatric *E. carunculatum* populations did not differ significantly from allopatric populations in sensilla number ($t_{16.3} = 0.98$, $P = 0.35$), proportion of the mesostigmal plate covered by sensilla ($t_{16.8} = 1.33$, $P = 0.20$), or sensilla density (overall density: $t_{9.7} = -0.26$, $P = 0.80$; mean distance between sensilla: $t_{18} = -1.31$, $P = 0.21$). In addition to divergence of mean trait values, RCD can also result in reduced trait variance in sympatry without affecting the mean (Pfennig and Pfennig 2009). Sympatric *E. carunculatum* populations displayed less variance in both sensilla number (Figure 2A) and the proportion of the plate covered by sensilla (Figure 2B). However, these trends were not statistically significant (sensilla number with locally allopatric outlier removed: Bartlett's $K^2_1 = 0.75$, $P = 0.39$; proportion of plate covered by sensilla: Bartlett's $K^2_1 = 2.5$, $P = 0.11$). KDE comparisons also did not reveal significant differences in sensilla distributions between sympatric and allopatric *E. carunculatum* populations (Table S2). However, the analysis revealed significant differences in sensilla distributions between several pairs of populations that are not sympatric with *E. anna* (Figure 3E). This result is consistent with those described above that indicated higher variance in sensilla traits among allopatric 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 $P < 0.05$, ** $P < 0.001$).
Interestingly, although mean trait values did not differ significantly between sympatric and allopatric populations, sensilla traits displayed considerable variation within the populations we sampled. For example, within a single population, a particular female might have twice as many sensilla than another female (Figure S2). This pattern was also observed in the *E. anna* populations we studied.

**Discussion**

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

When species make secondary contact after initial divergence in allopatry, the possible outcomes are increased species divergence (e.g., Sætre et al. 1997; Noor 2000; Naisbit et al. 2001; Yukilevich 2012; Dyer et al. 2014), decreased divergence (e.g., Ritchie et al. 1989; Shurtliff et al. 2013; Yang et al. 2016), one species goes locally extinct due to reproductive exclusion (Hochkirch et al. 2007, Groning and Hochkirch 2008), or no change in either direction (Abbott et al. 2013). Because *E. anna* and *E. carunculatum* produce reproductively disadvantaged hybrids (Barnard et al. 2017), selection is expected to favor increased premating
isolation. Within each species, we predicted that female sensilla traits would show character
displacement in sympatric populations, which could indicate a shift in female preferences to
avoid mating with heterospecifics. Contrary to this prediction, *E. carunculatum* sympatric and
allopatric populations were not significantly different in mean sensilla trait values (Figure 2) or
sensilla density distributions (Figure 3E). Although we observed a trend toward more sensilla in
sympatric *E. anna* populations relative to allopatric populations (Figure 2A), it is difficult to
conduct a robust comparison for this species because (1) *E. anna*’s entire geographic range
overlaps with *E. carunculatum*’s range and (2) *E. anna* are often relatively rare (Acorn 2004; A.
Barnard, personal obs.). It was therefore difficult to collect sufficient *E. anna* samples from
populations that do not co-occur with *E. carunculatum*. We do, however, expect a stronger
pattern of RCD in sympatric *E. anna* females because *E. carunculatum* males can take them in
tandem relatively easily, whereas *E. anna* males are typically unsuccessful at taking *E.
carunculatum* females in tandem (Barnard et al. 2017). This means that *E. anna* females may
have more opportunities for mating mistakes than *E. carunculatum* females, which can result in
stronger asymmetric RCD (Lemmon 2009; Pfennig and Pfennig 2009).

There are at least four potential explanations for the absence of RCD in the form of
significant differences in the sensilla traits we measured between sympatric and allopatric
populations of *E. carunculatum*. First, species-specific sensilla distributions may be sufficiently
different to allow females to recognize when they are taken in tandem by heterospecific or
conspecific males. If this is true, small degrees of variation within the overall species pattern
among females might not affect females’ species-recognition abilities. RCD is most easily
facilitated when the trait under selection already differs between species (Pfennig and Pfennig
However, these traits may have already diverged enough sufficiently to preclude strong selection for further divergence.

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

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

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

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

Female preference can drive sexual selection, promote trait divergence, and cause RI
between species (Ritchie 1996). A longstanding presumption in the literature on genital
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.

**References cited**

Abramoff, M. D., P. J. Magalhaes, and S. J. Ram. 2004. Image processing with ImageJ. Biophotonics International 11:36-42.

Acorn, J. 2004. Damselflies of Alberta: flying neon toothpicks in the grass. The University of Alberta Press, Edmonton, Alberta, Canada.

Adams, D. C. and E. Otarola-Castillo. 2013. geomorph: an R package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution 4:393-399.

Ah-King, M., A. B. Barron, and M. E. Herberstein. 2014. Genital evolution: why are females still understudied? PLoS Biology 12:e1001851.

Arthur, B. J., T. Sunayama-Morita, P. Coen, M. Murthy, and D. L. Stern. 2013. Multi-channel acoustic recording and automated analysis of *Drosophila* courtship songs. BMC Biology 11:1-11.

Barnard, A. A., O. M. Fincke, M. A. McPeek, and J. P. Masly. 2017. Mechanical and tactile incompatibilities cause reproductive isolation between two young damselfly species. Evolution 71:2410–2427.

Battin, T. J. 1993a. The odonate mating system, communication, and sexual selection: A review. Bulletin of Zoology 60:353-360.

Battin, T. J. 1993b. Revision of the puella group of the genus Cuenagrion Kirby, 1890 (Odonata, Zygoptera), with emphasis on morphologies contributing to reproductive isolation. Hydrobiologia 262:13-29.

Boughman, J. W., H. D. Rundle, and D. Schluter. 2005. Parallel evolution of sexual isolation in sticklebacks. Evolution 59:361-373.

Briceño, R., W. Eberhard, and A. Robinson. 2007. Copulation behaviour of *Glossina pallidipes* (Diptera: Muscidae) outside and inside the female, with a discussion of genitalic evolution. Bulletin of entomological research 97:471-488.

Briceño, R. D. and W. G. Eberhard. 2009a. Experimental demonstration of possible cryptic female choice on male tsetse fly genitalia. Journal of Insect Physiology 55:989-996.
Briceño, R. D. and W. G. Eberhard. 2009b. Experimental modifications imply a stimulatory function for male tsetse fly genitalia, supporting cryptic female choice theory. Journal of evolutionary biology 22:1516-1525.

Brown, W. L. and E. O. Wilson. 1956. Character displacement. Systematic zoology 5:49-64.

Castillo, D. M., M. K. Burger, C. M. Lively, and L. F. Delph. 2015. Experimental evolution: Assortative mating and sexual selection, independent of local adaptation, lead to reproductive isolation in the nematode *Caenorhabditis remanei*. Evolution 69:3141-3155.

Coleman, S. W. 2008. Taxonomic and sensory biases in the mate-choice literature: there are far too few studies of chemical and multimodal communication. Acta Ethologica 12:45-48.

Corbet, P. S. 1999. Dragonflies: Behaviour and ecology of Odonata. Cornell University Press, Ithaca, NY.

Cordoba-Aguilar, A. 2005. Possible coevolution of male and female genital form and function in a calopterygid damselfly. J Evol Biol 18:132-137.

Costanzo, K. and A. Monteiro. 2007. The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. Proceedings of the Royal Society of London B: Biological Sciences 274:845-851.

Coyne, J. A. 1993. The genetics of an isolating mechanism between two sibling species of *Drosophila*. Evolution 47:778-788.

Coyne, J. A., A. Crittenden, and K. Mahi. 1994. Genetics of a pheromonal difference contributing to reproductive isolation in *Drosophila*. Science 265:1461-1464.

Dobzhansky, T. 1937. Genetics and the Origin of Species. Columbia University press.

Donnelly, N. T. 2008. A Hybrid Complex in *Enallagma*. Argia 20:10-11.

Dopman, E. B., P. S. Robbins, and A. Seaman. 2010. Components of reproductive isolation between North American pheromone strains of the European corn borer. Evolution 64:881-902.

Dougherty, L. R. and L. W. Simmons. 2017. X-ray micro-CT scanning reveals temporal separation of male harm and female kicking during traumatic mating in seed beetles. Proceedings of the Royal Society of London B: Biological Sciences 284: 20170550.

Dufour, L. 1844. Anatomie générale des diptéres. Annales des Sciences Naturelles 1:244-264.

Duong, T. 2016. ks: Kernel Smoothing. R package version 1.10.4.

Dyer, K. A., B. E. White, J. L. Sztepanacz, E. R. Bewick, and H. D. Rundle. 2014. Reproductive character displacement of epicuticular compounds and their contribution to mate choice in *Drosophila subquinaria* and *Drosophila recens*. Evolution 68:1163-1175.

Eberhard, W. G. 1992. Species isolation, genital mechanics, and the evolution of species-specific genitalia in three species of *Macrodactylus* Beetles (Coleoptera, Scarabeidae, Melolonthinae). Evolution 46:1774-1783.

Eberhard, W. G. 1994. Evidence for widespread courtship during copulation in 131 species of insects and spiders, and implications for cryptic female choice. Evolution 48:711-733.

Eberhard, W. G. 2001. The functional morphology of species-specific clasping structures on the front legs of male sepsid flies. Zoological Journal of the Linnean Society 133:335-368.

Eberhard, W. G. 2002. Physical restraint or stimulation? The function(s) of the modified front legs of male *Archisepsis diversiformis* (Diptera, Sepsidae). Journal of Insect Behavior 15:831-850.

Eberhard, W. G. 2010. Evolution of genitalia: theories, evidence, and new directions. Genetica 138:5-18.
Edvardsson, M. and A. Göran. 2000. Copulatory courtship and cryptic female choice in red flour beetles Tribolium castaneum. Proceedings of the Royal Society of London B: Biological Sciences 267:559-563.

Ewing, A. W. and H. Bennet-Clark. 1968. The courtship songs of Drosophila. Behaviour 31:288-301.

Fincke, O. M., A. Fargevieille, and T. D. Schultz. 2007. Lack of innate preference for morph and species identity in mate-searching Enallagma damselflies. Behavioral Ecology and Sociobiology 61:1121-1131.

Frazee, S. R. and J. P. Masly. 2015. Multiple sexual selection pressures drive the rapid evolution of complex morphology in a male secondary genital structure. Ecology and Evolution 5:4437-4450.

Gaffin, D. and B. Brayfield. 2017. Exploring the chemo-textural familiarity hypothesis for scorpion navigation. Journal of Arachnology 45:265-270.

Gerhardt, H. C. and F. Huber. 2002. Acoustic Communication in Insects and Anurans: Common Problems and Diverse Solutions. University of Chicago Press, Chicago, IL.

Girard, M. B., D. O. Elias, and M. M. Kasumovic. 2015. Female preference for multi-modal courtship: multiple signals are important for male mating success in peacock spiders. Proceedings of the Royal Society of London B: Biological Sciences 282.

Howard, D. J. 1993. Reinforcement: origin, dynamics, and fate of an evolutionary hypothesis. Pp. 46–69 in R. G. Harrison, ed. Hybrid zones and the evolutionary process. Oxford University Press, New York.

Ingram, K. K., T. Laamanen, N. Puniamoorthy, and R. Meier. 2008. Lack of morphological coevolution between male forelegs and female wings in Themira (Sepsidae: Diptera: Insecta). Biological Journal of the Linnean Society 93:227-238.

Jiggins, C. D., R. E. Naisbit, R. L. Coe, and J. Mallet. 2001. Reproductive isolation caused by colour pattern mimicry. Nature 411:302–305.

Johnson, D. M. and P. H. Crowley. 1980. Habitat and seasonal segregation among coexisting odonate larvae. Odonatologica 9:297–308.

Johnson, J. 2009. Presumed Enallagma anna Williamson × carunculatum Morse Hybrids from Oregon and California. Bulletin of American Odonatology 11:8-10.

Jurzitza, G. 1974. Rasterelektronenmikroskopische Untersuchungen des Zangengriffes und der Laminae mesostigmale einiger Coenagrionidae (Odonata, Zygoptera). Forma Functio 7:377-392.

Jurzitza, G. 1975. Rasterelektronenmikroskopische Untersuchungen an den Appendices und den Laminae mesotigmate einiger Enallagma-Arten (Odonata. Zygoptera). Forma Functio 8:33-48.

Kamimura, Y. and H. Mitsumoto. 2011. Comparative copulation anatomy of the Drosophila melanogaster species complex (Diptera: Drosophilidae). Entomological science 14:399-410.

Keil, T. A. 1997. Functional morphology of insect mechanoreceptors. Microscopy Research and Technique 39:506–531.

Krieger, F. and E. Krieger-Loibl. 1958. Beiträge zum Verhalten von Ischnura elegans und Ischnura pumilio (Odonata) 1. Zeitschrift für Tierpsychologie 15:82-93.

Kronforst, M. R., L. G. Young, D. D. Kapan, C. McNeely, R. J. O'Neill, and L. E. Gilbert. 2006. Linkage of butterfly mate preference and wing color preference cue at the genomic location of wingless. Proc Natl Acad Sci U S A 103:6575-6580.
Lemmon, E. M. 2009. Diversification of conspecific signals in sympatry: geographic overlap drives multidimensional reproductive character displacement in frogs. Evolution 53:1155-1170.

Loibl, E. 1958. Zur Ethologie und Biologie der deutschen Lestiden (Odonata) 1. Zeitschrift für Tierpsychologie 15:54-81.

Masly, J. P. 2012. 170 Years of "Lock-and-Key": Genital Morphology and Reproductive Isolation. International Journal of Evolutionary Biology 2012:247352.

Mayr, E. 1942. Systematics and the origin of species, from the viewpoint of a zoologist. Harvard University Press, Cambridge, MA.

McIver, S. B. 1975. Structure of cuticular mechanoreceptors of arthropods. Annual Review of Entomology 20:381-397.

McMillan, W. O., C. Jiggins, and J. Mallet. 1997. What initiates speciation in passion-vine butterflies? Proceedings of the National Academy of Sciences USA 94:8628–8633.

McPeek, M. A. 1998. The consequences of changing the top predator in a food web: a comparative experimental approach. Ecological Monographs 68:1-23.

McPeek, M. A., L. Shen, and H. Farid. 2009. The correlated evolution of three-dimensional reproductive structures between male and female damselflies. Evolution 63:73-83.

McPeek, M. A., L. Shen, J. Z. Torrey, and H. Farid. 2008. The tempo and mode of three-dimensional morphological evolution in male reproductive structures. Am Nat 171:E158-178.

McPeek, M. A., L. B. Symes, D. M. Zong, and C. L. McPeek. 2011. Species recognition and patterns of population variation in the reproductive structures of a damselfly genus. Evolution 65:419-428.

Mendelson, T. C. and K. L. Shaw. 2012. The (mis) concept of species recognition. Trends in ecology & evolution 27:421-427.

Mendelson, T. C. and G. Wallis. 2003. Sexual isolation evolves faster than hybrid inviability in a diverse and sexually dimorphic genus of fish (Percidae: Etheostoma). Evolution 57:317-327.

Myers, S. S., T. R. Buckley, and G. I. Holwell. 2016. Male genital claspers influence female mate acceptance in the stick in sect Clitarchus hookeri. Behavioral Ecology and Sociobiology 70:1547-1556.

Naisbit, R. E., C. D. Jiggins, and J. Mallet. 2001. Disruptive sexual selection against hybrids contributes to speciation between Heliconius cydno and Heliconius melpomene. Proceedings of the Royal Society of London B: Biological Sciences 268:1849-1854.

Noor, M. and J. A. Coyne. 1996. Genetics of a difference in cuticular hydrocarbons between Drosophila pseudoobscura and D. persimilis. Genetics Research 68:117-123

Noor, M. F. 2000. On the evolution of female mating preferences as pleiotropic byproducts of adaptive evolution. Adaptive Behavior 8:3-12.

Patterson, B. D. and C. S. Thaeler Jr. 1982. The mammalian baculum: hypotheses on the nature of bacular variability. Journal of Mammalogy 63:1-15.

Paulson, D. R. 2009. Dragonflies and Damselflies of the West. Princeton University Press, Princeton, New Jersey.

Paulson, D. R. 1974. Reproductive isolation in damselflies. Systematic Zoology 23:40-49.

Paulson, D. R. 2011. Dragonflies and Damselflies of the East. Princeton University Press, Princeton, New Jersey.
Pfennig, K. S. and D. W. Pfennig. 2009. Character displacement: ecological and reproductive responses to a common evolutionary problem. The Quarterly Review of Biology 84:253-276.

Plath, M., J. Parzefall, K. E. Körner, and I. Schlupp. 2004. Sexual selection in darkness? Female mating preferences in surface- and cave-dwelling Atlantic mollys, Poecilia mexicana (Poeciliidae, Teleostei). Behavioral Ecology and Sociobiology 55:596-601.

Price, T. D. and M. M. Bouvier. 2002. The evolution of F1 postzygotic incompatibilities in birds. Evolution 56:2083-2089.

Rafferty, N. E. and J. W. Boughman. 2006. Olfactory mate recognition in a sympatric species pair of three-spined sticklebacks. Behavioral Ecology 17:965-970.

Rebora, M., F. Frati, S. Piersanti, G. Salerno, R. Selvaggini, and O. M. Fincke. 2018. Field tests of multiple sensory cues in sex recognition and harassment of a colour polymorphic damselfly. Animal Behaviour 136:127-136.

Ritchie, M. G. 1996. The shape of female mating preferences. Proceedings of the National Academy of Sciences USA 93:14628-14631.

Ritchie, M. G., R. K. Butlin, and G. M. Hewitt. 1989. Assortative mating across a hybrid zone in Chorthippus parallelus (Orthoptera: Acrididae). Journal of Evolutionary Biology 2:339–352.

Robertson, H. M. and H. E. H. Paterson. 1982. Mate Recognition and Mechanical Isolation in Enallagma Damselflies (Odonata: Coenagrionidae). Evolution 36:243-250.

Rohlf, F. J. 1999. Shape statistics: Procrustes superimpositions and tangent spaces. Journal of Classification 16:197-223.

Rüschenbaum, S. and I. Schlupp. 2013. Non-visual mate choice ability in a cavefish (Poecilia mexicana) is not mechanosensory. Ethology 119:368-376.

Sætre, G.-P., T. Moum, S. Bureš, M. Král, M. Adamjan, and J. Moreno. 1997. A sexually selected character displacement in flycatchers reinforces premating isolation. Nature 387:589-592.

Sánchez-Guillén, R. A., A. Cordoba-Aguilar, A. Cordero Rivera, and M. Wellenreuther. 2014. Rapid evolution of prezygotic barriers in non-territorial damselflies. Biological Journal of the Linnean Society 113:485-496.

Sánchez-Guillén, R. A., M. Wellenreuther, and A. Cordero Rivera. 2012. Strong asymmetry in the relative strengths of prezygotic and postzygotic barriers between two damselfly sister species. Evolution 66:690-707.

Shapiro, A. M. and A. H. Porter. 1989. The lock-and-key hypothesis: Evolutionary and biosystematic interpretation of insect genitalia. Annual Review of Entomology 34:321-345.

Shaw, K. L. 2000. Interspecific genetics of mate recognition: inheritance of female acoustic preference in Hawaiian crickets. Evolution 54:1303–1312.

Shurtleff, Q. R., P. J. Murphy, J. D. Yeiter, and M. D. Matocq. 2013. Experimental evidence for asymmetric mate preference and aggression: behavioral interactions in a woodrat (Neotoma) hybrid zone. BMC Evolutionary Biology 13:220-233.

Simmons, L. W. 2014. Sexual selection and genital evolution. Austral Entomology 53:1-17.

Stratton, G. E. and G. W. Uetz. 1986. The Inheritance of Courtship Behavior and Its Role as a Reproductive Isolating Mechanism in Two Species of Schizocosa Wolf Spiders (Araneae; Lycosidae). Evolution 40:129-141.
Svensson, E. I., A. Runemark, M. N. Verzijden, and M. Wellenreuther. 2014. Sex differences in developmental plasticity and canalization shape population divergence in mate preferences. Proceedings of the Royal Society of London B: Biological Sciences 281:1-8.

Team, R. C. 2015. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Tennesen, K. J. 1975. Reproductive behavior and isolation of two sympatric Coenagrionid damselflies in Florida. University of Florida, Gainesville, FL.

Trabalon, M., A. G. Bagnères, and C. Roland. 1997. Contact sex signals in two sympatric spider species, *Tegenaria domestica* and *Tegenaria pagana*. Journal of Chemical Ecology 23:747–758.

Wells, M. M. and C. S. Henry. 1998. Songs, reproductive isolation, and speciation in cryptic species of insects: A case study using green lacewings. Pp. 217-233. Endless forms: species and speciation. Oxford University Press, New York, NY.

Wiernasz, D. C. and J. G. Kingsolver. 1992. Wing melanin pattern mediates species recognition in *Pieris occidentalis*. Animal Behaviour 43:89–94.

Williams, T. H. and T. C. Mendelson. 2014. Quantifying reproductive barriers in a sympatric pair of darter species. Evolutionary Biology 41:212-220.

Yang, Y., C. L. Richards-Zawacki, A. Devar, and M. B. Dugas. 2016. Poison frog color morphs express assortative mate preferences in allopatry but not sympatry. Evolution 70:2778–2788.

Yassin, A. and V. Orgogozo. 2013. Coevolution between male and female genitalia in the *Drosophila melanogaster* species subgroup. PLoS One 8:e57158.

Yukilevich, R. 2012. Asymmetrical patterns of speciation uniquely support reinforcement in *Drosophila*. Evolution 66:1430-1446.
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     |
|                 | 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     |
|                 | 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     |
|                 | Pondera Coulee, MT (12)  | *E. anna*              | 2015           | 1  | AB     |
| Locally         | Beaver Creek, WY (13)    | *E. anna*              | 2015           | 1  | AB     |
| allopatric      | 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     |