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Divergence in female damselfly sensory structures is consistent with a species recognition function but shows no evidence of reproductive character displacement

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Abstract

Males and females transmit and receive signals prior to mating that convey information such as sex, species identity, or individual condition. In some animals, tactile signals relayed during physical contact between males and females before and during mating appear to be important for mate choice or reproductive isolation. This is common among odonates, when a male grasps a female's thorax with his terminal appendages prior to copulation, and the female subsequently controls whether copulation occurs by bending her abdomen to complete intromission. It has been hypothesized that mechanosensory sensilla on the female thoracic plates mediate mating decisions, but is has been difficult to test this idea. Here, we use North American damselflies in the genus *Enallagma* (Odonata: Coenagrionidae) to test the hypothesis that variation in female sensilla traits is important for species recognition. *Enallagma anna* and *E. carunculatum* hybridize in nature, but experience strong reproductive isolation as a consequence of divergence in male terminal appendage morphology. We quantified several mechanosensory sensilla phenotypes on the female thorax among multiple populations of both species and compared divergence in these traits in sympatry versus allopatry. Although these 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. Our results suggest that species-specific placement of female mechanoreceptors may be sufficient for species recognition, although other female sensory phenotypes might have diverged in sympatry to reduce interspecific hybridization.

KEYWORDS
damselfly, mating, mechanosensor, reproductive character displacement, reproductive isolation

1 | INTRODUCTION

For sexual organisms, maintenance of species boundaries relies on reproductive isolation (RI) between recently diverged species (Mayr, 1942). Premating reproductive isolating barriers, including behavioral isolation, often evolve earlier in the speciation process than postmating barriers in a variety of animal taxa (e.g., Barnard, Fincke, McPeek, & Masly, 2017; Castillo, Burger, Lively, & Delph, 2015; Coyne & Orr, 1989, 1997; Dopman, Robbins, & Seaman, 2010; McMillan, Jiggins, & Mallet, 1997; Mendelson & Wallis, 2003; Price & Bouvier, 1997; Sánchez-Guillén, Wellenreuther, & Cordero-Rivera, 2016; Williams & Mendelson, 2014). Behavioral isolation requires that mate recognition signals and/or preferences diverge between populations, which ultimately results in the ability of individuals to discriminate conspecifics from heterospecifics. Species recognition signals may rely on a variety of sensory modalities such as color (Boughman, Rundle, & Schluter, 2005; Jiggins, Naisbit, Coe, & Mallet, 2001; Kronforst et al., 2006; Sætre et al., 1997; Wiernasz & Kingsolver, 1992; Williams & Mendelson, 2014), courtship behavior (Stratton & Uetz, 1926), sound/vibration (Arthur, Sunayama-Morita, Coen, Murthy, & Stern, 2013; Ewing & Bennet-Clark, 1968; Gerhardt & Huber, 2002; Wells & Henry, 1998; Shaw, 2012), and volatile chemicals (Coyne, Crittenden, & Mahi, 1994; Noor & Coyne, 1996; Rafferty & Boughman, 2016; Trabalon et al., 1997). Often, multiple signals act in concert to affect species recognition (e.g., Costanzo & Monteiro, 2007; Girard, Elias, & Kasumovic, 2015).

Although much is known about the importance of visual, auditory, and chemical 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 to contribute to mating decisions (Mendelson & Shaw, 2012), but it is unclear whether tactile cues could represent a primary species recognition signal, given that visual, auditory, and chemical cues usually act earlier during the mating sequence. Research on the prevalence of tactile signals in mating decisions is limited (Coleman, 2008) partly 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, understanding the role of tactile signals along the continuum between intraspecific mate choice and interspecific RI is important 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 (Eberhard, 1985). 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 (Masly, 2012; Shapiro & Porter, 2013; Simmons, 2018). However, observations both within (Briceño & Eberhard, 2009; Briceño & Eberhard, 2009; Eberhard, 1994; Edvardsson & Göran, 2000; Frazee & Masly, 2015) and between species (Barnard et al., 2017; Coyne, 1993; Eberhard, 1992; Patterson & Thaeler, 1982; Robertson & Paterson, 1982) suggest that male reproductive structures 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 & Porter, 2013), subtle morphological differences (e.g., Kamimura & Mitsumoto, 2011; Yassin & Orgogozo, 2013) could enable females to detect variation among males' morphology. Female variation in detection ability could also occur in signal processing at the level of neurons, neural networks, and/or in the distribution and morphology of sensory structures that receive male tactile signals. These sensory structures may exist not just in the female genitalia or reproductive tract, but in any region of the female that receives contact from male structures.

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, Laamanen, Puniamoorthy, & Meier, 2008) and damselflies (Battin, 1993, 1993 ; Córdoba-Aguilar, 1999, 2002, 2005; Jurzitza, 1974, 1975; Robertson & Paterson, 1982; Tennessen, 1975). Additional studies have demonstrated that tactile cues from male organs influence female mating responses, via experimental manipulation of male structures and desensitization of females (Briceño & Eberhard, 2009; Briceño, Eberhard, & Robinson, 2007; Eberhard, 2002, 2010 ; Myers, Buckley, & Holwell, 2016; Wulff, Schöneich, & Lehmann, 2018). 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, Parzefall, Körner, & Schlupp, 2004; Plath, Wiedemann, & Parzefall, 2004; but see Rüschenbaum & Schlupp, 2014).

Tactile signals appear to be a significant cause of RI in Zygoptera, the damselfly suborder of Odonata (Corbet, 1999; Krieger & Krieger-Loibl, 1958; Loibl, 1958; Robertson & Paterson, 1982). Concentrations of cuticular mechanoreceptors (sensilla) on the female thorax have been described in several coenagrionid damselfly genera (Battin, 1993, 1993 ; Jurzitza, 1974, 1975 ; Robertson & Paterson, 1982; Tennessen, 1975). The morphology of these sensilla is consistent with a mechanosensory function and does not indicate that they conduct signals related to olfaction, hygroreception, or temperature reception (McIver, 1975). These sensilla reside in areas where males' grasping appendages contact the female thorax before and during mating, which has led to speculation that they allow females to evaluate male morphologies and discriminate conspecific from heterospecific males. This idea is based on demonstrated reductions in female receptivity when grasped by males with manipulated appendages (Loibl, 1958; Robertson & Paterson, 1982) or heterospecific or hybrid males (Barnard et al., 2017; Sánchez-Guillén et al., 2016; Sánchez-Guillén et al., 2016; Tennessen, 1975). In African *Enallagma*, species-specific placement of sensilla within the female mesostigmal plates appears to correspond to where they are grasped by the male (Robertson & Paterson, 1982), which further suggests that the sensilla receive tactile cues based on male morphology that aid in species recognition.

In insects, each cuticular mechanoreceptor is associated with a single sensory neuron (McIver, 1975; Keil, 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 are expected to enhance a female's sensory resolution by increasing the combinatorial complexity of tactile signals that she 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 tactile 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 (e.g., Gaffin & Brayfield, 2017). Female damselfly thoracic sensilla thus present an external, quantifiable phenotype in which 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 & Crowley, 1980; McPeek, 1998), and do not engage in premating courtship (Barnard et al., 2017; Fincke, Fargevieille, & Schultz, 2007) or use chemical cues for mate selection (Rebora et al., 1998). 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 thorax to form "tandem," the premating position. The male superior grasping appendages (cerci) have species-specific morphologies, and differences in the morphology of these structures are the primary cause of RI in this genus (Paulson, 1974; Barnard et al., 2017). Two species, *Enallagma anna* and *Enallagma carunculatum*, have strikingly different cercus morphologies, yet occasionally hybridize in nature to produce males and females with reproductive structure morphologies that are intermediate to each of the pure species (Barnard et al., 2017; Donnelly, 2008; Johnson, 2009; Miller & Ivie, 1995). Females of both pure species discriminate strongly against both heterospecific and interspecific hybrid males that take them in tandem, which indicates that female *E. anna* and *E. carunculatum* can detect not only large differences in species-specific male stimulation, but also more subtle differences such as those that distinguish conspecific and hybrid males (Barnard et al., 2017).

Because it appears that mesostigmal sensilla mediate species recognition, they might be expected to show signs of reproductive character displacement (RCD): increased divergence of traits involved in RI in regions of sympatry between *E. anna* and *E. carunculatum* relative to regions of allopatry (Brown & Wilson, 1956; Howard, 1993; Pfennig & Pfennig, 2009). RCD can manifest phenotypically as divergence in either signaling traits or mate preferences in which sympatric females display stronger discrimination against heterospecific males than do allopatric females of the same species (e.g., Gerhardt, 1994; Gabor & Ryam, 2001; Albert & Schluter, 2004; Wheatcroft & Qvarnström, 2017). This strengthening of preference in sympatry may evolve via direct selection on adult prezygotic phenotypes, or via reinforcement, where selection against interspecific hybrids gives rise to selection for enhanced premating isolation between species (Dobzhansky, 1937). *Enallagma anna* and *E. carunculatum* can interbreed, but their hybrids experience significantly reduced fitness (Barnard et al., 2017). Female *Enallagma* experience frequent mating attempts from heterospecific males where both species co-occur (Paulson, 1974; Fincke et al., 2007; Barnard et al., 2017). These findings suggest that in sympatry, females may experience selection for stronger species discrimination ability. Studies of several *Enallagma* species (not including *E. anna* or *E. carunculatum*) have revealed that male cercus shape varies little among populations, even across large geographical regions (McPeek, Symes, Zong, & McPeek, 2011; Siepielski, McPeek, & McPeek, 2018). *Enallagma anna* and *E. carunculatum* appear to show similar patterns, at least in the western part of their distributions (Figure S1, Supporting information). It is possible, however, that females in sympatry with other species are more sensitive to variation among males than are females of the same species in regions of allopatry, and this variation in sensitivity may be reflected in female sensilla traits.

Here, we use sensilla number, density, and location as proxies for female preference, to test the hypothesis that variation in female sensilla phenotypes supports a function in species recognition. We tested this hypothesis by quantifying sensilla 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. We predicted that in sympatric populations, females would possess higher sensilla numbers, higher sensilla density, and/or different spatial distributions of sensilla within their mesostigmal plates when compared to females from allopatric populations.

2 | MATERIALS AND METHODS

2.1 | Population sampling

We measured the sensilla traits of 29 *E. anna* females across 13 populations, and 74 *E. carunculatum* females across 28 populations (Figure 1, Table 1). We classified each population as 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 (Figure 1), only *E. carunculatum* has completely allopatric populations. We designated populations as locally allopatric at sites within the area of range overlap, but where only one species is known to occur based on occurrence data from OdonataCentral.org. Specimens were either dried or preserved in ethanol; neither preservation method alters the morphology of the hard cuticle that comprises the mesostigmal plates, nor the ability to visualize sensilla. Although some specimens were collected as early as 1945, the majority of samples (82 of 103) we studied were collected between 2012 and 2016.

2.2 | 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 (Figure 2). Specimens were mounted on aluminum stubs with

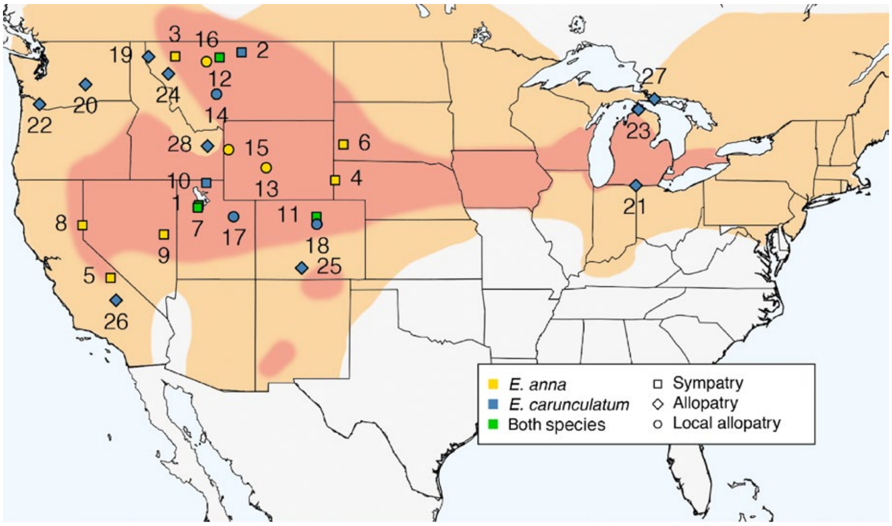

FIGURE 1 Sampling sites and species ranges. *Enallagma anna*'s geographic range (red) occurs within *Enallagma carunculatum*'s geographic range (orange). Names of sites associated with each number are described in Table 1. Symbol color indicates the species sampled and symbol shape indicates the population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

carbon tape, sputter-coated with gold-palladium, and imaged at, 200× magnification and 3 kV using a Zeiss NEON scanning electron microscope.

To avoid any potential bias during measurements, we blind-coded image files before measuring all sensilla traits. We measured abdomen length (abdominal segments 1–10, excluding terminal appendages) on the full-body photographs as an estimate for body size using the segmented line tool in ImageJ (Abramoff, Magalhaes, & Ram, 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 ($n = 20$). Sensilla counts on a subset of 57 females showed that left plate and right plate sensilla counts were highly correlated within individual females ($r = 0.85$). 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 the image 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 multipoint selection tool. When a sensillum breaks, it leaves behind distinctive round area with a central pore. In these cases, we recorded the area where the sensillum had been (Figure 2b-c).

We traced an outline around the plate image, excluding the lateral carina (Figure 2f), using a Wacom Cintiq 12WX tablet and stylus (Wacom, Saitama, Japan) and the freehand selection tool in ImageJ. This procedure produced x and y coordinates that described the plate outline. We performed all measurements twice for each specimen. Measurements across the two technical replicates were highly correlated ($r_{\text{abdomen}} = 0.95$, $n = 78$; $r_{\text{count}} = 0.95$, $n = 103$; $r_{\text{plate area}} = 0.99$, $n = 86$), so we used the mean trait values of the two replicates in subsequent analyses. Seventeen samples were imaged at angles that allowed counting of the sensilla, but distorted the plate shape or distances between the sensilla. Those samples are included in analyses of sensilla number but were not included in the analyses of sensilla density or distribution.

2.3 | Sensilla trait analyses

We conducted all morphometric and statistical analyses using R v. 3.4.1 (R Core Team, 2015). We used the mesostigmal 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 was 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 ($r_{E.\ carunculatum} = 0.83$; $r_{E.\ anna} = 0.81$), so we used the mean values for these measures in our analyses.

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*: $R^2_{\text{adj}} = -0.007$, $F_{1,25} = 0.82$, $p = 0.737$; *E. carunculatum*: $R^2_{\text{adj}} = 0.01$, $F_{1,48} = 0.52$, $p = 0.47$). We thus present the results that compare sensilla counts without correcting for differences in body size.

2.4 | Sensilla spatial analyses

To quantify sensilla distributions within each plate, we generated kernel density estimates (KDEs) for populations with at least four sampled individuals (six *E. carunculatum* populations, Table 3; and two *E. anna* populations, both sympatric) 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

TABLE 1 Sampling sites for *Enallagma anna* (Ea) and *Enallagma carunculatum* (Ec) populations

Type	Site (site number[a])	Species	Latitude	Longitude	Year collected	*N*[b]	Source[c]
Sympatric	Big Spring, UT (1)	Ea	40.7407	−112.6472	2016	10	AB
		Ec				4	
	Big Sandy Creek, MT (2)	Ec	48.4519	−109.9199	2015	1	AB
	Creston, MT (3)	Ea	48.2437	−114.1406	1972	1	BM
	Dry Sheep Creek, NE (4)	Ea	41.9999	−103.9706	2012	1	BM
	Fish Springs Run, CA (5)	Ea	37.0794	−118.2539	1998	2	BM
	Grace Coolidge Creek, SD (6)	Ea	43.8072	−103.4502	1969	1	BM
	Horseshoe Springs, UT (7)	Ea	40.6203	−112.7099	2016	1	AB
		Ec				1	
	Long Valley Creek, CA (8)	Ea	39.7315	−120.0434	1973	5	DP
	Murray Creek, NV (9)	Ea	39.2669	−114.8687	2001	1	
	Malad River, UT (10)	Ec	41.8652	−112.1692	1983	2	BM
	Niwot Ditch, CO (11)	Ea	40.1632	−105.1544	2015	2	AB
		Ec				1	
	Pondera Coulee, MT (12)	Ea	48.1892	−111.3268	2015	1	AB
		Ec				1	
Locally allopatric	Beaver Creek, WY (13)	Ea	42.6417	−108.3475	2015	1	AB
	Indian Road Camp, MT (14)	Ec	46.3336	−111.5254	2015	4	AB
	Jackson, WY (15)	Ea	43.5363	−110.7629	1971	2	BM
	Muddy Creek, MT (16)	Ea	47.9796	−112.1565	2015	1	AB
	Strawberry River, UT (17)	Ec	40.1692	−110.4229	2016	1	AB
	West Greenbelt, CO (18)	Ec	39.7742	−105.1350	2014	9	AB
Allopatric	Bull Lake, MT (19)	Ec	48.2262	−115.8404	2015	1	AB
	Crab Creek, WA (20)	Ec	46.8317	−119.8431	2016	20	DP
	Clear Lake, IN (21)	Ec	41.7360	−84.8397	1945	1	BM
	Columbia River, WA (22)	Ec	45.83	−122.77	1952	2	BM
	Douglas Lake, MI (23)	Ec	45.5606	−84.6741	2016	17	OF
	Flathead River, MT (24)	Ec	47.3678	−114.5776	2015	4	AB
	Home Lake, CO (25)	Ec	37.5756	−106.0937	2015	1	AB
	Little Lake, CA (26)	Ec	35.9490	−117.9023	1967	1	DP
	Drumond Island, MI (27)	Ec	46.00	−83.66	2002	1	BM
	Snake River, ID (28)	Ec	43.7231	−112.0865	1983	2	BM

[a] Site number corresponds to the numbered locations in Figure 1.

[b] *N*: number of females that were imaged and measured for this study.

[c] Sources of damselfly specimens: A. Barnard (AB), Ola Fincke (OF), Bill Mauffray (BM), and Dennis Paulson (DP).

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.

We compared sensilla distributions among *E. carunculatum* populations using pairwise KDE tests using the function kde.test with the default settings. This test returns a *p*-value that reflects the probability of generating the two respective KDEs 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 did not compare the two sympatric *E. anna* populations, but computed their KDEs for illustrative purposes.

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 to 1,078 for *E. anna* and 688 to 1,028 for *E. carunculatum*. We standardized the number of coordinates representing each plate by retaining the points at the upper and lower medial corners and randomly sampling 198 points in between. We then treated these 200 points as landmarks (the two corners represented fixed landmarks

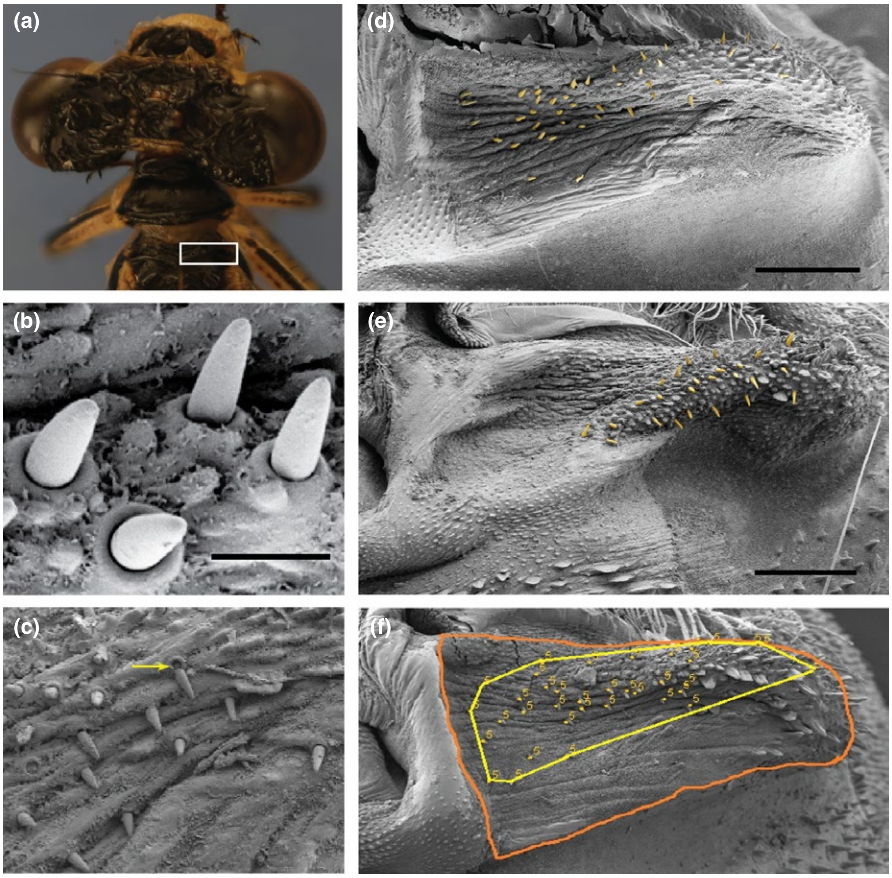

FIGURE 2 Sensilla locations. (a) White box indicates the location of right mesostigmal plate on the damselfly thorax. (b) Ultrastructural details of individual sensilla. (c) Evidence of broken sensilla. Yellow arrow indicates pore and broken-off sensillum next to it. (d, e) Scanning electron micrographs show the locations of sensilla (highlighted in yellow) on the mesostigmal plates of *Enallagma anna* (d) and *Enallagma carunculatum* (e). (f) Method to obtain (*x*, *y*) coordinates of mesostigmal plate outline and individual sensilla from scanning electron microscope images. The orange line shows the outline that represents the boundaries of the mesostigmal plate. Yellow dots indicate individual sensilla. The yellow line around the sensilla shows the polygon generated by connecting the outermost sensilla. Scale bars represent 10 μm in panel (b) and 100 μm in panels (e) and (f)

and the remaining 198 points were designated as sliding semilandmarks). We used the R package geomorph (Adams & Otarola-Castillo, 2013) to obtain an average two-dimensional plate shape for each population via general Procrustes analysis (Rohlf, 2011), which calculates a mean shape from the landmarks of a set of superimposed shapes.

2.5 | Statistical analyses

Some populations were well sampled, whereas others were represented by a single female (Table 1). To avoid pseudoreplication, for each population with $N > 1$, our analyses of sensilla number, density, and area of each mesostigmal plate covered by sensilla used the population mean of each trait value, so that each population was represented by a single measurement. For populations with $N = 1$, we treated this single value as the population mean. However, because these samples may not be representative of their respective populations, we analyzed *E. carunculatum* populations using all 19 populations, as well a separate analysis using only the six populations with $N < 4$. Both analyses yield similar results; we report the results for the analysis using all samples in the main text and provide results from the subset of samples with $N \geq 4$ in the Supporting information Table S1. We arcsin transformed proportion data prior to analysis. To compare traits between *E. anna* and *E. carunculatum*, we used Welch's *t*-tests. We compared traits among sympatric, locally allopatric, and fully allopatric *E. carunculatum* populations using Kruskal–Wallis tests, and between sympatric and locally allopatric *E. anna* populations using Welch's *t*-tests. To understand the relationships between sensilla number, sensilla density, and the area of the plate occupied by sensilla, we performed linear regressions between each pair of traits.

3 | RESULTS

3.1 | *Enallagma anna* and *E. carunculatum* females possess distinct sensilla traits

Enallagma anna females possessed significantly more sensilla per plate ($\bar{x} = 49 \pm 2$) than *E. carunculatum* females ($\bar{x} = 28 \pm 1$, $t_{35.1} = 11.13$, $p = 4.6 \times 10^{-13}$; Figure 3a). *Enallagma anna* females also possessed sensilla distributed over a larger proportion of each plate ($t_{39.7} = 11.1$, $p = 8.6 \times 10^{-14}$; Figure 3b), and larger mean distances between sensilla ($t_{54} = 6.7$, $p = 1.3 \times 10^{-8}$; Figure 3c). This ultimately results in a lower density of sensilla per unit area in *E. anna* compared to *E. carunculatum* ($t_{99.6} = -12.96$, $p = 2.2 \times 10^{-16}$; Figure 3d). The sensilla also occurred in different locations on the mesostigmal plates of each species: They were more medially located in *E. anna* and more laterally located in *E. carunculatum* (Figures 3 and 4).

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,

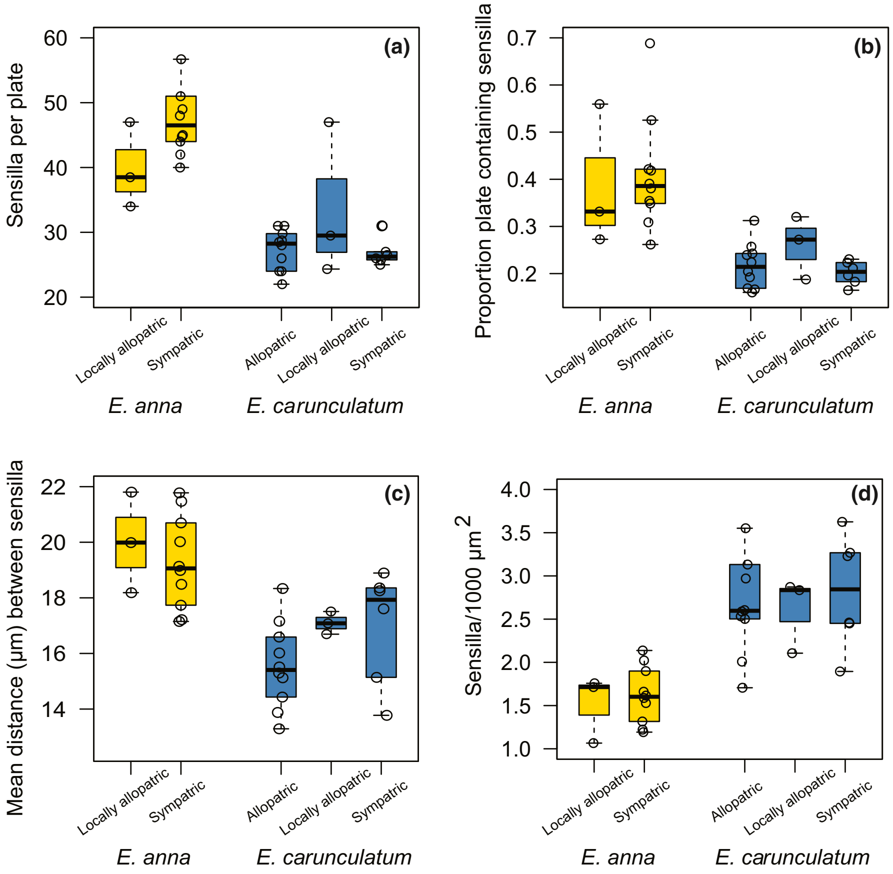

FIGURE 3 *Enallagma anna* and *Enallagma carunculatum* sensilla traits by population type. (a) The number of sensilla on one mesostigmal plate. (b) Proportion of the plate that contains sensilla. (c) Mean nearest neighbor distances between sensilla. (d) Sensilla density in the region of the plate that contains sensilla. Within each panel, each open circle represents the mean of one population or the single measured value for populations with $N = 1$. 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

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 show that a greater number of sensilla were more strongly associated with a sensilla distribution that covers a larger area of the mesostigmal plate rather than a greater concentration sensilla within in a smaller area.

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

We made several nonmutually exclusive predictions expected under RCD for the sensilla traits we measured 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: (a) more numerous sensilla, (b) denser sensilla, and (c) 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 2). 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, locally allopatric, and fully allopatric *E. carunculatum* populations did not differ significantly from one another in sensilla number (Kruskal–Wallis $\chi^2_2 = 0.69$, $p = 0.71$), proportion of the mesostigmal plate covered by sensilla (Kruskal–Wallis $\chi^2_2 = 2.16$, $p = 0.34$), or sensilla density (overall density: Kruskal–Wallis $\chi^2_2 = 0.12$, $p = 0.94$; mean distance between sensilla: Kruskal-Wallis $\chi^2_2 = 3.53$, $p = 0.17$). In addition to divergence of mean trait values, RCD can also result in reduced trait variance in sympatry without affecting the mean (Pfennig & Pfennig, 2009). Sympatric *E. carunculatum* populations displayed less interpopulation variance than allopatric populations in both mean sensilla number (Figure 3a) and mean proportion of the plate covered by sensilla (Figure 3b).

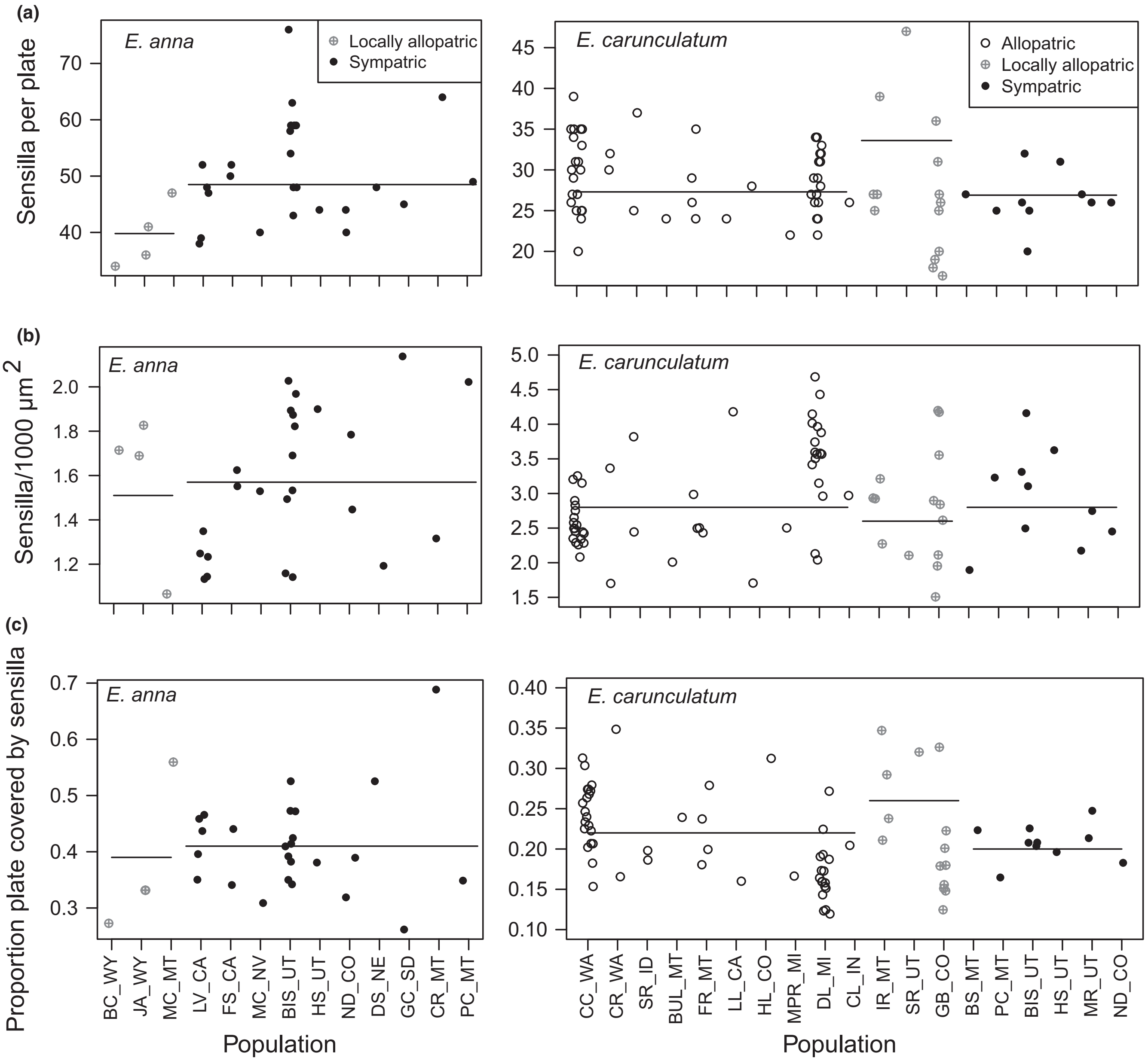

FIGURE 4 Individual trait values for sensilla number, sensilla density, and proportion of plate containing sensilla. Each symbol represents a single female, separated by population along the *y*-axis. Horizontal lines indicate the mean value for each population type (completely allopatric, locally allopatric, or sympatric), calculated from population means. Populations are described in Table 1

TABLE 2 Statistical comparison of sensilla traits in locally allopatric and sympatric *Enallagma anna* populations

	Mean ± *SEM*				
Trait	Local allopatry ($N^a = 3$)	Sympatry ($N = 10$)	*t*	*df*	*p*
Sensilla number	39.8 ± 3.8	48.5 ± 2.3	−1.93	3.6	0.13
Proportion plate containing sensilla	0.67 ± 0.27	0.69 ± 0.15	−0.25	2.8	0.82
Sensilla density (sensilla/1000 μm^2)	1.5 ± 0.2	1.6 ± 0.1	−0.43	2.9	0.70
Mean distance (μm) between sensilla pairs	20.0 ± 1.0	19.3 ± 0.5	0.62	3.13	0.58

[a] *N*: number of populations analyzed.

However, these trends were not statistically significant (sensilla number: Bartlett's $K_1^2 = 0.83$, $p = 0.36$; proportion of plate covered by sensilla: Bartlett's $K_1^2 = 1.86$ $p = 0.17$).

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 4). This pattern was also observed in the *E. anna* populations we studied.

Kernel density estimates comparisons did not reveal significant differences in sensilla distributions between sympatric and allopatric *E. carunculatum* populations (Table 3). However, the analysis revealed significant differences in sensilla distributions between several pairs of allopatric *E. carunculatum* populations (Figure 5e), which indicates that populations isolated from *E. anna* vary more among themselves than do populations sympatric with *E. anna*, which share similar sensilla patterns. The population with the most distinct sensilla distribution was in Michigan, which is geographically isolated from the other populations. Excluding this population from the analysis changed the FDR-adjusted alpha level for significance, but did not change the overall result, in which two allopatric populations in Montana and Washington still had significantly different sensilla distributions. These results are consistent with those described above that indicated higher variance in sensilla traits among allopatric populations compared to sympatric populations.

4 | DISCUSSION

Enallagma anna and *E. carunculatum* females possess 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 & Paterson, 1982), and our results show a similar pattern for two North American species. *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 likelihood that *E. anna* male cerci make greater spatial contact with the mesostigmal plates.

When species make secondary contact after initial divergence in allopatry, the possible outcomes are increased species divergence (e.g., Dyer, White, Sztepanacz, Bewick, & Rundle, 2014; Naisbit, Jiggins, & Mallet, 2001; Noor, 2000; Sætre et al., 1997; Yukilevich, 2012), decreased species divergence (e.g., Ritchie, Butlin, & Hewitt, 1992; Shurtliff, Murphy, Yeiter, & Matocq, 2002; Yang, Richards-Zawacki, Devar, & Dugas, 2016), local extinction of one species due to reproductive exclusion (Gröning & Hochkirch, 2008; Hochkirch, Gröning, & Bucker, 2007), 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 when the species are sympatric. Within each species, we predicted that female sensilla traits in sympatric populations would diverge from those of allopatric populations indicative of a shift in female preferences to avoid mating with heterospecifics. Contrary to this prediction, sympatric and allopatric *E. carunculatum* populations were not significantly different in mean sensilla trait values (Figure 3) or sensilla density distributions (Figure 5e).

Although we observed a trend toward more sensilla in sympatric *E. anna* populations relative to allopatric populations (Figures 3a and 4a), it is difficult to conduct a robust comparison for this species because *E. anna*'s entire geographic range overlaps with *E. carunculatum*'s range and *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 might, 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 usually 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 & Pfennig, 2009).

There are at least three potential explanations for the absence of RCD in the form of significant differences in the sensilla traits we

TABLE 3 Results of pairwise comparisons of sensilla kernel density estimates for *Enallagma carunculatum* populations. False discovery rate-adjusted *p*-values are reported[b]

Population	Big Springs, UT	Crab Creek, WA	Douglas Lake, MI	Flathead River, MT	West Greenbelt, CO	N[a]	Population type
Big Springs, UT	1					4	Sympatric
Crab Creek, WA	1	1				20	Allopatric
Douglas Lake, MI	0.263	**$2.53e^{-10}$**	1			17	Allopatric
Flathead River, MT	1	**0.0103**	0.263	1		4	Allopatric
West Greenbelt, CO	1	0.0625	0.3835	0.502	1	4	Locally allopatric
Indian River, MT	1	1	**0.0103**	0.0625	0.3115	4	Locally allopatric

[a] *N*: number of females whose sensilla coordinates were used to calculate KDEs.
[b] Bold values indicate $p < 0.05$.

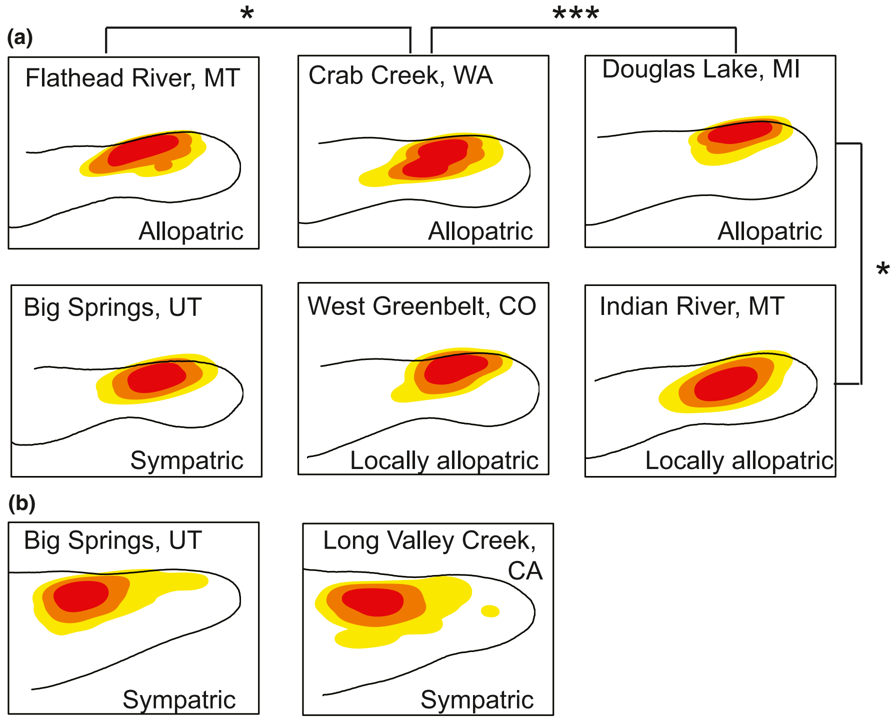

FIGURE 5 Population kernel density estimates for *E. carunculatum* (a) and *E. anna* (b) sensilla. The shading indicates different regions of sensilla density: red represents the 75–99th percentile of sensilla density, orange represents the 50–74th percentile, and yellow represents the 25th-49th percentile. Each outline represents the average mesostigmal plate shape for the population. Asterisks indicate *E. carunculatum* populations whose KDEs are significantly different ($*p < 0.05$, $***p < 0.001$).

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. Indeed, a recent study found that intraspecific variation in male cercus morphology appears too minor for *Enallagma* females to show strong discrimination among conspecific males that grasp them (Siepielski et al., 2018). Although RCD is most easily facilitated when the trait under selection already differs between species (Pfennig & Pfennig, 2009), these sensilla traits may have already diverged sufficiently enough to preclude strong selection on further divergence.

Second, it is possible that the external sensilla phenotypes we measured are not representative of proximate female sensory traits, and the variation that directs mating decisions occurs elsewhere within the female nervous system. For example, individual sensilla might differ in firing rate or sensitivity to pressure applied by the cerci. Any of these variables could differ between species or within the same species in allopatry and sympatry without noticeable differences in sensilla morphology.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 the relative frequencies with which females encounter heterospecific and conspecific males and female sexual experience (e.g., Svensson, Runemark, Verzijden, & Wellenreuther, 1989).

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 & Brayfield, 2017), then females in a population with a seemingly minor upward shift in sensilla number could gain a substantial 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. Small spatial differences within largely similar patterns may not contribute a signal large enough to be captured in a statistical test, but still reflect salient variation in the way females receive tactile stimuli. This might include three-dimensional spatial differences that we were unable to measure here. Additionally, small sample sizes from some populations and high variation within populations may have limited our ability to detect evidence of RCD. We pooled geographically widespread populations because fragmenting the samples for regional comparisons would have compromised our statistical power to detect differences between sympatry and allopatry.

These possible explanations highlight the interesting avenues that female damselfly sensilla provide for investigating the mechanisms underlying 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 (Barnard et al., 2017; McPeek et al., 2011; McPeek, Shen, & Farid, 2009; McPeek, Shen, Torrey, & Farid, 2008). Females of both species display substantial intrapopulation variation in sensilla traits (Figure 4), 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 whether and 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 & 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 long-standing presumption in the literature on genital evolution and speciation has been that female reproductive morphologies are less variant or species-specific than male genitalia (reviewed in Shapiro & Porter, 2013). However, recent studies of variation in female reproductive structures suggest that variation does exist among individuals and species (Ah-King, Barron, & Herberstein, 2014), and our data highlight the importance of looking beyond the easily quantified external morphologies. When male reproductive structure morphologies are obviously divergent, but female morphologies are not, females may possess important variation at neurophysiological levels that affect how they evaluate male tactile signals, similar to the way females evaluate signals in other sensory modalities.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR' CONTRIBUTIONS

AAB and JPM planned the study; AAB collected the data and performed the analyses; AAB and JPM wrote the paper.

DATA ACCESSIBILITY

Data associated with this manuscript have been archived in Dryad Digital Repository (provisional https://doi.org/10.5061/dryad.vf807sd).

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SUPPORTING INFORMATION

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