Individual variation in male pheromone production in carpenter bees correlates with size and sexual maturity.

ANDREW J. GOFFINET[1+], KATHY DARRAGH[1+], MADELEINE M. OSTWALD[2], STEPHEN L. BUCHMANN[3], SANTIAGO R. RAMIREZ[1*]

[1]*Department of Evolution and Ecology, University of California, Davis, CA, 95616*

[2]*Cheadle Center for Biodiversity & Ecological Restoration, University of California, Santa Barbara, CA, 93106*

[3]*Department of Ecology & Evolutionary Biology, University of Arizona, Tucson, AZ, 85721*

ORCID Kathy Darragh 0000-0002-9215-014X

ORCID Madeleine M. Ostwald 0000-0002-9869-8835

ORCID Santiago R. Ramírez 0000-0003-1306-1315

+ These authors contributed equally

*Corresponding author (Email: sanram@ucdavis.edu)

Acknowledgements

We thank Nicholas Saleh for brainstorming and giving feedback throughout the project. We also acknowledge the support of the Ramirez lab with this project.

Abstract- Sex pheromones are species-specific chemical signals that facilitate the location, identification, and selection of mating partners. These pheromones can vary between individuals, and act as signals of mate quality. Here, we investigate the variation of male pheromones in the mesosomal glands of the large carpenter bee *Xylocopa sonorina*, within a Northern California population. We tested the hypothesis that morphological traits are correlated with the observed variation in chemical blend composition of these bees. We also conducted behavioral assays to test whether these male pheromones act as long-range attractants to conspecifics. We found that larger males with darker mesosomal glands, an indicator of sexual maturity, have a higher pheromone amount in their glands. Our analysis also shows initial evidence that this pheromone blend serves as a long-range attractant to both males and females. We show that both male body size and sexual maturation are important factors influencing pheromone abundance, and that this 3-component pheromone blend acts as a long-range attractant. We hypothesize that females are more likely to mate with males releasing more pheromone, either due to increased ability to detect the blend or through female preference for pheromone abundance.

Key words- Pheromones, sexual selection, mate choice, chemical communication.

INTRODUCTION

Detection of chemical signals is considered to be amongst the most ancient and widespread of sensory systems, playing a major role in both inter- and intraspecific communication (Ache and Young 2005; Amo and Bonadonna 2018). In insects, pheromones are interspecific chemical signals, which are important for a diverse range of behaviors including aggregation (Wertheim et al. 2005), trail recruitment, and social organization (Czaczkes et al. 2015), and mating (Wyatt 2014). The chemical signals themselves can be formed of a single compound (Daimon et al. 2012) or a mix of multiple compounds (Andersen et al. 1988). A particularly well-studied group of chemical signals are those involved in mating, known as sex pheromones (Wyatt 2014). However, a less well-studied topic is what drives variation between individuals or which traits co-vary with sex pheromones.

Sex pheromones can serve a multitude of purposes during mating and mate choice. In some cases they are important for the initial step of mate attraction, acting over long ranges to attract individuals of the opposite sex (Itagaki and Conner 1988; Baker 1989). They are often species-specific signals, maintaining reproductive isolation by reducing hybridization between sympatric species (Daimon et al. 2012). They can also play a role in intraspecific mate choice, signaling mate quality to the receiver (Juárez et al. 2015). Sex pheromones are thought to act as honest signals of mate quality, as diet can impact pheromone production (Darragh et al. 2019), with males fed a higher quality diet showing increased reproductive success (Rantala et al. 2003; Liedo et al. 2013). Therefore, while pheromones are often species-specific, they also show variation both between and within populations due to genetic drift, selection, or variation in diet quality or environmental conditions (Allison and Cardé 2016).

The large carpenter bees (Apidae: *Xylocopa*) are a cosmopolitan genus of wood nesting bees, which display a range of mating behaviors and male secondary sexual traits. Ancestrally, *Xylocopa* males defend resources to gain access to females, which is correlated with small or no mesosomal glands and a lack of sexual dimorphism (Leys and Hogendoorn 2008). However, in some subgenera, males defend non-resource based territories (Minckley 1994). These species tend to have sexually dimorphic males, with males exhibiting enlarged mesosomal glands, potentially some of the largest exocrine glands in insects (Minckley 1994). These glands release long-range sex pheromones to attract females and are thought to signal indirect benefits. An especially well-

studied species in this group is *Xylocopa sonorina*, the Valley Carpenter Bee, found from California, Arizona, New Mexico, to Texas and into Sonora, Mexico, and introduced to Hawaii and various Pacific Islands. This species displays extreme sexual dimorphism, with all-black females and golden males with green eyes (Figure 1) (Marshall and Alcock 1981). In early spring, males leave their nests daily in the late afternoon, where they defend a non-resource based territory (Marshall and Alcock 1981; Alcock and Smith 1987). These are often referred to as a lek mating system (as in some birds) where one or more male carpenter bees simultaneously display to arriving females as potential mates (Marshall and Alcock 1981). Despite female interactions being relatively infrequent, the majority of these interactions do not result in copulation (Marshall and Alcock 1981; Alcock and Johnson 1990). Additionally, given that females must land to commence copulation, and that the males do not pursue unmated females, mate choice appears to be driven primarily by the female (Alcock and Smith 1987; Alcock and Johnson 1990).

Sex pheromones play an important role in male *X. sonorina* mating displays. Males choose a focal point, either a branch or a sprig of a non-flowering shrub or tree, which they scent-mark and hover above, either chasing away or flying in loops with conspecific males (Marshall and Alcock 1981; Alcock and Smith 1987). The pheromone released by males is easily detectable to human observers (Marshall and Alcock 1981), who perceive it as a distinctive rose-scent, with the primary constituent being a common compound found in rose volatiles (Dani et al. 2021). One hypothesis is that the similarity of this pheromone to floral scents could have evolved to exploit pre-existing sensory systems used to find flowers for nectar sources (Schiestl 2010). The pheromone itself is a blend of three primary compounds: all trans-geranylgeraniol, all trans-farnesal and 3,7,11-methyl-2,7,10-dodecatrienal in a 9:6:1 ratio (Andersen et al. 1988). In a previous field bioassay, the two most abundant components seem to attract females as successfully as wild males (Minckley et al. 1991). Producing the pheromone is thought to be costly as the mesosomal glands, which are lacking in females, are very large, occupying almost 20% of thoracic volume, otherwise occupied by the indirect flight muscles in other bee species (Ostwald et al. 2022). The quantity of male sex pheromone produced potentially indicates male quality, with females seeming to assess several males before making a decision (Marshall and Alcock 1981).

In this study, we investigate how the chemical profile of *Xylocopa sonorina* males varies within its population from Northern California. We analyze the mesosomal gland contents released by displaying males during their active season. We describe the pheromone blend found within the glands and identify factors which are correlated with variation in pheromone composition and amount. We also carry out field behavioral assays to test whether male pheromones serve as long range attractants for both males and females.

METHODS AND MATERIALS

Sampling

Male bees were collected between April 2020 and May 2022 while actively displaying in the late afternoon. Samples were collected throughout the city of Davis, California, USA. For a detailed list of sites, see supplementary materials (Table S1). In addition, two males were collected by hand from their nest in a log prior to the commencement of their mating season. These latter samples were excluded from the overall analysis, since these individuals had not yet reached sexual maturity. Upon collection, the samples were frozen at at -20ºC. First, we measured the intertegular distance, which we use as a proxy for body size (Cane 1987). We also recorded the wing wear (nicked wing edges), which we used as a proxy for male age (Mueller and Wolf-Mueller 1993). To dissect the thoracic pheromone gland from the mesothorax, the recently frozen bees were pinned onto an agar dissection dish, with one pin placed through the prothorax and an additional pin placed through the abdomen. Using forceps, the dorsal mesosomal cuticle was cut away, exposing the mesosomal gland below. The glands were removed from the thorax, placed onto a white microscope stage, and photographed, to measure their color.

Coloration Analysis

The Fiji distribution of ImageJ was used to collect color data (Schindelin et al. 2012). In each photo, the gland was hand selected, five random points were chosen across the gland, and across these five points, the program provided both the RGB (red-green-blue color model) values and the HSB (hue-saturation-brightness color model) values for the image. These values were then averaged. To assess color, a ratio of R (red) and G (green) was calculated, as the different color values are only meaningful relative to the other values, and this ratio was used in our analysis (Bergman and Beehner 2008).

Chemical Analysis

Each thoracic gland was placed in a glass vial containing 500µl hexane with 5µg of 2-undecanone as an internal standard and solvent extracted for one hour. Following this, the solvent was transferred to a new vial, and stored at -20°C. Samples were analyzed using an Agilent model 5977A mass-selective detector connected to Agilent GC model 7890B, with a HP-5 Ultra Inert column (Agilent, 117 30 m × 0.25 mm, 0.25 µm). 1µl of each sample was injected using Agilent ALS 7694 autosampler in split mode with a 5:1 ratio with helium as the carrier gas (250°C injector temperature, split flow of 3.5 ml/min). The temperature program started at 80°C for 3 minutes, and then rose at 10°C/min to 300°C. The temperature was held at 300°C for 3 minutes and 315°C for 3 minutes. We quantified components using an internal standard of 2-undecanone, and compounds not found in at least 10% of individuals were disregarded.

Behavioral Assay

We conducted field behavioral bioassays in the spring of 2023 at a pair of test sites in the UC Davis Arboretum (38.5328, -121.7547). The sites were 10 meters apart, on the same species of shrubby plant. On each study day, a coin was flipped to randomly assign one of two treatments to each site. During the study, each site had qualitative cellulose filter paper disc, with a diameter of 9 cm, suspended from a branch of the shrub approximately 1.5 meters off the ground using an odorless, green lab tape. To create the experimental mixture, we used a mixture of the actual extracts from the top 75% of males in terms of total pheromone abundance (n=30) from the samples included in this study. We applied 1ml of this mixture to the filter paper in the field. The second treatment was a control, which consisted of 1 ml of pure hexane, also applied to the filter paper discs in the field.

Following the application of the two treatments, the site was observed for the following 60 minutes and all interactions from both male and female bees were recorded. Interactions were counted if an individual altered their flight path and approached the treated papers within 1 meter. We also noted if a “strong” interaction occurred, in which case the individual stopped and hovered within 0.5 meter of the filter paper for any length of time. Initially, observations took place from 16:00-17:00 in the afternoon, coinciding with male *X. sonorina* activity. However, throughout the season, this shifted back to 16:30-17:30 and eventually 17:00-18:00, following the observed male display behavior, which progressed later back into the afternoon as the season

advanced (Table S7). The assays commenced in mid-May and continued until the end of June. Previous observations found that male activity only happens at higher temperatures (>26.5ºC). We, therefore, only carried out the assay on days with temperatures high enough to observe males displaying on nearby plants.

Statistical Analysis: Variation in Compound Amount

Firstly, we were interested in determining the factors driving variation in total compound amount produced by the thoracic gland. We tested the effects of intertegular distance, wing wear, and various characteristics of the glands appearance, including color (R:G ratio), hue, saturation, and brightness, on total compound abundance, using ANOVA. We then evaluated our model fit with Akaike's information criteria (AIC) using the *AICcmodavg* package (Mazerolle 2020). The model with the lowest AIC score was chosen as the final model, containing the most likely explanatory variables.

Statistical Analysis: Variation in Chemical Profile Composition

The univariate analysis only includes total compound amount and does not consider *which compounds are present in the chemical profile. To include this information in the analysis,* we carried out a multivariate analysis to analyze the chemical mixture of the males, taking all the compounds into account. We visualized divergence across the samples using nonmetric multidimensional scaling (NMDS) in three dimensions, based on a Bray–Curtis similarity matrix created using absolute peak areas. We created the NMDS with the "metaMDS" function in *vegan,* and this was plotted using the *ade4* package (Thioulouse et al. 2018; Oksanen et al. 2022).

To investigate factors that drove pheromone composition, we used a PERMANOVA (permutational multivariate analysis of variance) on a Bray-Curtis similarity matrix, using the "adonis2" function in *vegan* with 1000 permutations (Oksanen et al. 2022). We assessed each term individually using the option by="margin", this ensures that the order of terms in the model does not affect the results (in contrast to the by="sequential" option where each term is considered in order). Model fit was evaluated using AIC scores, with each model being tested sequentially. The model with the lowest AIC score was selected as the final model and identified as the model containing the explanatory factors. However, we chose the simplest model as the best fit if we

found that two models were within two AIC values of each other. We also repeated this analysis with the relative compound abundances.

Statistical Analysis: Variation in the Most Abundant Compounds

The chemical profile of the bees is dominated by three main compounds, as described previously (Andersen et al. 1988). These compounds appear to be biologically important to the reproduction of *X. sonorina*, since an artificial mixture of two of these compounds, all trans-farnesal and all trans-geranylgeraniol, seems to be sufficient to attract females (Minckley et al. 1991). Therefore, we re-analyzed our data including just these three primary compounds to determine which factors drive their abundance and composition as described for the analysis of the entire dataset.

Plotting and Data Manipulation

To make plots we used the following packages: *ggpubr* (Kassambara 2020), *cowplot* (Wilke 2020) and *ggplot2* (Wickham 2016). In addition we used *tidyr* (Wickham and Girlich 2022), *plyr* (Wickham 2011), and *readr* (Wickham et al. 2023) for data transformation. Analyses were carried out in R version 4.2.1 (R Core Team 2022).

Behavioral Assay Analysis

To determine if the likelihood of interaction was affected by either sex of the individuals or treatment (male gland extract vs. hexane control), we used a binomial GLM (generalized linear model), with the package *MASS* (Venables and Ripley 2002). The response variable was whether at least one interaction was observed in a given trial. We included sex and treatment as explanatory variables as well as an interaction term between the two. To test significance of factors, we used likelihood ratio tests on models with and without the variable of interest.

All raw data and analyses are available on the OSF (https://osf.io/uf87d/?view_only=ab07af2f3c4e4c05a5f9f5fad680dd64).

RESULTS

We sampled a total of 40 males and identified 43 different compounds in their chemical profile. On average, 284.5±168.7 μg of total compound were present in each sample.

Across all the samples, the three main compounds described by prior studies in an Arizona population were also found in the Davis population, with all trans-geranylgeraniol, all trans-farnesal, and 3,7,11-trimethyl-2,7,10-dodecatrienal being the three most abundant compounds (Figure 1, Panel B, labels 1, 2, 3) (Andersen et al. 1988).

In addition to the displaying males, we also sampled two adult males which were collected from their nest in a log prior to the start of the mating season. Upon dissection, we observed that these males had extremely pale, almost white mesosomal glands, and when chemically analyzed, these males had an average total compound amount of 2.08±1.1 ug. Additionally, these males lacked the three main compounds seen in the actively displaying males.

Variation in Compound Amount

To investigate the impact of different factors on total compound amount, we carried out a univariate ANOVA analysis. We found that both brightness and size were significantly related to the total amount of compound (Brightness: ANOVA, $F_{1,36}$= 10.99, p=0.002094; Size: ANOVA, $F_{1,36}$=14.02, p=0.000632) (Figure 2, Table S2). We found that males with brighter glands have an overall lower total compound amount. Similarly, smaller males, measured by their intertegular distance, produce a lower total compound amount. These results agree with similar findings about the glands progressing through a pre-secretory (lightest = brightest color) phase, then into an active secretor phase, and finally a dark reddish brown storage phase ((Ostwald et al. 2022); Buchmann, unpubl.).

Variation in Chemical Profile Composition

To identify which factors drive overall compositional differences in the chemical profile of male glands, we carried out a multivariate PERMANOVA analysis. As in the univariate analysis, the best model included both brightness (PERMANOVA, Brightness, $F_{1,36}$ = 5.1835, p =0.001998) and size (PERMANOVA, Size, $F_{1,36}$ = 7.5555, p =0.000999) (Figure 3, Table S3). We found that size accounts for 16% of observed variation in chemical profile, with a further 11% explained by gland brightness.

When we reanalyzed the data using relative compounds abundances, we found that none of our variables explained variation in chemical profile (Table S4). This indicates that the relative

ratios of the pheromone's components remain consistent and are not dependent on body size or color of the dissected gland.

Variation in the Most Abundant Compounds

The prior two analyses included every compound which was present in at least 10% of sampled males. However, prior studies have identified three main compounds, two of which can attract females, and so we re-ran the previous analyses on a reduced dataset of these three main compounds (Andersen et al. 1988; Minckley et al. 1991). Combined, these three main compounds made up an average of 250.9 ± 157.7 μg in each male gland, which is 86.4% ± 6.02% of total gland pheromone amount.

We found that both brightness and size affected the total amount of compound (Brightness: ANOVA, $F(_{1,36})$= 11.32, p=0.001832; Size: ANOVA, $F(_{1,36})$=13.66, p=0.000724)(Figure S1, Table S5). These results were coincident with the entire chemical blend, where we found that males with brighter glands had lower amounts of the three main compounds, while smaller males also had lower amounts of the same three compounds.

To identify which factors drive overall compositional differences in the three main compounds, we carried out a PERMANOVA analysis. The best model fit again included both brightness (PERMANOVA, Brightness, $F_{1,36}$ = 5.1614, p =0.003996) and male bee size (PERMANOVA, Size, $F_{1,36}$ = 7.9076, p =0.000999), with size explaining 17% of observed variation in the three main compounds, and brightness explaining a further 11% (Figure S2, Table S6).

Behavioral Assay Results

In total, we conducted 14 behavioral field trials (bioassays), and interactions by either males or females occurred in 13 of the 14 trials, resulting in 37 total interactions (Table 1, Table S7). Females interacted with the control treatment on 4/14 days, while males interacted with the control on 1/14 days. These numbers were much higher for the filter paper treated with extract, with females interacting 11/14 days, and males 8/14 days. Treatment was found to significantly explain the likelihood of interaction (2ΔlnL= 16.016, d.f.=1, p=0.000063).

266 We also observed what we have termed "strong responses". These consist of individuals
267 stopping and hovering within 0.5 meters of the filter paper. This occurred on 3 separate days for
268 the males, and 5 separate days for the females, and was observed solely on the filter paper with
269 the male's extract applied, while this hovering behavior did not occur towards the control (Table
270 S7).

271 DISCUSSION

272 *Xylocopa sonorina* males release large amounts of their sex pheromone during mating
273 displays which are detectable to humans. Here, we investigated the chemical ecology of *X.*
274 *sonorina*, describing pheromone variation within a population in California and identified
275 morphological factors which could explain the observed variation. As previously identified in
276 Arizona individuals, we found that the three main constituents of this gland were all trans-
277 geranylgeraniol, all trans-farnesal, and an isomer of 3,7,11-trimethyl-2,7,10-dodecatrienal
278 (Andersen et al. 1988). We show that larger males with darker gland coloration (*i.e.* mature glands)
279 have more total pheromone in their glands. We found that these same factors (size and gland
280 brightness) also affect the overall chemical profile of the mesosomal gland. Our results were
281 consistent when focusing on just the three primary compounds, which have previously been
282 found to have been sufficient in attracting females (Minckley et al. 1991). However, we did not
283 find any factors which explained variation in relative compound amounts, suggesting that these
284 effects on variation are due to changes in compound amount rather than compositional changes
285 of the chemical profile.

286 We found that male size explained the most variation in terms of both total compound
287 amount and chemical profile variation of *X. sonorina*. In bees, as with other holometabolous
288 insects, the male's adult body size is determined during the larval stage, and is heavily impacted
289 by both abiotic (temperature, latitude, altitude, season) and biotic (primarily diet) factors (Chole
290 et al. 2019). Larger male body size is thought to signal a higher quality diet during development,
291 which corresponds to increased mating success (Agosta 2010). We found that larger *X. sonorina*
292 produce more sex pheromone, which we hypothesize could be related to a higher mating success,
293 as seen across a range of insects. For example, in the Tobacco Moth, *Ephestia elutella*, larger
294 males produce more pheromone when compared to smaller males, and these pairings result in
295 larger offspring (Phelan and Baker 1986). This is also observed across the order Hymenoptera. In

Siricid sawflies, larger males had increased mating success when compared to smaller individuals (Lu et al. 2022). However, the relationship between pheromone production and female choice is not always simple. In Ptermalid wasps, larger males release greater amounts of pheromones when compared to smaller males, and females are preferentially attracted to this increased level of pheromone. However in direct competition, females showed no preference for larger or smaller males, indicating that while body size plays a role in female attraction, it is not the only factor affecting female choice (Blaul and Ruther 2012). We hypothesize that larger male *X. sonorina* which produce a greater abundance of their sex pheromones could allow these larger males to attract females from a greater distance than smaller males. However, further work is required to discern if females show a preference for these larger males.

We also found that male sexual maturity affected pheromone amount and chemical profile composition. Individuals are predicted to mate with older, more experienced mates as this indicates their increased ability to survive longer (Brooks 2001). In some cases, age could be signaled by sex pheromones, ensuring that mating occurs in the window between sexual maturation and senescence (Gomez-Diaz, Carolina and Benton, Richard 2013). In the butterfly *Bicyclus anynana*, the male's sex pheromone composition changes as they age, and females exhibit preferences for the pheromones of middle aged males (Nieberding et al. 2012), with similar patterns documented in other species (Lassance and Löfstedt 2009; Chemnitz et al. 2015). We did not find a relationship between male *X. sonorina* sex pheromone and age when using wing wear as a proxy; however this may be a poor indicator of age due to variability in wing degradation (Eltz et al. 1999). We decided to use the color of the gland as a proxy for male maturation as previous work on *X. sonorina* has found the gland is initially light in color and subsequently darkens into a sclerotized storage organ with many hair lined tubules (Minckley et al. 1991; Ostwald et al. 2022). During our dissections, we observed a range of colors of the mesosomal glands, ranging from near white in some that were collected prior to the commencement of the males display season to very dark red-brown in some displaying males. The two males collected from their nests before their display season began had nearly white mesosomal gland tissues containing only trace amounts of pheromone. This contrasted with the actively displaying males we collected, which always had darker glands. We found that the brightness of the gland correlates with pheromone amount, with darker glands containing higher amounts of pheromone, in

agreement with the prediction that older, more sexually mature males have higher pheromone amounts.

The effect of higher sex pheromone production by larger, more sexually mature male *X. sonorina* on female choice is unclear. Two ways in which sex pheromone production could impact female choice are through the effectiveness of the pheromone as a long-range attractant or as an indicator of mate quality. Based on our behavioral assays, we present preliminary evidence that these compounds serve as a long-range attractant for both males and females. In hymenopterans, it has long been accepted that sex pheromones serve as important facilitators of sexual reproduction by acting as long-range attractants (Ayasse et al. 2001). Within *X. sonorina,* an early study showed that the primary constituents from the male gland seemed to be sufficient to attract females (Minckley et al. 1991). The results from our behavioral assay provide further evidence to support those observations.

Nonetheless, this does not discount the possibility that females are assessing male quality through their sex pheromones. *Xylocopa sonorina* exhibits a non-resource-based mating system. This is in contrast to the mating system of two other *Xylocopa* species found in California, *X. tabaniformis* and *X. californica.* In these species, males defend resource-based territories, have enlarged eyes and lack the large mesosomal glands found in *X. sonorina.* This suggests that as *Xylocopa* bees shifted from a resource based, territorial mating strategy to a non-resource-based mating strategy, they shifted from a visual based mating system to one that is facilitated by pheromones. The mating system seen in *X. sonorina* is also very similar to the non-resource-based display that is exhibited by orchid bees (Apidae: *Euglossa*). In this genus of bees, males collect volatile organic compounds from a variety of sources, which they store and later display at a central perch site, where they release their chemical mixture to attract females (Eltz et al. 2003, 2005; Pokorny et al. 2017). In the *X. sonorina* system, females must land for mating to commence (Marshall and Alcock 1981), and mating occurs relatively infrequently, with only 24% of female interactions with displaying males resulting in copulation (Alcock and Johnson 1990). In both orchid bees and *X. sonorina*, it appears that females are assessing multiple males, often in a lekking display, with status communicated through chemical signals. However, further studies are required to test this hypothesis and determine the effect that the male's mesosomal glands play in the female's assessment of multiple males along a display site.

While males do engage in interactions with conspecific males, these interactions primarily consist of chasing off rival males, and physical contact is extremely rare (Alcock and Smith 1987). The pheromones released by displaying males provides the required information to females to select their mates. This is contrasted with what has been seen in several other bees. In several subgenera of *Xylocopa,* males defend a territory which contains resources for females (Leys and Hogendoorn 2008). This ancestral behavior is correlated with small mesosomal glands, enlarged eyes and the lack of sexual dimorphism (Leys and Hogendoorn 2008). The two other *Xylocopa* species found in California, *X. tabaniformis* and *X. californica* both defend resource-based territories and lack sexual dimorphism, have enlarged eyes, and non-enlarged mesosomal glands (Leys and Hogendoorn 2008). The lack of large mesosomal glands and enlarged eyes suggests that vision is an important factor, with males chasing off conspecific males and slowly pursuing potential females (Frankie et al. 1979; Minckley 1994). There is also little evidence of pheromone involvement in the mesosomal reservoirs of *Xylocopa* species with "type 1" glands, the smallest class of mesosomal glands (Minckley 1994). This suggests that as *Xylocopa* bees shifted from a resource based, territorial mating strategy to a non-resource based mating strategy, they shifted from a visual based mating system to one that is facilitated by pheromones. The mating system seen in *X. sonorina* is also very similar to the symbolic (non-resource based) display that is exhibited by orchid bees (Apidae: *Euglossa*). In this genus of bees, males collect volatile organic compounds from a variety of sources, which they store and later display at a central perch site, where they release their chemical mixture to attract females (Eltz et al. 2003, 2005; Pokorny et al. 2017). This similarity suggests that similar mating systems, being primarily facilitated by pheromones, exists across the broader group of bees.

As well as being used by females to locate and assess mate quality, sex pheromones may also be used to facilitate male-male interactions. When conspecific, displaying *X. sonorina* males encounter each other at the same display location, they may exhibit one of two different behavioral responses. Sometimes, one male will emerge dominant and chase off their competitor, while in other cases, up to several males may share a display site, flying in loose circles around each other and the focal point for some length of time (Alcock and Smith 1987). Further studies are needed to determine if this variation in behavior is reflected in the chemistry of the pheromones released by displaying male *X. sonorina*. For example, electroantennogram (EAG) laboratory assays on both male and female antennae using individual pheromone components and natural

ratio 3-component blends would be especially informative. Based on results from our behavioral assay, we provide evidence that male *X. sonorina* are attracted to the mesosomal chemical blend released by males during their mating display. Additionally, similar to what has been described in other hymenopterans, such as sphecid (Coelho and Holliday 2001) and pompilid wasps (Alcock and Kemp 2006), larger males may be better fliers or a have a better ability to defend their territory. In the colletid bee, *Hylaeus alcyoneus*, larger males are more successful in male-male interactions, with them securing higher up perch sites, which corresponds to increased mating success (Alcock 1994). While male pheromones were not described in this study, the author suspected that these interactions may be facilitated by pheromone release based on male behavior, with males marking nearby branches and whirring their wings. *Xylocopa sonorina* may provide a viable system to investigate how sex pheromones facilitate male-male interactions during their complex mating displays.

Our study reveals variation in sex pheromone amounts (total glandular volumes), but not relative ratios, are correlated with both body size and sexual maturity in male *X. sonorina*. Larger, more sexually mature males produce higher amounts of sex pheromone. In addition, we show preliminary evidence that this blend of male pheromones serves as a long-range attractant *to both the opposite and same sex. Future studies could clarify the fitness consequences of* variation in pheromone amount, whether by impacting attraction range, female choice, or male-male competitive interactions.

REFERENCES

Ache BW, Young JM (2005) Olfaction: Diverse Species, Conserved Principles. Neuron 48:417–430. https://doi.org/10.1016/j.neuron.2005.10.022

Agosta SJ (2010) Male Body Size and Mating Success and Their Relation to Larval Host Plant History in the Moth *Rothschildia lebeau* in Costa Rican Dry Forest: Body Size and Mating Success. Biotropica 42:201–207. https://doi.org/10.1111/j.1744-7429.2009.00556.x

Alcock J (1994) Body size and its effect on male-male competition in *Hylaeus alcyoneus* (Hymenoptera: Colletidae). J Insect Behav 8:149–159. https://doi.org/10.1007/BF01988901

Alcock J, Johnson MD (1990) Female mate choice in the carpenter bee *Xylocopa varipuncta* (Hymenoptera: Anthophoridae). J Zool 221:195–204. https://doi.org/10.1111/j.1469-7998.1990.tb03991.x

Alcock J, Kemp DJ (2006) The Behavioral Significance of Male Body Size in the Tarantula Hawk Wasp *Hemipepsis ustulata* (Hymenoptera: Pompilidae). Ethology 112:691–698. https://doi.org/10.1111/j.1439-0310.2006.01204.x

Alcock J, Smith AP (1987) Hilltopping, leks and female choice in the Carpenter bee *Xylocopa (Neoxylocopa) varipuncta*. J Zool 211:1–10. https://doi.org/10.1111/j.1469-7998.1987.tb07448.x

Allison JD, Cardé RT (eds) (2016) Pheromone communication in moths: evolution, behavior, and application. University of California Press, Oakland, California

Amo L, Bonadonna F (2018) Editorial: The Importance of Olfaction in Intra- and Interspecific Communication. Front Ecol Evol 6:71. https://doi.org/10.3389/fevo.2018.00071

Andersen JF, Buchmann SL, Weisleder D, et al (1988) Identification of thoracic gland constituents from male *Xylocopa spp. latreille* (Hymenoptera: Anthophoridae) from Arizona. J Chem Ecol 14:1153–1162. https://doi.org/10.1007/BF01019343

Ayasse M, Paxton RJ, Tengö J (2001) Mating Behavior and Chemical Comminication in the order Hymenoptera. Annu Rev Entomol 46:31–78. https://doi.org/10.1146/annurev.ento.46.1.31

Baker TC (1989) Sex pheromone communication in the lepidoptera: New research progress. Experientia 45:248–262. https://doi.org/10.1007/BF01951811

Bergman TJ, Beehner JC (2008) A simple method for measuring colour in wild animals: validation and use on chest patch colour in geladas (*Theropithecus gelada*): MEASURING COLOUR IN WILD ANIMALS. Biol J Linn Soc 94:231–240. https://doi.org/10.1111/j.1095-8312.2008.00981.x

Blaul B, Ruther J (2012) Body size influences male pheromone signals but not the outcome of mating contests in *Nasonia vitripennis*. Anim Behav 84:1557–1563. https://doi.org/10.1016/j.anbehav.2012.10.001

Brooks R (2001) Can older males deliver the good genes? Trends Ecol Evol 16:308–313. https://doi.org/10.1016/S0169-5347(01)02147-4

Cane (1987) Estimation of Bee Size Using Intertegular Span (Apoidea). J Kans Entomol Soc 60:145–147

Chemnitz J, Jentschke PC, Ayasse M, Steiger S (2015) Beyond species recognition: somatic state affects long-distance sex pheromone communication. Proc R Soc B Biol Sci 282:20150832. https://doi.org/10.1098/rspb.2015.0832

Chole H, Woodard SH, Bloch G (2019) Body size variation in bees: regulation, mechanisms, and relationship to social organization. Curr Opin Insect Sci 35:77–87. https://doi.org/10.1016/j.cois.2019.07.006

Coelho JR, Holliday CW (2001) Effects of Size and Flight Performance on Intermale Mate Competition in the Cicada Killer, *Sphecius speciosus* Drury (Hymenoptera: Sphecidae). J Insect Behav

Czaczkes TJ, Grüter C, Ratnieks FLW (2015) Trail Pheromones: An Integrative View of Their Role in Social Insect Colony Organization. Annu Rev Entomol 60:581–599. https://doi.org/10.1146/annurev-ento-010814-020627

Daimon T, Fujii T, Fujii T, et al (2012) Reinvestigation of the Sex Pheromone of the Wild Silk Moth *Bombyx mandarina* : The Effects of Bombykal and Bombykyl Acetate. J Chem Ecol 38:1031–1035. https://doi.org/10.1007/s10886-012-0164-0

Dani KGS, Fineschi S, Michelozzi M, et al (2021) Diversification of petal monoterpene profiles during floral development and senescence in wild roses: relationships among geraniol content, petal colour, and floral lifespan. Oecologia 197:957–969. https://doi.org/10.1007/s00442-020-04710-z

Darragh K, Byers KJRP, Merrill RM, et al (2019) Male pheromone composition depends on larval but not adult diet in *Heliconius melpomene*. Ecol Entomol 44:397–405. https://doi.org/10.1111/een.12716

Eltz T, Roubik DW, Whitten MW (2003) Fragrances, male display and mating behaviour of *Euglossa hemichlora*: a flight cage experiment. Physiol Entomol 28:251–260. https://doi.org/10.1111/j.1365-3032.2003.00340.x

Eltz T, Sager A, Lunau K (2005) Juggling with volatiles: exposure of perfumes by displaying male orchid bees. J Comp Physiol A 191:575–581. https://doi.org/10.1007/s00359-005-0603-2

Eltz T, Whitten WM, Roubik DW, Linsenmair KE (1999) Fragrance Collection, Storage, and Accumulation by Individual Male Orchid Bees. J Chem Ecol 25:157–176

Frankie GW, Vinson SB, Lewis (1979) Territorial Behavior in Male *Xylocopa micans* (Hymenoptera: Anthophoridae). J Kans Entomol Soc 52:313–323

Gomez-Diaz, Carolina, Benton, Richard (2013) The joy of sex pheromones. Eur Mol Biol Organ 14:874–883. https://doi.org/10.1038/embor.2013.140

Itagaki H, Conner WE (1988) Calling Behavior of *Manduca sexta* (L.) (Lepidoptera: Sphingidae) with Notes on the Morphology of the Female Sex Pheromone Gland. Ann Entomol Soc Am 81:798–807. https://doi.org/10.1093/aesa/81.5.798

Juárez ML, Devescovi F, Břízová R, et al (2015) Evaluating mating compatibility within fruit fly cryptic species complexes and the potential role of sex pheromones in pre-mating isolation. ZooKeys 540:125–155. https://doi.org/10.3897/zookeys.540.6133

Kassambara A (2020) ggpubr: “ggplot2” Based Publication Ready Plots

Lassance J-M, Löfstedt C (2009) Concerted evolution of male and female display traits in the European corn borer, *Ostrinia nubilalis* . BMC Biol 7:10. https://doi.org/10.1186/1741-7007-7-10

Leys R, Hogendoorn K (2008) Correlated evolution of mating behaviour and morphology in large carpenter bees (*Xylocopa*). Apidologie 39:119–132. https://doi.org/10.1051/apido:2007044

Liedo P, Orozco D, Cruz-López L, et al (2013) Effect of post-teneral diets on the performance of sterile *Anastrepha ludens* and *Anastrepha obliqua* fruit flies: Pre-release feeding on sterile *Anastrepha* male performance. J Appl Entomol 137:49–60. https://doi.org/10.1111/j.1439-0418.2010.01568.x

Lu P, Hao E, Bao M, et al (2022) Mating Behavior and Identification of Male-Produced Pheromone Components in Two Woodwasps, *Sirex noctilio* and *Sirex nitobei* , in China. Insects 13:966. https://doi.org/10.3390/insects13100966

Marshall LD, Alcock J (1981) The evolution of the mating system of the Carpenter bee *Xylocopa varipuncta* (Hymenoptera: Anthophoridae). J Zool 193:315–324. https://doi.org/10.1111/j.1469-7998.1981.tb03447.x

Mazerolle MJ (2020) AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c)

Minckley (1994) Comparative morphology of the mesosomal 'gland' in male large carpenter bees (Apidae: Xylocopini). Biol J Linn Soc 53:291–308. https://doi.org/10.1111/j.1095-8312.1994.tb01014.x

Minckley RL, Buchmann SL, Wcislo WT (1991) Bioassay evidence for a sex attractant pheromone in the large carpenter bee, *Xylocopa varipuncta* (Anthophoridae: Hymenoptera). J Zool 224:285–291. https://doi.org/10.1111/j.1469-7998.1991.tb04805.x

Mueller UG, Wolf-Mueller B (1993) A method for estimating the age of bees: Age-dependent wing wear and coloration in the Wool-Carder bee *Anthidium manicatum* (hymenoptera: Megachilidae). J Insect Behav 6:529–537. https://doi.org/10.1007/BF01049530

Nieberding CM, Fischer K, Saastamoinen M, et al (2012) Cracking the olfactory code of a butterfly: the scent of ageing: The scent of ageing in a butterfly. Ecol Lett 15:415–424. https://doi.org/10.1111/j.1461-0248.2012.01748.x

Oksanen J, Simpson GL, Blanchet FG, et al (2022) vegan: Community Ecology Package

Ostwald MM, Alba-Tercedor J, Minckley RL, Buchmann SL (2022) Three-dimensional morphology of the hypertrophied sex pheromone gland in a lek-mating carpenter bee (*Xylocopa sonorina*) revealed by micro computed tomography and scanning electron microscopy. Apidologie 53:60. https://doi.org/10.1007/s13592-022-00967-w

Phelan PL, Baker TC (1986) Male-size-related courtship success and intersexual selection in the tobacco moth, *Ephestia elutella* . Experientia 42:1291–1293. https://doi.org/10.1007/BF01946428

Pokorny T, Vogler I, Losch R, et al (2017) Blown by the wind: the ecology of male courtship display behavior in orchid bees. Ecology 98:1140–1152. https://doi.org/10.1002/ecy.1755

R Core Team (2022) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Rantala MJ, Kortet R, Kotiaho JS, et al (2003) Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. Funct Ecol 17:534–540. https://doi.org/10.1046/j.1365-2435.2003.00764.x

Schiestl FP (2010) The evolution of floral scent and insect chemical communication: Evolution of floral scent. Ecol Lett 13:643–656. https://doi.org/10.1111/j.1461-0248.2010.01451.x

Schindelin J, Arganda-Carreras I, Frise E, et al (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9:676–682. https://doi.org/10.1038/nmeth.2019

Thioulouse J, Dray S, Dufour A-B, et al (2018) Multivariate Analysis of Ecological Data with ade4. Springer

Venables WN, Ripley BD (2002) Modern Applied Statistics with S, Fourth. Springer, New York

Wertheim B, van Baalen E-JA, Dicke M, Vet LEM (2005) PHEROMONE-MEDIATED AGGREGATION IN NONSOCIAL ARTHROPODS: An Evolutionary Ecological Perspective. Annu Rev Entomol 50:321–346. https://doi.org/10.1146/annurev.ento.49.061802.123329

Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York

Wickham H (2011) The Split-Apply-Combine Strategy for Data Analysis. J Stat Softw 40:1–29

Wickham H, Girlich M (2022) tidyr: Tidy Messy Data

Wickham H, Hester J, Bryan J (2023) readr: Read Rectangular Text Data

Wilke CO (2020) cowplot: Streamlined Plot Theme and Plot Annotations for “ggplot2”

Wyatt TD (2014) Pheromones and animal behavior: chemical signals and signatures, 2nd edition. Cambridge University Press, Cambridge

543

STATEMENTS AND DECLARATIONS

Funding

S.R.R. was funded by the David and Lucile Packard Foundation (2014-40378).

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Andrew J. Goffinet and Kathy Darragh. The first draft of the manuscript was written by Andrew J. Goffinet, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

FIGURE LEGENDS

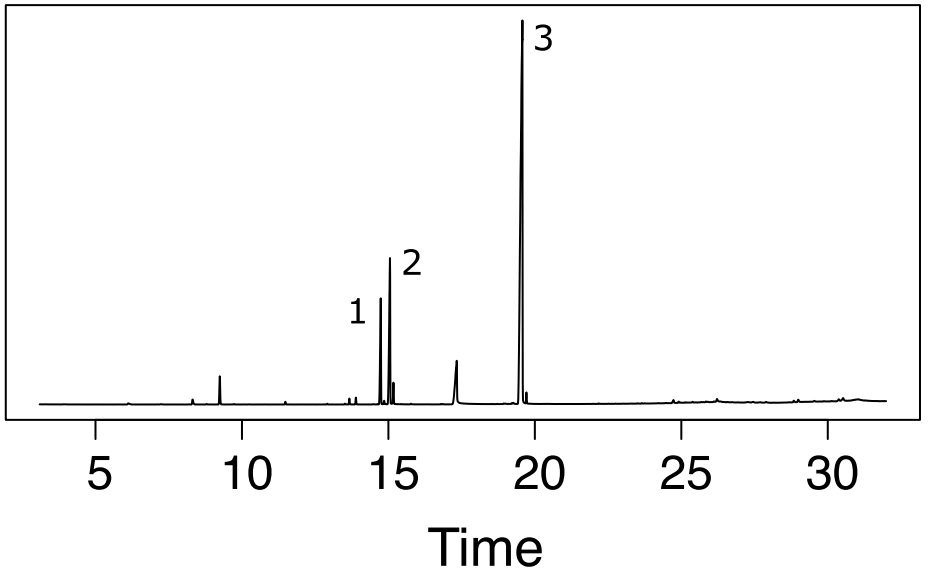
Figure 1: (a) Male *X. sonorina*. Photo credit: Sebastian Scofield. (b) Representative chromatogram of a male's mesosomal gland extract, showcasing the three primary compounds and the range of minor constituents. The peak labeled as 1 is, the peak labeled as 2 is 3,7,11-methyl-2,7,10-dodecatrienal, all trans-farnesal, and the peak labeled as 3 is all trans-geranylgeraniol.

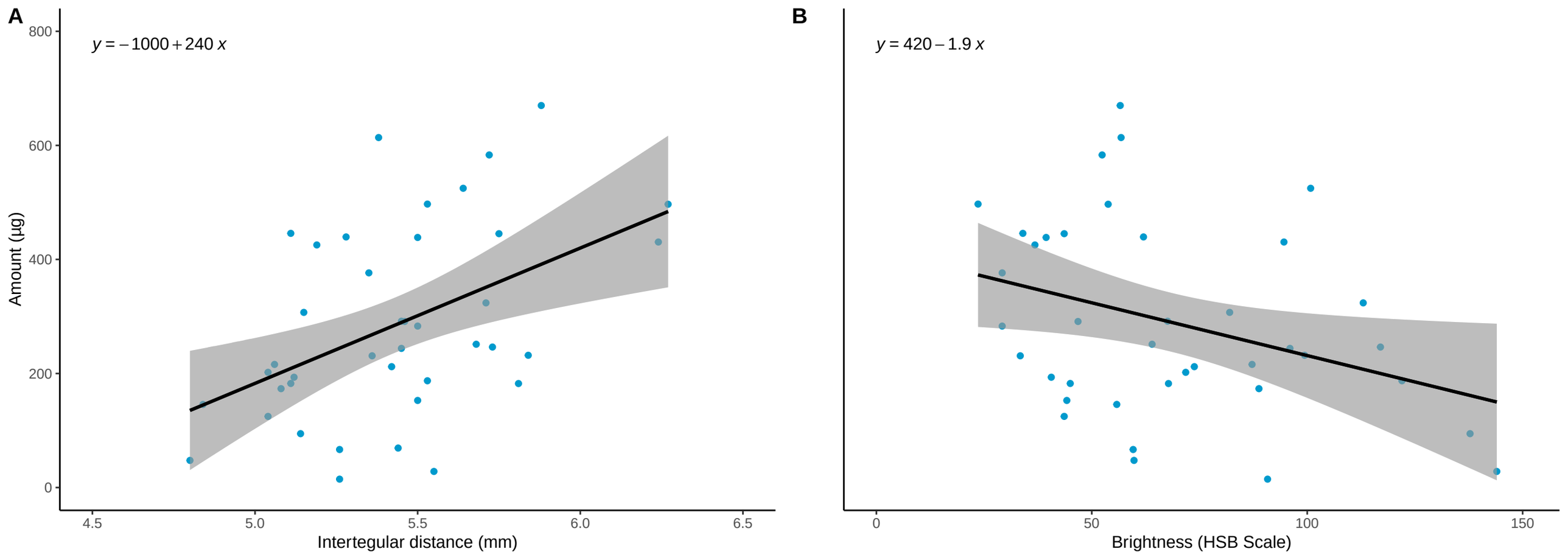
Figure 2: (a) Amount of pheromone is positively related to male *X. sonorina* body size (ANOVA, $F_{1,36}$=14.02, p=0.000632), using intertegular distance as a proxy. (b) Amount of pheromone is negatively related with gland brightness (ANOVA, $F_{1,36}$= 11.32, p=0.001832), according to the HSB scale. A higher value indicates a lighter color of the gland.

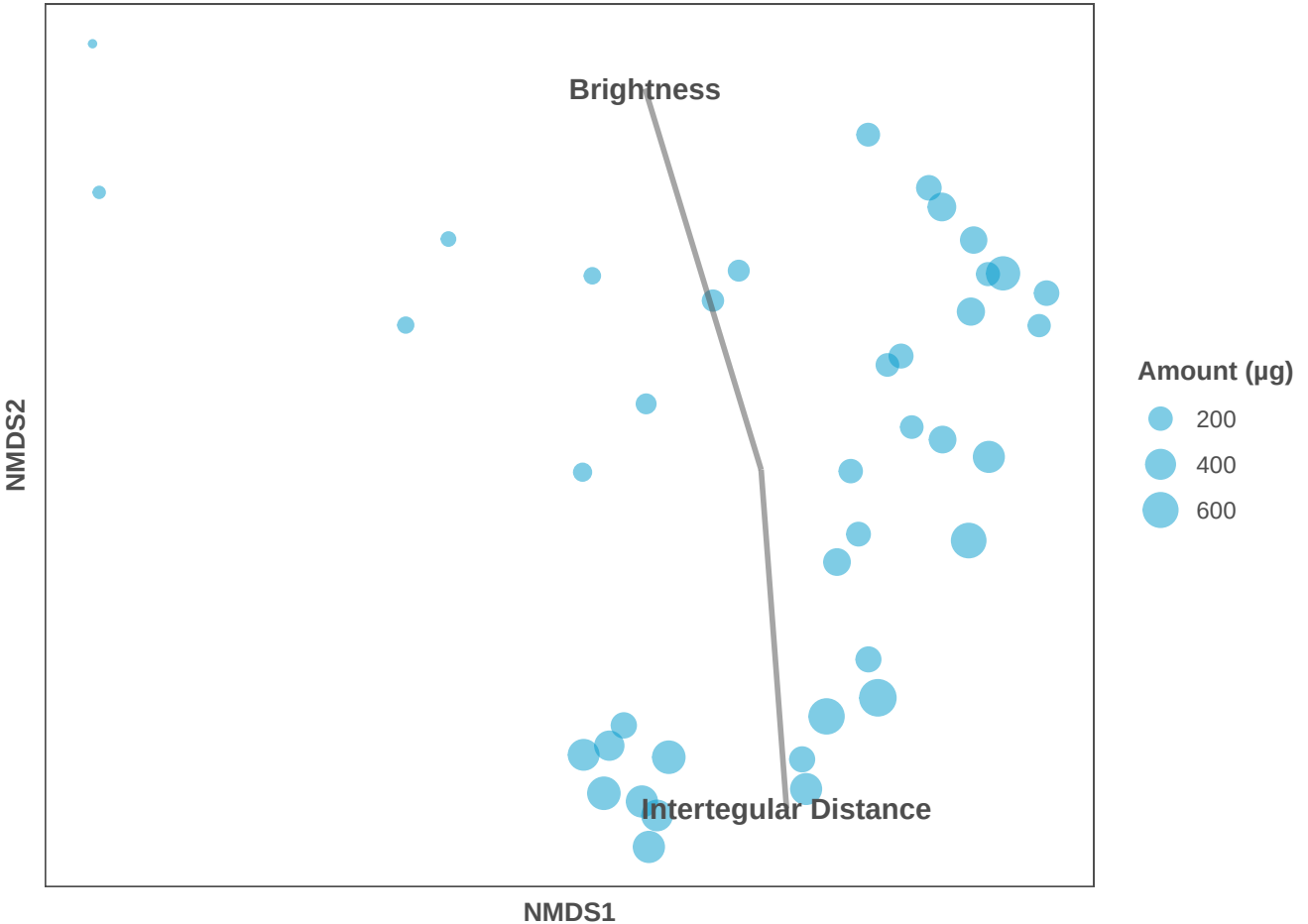
Figure 3: NMDS (nonmetric multidimensional scaling) plot illustrating in two dimensions the variation in mesosomal gland contents of male *X. sonorina.* Size of each circle corresponds to the absolute amount of pheromone. The impact of body size (with intertegular distance as a proxy) and brightness are shown.

A

B




A
y = −1000 + 240 x
Amount (µg)
800
600
400
200
0
4.5
5.0
5.5
6.0
6.5
Intertegular distance (mm)
B
y = 420 − 1.9 x
0
50
100
150
Brightness (HSB Scale)


Brightness
Intertegular Distance
NMDS2
NMDS1
Amount (µg)
200
400
600