Species-typical chemical signals mediate reproductive isolation in a teleost fish.

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Abstract

Pheromones play an important role in conspecific mate preference across taxa, but the mechanisms underlying the pheromonal basis of reproductive isolation in vertebrates are poorly understood. In swordtail fish (genus *Xiphophorus*), conspecific mate preference depends on female perception of male urine-borne pheromone signals. We focused on interspecific differences between the sympatric *X. birchmanni* and *X. malinche,* which form natural hybrid zones as a consequence of changes in water chemistry. Here we show that pheromones are localized to the testis and use solid phase extraction followed by high performance liquid chromatography / mass spectrometry (HPLC/MS) to characterize pheromone chemical composition. Analyzing HPLC/MS readouts for pure peaks with high relative intensity identified two distinct chemical components present in *X. birchmanni* but absent in *X. malinche.* Experimental manipulation of signal composition showed that the presence of these components is critical to conspecific mate preference by *X. birchmanni*. Characterizing chemical signals allows for powerful tests of how they interact with the environment and receiver perception, thereby contributing to both the maintenance and breakdown of reproductive isolation.

Introduction

Chemical communication constitutes a widespread barrier to gene flow between species ([1-7]). This is because the large repertoire of olfactory receptors allows for specialization and diversification of signals ([8-10]). Chemoreception can thus be narrowly tuned to attend to subtle specifics of signal chemistry ([11-12]), like different isomers of the same molecule ([13-15]), to changes

in the ratios of different molecules ([9, 16-18]), and to the presence or absence of distinct components ([19-21]). These mechanisms of coupling species-typical chemoreception and chemical signal production give chemosignals a key role in speciation. This is because specific odorant receptor proteins can be narrowly tuned to species-typical chemical signals ([8,22-24]). However, some sympatric species hybridize despite chemical-based conspecific mate preference ([17,25-27]). This may be because of overlap between conspecific and heterospecific signals ([23, 28,29]), or through interactions with the chemical environment that cause interference with communication ([30-32]). Understanding the chemical basis of pheromone signals is therefore critical to understanding their evolutionary role. The chemosignals involved in reproductive isolation have been extensively characterized in a number of systems, primarily in invertebrates. Despite the importance of pheromones to mate choice and reproductive isolation in vertebrates, the mechanisms linking chemical communication to conspecific mating preference remain poorly understood.

Behavioral data indicate that chemical communication is a primary isolating mechanism between two parapatric sister species of swordtail fish (Poeciliidae), *Xiphophorus birchmanni* and *X. malinche* and that conspecific mate preference can be abolished by interference with the chemical environment ([31,34,40]). However, *Xiphophorus birchmanni* and *X. malinche* form at least six natural hybrid zones along elevation gradients in the eastern Sierra Madre Oriental in Hidalgo state, Mexico ([25,41]). To date, however, all our insight on chemical communication in *Xiphophorus* comes from the behavioral responses of females; we have had no means to quantify or characterize chemical signals. Here we use analytical chemistry techniques in combination with behavioral assays to characterize species-typical differences in chemical signal composition that are meaningful to conspecific mate preference.

Results

Female preferences

Testis (N=12, Wilcoxon signed-rank, (WSR) two tailed test, Z = 1.84, p = 0.033), but not non-reproductive tissues (N=24, WSR test, Z = 0.6418, p = 0.261), elicited a female preference over aquarium-water controls. Testis was at least as effective as water collected from live courting males, as used in previous studies (N=16, WSR test, Z = 1.51, p = 0.066, (Figure 1B), and female *X. birchmanni* strongly preferred testis of conspecifics over *X. malinche* (N=20, WSR test, Z = 1.99, p = 0.023, Figure 1A).

Eluate following solid phase extraction (SPE) was at least as effective as signals from live males at eliciting conspecific preference (N=12, WSR test, Z = 1.38, p = 0.083), and *X. birchmanni* females preferred SPE of conspecific over heterospecific males (N=14, WSR test, Z = 1.95, p = 0.026, Figure 1C).

HPLC/MS analysis

High performance liquid chromatography (HPLC) and mass spectrometry (MS) identified peaks found in male testis eluate (Figure 2). Peaks 1-4 were conserved between the two species, showing no significant difference in retention time between species (N=24 *X. birchmanni,* N=16 *X. malinche*, unpaired t-test, (Peak 1 p = 1.0, t = 0.5, Peak 2 p = 0.5782, t = 0.2891, Peak 3 p = 0.5999, t = 0.2999, Peak 4 p = 0.8589, t = 0.5705, Figure 3). Two peaks, A

and B in Figure 2, were specific to *X. birchmanni.* HPLC readouts of *X. birchmanni* and *X. malinche* show minimal variation among males within a species (Figure 3,Table1).

We measured each peak by analyzing HPLC retention time and peak area to validate peaks were consistently repeated between males (Figure 2 & 3). Next, MS fragmentation patterns and *m/z* ratio were analyzed to verify that each peak was comprised of redundant compounds. MS/MS data was used to search databases (mzcloud, the Human Metabolome Database, MassBank) for signature fragmentation and m/z patterns that identify the compounds found in HPLC peaks. We filtered data searches by making conservative assumptions about the composition of candidate compounds: 1) a natural product, 2) synthesis along the urogenital tract, 3) a semi – to highly polar molecule and 4) a stable compound with a balanced charge ([21,33]).

The internal standard *m/z* was verified by a dominant negative ion 391.28, tolerance 0.001, matching the chemical in the database. The *m/z* ratios for conserved peaks 1 and 2 were highly variable and yielded no database matches. *m/z* peak 3 showed a dominant negative ion of 512.49 and MS/MS showed a similar dominant negative ion of 512.41, tolerance 0.02. Database searches noted a similar pattern to L-tyrosine 4-hydroxyphenylalanine, an amino acid. Peak 4 showed a negative dominant ion at 514.47, and MS/MS showed two negative ions at 255.21 and 273.22, tolerance 0.01, comparable to testosterone glucuronic acid.

Adjacent to conserved peak 4, *birchmanni*-typical peak A shows a profile consistent with a possible testosterone conjugate. This peak showed two low intensity negative ions at 269.10 and 287.20 with a dominant negative ion 367.15, tolerance 0.02. Data base searches suggest a testosterone sulfate compound. The second, *m/z* peak B, showed a dominant negative ion at 407.48 and a smaller ion at 815.78, tolerance 0.03. This pattern gave an equivalent database result of a small urinary conjugated bile acid, cholanic acid.

Chemical composition and mating preference

Female *X. birchmanni* preferred the *X. malinche* signal with *X. birchmanni* peaks over the *X. birchmanni* signal with peaks removed (N=14, WSR test, Z = -1.80933, p = 0.0352, Figure 4A). These components are therefore both necessary to elicit preference for conspecifics. Females did not show a preference between signals containing peak A alone versus peak B alone (N=22, WSR test, Z = -0.4383, p = 0.3299, Figure 4B&C). Females preferred the *X. malinche* signal with peak B added (A-B+) over *X. malinche* signal (N=22, WSR test, Z = -1.769, p = 0.0383) and showed the same trend for *X. malinche* signal with peak A added (A+B-; N=19, WSR test, Z = -1.4085, p = 0.0792.) There was no difference in response relative to *X. malinche* between adding peak A and peak B (Kruskal-Wallis test, H =0.2468, p = .61937).

Discussion

We localized pheromone production to the testis (Figure 1A), which allowed us to harvest and purify concentrated samples for solid phase extraction (SPE). Analysis of reconstituted eluates with HPLC/MS showed four distinct peaks (Figure 2) shared between *X. birchmanni* and *X. malinche,* and two peaks unique to *X. birchmanni* (Figure 2). The shared peaks showed similar retention times and *m/z* patterns between *X. birchmanni* and its sister species, *X. malinche* (Figure 3). By contrast, fractions A and B were found only in *X. birchmanni.* When these species-typical fractions were removed from *X. birchmanni* signal and

added to *X. malinche* signal, females reversed their preference (Figure 4D). Testing combinations of A and B suggests that these components may combine additively to elicit conspecific mating preference ([44]; Figure 4). It is unlikely that important compounds were lost during SPE, as SPE eluate was just as effective as unextracted testis and the compounds present in peaks A and B were sufficient to reverse the preference.

Communication between senders and receivers constitutes a key mechanism of reproductive isolation ([1]). Pheromones play an important role in conspecific mate preference across taxa ([2]). This is one of the first studies to characterize the chemical signals involved in reproductive isolation among any vertebrate species. Among insects, mutations to pheromone-production pathways and to pheromone receptor proteins are sufficient to generate behavioral isolation between species ([3]). In vertebrates, chemical communication is just as important to conspecific mate preference, but we know far less about the mechanisms underlying signal production and reception ([4]).

Surprisingly, pheromone profiles in these two species appear to be highly conserved, with two additional *birchmanni*-specific components responsible for the difference in how signals are perceived by *X. birchmanni* females. An intriguing possibility is that this greater complexity may have arisen as a result of stronger selection pressures on conspecific mate preferences. Upstream headwater habitats of *X. malinche* either contain no other fishes or the distantly related *Pseudoxiphophorus jonesii,* while *X. birchmanni* co-occur both with occasional *X. malinche* migrants from upstream as well as *X. variatus.* Selection may therefore favor the evolution of stereotyped, species-typical signal components as well as preferences for these components.

Hybridization is pervasive throughout the evolutionary history of *Xiphophorus*, and early-generation hybrids are readily generated in the laboratory. Nevertheless, early-generation hybrids are extremely rare in the wild, even between hybridizing species like *X. malinche and X. birchmanni*. In most species studied, females show preferences for conspecific pheromones over those of heterospecifics, and in at least one species preferences for male pheromone cues override those for sexually-dimorphic visual traits ([34]). Hybridization between *X. malinche* and *X. birchmanni,* specifically, was likely triggered by a breakdown in pheromone-mediated communication as a result of disturbance to the chemical environment ([31]). Pheromonal communication may be a robust mechanism for reproductive isolation under typical environmental conditions, but in aquatic organisms may be uniquely susceptible to environmental interference. Understanding the nature of chemical signals will be helpful in predicting the consequences of altered communication in a changing world.

Methods

Collection and dissection

All specimens were adult males and females collected from allopatric populations using baited minnow traps. *X. birchmanni* males (*N*=24) and females (*N* =98) were collected from the Rio Garces (20°57'22 N, 098°16'48 W) and the Rio Coacuilco (21°5'50.85 N, 98°35'19.46 W). *X. malinche* (*N*=16) were collected from the Rio Xontla (20°55'27.24"N 98°34'34.50W). Males were euthanized using buffered MS-222 and kept on ice prior to dissection. In order to identify the organ of pheromone production, we dissected out kidney, liver, testis and muscle tissue. Organs were stored individually in 1mL of distilled water at -20°C.

Pheromone SPE and HPLC-MS

We purified and concentrated candidate chemical cues using solid phase extraction (SPE). Candidate tissue was suspended in 0.5 mL of distilled (DI) water and stored at -20°C prior to use. Tissue was prepared for extraction by thawing at 23°C and 300 rpm on a mixing tray. Tissue of *X. birchmanni* and *X. malinche* males and 1mL holding water was loaded into a C18 (Bond Elut-C18, 200mg, 3ml, Agilent Technologies, Wilmington, DE, USA) SPE column mounted on a vacuum manifold pressurized at 15 Pa. The C18 column was prepped with 2mL MeOH followed by 2 mL DI water. Tissue was then loaded and washed with 1mL DI water. Tissue elution was performed with 25% MeOH and DI water, 65% MeOH and 100% MeOH. We tested additional mobile phase washes: acetonitrile, methanol ethanol, hexane, and stationary phase columns: C18, C8, silica, cyanopropyl. We tested each as a pair from polar (acetonitrile/C8) to non-polar (hexane, silica). The most polar C18 and median pairing methanol, yielded the most repeatable and robust sample signal. Each eluate was captured in a separate test tube along the manifold then each eluate was split for behavior trials and HPLC. For HPLC, an internal standard of 1µg of the unconjugated bile acid chenodeoxycholic acid (CDCA; Steraloids Inc, Newport, RI, USA) was added to each of the eluates to standardize retention times and injection volumes between samples and to a blank sample of 35% DI water/65% MeOH (control). Eluates were dried under a stream of 99.9% pure nitrogen gas at 1.5 LPM (Cal Gas Inc., Huntington Beach, CA, USA), reconstituted in 2 ml MeOH /water (60/40, v/v) and stored at -20°C till HPLC/MS. The LC column (Nova-Pak reversed-phase C18, 4µm, Waters Chromatography Division, Milford, MA, USA) was coupled to a mass spectrometer with electrospray ionization (ESI; LCQ-DECA, Thermo Electron Corporation, Houston, TX, USA). The mobile phase was isocratic at 15% MeOH for 4 minutes, increased linearly from 15% to 100% MeOH from 4 to 31 minutes, and allowed to run at 100% MeOH for 5 minutes before increasing back to 15% MeOH for the next sample. Additional run times extended to an hour showed no new additional compounds in HPLC readouts. Peaks were identified if maximum relative intensity was at least 50% above background, not dependent on another compound for presence (including IS) and found in all conspecific samples found in male testis. We used unpaired *t*-tests to evaluate the variation of HPLC readouts among males within *X. birchmanni* and *X. malinche.*

We then used a PE SCIEX QSTAR (Applied Biosystems, Foster City, CA, USA) to perform secondary mass spectrometry (MS/MS) using negative and positive-ion high-resolution electrospray ionization to identify compounds. The ion trap was operated in the negative and subsequent positive-ion mode with a spray voltage of 5kV. A stream of 99% pure nitrogen at 60 Pa was used as the sheath gas. Data were collected in the range *m/z* 250–950. The relative peak areas (normalized to the area of the internal standard, CDCA) in the HPLC fractions that elicited female conspecific mate preference in behavior trials were determined with Compass Data Analysis Viewer software (2014, Bruker Daltonik GmbH, Billerica, MA, USA) in conjunction with two open access databases: *m/z* cloud and MassBank, HMDB.

Creating chimeric signals

Having identified two peaks present in *X. birchmanni* and absent in *X. malinche* (A and B, see Results), we fractioned samples based on polarity and molecular weight to create chimeric signals, removing these peaks via SPE from the *X. birchmanni* signal and adding them to *X. malinche* and verified with HPLC. We then used these chimeric signals to evaluate the role of these chemical components in eliciting preferences.

Female preference trials

Organs were pooled in groups of the same tissue type from four individuals and suspended in 500ml of distilled water 24 hours prior to trials. Tissues were pooled to account for among-male variation. Live male cue stimuli were prepared by placing four males into a single 40 L collection aquarium for 6 hours adjacent to a tank containing four conspecific females to provide them visual stimulation. To assess females' responsiveness to SPE pheromone cue, eluates were pooled in groups of four males and suspended in 500ml 24 hours prior to trials. Live male cue stimuli, described above, were used for comparison to SPE cue.

Preference trials were carried out following established methods from Fisher et al 2006, and Rosenthal et al 2011. Trials were conducted in an aquarium (75x19x20 cm) divided into 3 equally divided zones defined in the Biobserve Viewer tracking system (Bonn, Germany). Each tank had a stimulus delivery system at each end and was controlled by two peristaltic pumps (VWR Scientific, Sugarland, Texas, USA) at a rate of 5 ml/minutes. Female *X. birchmanni* were acclimatized 20 minutes prior to trials in their individual test tank lane. To control for side bias, females were tested twice, switching the sides from which cues were presented. All trials ran for 600s each and females who did not respond (NR) or visit both sides by 300s were removed from analysis. We summed the association times in the two trials for analysis. Female *X. birchmanni* were tested on each conspecific organ tissue type versus a DI water control, and then with conspecific (*X. birchmanni*) versus heterospecific (*X. malinche*) candidate tissue cue (NR=2). Trials were carried out on *X. birchmanni* females: control (blank DI water) vs. *X. birchmanni* SPE, *X. birchmanni* vs. live male cue (NR=5), conspecific (*X. birchmanni*) and heterospecific (*X. malinche*) (NR=3), chimeric interchange of *X. birchmanni* and *X. malinche* species specific peaks (NR=3), and trials using A (NR=4) and B (NR=2) peaks isolated separately, as identified from HPLC (Figure.1, 2, & 4). To control for side bias, females were tested twice, switching the sides from which cues were presented. For each comparison, we used two-tailed Wilcoxon signed-rank tests to evaluate the null hypothesis of no difference in mean association time between paired stimuli. All analyses were conducted in JMP Pro.

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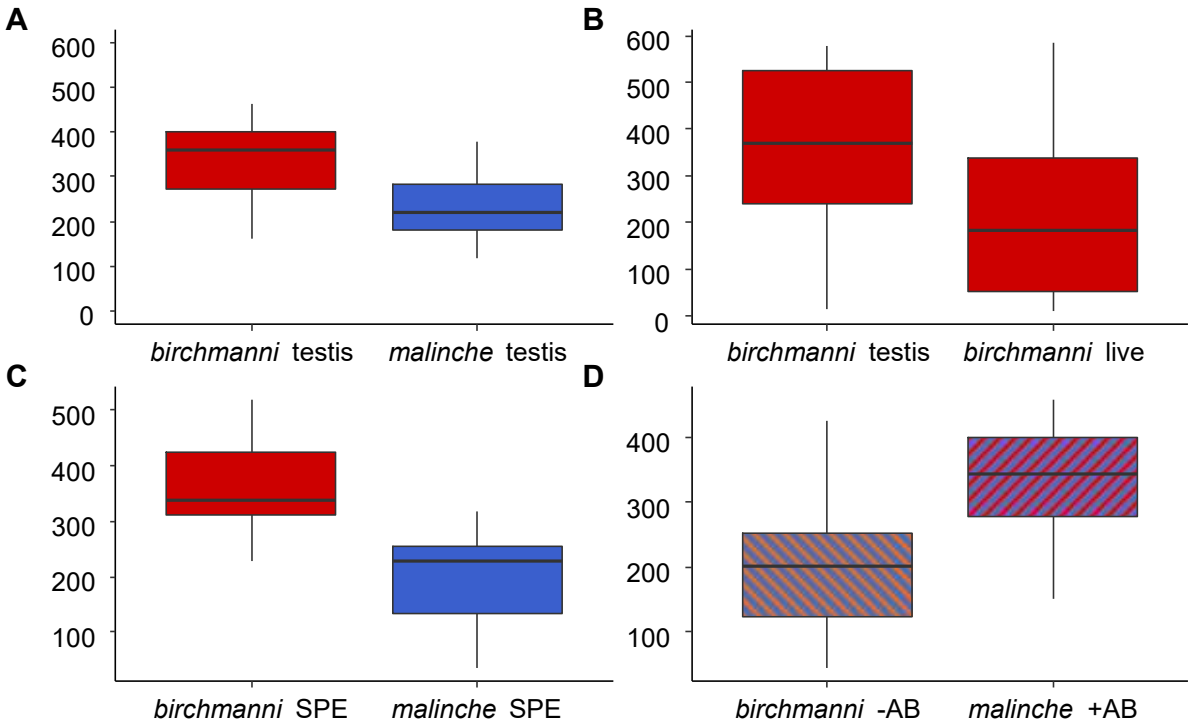

Figure 1. Association time (seconds, mean ± SEM) of female *X. birchmanni* with chemical signals. (A) Females prefer conspecific over heterospecific testis extract. (B) Testis extract is at least as attractive as cues from live male conspecifics. (C) Conspecific preference is maintained for reconstituted solid-phase extracts. (D) Chimeric interchange of *birchmanni*-typical elution peaks A and B reverses female preference.

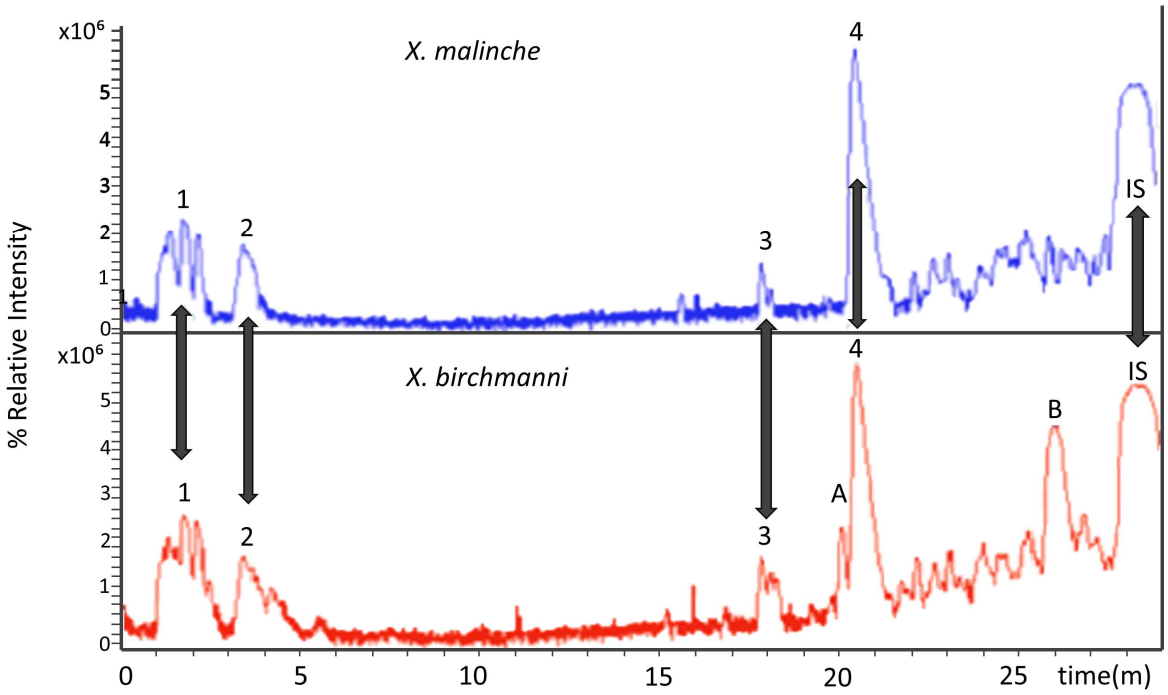

Figure 2. HPLC readouts of *X. malinche* (top) and *X. birchmanni* (bottom) SPE from testis. Peaks 1-4 are shared while A&B are found only in *X. birchmanni*. IS, internal standard, chenodeoxycholic acid.

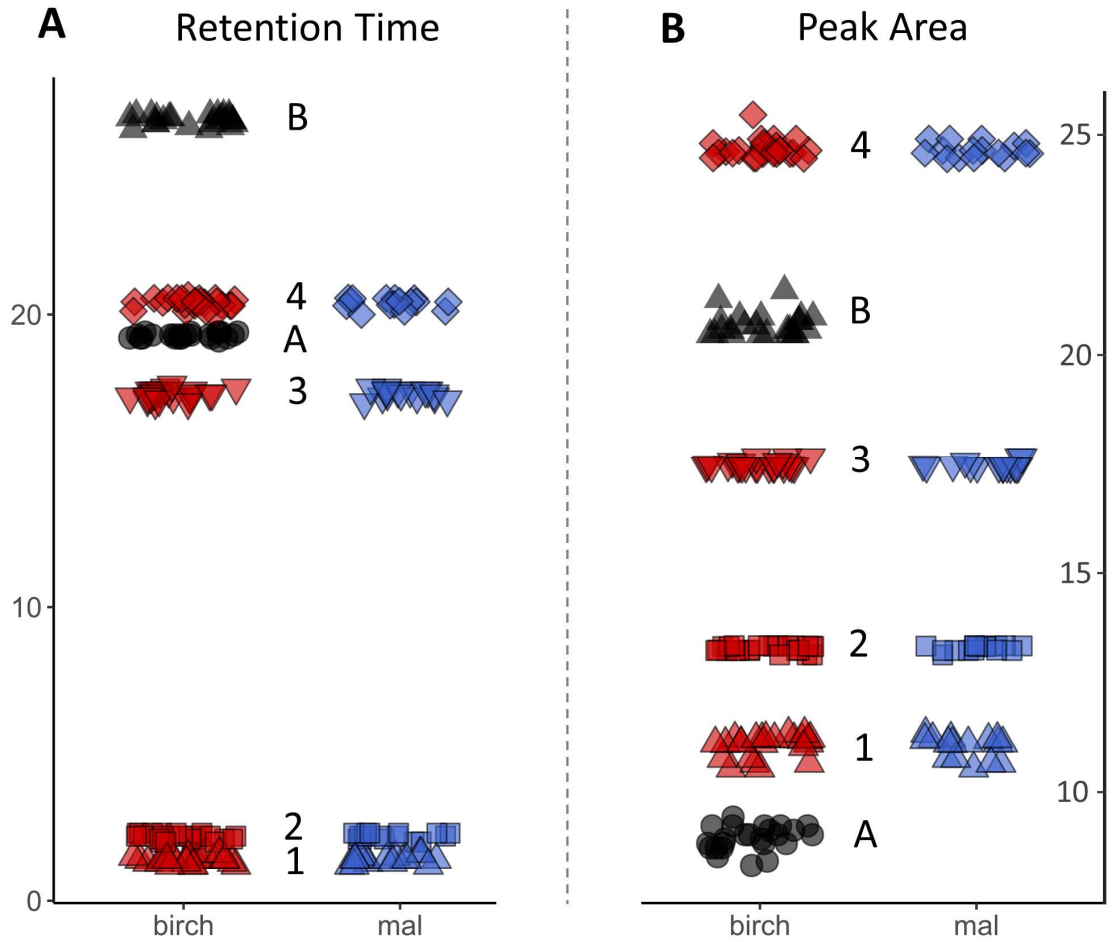

Figure 3. Scatterplot of *X. birchmanni* (red) and *X. malinche* (blue) peaks showing neither retention time (RT, minutes) nor peak area (PA, h*w) are significantly different for conserved peaks between species. Black circles are *X. birchmanni* specific peaks A & B. (Unpaired *t*-test, Peak 1 p = 1.0, $t = 0.5$, Peak 2 p = 0.5782, $t = 0.2891$, Peak 3 p = 0.5999, $t = 0.2999$, Peak 4 p = 0.8589, $t = 0.5705$).

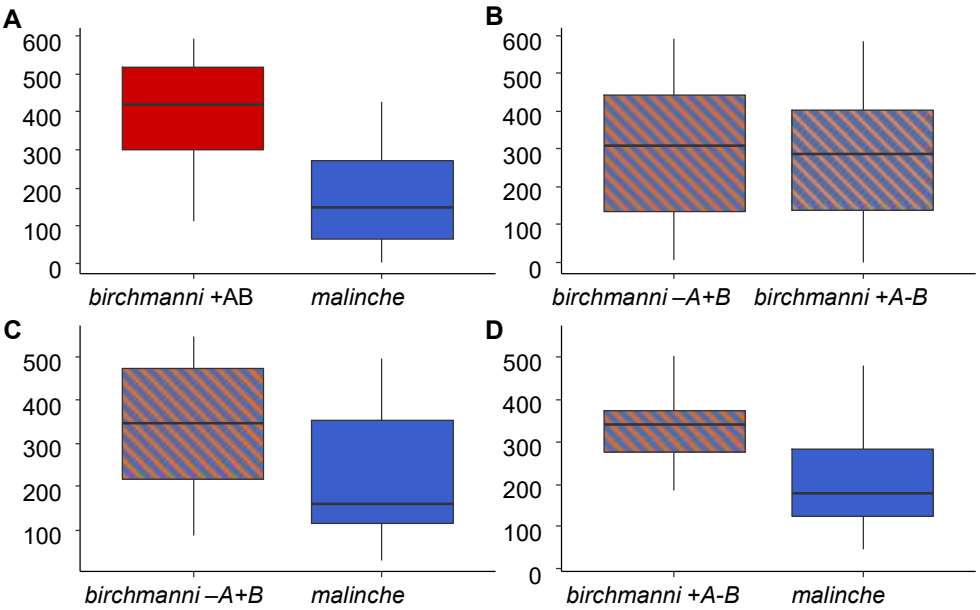

Figure 4. Additive preferences for components of conspecific signals. Association time (seconds, mean ± SEM) of female *X. birchmanni* with reconstituted solid phase extractions (SPE) of chemical signals. (A) Unmanipulated testis SPE (both *birchmanni* peaks present) elicits conspecific preferences. (B) Females do not show a preference for either peak when one is removed. (C) and (D) Females show marginal preferences for conspecific signals when either component is removed.

Table 1. HPLC analysis of *X. birchmanni (N=22) and X. malinche (N=16)* testis SPE. IS, internal standard

X. birchmanni			*X. malinche*	
Peak	Retention time (min)	Peak area	Retention time (min)	Peak area
1	1.3±0.11	10.877±0.242	1.5±0.31	10.867±0.312
2	2.1±0.18	12.995±0.113	2.3±0.19	12.845±0.143
3	17.1±0.43	17.076±0.194	17.5±0.53	17.076±0.124
A	20.1±0.32	8.336±0.261		
4	20.5±0.18	24.263±0.344	20.3±0.22	24.563±0.334
B	26.9±0.34	19.821±0.217		
IS	28.4 ±0.082	45.122±0.322	28.2 ±0.082	45.132±0.212