Perceived dominance status affects chemical signalling in the neriid fly *Telostylinus angusticollis*

Zachariah Wylde [a], *, Lewis Adler [b], Angela Crean [c], Russell Bonduriansky [a]

[a] *School of Biological, Earth and Environmental Sciences, Evolution and Ecology Research Centre, University of New South Wales, Sydney, NSW, Australia*
[b] *Bioanalytical Mass Spectrometry Facility, Mark Wainwright Analytical Centre, University of New South Wales, Sydney, NSW, Australia*
[c] *The University of Sydney, Sydney School of Veterinary Science, Sydney, NSW, Australia*

Chemical communication mediates many social interactions in insects but is still less well understood than other forms of communication. In particular, chemical signalling of social dominance is believed to play an important role in competitive interactions in both sexes, but much of the evidence is correlational. Here we manipulated social dominance and examined its effect on CHC profiles in *Telostylinus angusticollis*, a fly with a resource defence polygyny mating system. Focal individuals' perception of their own dominance status was manipulated by placing them in an arena with larger or smaller competitors to render them 'subordinate' or 'dominant.' We found that social dominance treatment affected males' and females' social status (quantified as proximity to the larval medium/oviposition dish), as well as their CHC profiles. Dominant individuals tended to have CHC profiles less similar to those of the opposite sex. Moreover, dominant females exhibited an overall elevation of all CHC expression, relative to subordinate females, whereas males that perceived themselves as subordinate exhibited a near-significant downregulation of male-limited CHCs. Our findings suggest that *T. angusticollis* males and females alter their CHC profiles in response to their self-perceived social dominance status. These chemical signals could play a role in social interactions both within and between the sexes.
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Highly social animals have evolved complex signalling strategies that quickly respond to social status changes but may not be so obvious to the human observer (Blomquist & Bagneres, 2010; Wyatt, 2003). One such strategy is chemical signalling which has been well documented in animals that mark their territories with scent, where odour can be regarded as a secondary sexual trait, like antlers and bird plumage, which often occurs with other ritualized and conspicuous traits (Blaustein, 1981; Dawkins, 1995). Chemical signals could also potentially function in intrasexual dominance signalling, but how such signals may be utilized as a sign of status is not well known, especially in insects that are not eusocial. Moreover, since any trait that conveys information to another individual can be regarded as a signal (Dawkins, 1995; Maynard Smith & Harper, 1995), chemical signals could occur as nonadaptive responses (e.g. to stress) that are exploited by other individuals. Although many noneusocial insects display aggregation behaviour (Waters, 1959) where individuals show no cooperation or division of labour but gather to breed, how the social and environmental context impacts chemical cues in such insects is poorly understood and even less is known about the roles these cues have in structuring dominance hierarchies (Gershman, Toumishey, & Rundle, 2014; Grillet, Dartevelle, & Ferveur, 2006; Lin & Michener, 1972; Savarit & Ferveur, 2002).

Cuticular hydrocarbons (CHCs) are mostly long-chained nonvolatile compounds that derive from fatty acid compounds found on the cuticle of various insect species (Everaerts, Farine, Cobb, & Ferveur, 2010). These complex chemicals have been implicated in desiccation resistance (Wigglesworth, 1933) while simultaneously acting as signalling molecules in short-range chemical communication (Gershman et al., 2014; Gibbs, 2007). Species vary greatly in the chemical compositions of their CHC signals (El-Sayed, 2009), and can use CHCs to identify conspecifics. For example, *Drosophila melanogaster* females use species-specific CHCs to locate egg-laying sites used by other members of their species (Duménil et al., 2016). However, a great deal of within-species variation in CHCs is also evident. For example, the expression of CHCs has been shown to be responsive to differences in age, diet, social environment and mating history (Curtis et al., 2013;

Everaerts et al., 2010; Gershman & Rundle, 2016b, 2016a; Kent, Azanchi, Smith, Formosa, & Levine, 2008; Petfield, Chenoweth, Rundle, Blows, & Avise, 2005; Yew, Cody, & Kravitz, 2008). CHCs can play a role in female mate choice (Johansson & Jones, 2007), and can evolve rapidly when natural and sexual selection pressures are altered (Blows, 2002; Chenoweth & Blows, 2008). There is mounting empirical evidence that sexual selection can promote the evolution of chemical traits but that the type and intensity of selection on chemical signals vary between species (for a review see Steiger & Stökl, 2014).

While CHCs can convey information about an individual's status and sex, most research has focused on signalling of fertility, reproductive status and dominance in social insects such as wasps and ants (Izzo, Wells, Huang, & Tibbetts, 2010; Smith, Hölldober, & Liebig, 2009). For example, male ants, *Cardiocondyla obscurior*, can avoid aggression from wingless males by mimicking the chemical bouquet of virgin female queens (Cremer, Sledge, & Heinze, 2002). Less is known about the role of CHCs in social signalling in nonsocial insects. Male *D. melanogaster* actively mark females during mating with anti-aphrodisiac pheromones, a form of chemical mate guarding that functions to decrease female attractiveness (Laturney & Billeter, 2016). There is also evidence that individuals can adjust their own CHC profiles in response to mating (Weddle et al., 2013).

Yet, it remains unclear whether individuals in noneusocial species can change their CHC signalling in response to their own social status within a group. It could be advantageous to do so to intimidate potential rivals, signal dominance to potential mates or avoid costly interactions with rivals. For example, in some species (e.g. flat lizards, *Platysaurus broadleyi*, Whiting, Webb, & Keogh, 2009; bluegill sunfish, *Lepomis macrochirus*, Dominey, 1980; giant cuttlefish, *Sepia apama*, Norman, Finn, & Tregenza, 1999), subordinate or sneaker males mimic females. Likewise, in rove beetles, *Aleochara curtula*, young and starved males can regain access to a carcass by producing the female sex pheromone (Peschke, 1987). This suggests that male insects that experience low social status may be able to utilize CHC profiles as a type of camouflage to avoid aggression from more dominant competitors. More generally, signals of dominance can be important, particularly in species with aggressive and damaging interactions, in allowing competitors to assess their rivals and thereby lower associated fighting costs (Maynard Smith, 1982). These dominance signals may be particularly important not just in eusocial species that have strict caste structures (i.e. ants and wasps), but also in noneusocial species where individuals compete for resources such as mate-searching territories, position in dominance hierarchies or mating opportunities (Izzo et al., 2010). It is clear that CHCs are remarkably dynamic, often changing (either as part of an adaptive strategy or nonadaptively as a side-effect of stress or changes in other traits) within short timeframes in response to experience or fluctuations in the social environment (Ingleby, 2015). Thus, CHC profiles could change to reflect individuals' social status, and serve as an important social signal in noneusocial insects.

An important limitation of the existing literature on the role of CHCs in status signalling is that much of the evidence is correlational. For example, some studies pair competing individuals in an arena to determine dominance status or to assess winner and loser effects, and report effects on chemical signals (e.g. Rillich & Stevenson, 2011; Thomas & Simmons, 2009). There is also some evidence in *Drosophila serrata* that CHC profiles correlate with mating success but not with an individual's ability to successfully defend a territory (White & Rundle, 2014). Yet, it is not clear whether differences in chemical signals between dominant and subordinate individuals reflect a perceived social status that can change dynamically, or whether these observed differences in both

dominance and chemical profile are invariant features of adult individuals that result from genetic or environmental differences during development. However, to determine whether individuals can adjust their own CHC profiles dynamically in response to their social environment, experimental studies that manipulate rather than simply assess individuals' social status are required. To our knowledge, only two experimental studies have been carried out that directly manipulated the social environment and examined the fine-scale effects on CHC signalling. Using the Australian field cricket, *Teleogryllus oceanicus*, Thomas, Gray, and Simmons (2011) showed that males increased expression of CHCs (some of which are sexually dimorphic, and have been shown to attract females) in the absence of acoustic signals from other courting males. Another study showed that social environment can alter the circadian rhythm of CHCs associated with male attractiveness in *D. serrata*, with the combination of CHCs that contributes to increased mating success varying over the course of a day as well as in response to social conditions (Gershman et al., 2014; Gershman & Rundle, 2016; Gershman & Rundle, 2016, 2016). However, we are not aware of any previous experimental study that has examined whether an individual's CHC profile can respond dynamically to its perception of its position in a dominance hierarchy. Examining such responses could reveal cryptic reproductive tactics. For example, subordinate males could change their CHC profiles to more closely align with female CHC profiles, facilitating sneak matings and reducing the risk of attacks by dominant males. Although much less is known about competition among females, it is possible that females could also alter their CHC profiles to intimidate rivals in competition for food or egg-laying sites, or to avoid costly sexual interactions such as male harassment.

Here we investigated the effects of individuals' perception of their own social status on CHC composition in *Telostylinus angusticollis* (Diptera: Neriidae). This fly forms large mating aggregations, in which females and males aggregate at oviposition sites on decaying tree bark (Adler & Bonduriansky, 2013; Kawasaki, Brassil, Brooks, & Bonduriansky, 2008). Individuals vary considerably in body size (Bonduriansky, 2006, 2007), and large males defend territories and frequently engage in combat while smaller males rarely fight and appear to employ nonterritorial tactics (Bath, Tatarnic, & Bonduriansky, 2012; Hooper, Spagopoulou, Wylde, Maklakov, & Bonduriansky, 2017). Combat success is strongly related to body size (Bonduriansky & Head, 2007; Hooper et al., 2017). Small and subordinate males also exhibit increased mating duration (Fricke et al., 2015). Females vary considerably in body size as well and have been observed to interact aggressively at oviposition sites (Z. Wylde and R. Bonduriansky, personal observations). However, it is not known whether *T. angusticollis* males or females employ chemical signalling tactics or adjust their CHC profiles in response to their social status within an aggregation.

We manipulated the self-perceived dominance status of focal males and females by placing them into social environments consisting of same-sex competitors of either larger or smaller body size than the focal individual. This experiment enabled us to examine (1) whether CHC profiles of males and females are responsive to perceived dominance status; (2) whether subordinate individuals exhibit CHC profiles resembling those of the opposite sex; and (3) whether the sexes respond differently to cues of dominance status in their social environment.

METHODS

Fly Culturing

Flies for use in the chemical identification and quantitation of epicuticular compounds were derived from laboratory-reared

stocks of *T. angusticollis* that originated from individuals collected in 2017 at Fred Hollows Reserve, Randwick, NSW, Australia (33°54′44.04″S, 151°14′52.14″E) and were reared in the laboratory for four generations prior to this experiment. All laboratory-bred individuals were reared in climate chambers at 25 ± 2 °C with a 12:12 h photoperiod and provided with water every 2 days. We manipulated the adult body size of individuals used in the experiment by rearing larvae on a nutrient-rich, nutrient-intermediate (henceforth, 'standard') or nutrient-poor larval diet. Diets were based on Sentinella et al. (2013) and were selected to generate considerable body size differences between competitor flies used in dominance treatments. All diets consisted of a base of 170 g of cocopeat moistened with 600 ml of reverse osmosis-purified water. The 'rich' larval diet consisted of 32.8 g of protein (Nature's Way soy protein isolate; Pharm-a-Care, Warriewood, Australia) and 89 g of raw brown sugar, the 'standard' larval diet consisted of 10.9 g of protein and 29.7 g raw brown sugar, and the 'poor' larval diet consisted of 5.5 g of protein and 14.8 g raw brown sugar. These nutrients were mixed into the cocopeat and water using a handheld blender and frozen at -20 °C until the day of use.

Virgin adults were collected at emergence and separated by sex, larval diet treatment and emergence date to control for age (± 2 days) across all treatments. Age is known to affect CHC profiles in flies (Gershman & Rundle, 2016). All adult flies were allowed to mature in individual 120 ml containers fitted with a feeding tube containing a sugar-yeast mixture and a drinking tube containing water ad libitum, and a substrate of moistened cocopeat. All males were kept in these containers until 5 ± 2 days of age (when males are fully reproductively mature), whereas females were kept until 12 ± 2 days of age (the typical age of full ovary development), prior to dominance treatments and assays. All adults were housed, and dominance treatments applied; in a controlled-temperature room set at 25 °C and 60% humidity and a 14:10 h light:dark cycle.

Manipulation of Dominance Status

Males of this species engage in escalated combat interactions (foreleg strikes and headlock, 'chest' impacts; Fig. 1) with rivals of similar body size, but a male challenged by a larger rival usually withdraws and displays submissive behaviours (Bath et al., 2012). Large, dominant males defend oviposition sites or females by chasing away or flicking their wings at males that attempt a takeover. Females do not show the same aggression behaviours as males but can 'wing flick' or engage in brief bouts of foreleg boxing with other females. Thus, the mean body size of rivals with which a *T. angusticollis* individual interacts could determine its perception

of its own place in the dominance hierarchy. To examine the plasticity of CHC profiles in response to the social environment we placed focal individuals (all reared on a standard larval diet) into an arena for 48 h with three competitors of the same sex that were either reared on nutrient-rich larval diet (such that competitors were larger than the focal individual) or nutrient-poor larval diet (such that competitors were smaller than the focal individual). These social environment treatments enabled us to directly manipulate the focal individual's self-perception of its social dominance to render it 'subordinate' or 'dominant' within its social environment (Fig. 2).

Focal individuals were randomly assigned to 'dominant' or 'subordinate' treatment groups, and placed individually into competitive arenas for 48 h, which is a sufficient length of time for neriid flies to establish a dominance hierarchy (Bonduriansky & Head, 2007). The arenas consisted of 1 l cylindrical containers covered with mesh stockings. Each arena contained a layer of moistened cocopeat and a 3 cm diameter petri dish containing oviposition medium in the centre.

The oviposition medium stimulated males and females to engage in reproductive behaviour similar to that observed at natural oviposition sites in the wild (Fig. 1). At the end of the 48 h period, each focal individual was observed for 10 min and any aggressive interactions with competitors were recorded. The focal individual's distance from the oviposition site was also estimated in body lengths (see Appendix Fig. A1) every 2.5 min during the
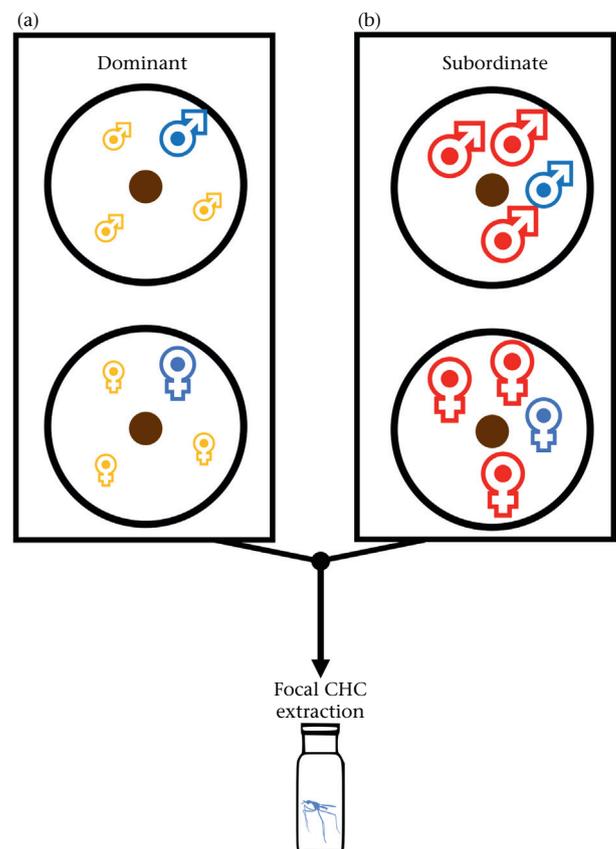

Figure 1. Male neriid flies engaged in escalated combat (on the left). On the right a male guards a female as she oviposits into a damaged area of a coral tree (*Erythrina* spp.). Photo courtesy of Russell Bonduriansky.

Figure 2. Experimental design. (a) Dominant treatment: focal male or female (blue) surrounded by three competitors reared on a poor larval diet, and therefore smaller than the focal individual (yellow). (b) Subordinate treatment: focal male or female (blue) surrounded by three competitors reared on a rich larval diet and therefore larger than the focal individual (red). All individuals were kept in these social environments for 48 h. Brown circle in the centre of arenas symbolizes the oviposition food used to elicit competitive behaviours. Only the CHCs of focal individuals were extracted.

behavioural observation time. The mean distance of the focal individual was then used for further analyses. These observations enabled us to assess focal individuals' dominance status relative to competitors, in order to determine whether the treatments were successful. Immediately following the 10 min observation period, entire arenas were placed in a -80 °C freezer to anaesthetize flies quickly and minimize any effects of stress that might affect CHC profiles. All focal individuals were then stored in Eppendorf tubes at -80 °C until chemical analysis. Additional flies, reared on each of the three larval diets, were kept individually (without competitors) in identical arenas to test for an effect of larval diet on CHC profile and determine whether dominance treatment effects could be explained simply as effects of competitors' CHCs transferring onto focal individuals.

Extraction of Epicuticle Hydrocarbons

Single fly extractions

Focal flies were thawed for 15 min at room temperature before hydrocarbons were extracted by immersing single flies in 100 µl of hexane (Sigma-Aldrich, Castle Hill, NSW, Australia, product no. 650552) spiked with a 10 µl/ml of hexacosane (Sigma-Aldrich, product no. 241687) internal standard. Individual flies were placed in 2 ml autosampler vials (Agilent Technologies, Mulgrave, Vic, Australia) and were immersed in hexane for 3 min and vortexed for 1 min before the fly was removed. Water was removed from each extraction by filtering the elution through a glass Pasteur pipette packed with silane-treated glass wool (Alltech, Australia) and a small amount of anhydrous sodium sulphate (Ajax Finechem, Seven Hills, NSW, Australia, product no. 503-500). Extracts were stored at -20 °C until analysis. Following the CHC extraction all flies were frozen at -20 °C for subsequent morphometric analysis. Thorax length is a reliable proxy for body size in this species (Bonduriansky, 2006) and was measured from images taken using a Leica MS5 stereoscope equipped with a Leica DFC420 digital microscope camera. Measurements were made using FIJI open source software (Schindelin et al., 2012).

Identification of CHC compounds

To aid in the identification of individual compounds present in the cuticle of *T. angusticollis*, CHCs from pooled samples (six flies) were extracted together in a single vial containing 400 µl of hexane (same extraction protocol as above). Individuals used in these extractions were pooled by larval diet (rich, poor and standard) and sex. For comparison, we also extracted CHCs from pooled wild-caught individuals of unknown mating status/age by sex that were trapped at Fred Hollows Reserve in early January 2018.

Chemical Analyses

Compound abundances

Chemical analysis of all single fly extracts was carried out on a 6890 Gas Chromatograph (GC) combined with an Agilent 5973 Mass Selective Detector (Agilent Technologies, Santa Clara, CA, U.S.A.). An Agilent 7673 autosampler fitted with a 10 µl syringe injected a sample volume of 2 µl, with the split/splitless injector set to 290 °C. Separations were carried out using a TRACE 260R-154P capillary column (60 m × 0.25 mm internal diameter, 0.25 µm film thickness, Thermo Fisher Scientific, Scoresby, Vic, Australia). Helium was used as the carrier gas at a flow rate of 1.0 ml/min with a splitless injection. The gas chromatography–mass spectrometry (GC-MS) data were processed with Agilent Chemstation software. The temperature program began at 150 °C increasing to 300 °C at a rate of 30 °C/min, holding at 300 °C for 0 min, then increasing to a final temperature of 330 °C at a rate of 3 °C/min. The run time for

this method was based on Curtis et al. (2013) and was optimized to maximize efficiency and keep the column clean. From these data, individual profiles of the CHC compounds were determined by integration of the area under peaks (20 peaks for males; 24 peaks for females, not all identified in Table 1). CHC values were converted to relative proportions by dividing the area under each peak by the area under the peak for the hexacosane internal standard present in each sample. This enabled us to correct for technical errors associated with GC-MS and any intrinsic changes to column integrity that might occur from high-throughput analysis.

To process the data from our samples pooled by diet, sex and wild-caught individuals, we utilised Progenesis QI Informatics software (Paglia et al., 2014). Each GC-MS run was imported as an ion-intensity map including *m/z* and retention time. These ion maps were then aligned to the retention times. From the aligned runs, an aggregate run was constructed and compared with all runs so that the same ions were detected in each run. Isotope and adduct deconvolution was used to reduce the number of features identified. All data were normalized to total ion intensity and then extracted for multivariate analysis.

Compound identification

Unambiguous identification of the chemical compounds present in the CHC extracts of *T. angusticollis* was not possible using regular GC-MS methods and comparison with the available NIST 11/Wiley 275 databases. To increase the resolution and obtain more precise chemical identification, pooled extractions were analysed using a Thermo Trace 1310 GC with a Thermo QExactive-GC orbitrap high resolution MS (Thermo Fisher Scientific, Dreieich, Germany). A Thermo TriPlus RSH autosampler fitted with a 10 µl syringe injected a sample volume of 2 µl with a split/splitless injector at 270 °C, using helium as a carrier gas (flow rate 1.0 ml/min) and a TG5silMS capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness) for separation (Thermo Scientific, Australia). The temperature program consisted of three ramps starting at 50 °C increasing at a rate of 30 °C/min to 90 °C, then at 10 °C/min to 180 °C and finally at 7 °C/min until a final temperature of 330 °C that was held for 12 min. Data were acquired initially in EI and PCI mode at a resolution of 60 000, and subsequently in PCI mode at 120,000. These analyses were conducted by the Central Analytical Research Facility at Queensland University of Technology on samples that were dried and reconstituted in GC grade hexane. The identities of CHCs were ascertained from the presence of molecular ions in their chromatographic peaks and identified using mass spectral fragmentation patterns from the PCI data. A 100 µg/ml sample of C_7–C_{40} saturated alkane mixture (Sigma-Aldrich, Australia product no. 49452) was used for identification of branching, using the same methods as above but with a split injection with a split ratio of 20. Most CHCs did not coelute with the C_7–C_{40} saturated alkane mixture demonstrating a relatively high abundance of branched to straight chained alkanes (Carlson, Bernier, & Sutton, 1998; Katritzky, Chen, Maran, & Carlson, 2000).

Statistical Analyses

All statistical analyses were carried out using R 3.4.3 (R Core Team, 2017) using the Modern Applied Statistics with S package 'MASS' (Venables & Ripley, 2002) and the Classification and Regression Training package 'caret.' (Kuhn, 2008) CHC signalling involves complex blends of compounds that probably function as a whole, so we did not analyse specific compounds or subsets of compounds (Bonduriansky et al., 2015; Everaerts et al., 2010). Instead, we utilized a multivariate approach to analyse differences in CHC profiles across our social treatment groups and between the sexes. First, the retention time was used to differentiate each peak,

Table 1
Epicuticular compounds of Telostylinus angusticollis, identified by CHC-specific ionic signatures

Peak ID	Compound	Molecular formula	Diagnostic ion	Sex
1	Unidentified CHC 1	–	–	♂♀
2	Unidentified CHC 2	–	–	♂♀
3[S]	Unidentified CHC 3	–	–	♂♀
4[S]	Unidentified CHC 4	–	–	♂♀
5[S]	Heptacosane	$C_{27}H_{56}$	381	♂♀
6	3-Methylheptacosane	$C_{28}H_{58}$	394	♂
7	3-Methyloctacosane	$C_{29}H_{60}$	408	♂
8[S]	2-Methylheptacosane	$C_{29}H_{60}$	408	♂♀
9[S]	2-Methylnonacosane	$C_{30}H_{62}$	422	♂♀
10[S]	3-Methylnonacosane	$C_{30}H_{62}$	422	♂♀
11[S]	Unidentified CHC 5	–	–	♂♀
12[S]	Unidentified CHC 6	–	–	♂♀
13[S]	3-Methyltriacontane	$C_{31}H_{64}$	436	♂♀
14[S]	Hentriacontane	$C_{31}H_{64}$	436	♂♀
15[#]	2-Methyltriacontane	$C_{31}H_{64}$	436	♂♀
16[S]	2-Methylhentriacontane	$C_{32}H_{66}$	450	♂♀
17[S]	Dotriacontane	$C_{32}H_{66}$	450	♂♀
18[#]	3-Methyldotriacontane	$C_{33}H_{68}$	464	♂♀
19[S]	3-Methylhentriacontane	$C_{32}H_{66}$	450	♂♀
20[S]	2,3-Dimethylhentriacontane	$C_{33}H_{68}$	464	♂♀
21	4-Methyltritriacontane	$C_{33}H_{68}$	478	♀
22[S]	Unidentified CHC 7	–	–	♂♀
23[S]	2-Methyltritriacontane	$C_{34}H_{70}$	478	♂♀
24	2-Methylpentatriacontane	$C_{36}H_{74}$	506	♂
25[S]	Unidentified CHC 8	–	–	♂♀
26	Unidentified CHC 9	–	–	♀
27	Unidentified CHC 10	–	–	♀
28	Unidentified CHC 11	–	–	♀
29	Unidentified CHC 12	–	–	♀

Compounds and their branching were identified by their GC Orbitrap-MS spectral fragmentation patterns and compared to a saturated alkane standard. Our samples also included compounds that were consistently present in male and/or female samples, but for which we were unable to determine the molecular formula and branch locations because of low signal, coelution or chain length > 40. The analyses include all sex-specific compounds, but only those shared compounds that were consistently present in all replicates for both sexes (indicated by the superscript 'S'). Shared compounds that could not be detected in some samples were excluded from the analyses.

with multiple samples overlaid to ensure peaks were consistent between samples. The standardized peak areas (calculated as the area of each peak divided by the internal standard) at each retention time were then analysed by multivariate analysis of variance (MANOVA) to test for effects of social treatment group ('dominant' or 'subordinate'), sex and their interaction.

Data transformation and preprocessing

All data were $\log_{10}$ transformed prior to analysis. Multi-collinearity can yield solutions that are numerically unstable or overfitted, impacting the generalizability of results, particularly in linear methods (Næs & Mevik, 2001). To avoid these problems, we constructed a correlation matrix among peaks and removed the columns that contributed a mean absolute correlation of > 0.75 from the data. We then performed recursive feature elimination (Breiman, 2001) to eliminate redundant features and improve our models' predictive accuracies using the random forest selection function with cross-validation (repeated 10 times). This algorithm is known to identify strong predictors in smaller data sets and produce optimal subsets of features that yield high classification accuracy (Darst, Malecki, & Engelman, 2018). Near-zero variance predictors can also cause the classifier to fail when training models (Kuhn & Johnson, 2013) so all predictors that had near-zero variance were also removed. Subsequently all data were centred, scaled and finally transformed using principal component analysis (PCA) to reduce dimensionality of the data.

Model training

We used linear discriminant analysis (LDA) on PC scores (see above) to investigate which combinations of CHCs discriminate focal individuals (within sex) into their social treatment groups. Subsequently, CHCs shared between the sexes (based on diagnostic ion and retention time) were analysed in the same way to investigate how shared CHCs discriminate between the sexes as well as 'dominant' and 'subordinate' groups within each sex, resulting in four sex∗treatment combinations. We interpreted factor loadings > 0.25 to have a significant contribution to the axes of variation for each discriminant function (Weddle et al., 2013).

LDAs were limited to principal components that accounted for 99% of the total variation in the data. We used a 10-fold repeated cross-validation to assess each model's accuracy. This method partitions the data into 10 subsets but maintains the proportionality of each treatment representation (Valetta et al., 2017). The models were trained on nine of the subsets and the remaining subset was used to assess its accuracy. This process was repeated until all subsets had been utilized as train and test sets. Because this method can sometimes overestimate accuracy, we used 90% of the data for cross-validation (as described above), and the remaining 10% of each original data set to test the accuracy of our final models. Also, to determine whether the resulting LDA models performed better than random, we reran the LDA analysis on 1000 randomly generated training sets, each consisting of the actual data with randomly assigned group labels (similar to the method used by Nehring, Evison, Santorelli, d'Ettorre, & Hughes, 2011). This generated a null distribution of LDA model accuracy values for comparison with our actual LDA results. LDA yields $N_{classes} - 1$ discriminant functions. Therefore, analyses of within-sex differences only yielded one discriminant function while analyses of both sex and treatment yielded three discriminant functions.

To determine whether the degree of male–female similarity in CHC profile was affected by perceived dominance status, we utilized the 'bayesboot' package (Baath, 2016) to calculate the posterior differences in bootstrapped LD1 mean scores between treatment groups, and to calculate confidence intervals, CI, for

these posterior differences. We calculated the mean difference (δ) between LD1 scores of individuals from each dominance treatment and individuals of the opposite sex and bootstrapped the posterior difference (posterior draws $= 10,000$) separately for each sex as $\delta = (DI\text{-}OS) - (SI\text{-}OS)$, where DI is the bootstrapped dominant LD1 mean of the focal sex, OS is the opposite-sex LD1 mean (treatments pooled) and SI is the bootstrapped subordinate LD1 mean of the focal sex.

We analysed the effects of sex, social environment and their interaction on mean distance to food source and the mean number of aggressive behaviours using a one-way ANOVA test. Sex-limited CHCs provide the most unambiguous signals of sex and could therefore be especially important in signalling sex and status. We therefore also examined whether our social treatments affected the relative expression of sex-limited CHCs by MANOVA. To investigate whether the sexes and dominance treatments differed in total CHC expression, all peak areas were summed per individual and compared between dominance status and sex using two-way ANOVA.

For comparison, we also examined the effects of rich, standard and poor larval diets on CHC profile using a PCA of relative peak areas (with data pooled across sexes). PCA was also carried out on samples of wild-caught females and males. CHC profiles from individuals reared on rich and poor larval diets were used to gauge whether dominance treatment effects could plausibly be explained as a simple transfer of CHCs from competitors to focal individuals (see Discussion).

Ethical Note

All insects used in this study were housed with ad libitum access to food and water throughout the experiments. Individuals were killed humanely and quickly in a -80 °C freezer to minimize prolonged stress. No licences or ethics approval were required for the experiments. Small pilot tests were used to optimize experimental protocol and minimize welfare impact on subjects.

RESULTS

Social dominance treatment was found to affect the mean distance of a focal male from the oviposition site (Fig. 3a) where 'dominant' individuals were significantly closer than their 'subordinate' counterparts (ANOVA: $F_{1, 84} = 62.26$, $P < 0.001$). Females, however, did not show a significant difference in mean distance to the oviposition site (ANOVA: $F_{1, 61} = .252$, $P = 0.139$). Likewise, social dominance treatment affected the mean number of aggressive behaviours performed by males (ANOVA: $F_{1, 84} = 20.11$, $P < 0.001$; Fig. 3b), but did not affect aggression in females (ANOVA: $F_{1, 61} = 0.251$, $P = 0.618$).

GC-MS Analysis of CHC Extracts

We analysed cuticular hexane extracts for 84 males and 61 females and identified a total of 30 CHCs. Of these, we definitively identified the structural formula of a total of 17 CHCs (Table 1). Some peaks could not be identified due to their relatively weak signal or complex mixture of molecular ions but were consistently present in samples and were thus included in the semiquantitative analyses of CHC profiles given below. Unidentified CHCs 1 and 2 were consistently present in female samples but detected in only some male samples, and these compounds were thus excluded from semiquantitative analyses in males. Peaks 16 and 19 were not consistently present in either male or female samples and were therefore excluded from quantitative analyses in both sexes. Mass spectrometry revealed five female-limited and three male-limited CHCs with a diverse range of branched alkanes ranging from 27 to 36 carbons in length (Table 1).
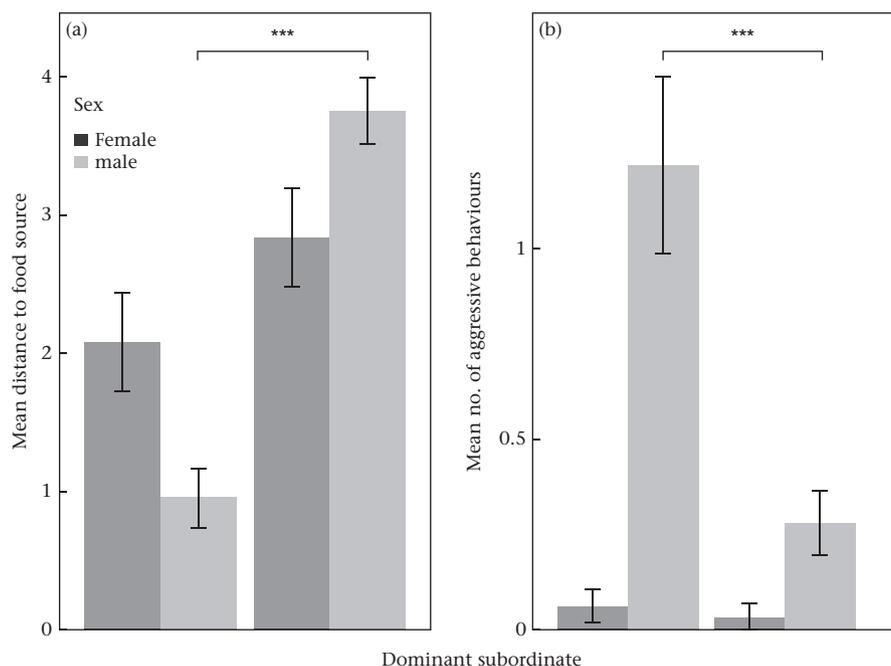

Figure 3. Effect of social dominance treatment on position within competitive arenas and behaviour for focal males and females. (a) Mean ($\pm$ SEM) distance of a focal individual measured as approximate body lengths from the petri dish containing a food/oviposition resource; (b) mean ($\pm$ SEM) number of aggressive behaviours performed by the focal individual, including 'wing flicks', 'chasing away a competitor' and higher 'escalated' combat interactions (headlock, foreleg strikes, chest impacts). ***$P < 0.001$ (one-way ANOVA models).
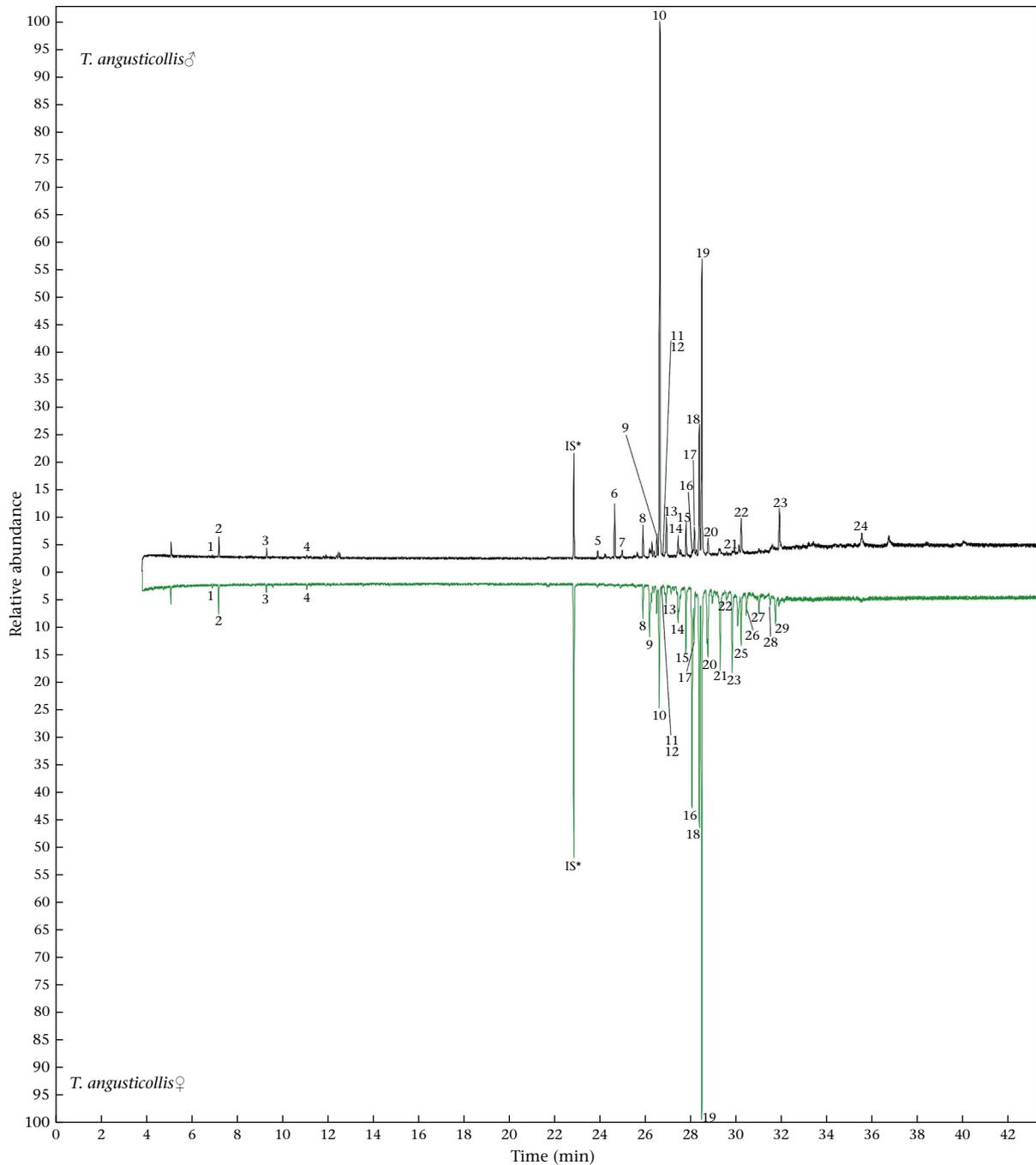
Figure 4. Mirrored GC chromatographic profile of a pooled male (black) and female (green) *T. angusticollis* (not adjusted for body-size). Relative abundance refers to the MS signal strength (arbitrary units) of a total ion chromatogram. Peaks 1 and 2 were excluded from quantitative analyses in males because of inconsistent presence between samples. Peaks 16 and 19 were also excluded from quantitative analyses because of inconsistency, but in both sexes. Un-numbered peaks in chromatograms represent analytical artefacts or non-CHC compounds based on their ionic signatures.

Of compounds present in both sexes, the most abundant was identified as 3-methylhentriacontane. The most abundant sex-limited compounds were 3-methylheptacosane and 4-methyltritriacontane in males and females, respectively. Hydrocarbon peaks clustered particularly from C_{27} to C_{36} with an increasingly complex number of peaks around C_{31}—C_{33}. Fig. 4 represents a typical gas chromatographic GC profile of CHC extracts of male and female *T. angusticollis*. Females tended to express a lower overall abundance of CHCs than males, a difference

that may simply reflect males' larger mean body size (Appendix Fig. A1).

Comparison of mean CHC abundances

We tested effects of treatment and sex using only those CHCs that were consistently present in all male and/or female samples (Table 1). GC-MS analysis revealed a total of 17 CHC peaks that were present in all female and male samples (i.e. shared between the sexes; Fig. 5). For these shared CHCs we found a significant effect of
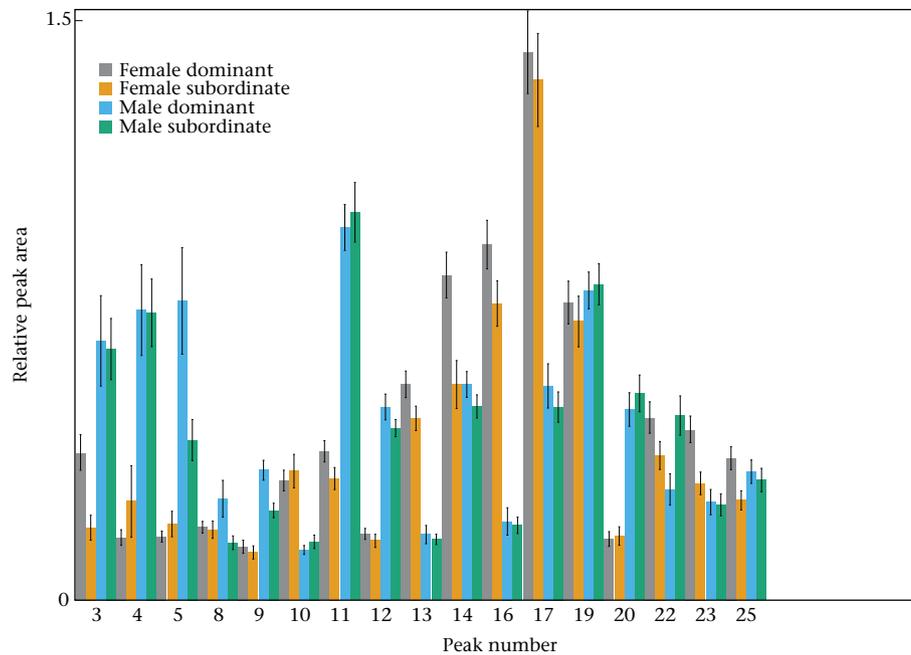
Figure 5. Relative peak areas for each shared CHC by sex and dominance treatment. The mean (± SEM) relative peak area (proportional to the internal standard) for each shared CHC peak detected by GC-MS is shown.

sex and social treatment and a significant interaction between them on relative peak area (Appendix Table A1). Of the 17 shared CHCs, 13 were significantly sexually dimorphic, five were affected by social treatment and six were affected by an interaction of sex and social treatment (Appendix Table A1). CHCs shared between the sexes did not differ in mean expression level ($t_{137} = 0.89$, $P = 0.38$). However, 'dominant' females showed significantly higher mean expression levels of shared CHCs than 'subordinate' females ($t_{57} = 2.27$, $P = 0.027$), whereas males showed no such difference ($t_{65} = 0.28$, $P = 0.78$).

Linear discriminant function analysis of CHC blend

The cuticular profiles differed between our treatment groups, and individuals could be classified by sex and social treatment using LDA. The LDA on CHC profiles within each sex (including shared and sex-limited CHCs) yielded one discriminant function that explained 99% of CHC variation ($N_{classes - 1}$) separately for each sex (Fig. 6). Model accuracies were high for both male and female LDAs, and although 95% CIs were wide, model accuracies for both sexes substantially exceeded the null expectation.

We also carried out an LDA based on the shared CHCs, yielding three discriminant functions that explained 89.79%, 5.47% and 4.74% of variation in CHCs between the four sex∗treatment combinations. Although this LDA model had somewhat lower accuracy than the sex-specific LDAs, its accuracy substantially exceeded the null expectation (Table 2, Fig. 7). As might be expected when comparing fine-scale variation of CHCs within sex, there was considerable overlap in hydrocarbon profiles between 'dominant' and 'subordinate' individuals (Fig. 6), and only some peaks contributed significantly to social treatment group separation (Appendix Table A2).

Based on individual scores for the main linear discriminant function (LD1) for shared CHCs (Fig. 7), if subordinate males were more similar than dominant males to the mean female CHC profiles, the mean δ value would be positive (i.e. > 0). Conversely, if subordinate females were more similar than dominant females to the mean male CHC profiles, the mean δ value would be negative
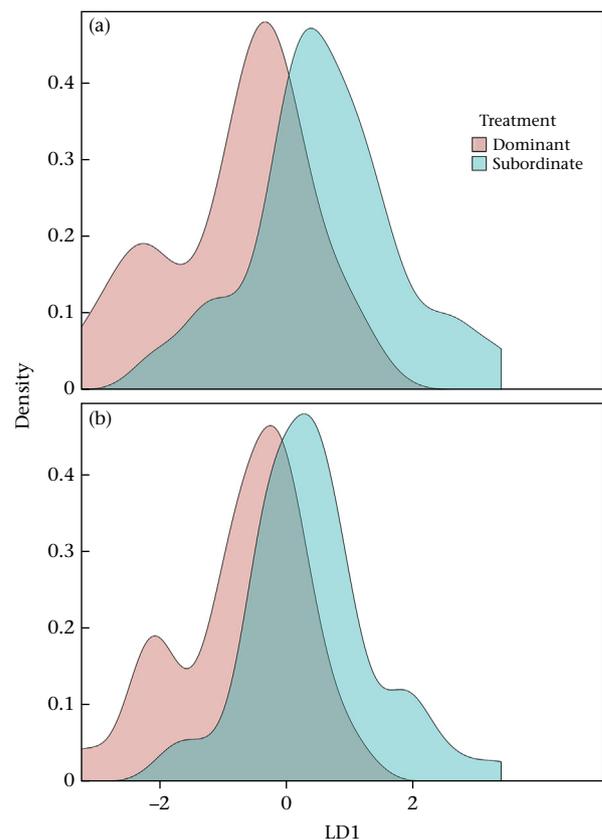

Figure 6. Results of linear discriminant function analyses of cuticular hydrocarbon extracts of (a) female and (b) male *T. angusticollis* from dominant and subordinate dominance status treatments. Density refers to the kernel density estimate, or proportion of data located there.

Table 2
The success of predicting dominance status within male, female and shared CHC data sets based on linear discriminant analysis

Data set	No. of PCs	Overall % model accuracy (95% CI)	Permutation test results % accuracy (95% CI)	% Correctly assigned	
				Dominant	Subordinate
All CHCs by sex					
Males	6	85.7 (0.4213, 0.9964)	58.8 (0.5860, 0.5901)	50	100
Females	6	70.8 (0.4891, 0.8738)	50.3 (0.4992, 0.5074)	100	68.2
Shared CHCs					
Overall	9	69.23 (0.3857, 0.9091)	30.3 (0.3009, 0.3045)		
Males				50	100
Females				33	80

The table shows the number of principal components (PCs) that accounted for 99% of the total variance; overall model accuracy with 95% confidence intervals, CI; null distribution for model accuracy based on a permutation test (1000 iterations) with corresponding 95% CI; the percentage of individuals that were assigned to the correct group (dominant or subordinate) in a repeated 10-fold cross-validation.
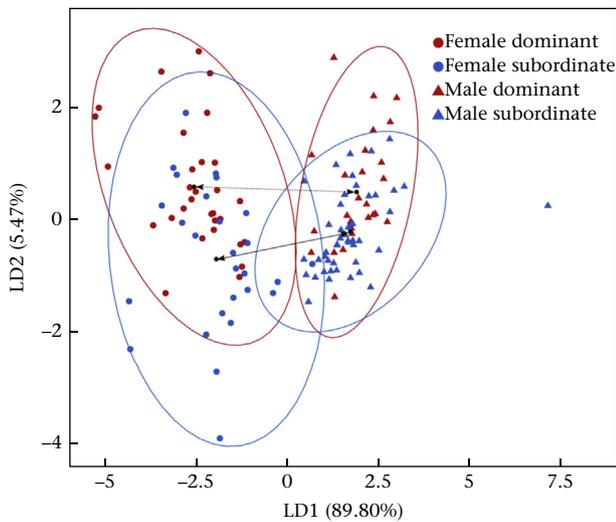

Figure 7. Scores for individual focal males and females on the first two discriminant functions of shared CHCs. Black points represent the centroids of each group. The dashed arrow indicates the relative distance (δ) between the centroids of dominant males and females and the solid arrow indicates the relative distance between centroids of subordinate males and females. Ellipses represent 95% confidence intervals.

(i.e. < 0). Bayesian bootstrap analysis supported these predictions for both males ($\delta = 0.169$, 95% CI $= 0.162$, 0.176) and females ($\delta = -0.608$, 95% CI $= -0.618$, -0.597). Thus, although there was considerable overlap in CHC profiles between dominance treatment groups and sexes, these results suggest that subordinate males and females tended to be more similar to each other in their CHC profile than dominant males and females were (Fig. 7).

Sex-limited CHCs

Social treatment significantly affected the expression of sex-limited CHCs in males (MANOVA: Pillai's trace $= 0.117$, $F_{1, 79} = 73.1$, $P = 0.022$) but not in females (MANOVA: Pillai's trace $= 0.105$, $F_{1, 61} = 61$, $P = 0.264$; Fig. 8, Appendix Table A3).

Larval diet effects on CHC profiles

For comparison, we also examined CHC profiles of males and females reared on rich, standard and poor larval diets, and individuals collected from the wild, by means of PCA on pooled samples (Fig. 9). PC1 and PC2 collectively described 90.9% of variation in CHC profiles. Males and females clustered by larval diet and laboratory versus wild origin along PC1, while PC2 separated laboratory-reared from wild-caught flies.
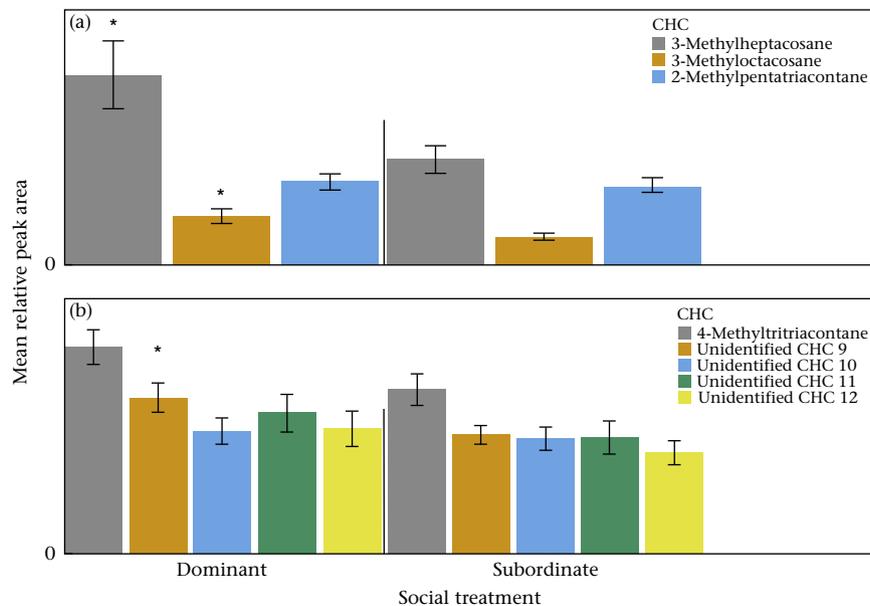

Figure 8. Effect of perceived dominance status on the expression of sex-limited CHCs in (a) males and (b) females. Bars show mean ± SEM relative peak area for sex-limited CHCs. An asterisk denotes significant differences between treatments (see Appendix Table A2).
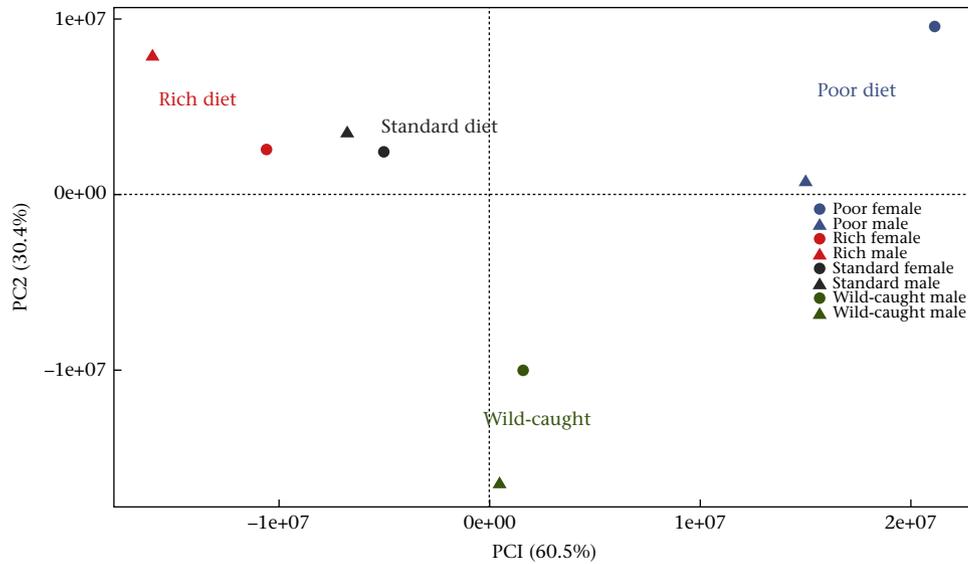
Figure 9. PCA factor scores for the first two PCs of the CHC profile for *T. angusticollis* males and females reared on different larval diets and for wild-caught individuals of each sex. Each point represents six individuals pooled by sex, diet and origin (laboratory, field).

DISCUSSION

Our results suggest that CHC profiles are sensitive to an individual's perceived dominance status within a group. Focal males and females placed with same-sex competitors of either larger or smaller body size achieved 'dominant' or 'subordinate' status within the group, and this treatment effect on social status was associated with differences in CHC profile. Our results thus suggest that an individual's performance in interactions with same-sex rivals (which is determined in part by its body size relative to the rivals' body sizes) influences the formation of an individual's own CHC profile, allowing an individual to signal his or her place in the dominance hierarchy. Our findings are consistent with work on chemical signalling in *D. melanogaster* by Kent et al. (2008) that showed individual CHC profiles to be modulated by their neighbours' genotypes and previous social context. An insect's ability to outcompete conspecifics can be determined by developmental resources such as larval diet (Amitin & Pitnick, 2007), which can affect body size and secondary sexual trait expression, and therefore influence social dominance (Bonduriansky, 2007; Moczek, 2002; Nijhout & Emlen, 1998). By rearing all focal individuals on the same larval diet and then placing them in contrasting adult competitive environments, we were able to control for effects of genetic variation and variation in developmental environment, to ask whether CHC profile can change in response to perceived dominance status. Our experiment is one of the first to attempt to directly manipulate this social context and our results suggest that males and females both adjust their chemical profiles, depending on whether they perceive themselves to be dominant or subordinate.

Our experimental treatments successfully manipulated focal individuals' social status. After 48 h in their social treatments, focal males in the 'dominant' treatment group were found to be closer on average to the oviposition site (petri dish with larval medium) than focal males in the 'subordinate' treatment group. Similar results (albeit weaker) were obtained for females. We therefore believe that the differences in CHC profile between social treatment groups within the sexes are most plausibly attributed to dynamic changes in chemical signalling associated with self-perception of social dominance within the group. A different (but nonexclusive)

explanation is that differences in CHC profiles resulted from differential access to food in our social treatments: 'subordinate' individuals may have been excluded by competitors from the petri dish of larval medium and altered their CHC profiles as a result of nutrient limitation. While we cannot exclude this possibility, our observations suggest that all flies had some access to the petri dish (and all flies had ad libitum access to water), and we believe that focal flies are therefore unlikely to have suffered substantial nutrient limitation. Moreover, if food limitation played a role in treatment effects on CHC signalling, this effect would represent a mechanism mediating the effects of social dominance on CHC signalling. Individual *T. angusticollis* in natural populations aggregate at oviposition sites, and adults also feed at these sites (Adler & Bonduriansky, 2013). If subordinate individuals suffer food limitation as a result of being excluded from these sites by dominant individuals, then the effects of nutrient limitation on individuals' CHC profiles could result in reliable chemical signalling of social dominance status. Such nutritional stress-mediated effects on CHC signalling could be nonadaptive (e.g. reducing individuals' sexual attractiveness or ability to intimidate same-sex rivals) but could still play important roles in social interactions via the information that such signals provide to other individuals. For example, nutrient-limited individuals could be rejected as potential mates or subjected to increased attacks by rivals.

We found some evidence that subordinate male CHC profiles were more female-like. Sex-limited CHCs in subordinate males showed expression levels that were significantly lower than those of dominant males. Subordinate males also had a shared CHC profile that more closely resembled that of a female. One plausible interpretation of this result is that subordinate *T. angusticollis* males employ a form of chemical mimicry by adopting a female-like CHC profile. By chemically mimicking a female, a male that perceives itself to be subordinate might reduce its risk of being damaged or threatened by dominant rivals. Equally, it could be advantageous for a dominant male to signal his dominance to help intimidate rival males and/or to make himself more attractive to females. Indeed, previous work showed that *T. angusticollis* males that previously won fights against rival males appeared to be preferred by females (Fricke et al., 2015). 'Subordinate' females were also observed to have CHC profiles more closely resembling those of

males. This could affect female—female competition for prime oviposition sites, perhaps by deterring rival females through the threat of male harassment. 'Subordinate' females may also chemically mimic males to avoid male harassment themselves, enabling them to spend more time feeding. A similar effect has been observed in the context of mating, where mated female *D. melanogaster* release a male-limited CHC that acts as an anti-aphrodisiac (Scott, 1986). A plausible alternative explanation of our findings is that subordinate males and females both have CHC profiles that primarily serve viability-related functions (such as desiccation resistance) while dominant males and females adopt CHC profiles that signal their sex and dominance status. A similar, viability-related role of CHCs in subordinate males and females might explain why their CHC profiles are more similar than those of dominant males and females.

Chemical signals can be transferred between mates. For example, male queen butterflies, *Danaus gilippus*, directly transfer crystals of danaidone (a sex pheromone) onto the antennae of a female during courtship to promote mating (Eisner & Meinwald, 1995). Similarly, *D. melanogaster* males have been shown to transfer cis-vaccenyl acetate (cVA) to females during copulation, perhaps to elicit aggressive (rather than sexual) reactions to the mated female from competitor males (Jallon, 1984). To our knowledge, there is no evidence that CHCs are passively transferred between individuals during aggressive interactions. Nevertheless, it is possible that competitors directly transferred CHCs to our focal individuals, an effect that could confound that of perceived dominance on CHC profiles. However, we believe that this is unlikely for several reasons. First, *T. angusticollis* males rarely engage in aggressive behaviours unless closely matched for body size (Adler & Bonduriansky, 2013). For this reason, we would expect individuals that are mismatched in body size to have little direct physical contact. Second, direct transfer of CHCs from competitors is not consistent with effects of larval diet on CHC profiles (Fig. 7). If the observed effect of social environment was driven by transfer of CHCs from competitors, then we would expect that dominant individuals should resemble the CHC profiles of their poor larval diet competitors, and vice versa. Since males and females reared on the poor larval diet clustered by CHC profile on PC1 (Fig. 9), this might be expected to result in similar CHC profiles in dominant females and males. Instead, we observed greater similarity between subordinate males and females than between dominant males and females, a pattern that cannot be readily explained by CHC transfer from competitors. Rather, our findings are more consistent with focal individuals actively changing CHC production in response to their social environment, a response that could be mediated by the visual and olfactory perception of competitors, similar to the feedback mechanisms utilized by *D. melanogaster* males to recognize conspecific competitors (Fernandez et al., 2010).

The clustering of pooled samples by dietary treatment aligns with the substantial body of evidence suggesting that CHCs are costly traits that can be influenced by dietary manipulations that affect condition (Blows, 2002; Bonduriansky et al., 2015; Delcourt & Rundle, 2011; Ferveur, 2005; Ingleby, 2015). Furthermore, in *D. melanogaster*, some dietary hydrocarbons have even been shown to be incorporated directly into the CHC profile (Blomquist, 2010). To our knowledge, no studies have examined the degree of sexual dimorphism in CHC profile as a function of the larval dietary environment, and this remains an interesting area for future research.

The CHCs extracted from *T. angusticollis* ranged from 26 to 36 in chain length. Such long molecular chain compounds tend to be more stable than short-chain CHCs, which are often volatile and involved in defensive secretions in insects (Blum, 1981). Because of this stability, at least some of these compounds are likely to be

involved in tactile chemical communication that contributes to social interactions in this species. On the other hand, because of the dual function of CHCs (Chung & Carroll, 2015), it is difficult to know which CHCs are more important for communication compared to desiccation avoidance. The functions of CHC signalling have not been investigated previously in neriid flies, and the fitness consequences of the observed changes in CHC profile in response to perceived dominance status remain to be determined.

In summary, current knowledge of communicative complexity in subsocial and nonsocial insects is limited, particularly in relation to chemical signalling (Nehring & Steiger, 2018). Our findings suggest that *T. angusticollis* individuals of both sexes adjust their chemical displays in response to self-perception of dominance status within a group. To our knowledge, this is the first evidence in a species of nonsocial insect that an individual's perception of its own status within a group can affect its CHC signalling. We also find qualitatively similar responses in both sexes, with subordinate males and females both showing greater resemblance than dominant individuals to the CHC profile of the opposite sex. These changes in CHC profile could affect performance in inter- and intrasexual interactions.

Acknowledgments

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Appendix

Table A1
Results of MANOVA and ANOVA for 17 CHC peaks shared between the sexes

Peak ID	Shared peak ID	Sex	Social treatment	Sex*Social treatment
		P	P	P
	Overall difference (MANOVA)	$2.2e^{-16}$*** (Pillai's trace = 0.909, $F_{1, 140}$ = 73.065)	0.001*** (Pillai's trace = 0.271, $F_{1, 140}$ = 2.716)	0.005** (Pillai's trace = 0.239, $F_{1, 140}$ = 2.293)
3[S]	SP1	$8.487e^{-6}$***	0.270	0.216
4[S]	SP2	$1.487e^{-8}$***	0.559	0.678
5[S]	SP3	$7.573e^{-7}$***	0.017*	0.009**
8[S]	SP4	0.983	0.016*	0.047*
9[S]	SP5	$4.661e^{-11}$***	0.002**	0.028*
10[S]	SP6	$1.383e^{-11}$***	0.343	0.991
11[S]	SP7	$2e^{-16}$***	0.945	0.353
12[S]	SP8	$2e^{-16}$***	0.099[†]	0.431
13[S]	SP9	$2e^{-16}$***	0.063[†]	0.108
14[S]	SP10	$5.191e^{-5}$***	0.001***	0.011*
16[S]	SP11	$2e^{-16}$***	0.095[†]	0.095[†]
17[S]	SP12	$2e^{-16}$***	0.495	0.847
19[S]	SP13	0.295	0.915	0.501
20[S]	SP14	$4.944e^{-15}$***	0.472	0.611
22[S]	SP15	0.711	0.200	0.002**
23[S]	SP16	$5.09e^{-5}$***	0.041*	0.028*
25[S]	SP17	0.098	0.053[†]	0.111

Superscript S refers to peaks consistently present in both sexes that were included in analyses of shared CHC peaks.
***$P<0.001$; **$P<0.01$; *$P<0.05$; [†]$P<0.1$.

Table A2
Summary of linear discriminant analysis (LDA) based on PCA transformation of CHC data

Principal component	Male	Female	Combined		
	LD 1	LD 1	LD1	LD2	LD3
% Variation	100	100	89.79	5.47	4.74
PC1	**0.557**	**0.582**	**-1.069**	0.062	0.086
PC2	**-0.438**	**-0.358**	**0.451**	**0.290**	0.128
PC3	**-0.523**	-0.139	-0.169	**0.290**	**-0.890**
PC4	**-0.438**	**-0.769**	**-0.362**	**0.702**	**0.371**
PC5	-0.113	0.175	**-0.393**	**-0.278**	-0.078
PC6	-0.118	0.231	**0.315**	0.129	0.031
PC7	–	–	-0.064	**1.095**	0.099
PC8	–	–	0.013	0.012	0.062
PC9	–	–	-0.242	**0.417**	**-0.530**

The analysis yielded one function that discriminates the two treatment groups within each sex (based on both shared and sex-limited CHCs) and three functions that discriminate the four sex*treatment combinations (based on shared CHCs only). Loadings >0.25 (i.e. ± 8% overlapping variance) were interpreted as contributing significantly to the axis of variation (bold). The number of principal components accounts for 99% of variation in all data sets.

Table A3
Results of ANOVA for CHCs within sexes

Peak ID	Compound	Male	Female
		P	*P*
	Overall difference	0.049*	0.017*
1	Unidentified CHC 1	–	0.002**
2	Unidentified CHC 2	–	0.001**
3[s]	Unidentified CHC 3	0.800	0.303
4[s]	Unidentified CHC 4	0.984	0.336
5[s]	Heptacosane	0.900	0.716
6	3-Methylheptacosane	0.008**	–
7	3-Methyloctacosane	-0.003**	–
8[s]	2-Methylheptacosane	0.010*	0.792
9[s]	2-Methylnonacosane	0.001**	0.547
10[s]	3-Methylnonacosane	0.296	0.622
11[s]	Unidentified CHC 5	0.652	0.073
12[s]	Unidentified CHC 6	0.152	0.384
13[s]	3-Methyltriacontane	0.702	0.056
14[s]	Hentriacontane	0.290	0.002**
16[s]	2-Methylhentriacontane	0.873	0.080
17[s]	Dotriacontane	0.544	0.654
19[s]	3-Methylhentriacontane	0.708	0.578
20[s]	2,3-Dimethylhentriacontane	0.489	0.820
21	4-Methyltritriacontane	–	0.074
22[s]	Unidentified CHC 7	0.009**	0.002**
23[s]	2-Methyltritriacontane	0.990	0.006**
24	2-Methylpentatriacontane	0.746	–
25[s]	Unidentified CHC 8	0.096	0.565
26	Unidentified CHC 9	–	0.039*
27	Unidentified CHC 10	–	0.668
28	Unidentified CHC 11	–	0.345
29	Unidentified CHC 12	–	0.274

We analysed each of the 20 and 24 peaks of males and females, respectively.
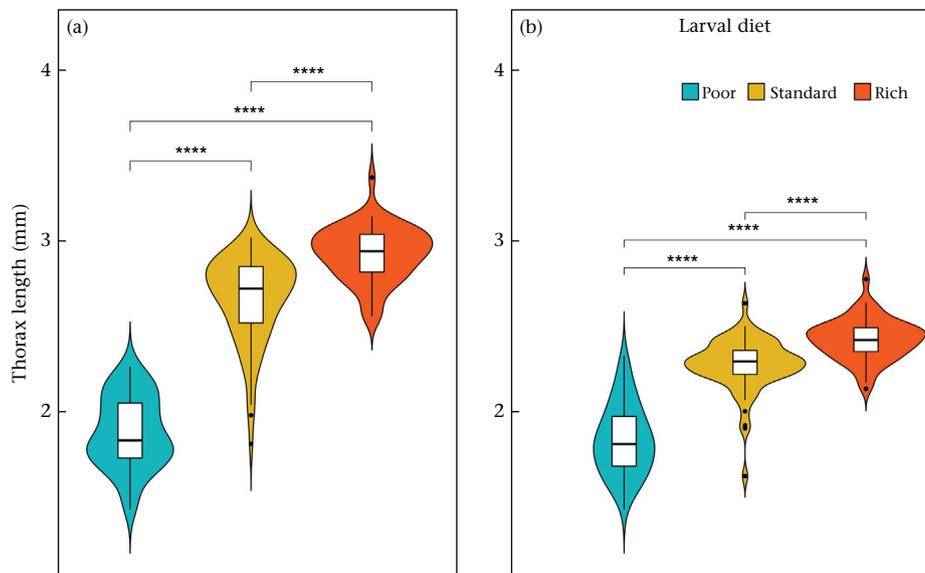**$P < 0.01$; *$P < 0.05$.

Fig. A1. The effects of larval diet quality on (a) male and (b) female thorax length. A subsample of 50 competitors per larval diet treatment were used to compare to our focal individuals. The violin plot outlines illustrate the kernel probability density (the width of the outlined area represents the proportion of the data located there). Within violin plots are box plots with median and interquartile range to illustrate data distribution. Asterisks indicate significant differences in body size between treatments within each sex (Kruskal–Wallis test: males: $\chi^2_2 = 126.7$; females: $\chi^2_2 = 106.6$). Males also differed from females (ANOVA: $F_{1, 347} = 26.15$, $P < 0.0001$).