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Single components of complex chemical signals convey sex identity and individual variation

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Chemical signals, such as those used in social communication, are often present as complex blends of compounds, suggesting that complexity is important in signal perception. Very few studies, however, have examined the interactions between different components of complex signals in social signalling. In the Mysore day gecko, *Cnemaspis mysoriensis*, secretions of males are sufficient to elicit a behavioural response in females and these male secretions differ from those of females in the presence of two key chemical compounds: cholesterol and squalene. This provided us with an opportunity to determine the functions and interactions of individual components in a complex multicomponent chemical signal. First, using tongue flick assays, we established that both components independently elicit a behavioural response in females, but not males. When presented as a multicomponent mix, the response levels of females were similar to those shown towards the individual components, thereby indicating that cholesterol and squalene are redundant components. Moreover, female responses towards these components matched their level of response towards natural male secretions, confirming that both cholesterol and squalene signal sex identity of males. When presented with a gradient of multicomponent stimulus concentrations, females, but not males, incrementally adjusted their tongue flick responses to different levels. Further, responses of females were similar regardless of whether cholesterol or squalene was at a higher relative concentration in the multicomponent stimulus. These last two sets of results indicate that the overall concentration, but not the relative ratio of cholesterol and squalene, has the potential to encode information about male quality. Lack of responses by males to these compounds across experiments strongly indicate the role of cholesterol and squalene in intersexual, and not intrasexual, communication. Overall, we show that two sex-specific compounds in a complex multicomponent chemical signal are effective in communicating complex sexual information from males to conspecific females.

Animal signals, especially those used in social interactions, often include multiple signal components spanning one or more sensory modalities. Multiple components are thought to have evolved to improve information transfer, making signals more effective (Hebets & Papaj, 2005; Rowe, 1999; Smith & Harper, 1995). As a consequence, components of complex signals can be either redundant or nonredundant depending on what information is conveyed and how receivers respond (Johnstone, 1996; Partan & Marler, 1999, 2005). Redundant components act as backups increasing the efficacy of signal transmission, whereas nonredundant components add information content to signals (Johnstone, 1996; Partan & Marler, 1999, 2005). Among the many modalities employed for social communication, chemical signalling is one of the most complex in the animal kingdom (Wyatt, 2014). Chemicals used in social interactions are often composed of multiple compounds which interact to beget complex multicomponent signals that can potentially convey detailed information to receivers. For example, different compounds in the mandibular gland secretions of the ponerine ant Bothroponera soror elicit different responses (Longhurst et al., 1980), while the species-specific chemical blends of pheromonal secretions of moths act as signals of individual quality (Linn & Roelofs, 1989). Although most studies on chemical signals acknowledge the presence of multiple components, interactions between these

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signalling components are rarely studied. In the Carpetan rock lizard, *Iberolacerta cyreni,* females show an elevated response towards ergosterol compared to oleic acid and show an even greater response towards these components when presented together (López & Martín, 2012). Because interactions between individual components can increase or modulate signal efficacy, it is important to understand how receivers respond to these components, individually as well as together (Hebets & Papaj, 2005). In doing so, we can obtain valuable insights into the design and evolution of multicomponent signals.

The Mysore day gecko, *Cnemaspis mysoriensis*, like most geckos, secretes chemicals that are involved in social communication (Kabir et al., 2019; Martín & López, 2014). Chemicals detected on the ventral surface of both males and females of *C. mysoriensis* are composed of at least 19 compounds, comprising fatty acids, fatty acid esters, cholesterol, squalene, glycerol and monolinoleoylglycerol (Kabir et al., 2019). Of these, steroids and fatty acids are major components in many other species of lizards, along with other minor components such as squalene, ketones, aldehydes, fatty acid esters and tocopherols (Weldon et al., 2008). Many of these secretory components play a role in sexual communication in different lizard species. For example, male dominance status is signalled through squalene in the amphisbaenian *Blanus cinereus* (López & Martín, 2009) and through hexadecanol in the Iberian rock lizard, *Lacerta monticola monticola* (Martín et al., 2007). Out of entire blends, though, only a few key compounds seem to play a significant role in receiver perception. For example, in the Yarrow's spiny lizard, *Sceloporus jarrovii*, cyclic dipeptide cyclo(L-Pro-L-Pro) elicits a response in conspecifics that is similar to that towards the entire chemical blend (Romero-Diaz et al., 2021). Among the chemical compounds in *C. mysoriensis*, two key compounds, cholesterol and squalene, are present in the gland secretions of males, but not females (Kabir et al., 2019). This sex difference in chemical secretions is present in other lizards (García-Roa et al., 2016) and can potentially be used as sex and/or individual recognition signals of individuals of the opposite sex. For *C. mysoriensis*, chemical secretions of males are both necessary and sufficient to elicit a behavioural response in females (Kabir et al., 2019). This system, therefore, allows us to test the functions and interactions of individual components of a multicomponent chemical signal in male–male and male–female sexual communication.

In a series of experiments, we examined the role of cholesterol and squalene (found only in males) in chemosensory signalling of *C. mysoriensis*. We first established the detection of cholesterol and squalene by quantifying the behavioural response of both sexes towards these individual compounds in a standard tongue flick assay (Cooper & Vitt, 1986; Martín et al., 2007). To examine the potential interaction between these components, we also quantified lizard responses towards cholesterol and squalene when presented together. Once we established that lizards could detect these compounds individually and together, we tested our hypothesis that cholesterol and squalene function as sex recognition signals of males. For this, we compared lizard responses towards cholesterol and squalene with those towards naturally secreted chemicals of males and females. We further tested the possibility that certain concentrations of these compounds could enable individual recognition, and thus we examined lizard responses to varying ratios of cholesterol and squalene in a concentration gradient. Our multilevel examination of receiver responses towards individual chemical components in different concentrations and combinations allow us to determine the relative importance and degree of redundancy of individual signalling components in a complex chemosensory sexual signal.

METHODS

General Capture and Housing Methods

Cnemaspis mysoriensis, a small diurnal lizard (snout-to-vent length ca. 29 mm), is found in the districts of Bangalore and Mysore in southern India. Individuals used for the study were captured from a forested campus of the Indian Institute of Science (13.02°N, 77.57°E, Bangalore, India), where they are found on trees, rocks and anthropogenic structures. *Cnemaspis mysoriensis* reproduces throughout the year (Biswas, 2005) and hence were expected to be sexually active throughout the study. Individuals were hand captured and carried in a cloth bag to a designated lizard experiment room within 2 h of capture. For all individuals, we measured snout-to-vent length (SVL), total length and mass. Lizards were then housed individually in plastic housing containers (30 × 20 cm and 10 cm high) that were prewashed with hydrogen peroxide (H_2O_2) and lined with damp tissue paper. Throughout their time in captivity, every individual was fed with four fruit flies, *Drosophila melanogaster*, per day. Hand gloves were worn during all handling procedures and experiments to avoid any contamination. All the experiments (described below) were carried out between 0900 and 1300 hours to avoid any changes induced by circadian rhythm variation. Immediately after the experiments, individuals were marked and released at their respective capture sites after 3 days in captivity.

Ethical Note

All the protocols employed in capturing, housing and behavioural assays were approved by the Animal Ethics Committee of the Indian Institute of Science (CAF/Ethics/489/2016). Care was taken to minimize stress to the captured individuals. Collection permits were not required since *C. mysoriensis* is not protected under the Schedules of the Indian Wildlife (Protection) Act, 1972.

Behavioural Assays

All the behavioural trials involved exposure to different chemical treatments and were carried out using the same protocol. Each set of trials (experiments A, B, C, D described below) were conducted with a different set of wild-caught lizards. All trials were conducted in testing tanks (30 × 20 cm and 10 cm high) that were cleaned with 3% H_2O_2 and lined with fresh tissue papers before every trial to eliminate the residual chemical secretions from previous individuals. Approximately 5 min before every trial, the focal individual was lightly wiped with 70% ethanol to avoid chemical residues in the experimental container and introduced into the experimental container under an opaque cup. During the 2 min of habituation to experimental conditions, 50 µl of the chemical stimulus (see details below for each experiment) was loaded onto a cotton swab and introduced into the arena on a small petri dish. Chemical solutions were freshly prepared before every set of behavioural trials. Behavioural trials started 10 s after uncovering the lizard, and lasted for 5 min, during which we recorded the number of tongue flick bouts and latency to the first tongue flick. A tongue flick is the protrusion of the tongue, either in air or on the substrate. Tongue flicks that were not separated by at least 3 s were considered within a single tongue flick bout. The number of tongue flick bouts (hereafter, Number of tongue flicks) reflects the intensity of the behavioural response or 'interest' towards the chemical stimulus, and latency to the first tongue flick is a measure of reaction time of that individual.

Experiment A: Single Chemical Components

We first created separate solutions of cholesterol and squalene (Sigma Aldrich, GC Grade) dissolved in dichloromethane (DCM) at a concentration of 60 mg/ml for each component. The working concentration of these compounds for the experiment was determined based on the minimum stimulus concentration that elicited a behavioural response in receivers according to our pilot data (see Supplementary material). Both these solutions were freshly prepared and mixed thoroughly using a vortex before every set of behavioural assays.

To examine receiver responses towards individual signal components, we exposed male and female lizards ($N = 30$ of each sex) to three treatments: (1) water, (2) DCM and (3) squalene or cholesterol. Water was used as a control for an individual's baseline behaviour and DCM provided a control for the solvent. The order of chemical treatments was randomized across individuals, with a 45 min gap between successive treatments.

Experiment B: Multicomponent Stimuli

To understand the potential interactions between signal components, we examined the number of tongue flicks towards cholesterol and squalene, when presented together. In this experiment, we presented each individual ($N = 30$ females and 20 males in each set) with seven treatments: (1) water, (2) DCM, (3) cholesterol (C), (4) squalene (S), (5) combination of cholesterol and squalene, (6) female (ventral secretions) and (7) male (ventral secretions). This experiment was carried out in two sets: in set 1 (1C:2S), the working concentrations used for cholesterol and squalene were 60 mg/ml and 120 mg/ml, respectively, whereas in set 2 (2C:1S), the working concentrations for cholesterol and squalene were reversed, at 120 mg/ml and 60 mg/ml, respectively, for both single and multicomponent stimuli. The relative ratios of cholesterol and squalene (1C:2S and 2C:1S) were used to represent the two ends of the spectrum of ratios of these components in natural chemical secretions of males of *C. mysoriensis* (see Appendix 1 and the Supplementary Material for details). To collect the ventral secretions of males and females that were used as stimuli, we lined tissue papers in the housing containers of a different set of lizards for 5 days, which allowed the ventral secretions to accumulate (similar to Kabir et al., 2019). These tissues were then collected in zip lock bags and stored at −20 °C until the behavioural trials. Each tissue paper was used as a treatment for not more than five individuals (as per Kabir et al., 2019; Kabir & Thaker, 2021). To avoid sensory overload and fatigue, the seven treatments were randomized and spread across 2 days, with a water control on both days to ensure the constancy of individual baseline response.

Experiment C: Concentration Gradient

We counted tongue flicks of lizards towards a concentration gradient of the multicomponent stimulus in two sets ($N = 30$ females and 20 males in each set), in which set 1 had higher concentrations of squalene relative to cholesterol and set 2 had the opposite. In set 1, individuals were presented with six treatments: (1) water, (2) DCM, (3) 45C|90S (cholesterol|squalene) (mg/ml), (4) 60C|120S (mg/ml), (5) 75C|150S (mg/ml) and (6) 90C|180S (mg/ml). In set 2, individuals were presented with six treatments: (1) water, (2) DCM, (3) 90C|45S (mg/ml), (4) 120C|60S (mg/ml), (5) 150C|75S (mg/ml) and (6) 180C|90S (mg/ml). Similar to experiment B, treatments in each set were presented to the focal individuals over 2 days with a water control on both days.

Experiment D: Different Component Ratios

In the final experiment, we counted tongue flicks by individuals ($N = 30$ females and 20 males) towards the multicomponent stimuli presented in different relative ratios. Individuals were presented with eight treatments: (1) water, (2) DCM, (3) 60C|120S (mg/ml) (1:2), (4) 60C|180S (mg/ml) (1:3), (5) 60C|240S (mg/ml) (1:4), (6) 120C|60S (mg/ml) (2:1), (7) 180C|60S (mg/ml) (3:1) and (8) 240C|60S (mg/ml) (4:1). These ratios were selected based on the actual ratios of cholesterol:squalene in male ventral secretions which ranged from 0.1 to 4.6 (Appendix Fig. A1, Supplementary Material). To test the effect of each component, the concentration of one component was held constant while the other was varied to create different ratios. The order of treatment presentation was randomized, and treatments were presented over 2 days, with a water control on both days.

Statistical Analyses

To determine the effect of single chemical treatments (Experiment A) on the number of tongue flicks, we used generalized linear mixed models (GLMM) with a Poisson distribution for all individuals together (using the R package glmmADMB; Skaug et al., 2014). In this model, chemical treatments and sex were fixed factors, with 'animal ID' and 'treatment order' as random effects to account for the fact that these are repeated measurements and to avoid any order effects, respectively. Post hoc pairwise differences in tongue flicks towards the treatments were determined using lsmeans (Lenth & Hervé, 2015) with Tukey's HSD corrections. For the multicomponent experiment (B), the concentration gradient experiment (C) and the experiment with different ratios (D), we first performed a two-tailed pairwise *t* test on the number of tongue flicks towards the water controls presented on both days to ensure that the individual baseline behavioural response was the same across days. Following this, the effect of other chemical treatments on the number of tongue flicks was determined using GLMM as described above. The analyses described above were also conducted for the latency to tongue-flick response (Appendix 2, Figs. A3, A4). All statistical analyses were done using R (version 3.6.3), and the tables of pairwise comparisons across treatments within experiments are reported in Appendix 2 (Tables A1–A10).

RESULTS

Experiment A: Single Chemical Components

All females and males responded to the treatment stimuli by tongue flicking. The GLMM revealed a significant interaction between chemical treatments and sex for both cholesterol ($\chi^2 = 6.56$, $P = 0.037$) and squalene ($\chi^2 = 16.88$, $P = 0.0002$). In both the squalene and the cholesterol trials, DCM alone elicited significantly more tongue flicks than the water control for both females (squalene trials: $Z = 4.145$, $P < 0.0001$; cholesterol trials: $Z = 4.767$, $P < 0.0001$) and males (squalene trials: $Z = 4.872$, $P < 0.0001$; cholesterol trials: $Z = 4.468$, $P < 0.0001$).

When presented with cholesterol, females showed significantly more tongue flicks compared to both water ($Z = 6.836$, $P < 0.0001$) and DCM controls ($Z = 2.575$, $P = 0.027$; Fig. 1a). Males, in contrast, showed no significant difference in their responses towards cholesterol and the DCM control ($Z = 0.605$, $P = 0.818$; Fig. 1a, Appendix Fig. A3a). For both females and males, latency to tongue flick were similar towards the DCM control and treatment stimuli (Appendix Fig. A3a).

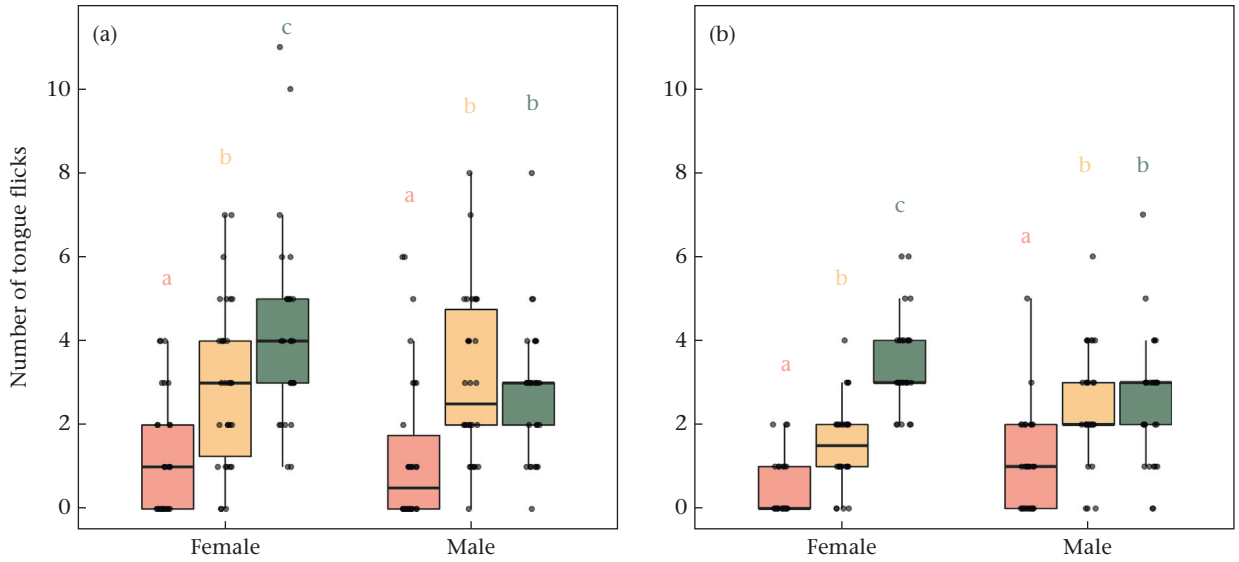

Figure 1. Number of tongue flicks by females and males when presented with (a) cholesterol (green) and (b) squalene (green). For both sets of trials, dichloromethane (DCM, yellow) and water (pink) were used as a solvent control and a baseline control, respectively. Box plots show the median and interquartile ranges (IQR). Dots represent data values. Vertical lines represent quartile 1–1.5 × IQR and quartile 3 + 1.5 × IQR. Sex-specific treatments that are significantly different at $P < 0.05$ are denoted by different colour-coded letters above the box plots.

When presented with squalene, females were significantly quicker to begin tongue flicks (Appendix Fig. A3b) and made significantly more tongue flicks compared to their response to both water ($Z = 7.083$, $P < 0.0001$) and the DCM control ($Z = 4.607$, $P < 0.0001$; Fig. 1b). Males, however, showed no significant difference in the number of tongue flicks towards squalene compared to the DCM control ($Z = 0.555$, $P = 0.844$; Fig. 1b, Appendix Fig. A3b). Thus, both cholesterol and squalene stimuli elicited a significantly greater tongue flick response in females, but not in males.

Experiment B: Multicomponent Stimuli

The baseline behavioural response of lizards, as measured by the number of tongue flicks towards the water control, were similar across days for females in both set 1 ($t = 0.257$, $P = 0.798$) and set 2 ($t = 0.614$, $P = 0.544$). The GLMM revealed a significant difference in individual response towards chemical treatments in both set 1 ($\chi^2 = 163.65$, $P < 0.0001$) and set 2 ($\chi^2 = 167.96$, $P < 0.0001$). In set 1 (1C:2S), where concentrations of squalene were double that of cholesterol, females showed more tongue flicks towards squalene ($Z = 5.439$, $P < 0.0001$) and cholesterol ($Z = 4.787$, $P < 0.0001$) compared to the DCM control (Fig. 2). In set 2 (2C:1S), where concentrations of cholesterol were double that of squalene, the number of tongue flicks by females towards cholesterol ($Z = 4.82$, $P < 0.0001$) and squalene ($Z = 4.918$, $P < 0.0001$) was also significantly greater than towards the DCM control. In both sets, the number of tongue flicks by females towards the combination of cholesterol and squalene was not significantly greater than that towards cholesterol (set 1: $Z = 2.645$, $P = 0.113$; set 2: $Z = 0.887$, $P = 0.975$) and squalene (set 1: $Z = 1.921$, $P = 0.466$; set 2: $Z = 0.781$, $P = 0.987$) alone (Fig. 2). Furthermore, natural male secretions elicited tongue flick responses in females that were not significantly different to those towards squalene (set 1: $Z = 0.487$, $P = 0.999$; set 2: $Z = 0.266$, $P = 1$) and cholesterol (set 1: $Z = 0.249$, $P = 1$; set 2: $Z = 0.372$, $P = 0.999$) when presented individually or together (set 1: $Z = 2.401$, $P = 0.198$; set 2: $Z = 0.516$, $P = 0.997$; Fig. 2). By contrast, ventral secretions of females elicited responses in females that were lower and not significantly different to those shown towards the DCM control (set 1: $Z = 1.356$, $P = 0.825$; set 2: $Z = 2.201$, $P = 0.295$). Latency to first tongue flick did not differ significantly between the DCM control and any of the chemical treatments (Appendix Fig. A4).

Males did not show a significant difference in the number of tongue flicks or the latency of the first tongue flick towards the chemical treatments compared to the DCM control (Appendix Fig. A5).

Experiment C: Concentration Gradient

In this experiment, females showed a similar number of tongue flicks towards the water controls across treatment days in both set 1 ($t = 0.123$, $P = 0.902$) and set 2 ($t = 0.928$, $P = 0.361$). The GLMM showed a significant difference in the number of tongue flicks by individuals as a response to chemical treatments in both set 1 ($\chi^2 = 136.37$, $P < 0.0001$) and set 2 ($\chi^2 = 134.56$, $P < 0.0001$). In set 1, where the relative ratio of cholesterol (C) to squalene (S) was 1:2, the number of tongue flicks by females towards the 60C|120S ($Z = 3.686$, $P = 0.003$), 75C|150S ($Z = 5.503$, $P < 0.0001$) and 90C|180S ($Z = 6.043$, $P < 0.0001$) treatments were significantly greater compared to the 45C|90S treatment (Fig. 3). Although not statistically significant, the average number of tongue flicks increased when the stimulus concentration was increased from 60C|120S to 75C|150S ($Z = 1.973$, $P = 0.358$) and 90C|180S ($Z = 2.577$, $P = 0.103$; Fig. 3). There was no significant difference in the number of tongue flicks by females towards the 75C|150S and 90C|180S treatments ($Z = 0.613$, $P = 0.990$).

Similarly, in set 2, where the relative ratio of cholesterol to squalene was 2:1, the number of tongue flicks by females towards the 120C|60S ($Z = 2.947$, $P = 0.038$), 150C|75S ($Z = 5.64$, $P < 0.0001$) and 180C|90S ($Z = 5.741$, $P < 0.0001$) treatments was significantly greater than to the 90C|45S treatment (Fig. 3). The average number of tongue flicks increased when the stimulus concentration was increased from 120C|60S to 150C|75S ($Z = 2.864$, $P = 0.048$) and 180C|90S ($Z = 2.969$, $P = 0.035$; Fig. 3). There was no significant difference in the number of tongue flicks by females towards the 150C|75S and 180C|90S treatments ($Z = 0.115$, $P = 1$).

Males, in contrast, did not show an elevated tongue flick response towards any concentration level compared to the DCM control (Appendix Fig. A6).

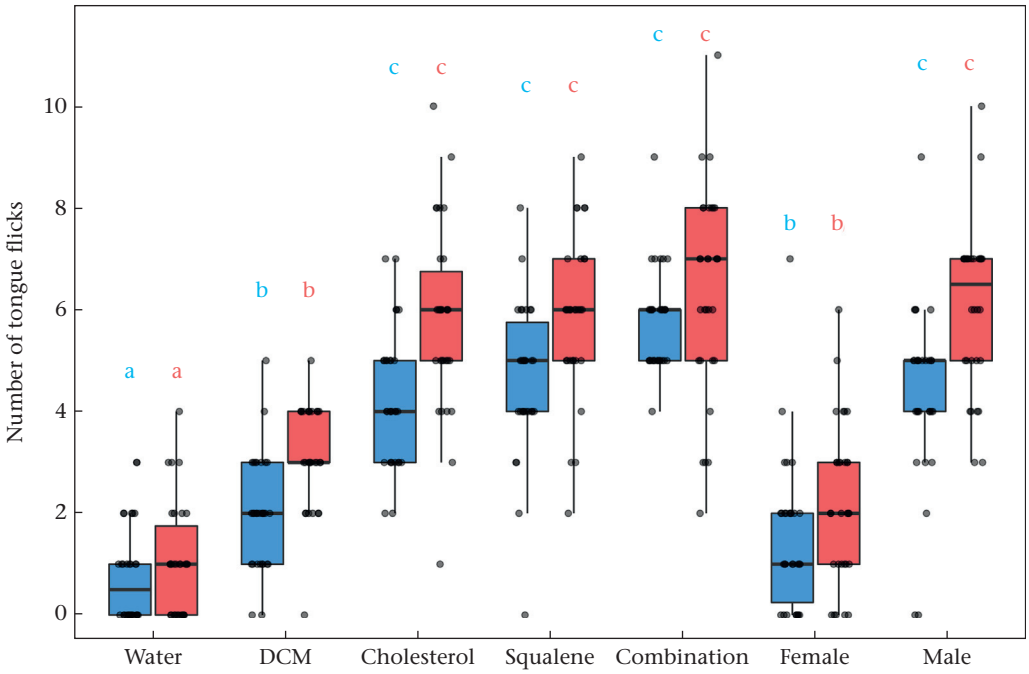

Figure 2. Number of tongue flicks of females directed towards water, dichloromethane (DCM) control, cholesterol alone, squalene alone, combination of cholesterol and squalene, and natural chemical secretions of females and males. Concentrations of cholesterol (C) and squalene (S) in trials shown in blue (1C:2S) are 60 mg/ml and 120 mg/ml, respectively, and those in red (2C:1S) are 120 mg/ml and 60 mg/ml, respectively. Box plots show the median and interquartile ranges (IQR). Dots represent data values. Vertical lines represent quartile 1−1.5 × IQR and quartile 3 + 1.5 × IQR. Sex-specific treatments that are significantly different at $P < 0.05$ are denoted by different colour-coded letters above the box plots. The letters only denote differences within, but not between, the two sets of trials.

Experiment D: Different Component Ratios

Females in this experiment did not differ in their baseline behavioural response towards the water controls on both days ($t = 0.479$, $P = 0.636$), but they did show a significant difference in the number of tongue flicks towards chemical treatments ($\chi^2 = 158.76$, $P < 0.0001$). The tongue flick response towards DCM was significantly lower than that towards all other treatments (Appendix 2). The number of tongue flicks towards the 60C|120S treatment was significantly lower than towards the 60C|180S ($Z = 3.349$, $P = 0.018$), 60C|240S ($Z = 3.755$, $P = 0.004$), 180C|60S ($Z = 4.39$, $P = 0.0003$) and 240C|60S ($Z = 3.037$, $P = 0.049$) treatments (Fig. 4). The number of tongue flicks towards the 60C|180S treatment did not differ significantly from that towards the 60C|240S ($Z = 0.42$, $P = 0.999$), 180C|60S ($Z = 1.084$, $P = 0.96$) or 240C|60S ($Z = 0.322$, $P = 1$) treatments (Fig. 4). Further, there was no significant difference between the number of tongue flicks towards

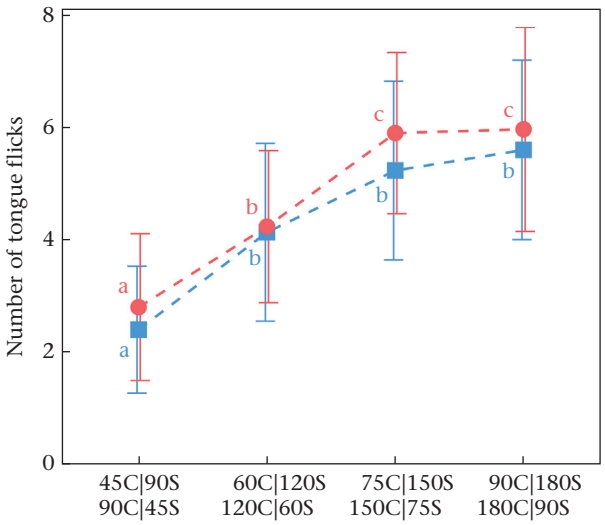

Figure 3. Number of tongue flicks (mean ± SE) by females towards a concentration gradient of multicomponent stimuli comprising cholesterol (C) and squalene (S) in mg/ml at a 1:2 (blue) or 2:1 ratio (red). Treatments that are significantly different at $P < 0.05$ are denoted by different colour-coded letters. The letters only denote differences within, but not between, the two sets of trials.

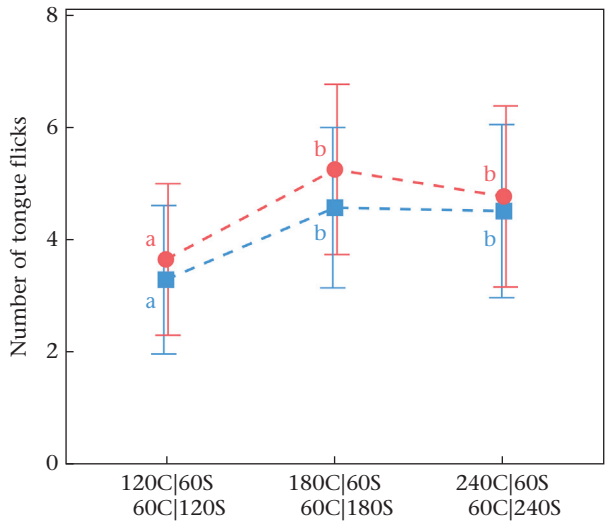

Figure 4. Number of tongue flicks (mean ± SE) by females in response to multicomponent stimuli comprising cholesterol (C) and squalene (S) in mg/ml presented in different relative ratios. Values in blue denote higher C to S concentrations and those in red denote higher S to C concentrations. Treatments that are significantly different at $P < 0.05$ are denoted by different colour-coded letters.

60C|120S and 120C|60S ($Z = 0.328$, $P = 1$), and towards 60C|240S and 240C|60S ($Z = 0.742$, $P = 0.996$). Similar to other experiments, male responses towards different chemical treatments were not significantly different to that towards the DCM control (Appendix 2 Fig. A7).

DISCUSSION

Chemical communication in many species involves a complex mixture of compounds, suggesting that complexity is necessary to elicit a response in receivers. In the diurnal gecko *C. mysoriensis*, cholesterol and squalene are found in the femoral gland secretions of males but not females (Kabir et al., 2019), potentially indicating that these compounds alone are key signalling components for intersexual interactions. Using a series of behavioural assays, we found that females, but not males, showed elevated tongue flick responses towards both cholesterol and squalene individually and together. Female responses towards these chemicals not only matched the level they showed towards natural male secretions, but also changed in response to different overall concentrations. Contrary to our expectation, the intensity of female responses (number of tongue flicks) did not change with the relative ratios of the two chemical components. These results strongly suggest that for females, cholesterol and squalene found in male secretions are likely to function redundantly as sex recognition signals, and potentially as signals of individual quality.

Receiver response is the principal means to understand the function of animal signals. In squamate reptiles (Order Squamata), tongue flicking has been associated with vomeronasal functioning during chemoreception and behavioural interest towards chemosensory stimuli (García-Roa et al., 2017; Schwenk, 1993). To narrow down the possible explanations for the function of multicomponent signals, Hebets and Papaj (2005) proposed an examination of the following questions: (1) are the individual signal components necessary and/or sufficient to elicit a receiver response and (2) does the presence of one signal influence the receiver's response to a second? Our tongue flick assays on *C. mysoriensis* showed that cholesterol and squalene were both sufficient, but not necessary to elicit a response in females. We also found that the female response towards these chemical components was similar when they were presented separately or together, regardless of the relative ratio, clearly highlighting the redundant function of cholesterol and squalene in this multicomponent signal.

Redundancy in signalling components has been reported extensively across taxa and seems to be more common than non-redundancy (Hebets & Papaj, 2005; Partan & Marler, 2005). For example, in the honeybee, *Apis mellifera*, structurally diverse queen pheromones are functionally redundant in inducing sterility in workers (Princen et al., 2019). Such redundant signal components often increase receiver responses when presented together, likely as a result of improvement in transmission accuracy of the signal (Partan & Marler, 2005). However, our results showed that the receiver response towards cholesterol and squalene did not change when they were presented together (Fig. 2). Additionally, the relative ratio of one component versus the other did not change the female's response if the total concentration remained constant (Fig. 4). Why then has complexity evolved when either cholesterol or squalene elicits an equivalent response in receivers? Rowe (1999) postulated that apart from the intensity of the receiver's response, multiple signal components also affect other measures of signal detectability, such as reaction time. For example, in the El Abra pygmy swordtail, *Xiphophorus nigrensis*, females are quicker to react to males that vary in both size and courtship than to males that vary in just one component (Reding & Cummings, 2017). Similarly, multicomponent signals increase the speed of detection of males by female wolf spiders, *Schizocosa ocreata* and *Schizocosa rovneri* (Uetz et al., 2009). In *C. mysoriensis*, however, neither females nor males showed significant differences in response time towards cholesterol and squalene, when they were presented separately or together (Appendix Figs. A3, A4). We speculate that the presence of additional chemical components in *C. mysoriensis*, may instead increase the likelihood of signal detection against the background noise, although this requires further testing under natural conditions.

Redundancy is often found in important signals, such as those used in sexual communication, given the potential costs of missing or misinterpreting information. Sex recognition, a type of sexual communication, is an important step in mate choice, since sexual efforts directed towards the wrong sex can be costly (Johansson & Jones, 2007). For example, the absence of a sex recognition mechanism in the common toad, *Bufo bufo*, may explain why males form amplexus with other males just as frequently as they do with females (Marco & Lizana, 2002). In our study, the similarity in female tongue flick responses towards cholesterol, squalene and male ventral secretions reveals that both chemicals act as redundant sex recognition signals in *C. mysoriensis*. In addition to cholesterol and squalene, the yellow colour patches on the ocular scales of males may also function as a sex recognition signal (Kabir et al., 2019). Such high redundancy in sex identification signals in *C. mysoriensis* across and within modality could be attributed to the cryptic and otherwise indistinguishable dorsal coloration of males and females in this species (Giri et al., 2009). Given the possible missed opportunity cost, such as losing potential mates or territories to potential competitors, information about sex identity is crucial. Efficient transmission of such important signals also depends on the chemical traits of their constituents. High saturation and aromaticity render chemical compounds more stable, making them persist for longer in the environment (Baeckens et al., 2015). In *C. mysoriensis*, many compounds in the femoral gland secretions have large molecular weights (Kabir et al., 2019, 2020), which allows for lower diffusion rates and longer persistence of these signals (Louw et al., 2007). This is true for both cholesterol (molecular weight = 386.65) and squalene (molecular weight = 410.73). Redundancy and longer persistence of chemical secretions of *C. mysoriensis* in the environment make them effective passive signals for intraspecific communication even in the absence of the signaller. Further studies examining costs manifested through information loss can yield useful insights into the evolution of redundant components, such as those found in *C. mysoriensis*.

Females of *C. mysoriensis* also displayed the ability to change the intensity of their response to different concentrations of the multicomponent chemical stimuli (Fig. 3). In two independent experiments (C and D) with females, we found that the number of tongue flicks initially increased with increasing concentration of the multicomponent chemical mix. Exposure to concentrations beyond 225 mg/ml yielded no further increase in number of tongue flicks. Similarly, concentrations below 60 mg/ml did not elicit a tongue flick response. These upper and lower concentrations could reflect sensory thresholds. This threshold-type behavioural response was consistent regardless of whether cholesterol or squalene was the more abundant chemical in the multicomponent mix. Thus, overall concentration, and not the relative ratio of squalene and cholesterol, may potentially function as a signal of male quality to females. Other studies with *C. mysoriensis* seem to suggest that females are able to assess male quality, determined by ectoparasite load and sprint speed, based on their chemical secretions alone (Joshi, 2020). Whether the ventral secretions of high- versus low-quality males vary in overall concentration or relative ratio of compounds is unknown. Quantitative signals, in many animals, can also serve in individual quality assessment and mate choice. For example, in the treefrog, *Hyla arborea*, females prefer male calls with lower peak

frequencies as they indicate larger male size (Plénet et al., 2010). Signals that encode individual quality are mostly cost-added to ensure reliability (Zahavi, 1975, 1993). Cholesterol is one of the most important compounds in the functioning of all the membranes in living cells (Simons & Ikonen, 2000). Similarly, squalene is a major antioxidant and a precursor in biosynthesis of all sterols, including cholesterol (Narayan Bhilwade et al., 2010). Because of this additional cost of allocation, cholesterol and squalene are strong contenders as male quality signals in this species. Further experiments examining information content of this multicomponent signal and its involvement in female mate choice are needed to ascertain the function of these secretion components.

Overall, the pattern of female responses indicates that this multicomponent signal comprising cholesterol and squalene is important in sexual communication in *C. mysoriensis*. Sex differences in communication strategies are not uncommon and often reflect different selection pressures acting on each sex based on their different social requirements. For example, in the rock hyrax, *Procavia capensis*, group-living females have a larger vocal repertoire than solitary males (Demartsev et al., 2019). The relevance of information content might also lead to the differences in perception of social signals (López & Martín, 2009). In *C. mysoriensis*, sexes differ not only in the expression of chemical signals, but also in their perception. We have conclusively shown that females, but not males, show an elevated tongue flick response towards both cholesterol and squalene. This supports previous findings that females and males of this species differ in their primary modality of communication. Chemical secretions are both necessary and sufficient to elicit a response in females of this species, while males respond only when both chemical and visual stimuli are presented (Kabir et al., 2019). Despite the complexity of the multicomponent chemical signal of lizards, two male-specific compounds, cholesterol and squalene, are sufficient to elicit responses in females. In this diurnal gecko species, we provide a rare example of sex recognition functions of equivalently redundant single components.

Author Contributions

M.J. and M.T. were involved in conceptualizing and designing the study. Behavioural trials were carried out by B.E. (one part of experiment A) and M.J. (all other experiments). Data were analysed by M.J. with inputs from M.T. The first draft was written by M.J. and all authors contributed to editing the manuscript.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.1016/j.anbehav.2022.02.013.

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Appendix 1

We determined the relative ratios of cholesterol and squalene in the femoral gland secretions of males of *Cnemaspis mysoriensis*. These chemicals are released through the femoral pores, where they spread as a thin layer on the ventral surface and on substrates. We used a disinfected spatula to gently scrape the secretions from the ventral surface. The secretions were then dissolved in 1 ml dichloromethane (DCM; Merck, HPLC grade). Since the geckos are small, the volume of secretions from a single scrape from an individual is not enough for the chemical analysis. We therefore collected the secretions in two ways: from (1) individuals and (2) groups. In the first category, we repeatedly collected the secretions of a single individual over 10 consecutive days and pooled these samples in a single GC vial ($N = 41$ individuals). Throughout the collection, geckos were individually housed in a disinfected plastic container and provided with a constant diet of fruit flies and water ad libitum. In category 2, the secretions from 10 different individuals were collected in a single GC vial over the course of 1 day, and these lizards were released on the same day of their capture and collection ($N = 80$ individuals/8 vials). All the GC vials were stored at −20 °C until the chemical analysis.

The samples were lyophilized to remove moisture and redissolved in pyridine. The protocol for chemical analysis in GC–M S was followed exactly as described in Kabir et al. (2019). Cholesterol and squalene were identified in the chromatograms by matching their mass spectra and retention times (cholesterol: 53.41 min; squalene: 49.24 min) with the respective standards. Peaks for both compounds were selected and the ratios of % TIC (total ion chromatogram) were calculated for each sample (% TIC − cholesterol/% TIC − squalene).

The ratio of cholesterol to squalene ranged from 4.602 to 0.096 for both the individual lizard samples and group samples (Fig. A1). The average cholesterol:squalene ratio was 1.43 ± 1 for individuals and 1.57 ± 1.11 for grouped samples (Fig. A2).

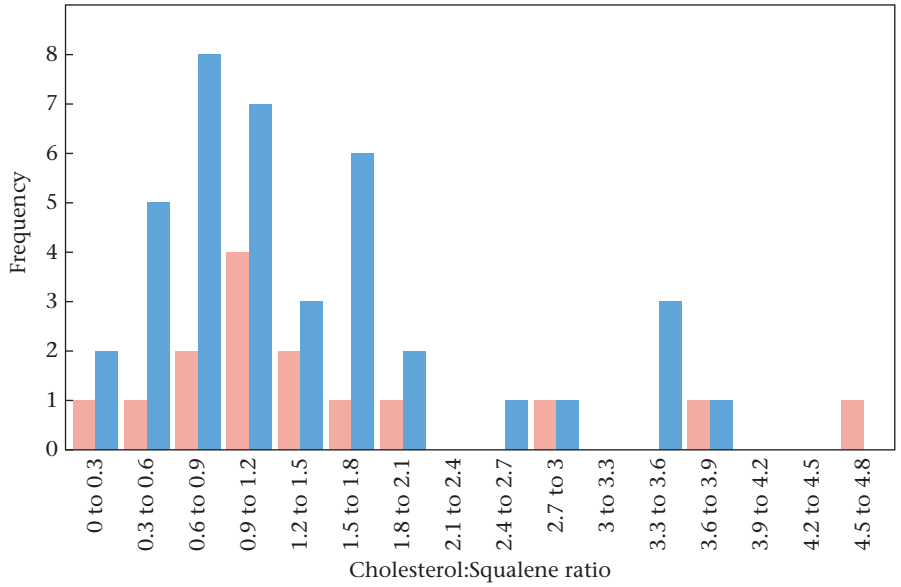

Figure A1. Frequency distribution of cholesterol:squalene ratios in secretions collected from individual males (blue) and groups of males (brown).

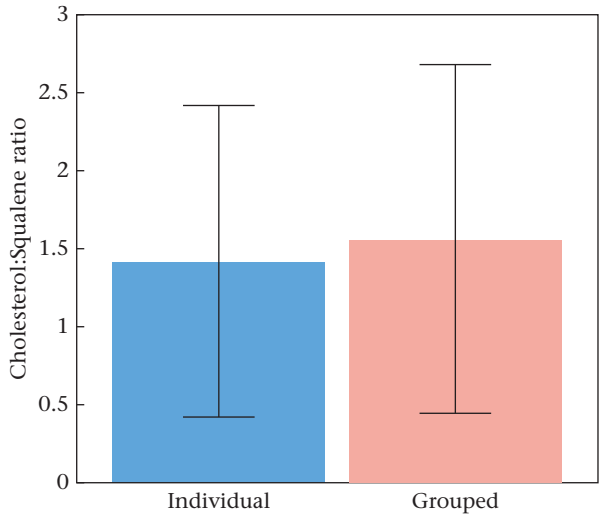

Figure A2. Cholesterol:squalene ratios (mean ± SE) in secretions collected from individual males (blue) and groups of males (brown).

Appendix 2

Table A1
Pairwise comparisons of the number of tongue flicks by females across all treatments (experiment B, set 1)

	Water	DCM	Cholesterol	Squalene	Combination	Female	Male
Water		**0.003**	**< 0.001**	**< 0.001**	**< 0.001**	0.131	**< 0.001**
DCM	3.794		**< 0.001**	**< 0.001**	**< 0.001**	0.825	**< 0.001**
Cholesterol	7.486	4.787		0.99	0.113	**< 0.001**	1.000
Squalene	7.946	5.439	0.736		0.466	**< 0.001**	0.999
Combo	9.068	7.068	2.645	1.921		**< 0.001**	0.198
Female	2.584	1.356	5.901	6.501	7.985		**< 0.001**
Male	7.644	5.009	0.249	0.487	2.401	6.106	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane.

Table A2
Pairwise comparisons of the number of tongue flicks by males across all treatments (experiment B, set 1)

	Water	DCM	Cholesterol	Squalene	Combination	Female	Male
Water		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.002**	**< 0.001**
DCM	6.080		0.284	0.999	0.999	0.089	0.986
Cholesterol	7.553	2.221		0.155	0.503	**< 0.001**	0.775
Squalene	5.870	0.296	2.511		0.995	0.179	0.935
Combo	6.332	0.362	1.866	0.658		**0.034**	0.999
Female	3.949	2.734	4.810	2.449	3.079		**0.009**
Male	6.619	0.783	1.448	1.079	0.422	3.478	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane.

Table A3
Pairwise comparisons of the number of tongue flicks by females across all treatments (experiment B, set 2)

	Water	DCM	Cholesterol	Squalene	Combination	Female	Male
Water		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.014**	**< 0.001**
DCM	5.269		**< 0.001**	**< 0.001**	**< 0.001**	0.295	**< 0.001**
Cholesterol	8.815	4.82		1	0.975	**< 0.001**	0.999
Squalene	8.882	4.918	0.106		0.987	**< 0.001**	1
Combo	9.363	5.634	0.887	0.781		**< 0.001**	0.998
Female	3.366	2.201	6.714	6.803	7.449		**< 0.001**
Male	9.048	5.164	0.372	0.266	0.516	7.025	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane.

Table A4
Pairwise comparisons of the number of tongue flicks by males across all treatments (experiment B, set 2)

	Water	DCM	Cholesterol	Squalene	Combination	Female	Male
Water		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
DCM	5.066		0.999	1	1	0.999	0.986
Cholesterol	5.312	0.335		0.999	0.998	0.988	0.921
Squalene	5.066	0.078	0.335		1	0.999	0.996
Combo	4.938	0.171	0.506	0.171		1	0.996
Female	4.739	0.432	0.766	0.432	0.261		0.999
Male	4.462	0.790	1.123	0.79	0.619	0.358	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane.

Table A5
Pairwise comparisons of the number of tongue flicks by females across all treatments of the concentration gradient (experiment C, set 1)

	Water	DCM	45|90	60|120	75|150	90|180
Water		**0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
DCM	3.891		0.983	**< 0.001**	**< 0.001**	**< 0.001**
45|90	4.473	0.689		**0.003**	**< 0.001**	**< 0.001**
60|120	7.276	4.317	3.686		0.358	0.103
75|150	8.532	6.079	5.503	1.973		0.990
90|180	8.895	6.601	6.043	2.577	0.613	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane. Ratios 45|90, 60|120, 75|150 and 90|180 are ratios of cholesterol to squalene in mg/ml.

Table A6
Pairwise comparisons of the number of tongue flicks by males across all treatments of a concentration gradient (experiment C, set 1)

	Water	DCM	45|90	60|120	75|150	90|180
Water		**< 0.001**	**< 0.001**	**0.004**	**< 0.001**	**< 0.001**
DCM	4.000		0.996	0.998	0.999	0.999
45|90	4.430	0.509		0.934	1.000	0.999
60|120	3.617	0.441	0.949		0.971	0.982
75|150	4.290	0.342	0.167	0.782		1.000
90|180	4.219	0.258	0.251	0.698	0.084	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane. Ratios 45|90, 60|120, 75|150 and 90|180 are ratios of cholesterol to squalene in mg/ml.

Table A7
Pairwise comparisons of the number of tongue flicks by females across all treatments of a concentration gradient (experiment C, set 2)

	Water	DCM	90|45	120|60	150|75	180|90
Water		**0.004**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
DCM	3.608		0.978	**0.004**	**< 0.001**	**< 0.001**
45|90	4.265	0.730		**0.037**	**< 0.001**	**< 0.001**
60|120	6.707	3.630	2.947		**0.048**	**0.035**
75|150	8.749	6.240	5.640	2.864		1.000
90|180	8.819	6.306	5.741	2.969	0.115	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane. Ratios 90|45, 120|60, 150|75 and 180|90 are ratios of cholesterol to squalene in mg/ml.

Table A8
Pairwise comparisons of the number of tongue flicks by males across all treatments of a concentration gradient (experiment C, set 2)

	Water	DCM	90|45	120|60	150|75	180|90
Water		**0.027**	0.307	0.299	0.175	**0.002**
DCM	3.148		0.946	0.998	0.989	0.978
45|90	2.179	1.035		0.999	0.999	0.489
60|120	2.198	0.511	0.332		1	0.851
75|150	2.457	0.743	0.294	0.100		0.683
90|180	3.923	0.863	1.886	1.301	1.599	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane. Ratios 90|45, 120|60, 150|75 and 180|90 are ratios of cholesterol to squalene in mg/ml.

Table A9
Pairwise comparisons of the number of tongue flicks by females towards different ratios of multicomponent stimuli (experiment D)

	Water	DCM	60|120	60|180	60|240	120|60	180|60	240|60
Water		**0.002**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
DCM	3.917		**0.008**	**< 0.001**	**< 0.001**	**0.022**	**< 0.001**	**< 0.001**
60|120	6.817	3.602		**0.018**	**0.004**	1	**< 0.001**	**0.049**
60|180	9.029	6.638	3.349		0.999	**0.006**	0.960	1
60|240	9.278	6.992	3.755	0.042		**0.001**	**0.997**	**0.996**
120|60	6.582	3.292	0.328	3.664	4.066		**< 0.001**	**0.018**
180|60	9.661	7.541	4.390	1.084	0.664	4.696		0.856
240|60	8.835	6.363	3.037	0.322	0.742	3.353	1.404	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane. Ratios 60|120, 60|180, 60|240, 120|60, 180|60 and 240|60 are ratios of cholesterol to squalene in mg/ml.

Table A10
Pairwise comparisons of the number of tongue flicks by males towards different ratios of multicomponent stimuli (experiment D)

	Water	DCM	60|120	60|180	60|240	120|60	180|60	240|60
Water		**0.004**	**0.006**	**0.002**	**0.014**	**0.033**	**< 0.001**	**0.002**
DCM	3.754		1	1	0.999	0.997	0.998	1
60|120	3.674	0.093		1	1	0.999	0.996	1
60|180	3.986	0.275	0.368		0.998	0.981	1	1
60|240	3.428	0.378	0.285	0.652		1	0.973	0.998
120|60	3.170	0.670	0.557	0.944	0.293		0.900	0.982
180|60	4.282	0.631	0.724	0.356	1.007	1.297		1
240|60	3.986	0.275	0.368	0.009	0.652	0.944	0.356	

The *Z* ratios from the Tukey's multiple comparison test are written below the diagonal and *P* values are written above the diagonal. *P* values of the pair of treatments showing significant difference at $P < 0.05$ are in bold. DCM: dichloromethane. Ratios 60|120, 60|180, 60|240, 120|60, 180|60 and 240|60 are ratios of cholesterol to squalene in mg/ml.

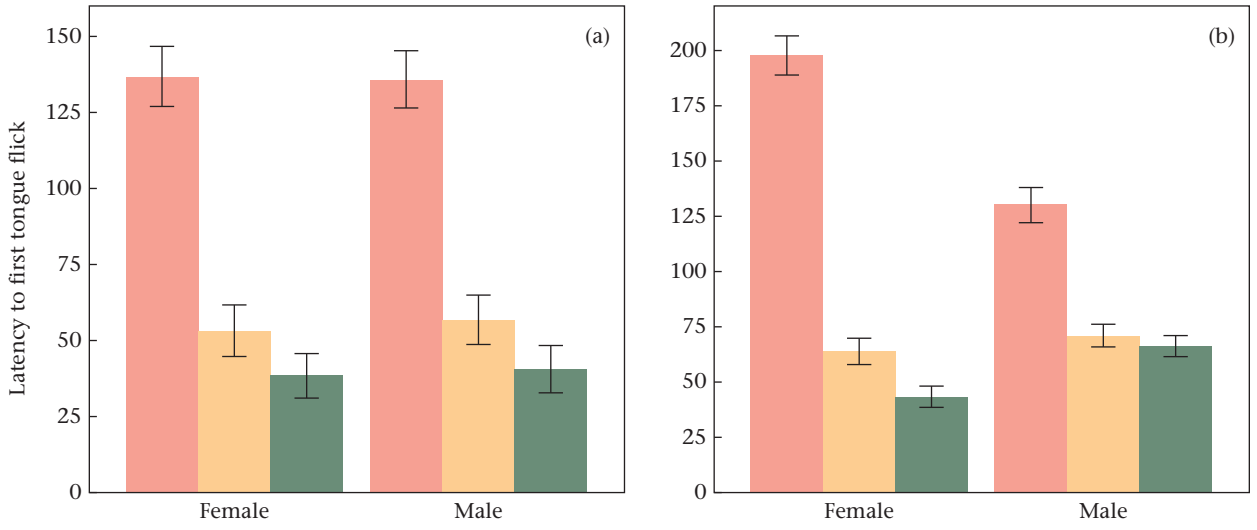

Figure A3. Latency to the first tongue flick (mean ± SE) by females and males when exposed to (a) cholesterol (green) and (b) squalene (green) in experiment A. Water (pink) and dichloromethane (DCM, yellow) are baseline control and solvent control, respectively.

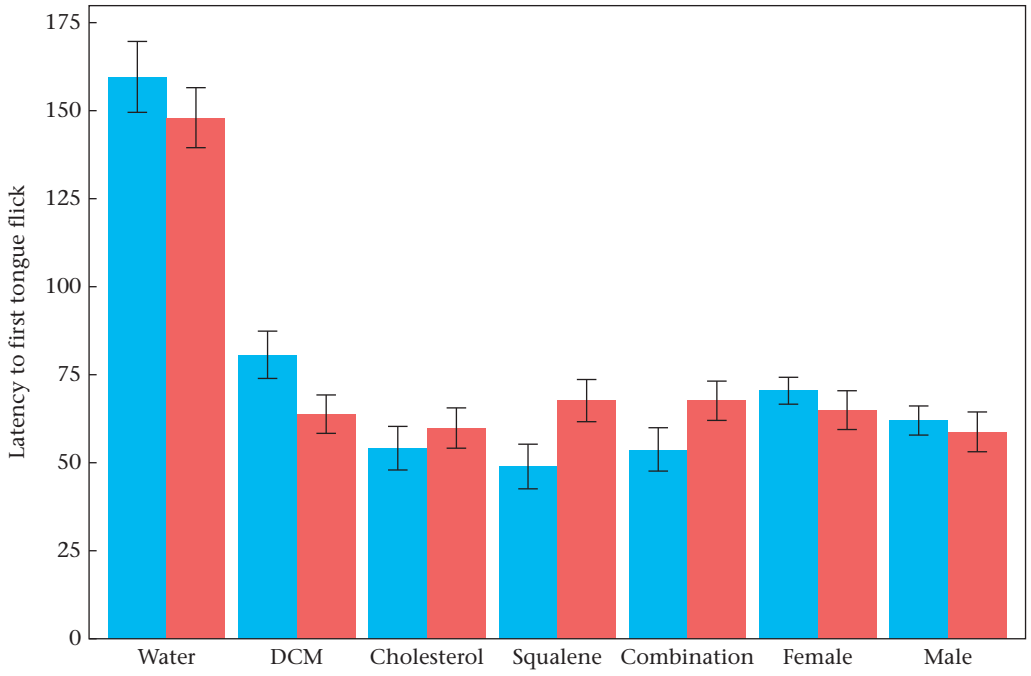

Figure A4. Latency to the first tongue flick (mean ± SE) of females towards cholesterol (C) alone, squalene (S) alone, combination of cholesterol + squalene with relative ratios of 1:2 (1C:2S; blue) and 2:1 (2C:1S; red) and natural chemical secretions of females and males in experiment B. Concentrations of cholesterol (C) and squalene (S) in trials shown in blue (1C:2S) are 60 mg/ml and 120 mg/ml, respectively, and those in red (2C:1S) are 120 mg/ml and 60 mg/ml, respectively.

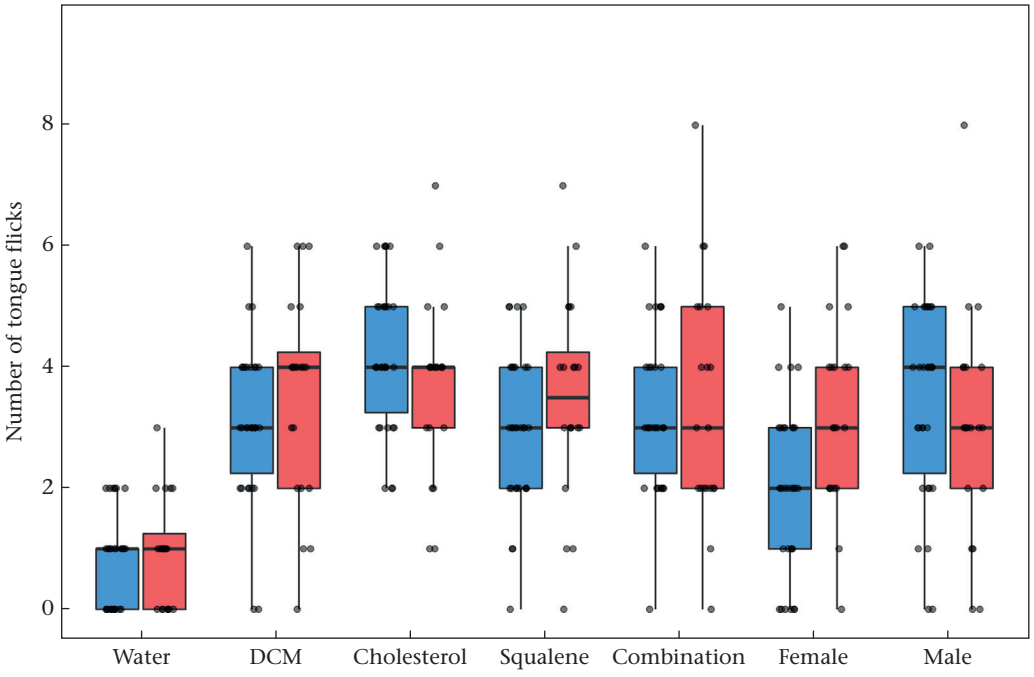

Figure A5. Number of tongue flicks of males in experiment B directed towards water, dichloromethane (DCM) control, cholesterol alone, squalene alone, combination of cholesterol and squalene, and natural chemical secretions of females and males. Concentration of cholesterol (C) and squalene (S) in trials shown in blue (1C:2S) are 60 mg/ml and 120 mg/ml, respectively, and those in red (2C:1S) are 120 mg/ml and 60 mg/ml, respectively. Box plots show the median and interquartile ranges (IQR). Dots represent data values. Vertical lines represent quartile 1−1.5 × IQR and quartile 3 + 1.5 × IQR.

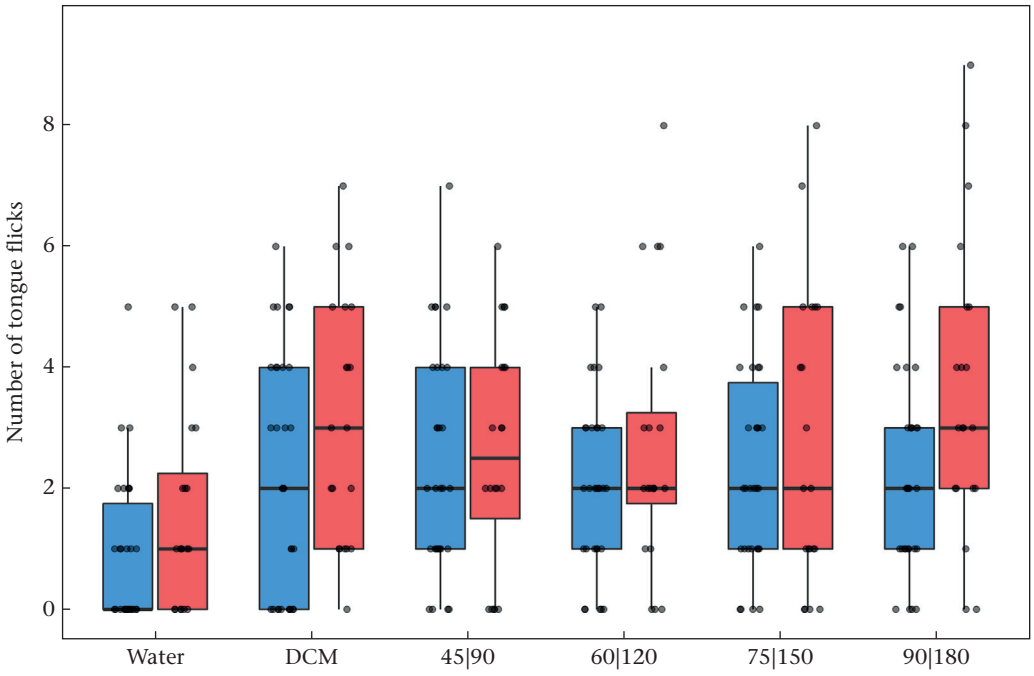

Figure A6. Number of tongue flicks by males in experiment C towards water, dichloromethane (DCM) control and a concentration gradient of multicomponent stimuli comprising cholesterol (C) and squalene (S) in relative ratios of 1:2 (1C:2S; blue) and 2:1 (2C:1S; red) in mg/ml. Box plots show the median and interquartile ranges (IQR). Dots represent data values. Vertical lines represent quartile 1−1.5 × IQR and quartile 3 + 1.5 × IQR.

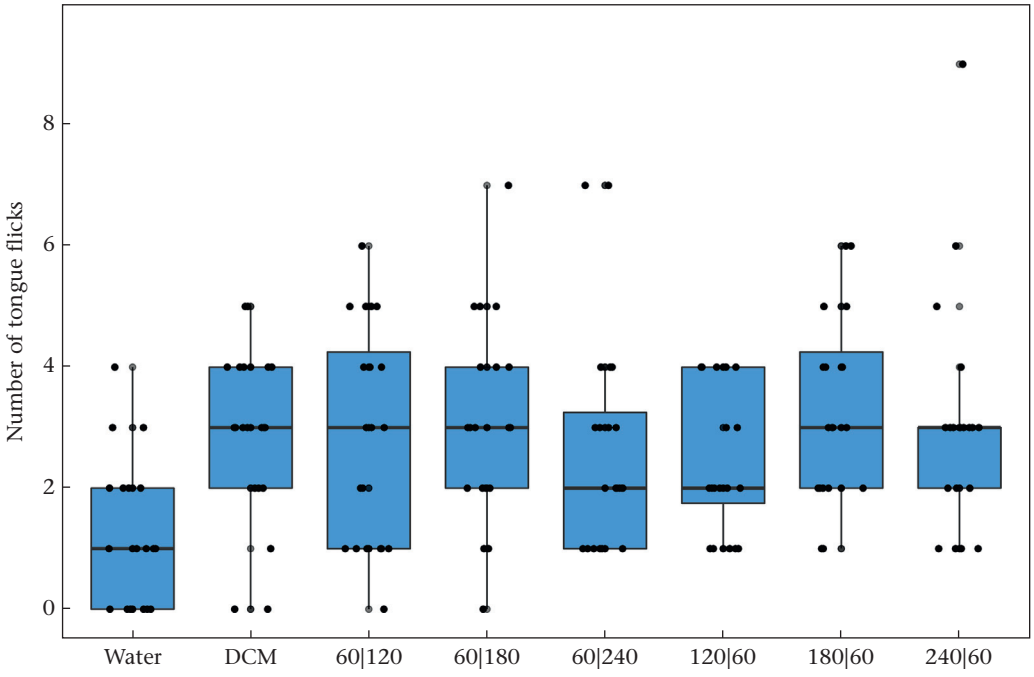

Figure A7. Number of tongue flicks by males in experiment D in response to water, dichloromethane (DCM) control and multicomponent stimuli comprising cholesterol (C) and squalene (S) presented in different relative ratios (C|S) in mg/ml. Box plots show the median and interquartile ranges (IQR). Dots represent data values. Vertical lines represent quartile 1−1.5 × IQR and quartile 3 + 1.5 × IQR.