The role of male chemical secretion components in sex recognition, mate assessment and mate choice in the diurnal gecko *Cnesmaspis mysoriensis*

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CERTIFICATE

This is to certify that this dissertation entitled “The role of male chemical secretion components in sex recognition, mate assessment and mate choice in the diurnal gecko *Cnesmaspis mysoriensis*” towards the partial fulfilment of the MS degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Joshi Mihir Makarand at IISc Bangalore under the supervision of Dr. Maria Thaker, Assistant Professor, Centre for Ecological Sciences, IISc Bangalore during the academic year 2019-2020.

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DECLARATION

I hereby declare that the matter embodied in the report entitled "The role of male chemical secretion components in sex recognition, mate assessment and mate choice in the diurnal gecko *Cnesmaspis mysoriensis*" are the results of the work carried out by me at the Centre for Ecological Sciences, Indian Institute of Science, under the supervision of Dr. Maria Thaker and the same has not been submitted elsewhere for any other degree.

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Abstract

Animals often use multicomponent signals in intraspecific communication to convey sexual information such as sex identity and quality of the signaler. In the day gecko *Cnemaspis mysoriensis*, two chemical components, squalene and cholesterol, are present in the femoral and precloacal gland secretions of males, but are absent in females. This multicomponent signal provides an opportunity to understand the function of each component of a complex signal. I quantified receiver responses towards squalene and cholesterol when presented individually and in combination. The tongue flick assay revealed that females, but not males, showed an elevated response towards squalene and cholesterol, as individual components and together. Females also showed increase in tongue flick response with the increase in squalene and cholesterol stimuli concentrations. I further examined female preferences towards natural male secretions in a Y-tube choice experiment. Female choice revealed that secretions of males with low ectoparasite load and high sprint speed were preferred. Based on the receiver tongue flick responses and female choices, I conclude that: 1) squalene and cholesterol independently and together act as sex recognition signals of males in this species, 2) females are able to differentiate between conspecifics males based on squalene and cholesterol concentrations and 3) male secretion components also contribute in mate assessment and females prefer males with low ectoparasite load and high sprint speed.

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Introduction

Animal signal can be defined as an action or structure that 'has evolved to indicate an otherwise unperceivable quality about the signaler or the signaler's environment' (Donath, 2007). Based on the information content of the signals, they can either be "self-reporting" or "other-reporting" (Smith and Harper, 1995). Self-reporting signals provide information to receivers about the signaller, while other-reporting signals encode information about other biotic or abiotic factors in the surroundings. For example, males performing sexual displays to attract mates and nestlings begging for food are self-reporting signals, whereas alarm calls towards predators and bee dances that indicate the direction of a food source are other-reporting signals. Although the information content based perception of animal signals is both common and convenient, it ignores the importance of factors shaping the functional signal design (Rendall et al., 2009).

From an evolutionary perspective, animal signals can be viewed as those actions or structures that increase the fitness of the signaller (and in most cases even the receiver) by changing the behavior of receivers and have evolved specifically for this reason (Smith and Harper, 1995). Based on evolutionary mechanisms, signals can either be a minimal, cost-added or index signal (Smith and Harper, 1995). For example, a *Drosophila subobscura* female, when mated, gives a courtship inhibiting signal. This is a minimal signal as it gets the information across to a courting male and is cost-free (Smith, 1956). The tail of peafowl, *Pavo cristatus,* is a cost-added signal as the cost associated with the signal is much more than what is required to transmit the information (Grafen, 1990). The added cost in such signals, by preventing cheating, ensures reliability of these signals. Lastly, tigers, *Panthera tigris*, scratch tree barks as high as possible to mark their territories (Thapar, 1986). This is classified as an index signal because of the physical association between the signal and the size of the individual, which is a virtue of interest for receivers. In spite of the variety of definitions and classifications of animal signals, two general utilitarian themes of signals are necessary: 1) signals transmit information and 2) signals elicit behavioral change in receivers (Markl, 1983; Zahavi, 1987). Also, irrespective of the category they fall under,

signals confer a fitness advantage to at least the signaller, but very often, even the receiver.

Due to the fitness advantage, signals used in communication are naturally selected for and have evolved in every known taxon from bacteria to mammals (De Cock and Matthysen, 2005; Dall et al., 2005; Federle and Bassler, 2003; Francke and Dettner, 2005; Sekimoto, 2005). Evolution has enabled animals to communicate complex information through complex signals (Baeckens et al., 2018). Whether complex or not, signals have to be transmitted through the environment so that they reach the receiver and elicit an appropriate behavioral response to be successful (Hebets and Papaj, 2005). Guildford and Dawkins (1991) describe two kinds of selection pressures on complex signals: 1) content-based selection and 2) efficacy-based selection. While content-based selection acts on those components of the signal that are associated with the information content or message of the signal, the efficacy-based selection acts on the components that ensure efficient passage of signals through the environment and reception by receivers (Hebets and Papaj, 2005). Essentially, the content-based selection relates to 'why' of the signal, whereas, efficacy-based selection relates to 'how' of the signal (Hebets and Papaj, 2005).

Content-based selection gives rise to multiple signal components, belonging to one or more sensory modalities, that can either be "redundant" (or "back-up") signals or "non-redundant" (or "multiple messages") signals (Bro-Jørgensen, 2010; Partan and Marler, 1999, 2005). Redundant signals do not provide different information, but increase the accuracy of transmission of information. For example, in the arctiid moth *Cycnia tenera*, males use ultrasonic clicks and pheromones as courtship signals. Females of the species respond similarly to both the signal components irrespective of whether they are presented together or separately (Conner, 1987). Non-redundant signals, on the other hand, provide different information that could either be different types of information or different forms of signaller quality. Further, multiple messages in a non-redundant signal could be targeted at one or more recipients. For example, in dart-poison frog *Epipedobates femoralis*, the bimodal signal elicits a different response in the receiver when each component is present separately or together (Narins et al.,

2003). The visual cue in this species (pulsing vocal sacs without sound) does not elicit any response from conspecifics, but the acoustic cue (audio playback of vocalizations) attracts males towards the audio source (Narins et al., 2003). When presented with both the visual and acoustic cues together, a large number of males get attracted and demonstrate aggressive behavior towards the signaling individual (Narins et al., 2003). Irrespective of whether the signals are redundant or non-redundant, animals use these highly evolved complex signals to communicate complex information such as food availability, presence of predators, social status and mating intention.

Sexual communication is one context in which animals have evolved extremely elaborate and complex signals. Sexual communication directly or indirectly influences mate choice, where mate choice is “any behavior that restricts the set of potential mates” (Wiley and Poston, 1996). Mate choice can either be direct or indirect. Direct mate choice needs direct discrimination between individuals of the opposite sex based on some traits. For example, in the great snipe *Gallinago media,* females choose specific males based on the position of territories they occupy in a lek. Indirect mate choice involves behaviors that restrict choice to a specific species, specific sex or specific mate quality (Johansson and Jones, 2007). For example, in bark beetles, belonging to genus *Ips*, the pheromones used for attracting conspecifics to mating sites are distinct in closely related species (Symonds and Elgar, 2004). Crayfish *Procambarus clarkii* show sex recognition based on presence or absence of carbohydrate pheromones (Ameyaw-Akumfi and Hazlett, 1975). In cockroach *Nauphoeta cinerea* male pheromones signal individual quality and attract females to allow for mate assessment (Moore and Moore, 1999). Species recognition, mate recognition and mate assessment signals are not mutually exclusive (Ryan and Rand, 1993) and have evolved to minimize the costs of searching for, courting and mating individuals that reduce reproductive fitness (Johansson and Jones, 2007). This fitness advantage has promoted use of sophisticated sexual communication signals using different sensory modalities involving visual, acoustic, chemical and seismic stimuli by animals. Amongst these, chemical signaling is the most primitive and widespread form of communication (Johansson and Jones, 2007).

The proposed role of chemicals in sexual communication in the 19th century (Darwin, 1872) was confirmed only after the discovery of sex pheromones in the silk moth *Bombyx mori* (Karlson, 1958). Sex pheromones are defined as the chemicals that influence the sexual behavior of conspecifics to benefit the emitter (Johansson and Jones, 2007). Although the contribution of chemical signaling in sexual communication is now extensively studied in multiple taxa (Brennan and Keverne, 2004; Chouinard, 2012; Dunham, 1978; Wyatt, 2003), the focus of these studies has been on sex pheromones involved in mate attraction (Svensson, 1996) and species or mate recognition (Ptacek, 2000). In lizards (Sub-order Lacertilia), sexual communication involving chemical signals is well documented (García-Roa et al., 2016; MacGregor et al., 2017). Chemicals secreted on the ventral surface from femoral and precloacal glands act as chemical signals for sexual communication (Cole, 1966). Their role as species recognition signals (Barbosa et al., 2006), sex recognition signals (Cooper and Pèrez-mellado, 2002) and mate assessment signals (López and Martín, 2005; Martin and Lopez, 2006; Martín et al., 2007) have been extensively studied in lizards, but the role of each component of these signals is hardly ever studied. Studying the roles of components of a chemical signal as either recognition or assessment signals can shed light on how sexual information is conveyed through chemical signals by emitters and how receivers perceive it.

Animals use a variety of chemicals as sex pheromones to communicate sexual information. For example, different species of moths are known to use saturated fatty alcohols such as Z-ll-tetradecenol, acetates like Z-9-tetradecenyl acetate and unsaturated fatty aldehydes like E10,Z12-Hexadecadienal as sex pheromones to attract mates (Ando et al., 1978; Kalinová et al., 2001). Several invertebrates as well as vertebrates are also known to use functional hormones as sex pheromones (Dunham, 1978; Singer, 1991). In lizards, the chemicals secreted from either skin or femoral and precloacal glands can act as sex pheromones (Martin and Lopez, 2014). Femoral and precloacal glands are homologous and are located in different positions in different species (Gabe and Saint Girons, 1965). These are holocrine glands, controlled by androgenic hormones (Fergusson et al., 1985), which secrete chemicals on the ventral body surface. As the gland secretions are stimulated by testosterone and other related

hormones, the volume of secretion is more abundant in males and during the breeding season (Alberts et al., 1993). Femoral and precloacal glands produce lipidophilic and proteinaceous chemicals, which along with performing their main function of preventing water loss, also contribute in sexual communication (Martin and Lopez, 2014). Although proteins have the potential to act as sex pheromones, lipids are highly volatile and have a greater molecular diversity, potentially increasing their information content (Martin and Lopez, 2014). Steroids are among the most abundant chemicals found in femoral and precloacal gland secretions (Weldon et al., 2008). Cholesterol and related steroids like campesterol and cholestanol are the main steroids, but others, such as cholesta-3,5-diene, stigmasterol and sitosterol are also found in these secretions (Weldon et al., 2008). The role of these steroids as recognition or assessment signals, however, has not been studied in lizards.

Cnemaspis is a genus of diurnal geckos distributed throughout Asia and Africa (Sayyed et al., 2018). Similar to other species of diurnal geckos, it is secondarily diurnal and has evolved from nocturnal ancestors (García-Roa et al., 2017; Röll, 2001). A shift to diurnal habit has allowed for the evolution of visual signaling traits along with the more primitive olfactory traits (Ellingson et al., 1995; Mayerl et al., 2015). The Mysorean day gecko *Cnemaspis mysoriensis* is endemic to the southern Indian regions of Bangalore and Mysore (Giri et al., 2009). Males and females of this species have chevron-like brown and white dorsal colour pattern (Jerdon, 1853). Some males of this species show yellow coloration on their gular region while females do not have any yellow colour patches. Males of this species, but not females, possess femoral and precloacal glands. Secretions from these glands get passively deposited on the surfaces males move on. Males, during courtship, expand the gular region making it conspicuous to females. This visual trait, along with the femoral and precloacal gland secretions make up a multimodal signal that involves both visual and chemical stimuli. In a previous study that examined multimodal signals in *C. mysoriensis* from both the signaller and receiver perspectives, female response towards male chemical secretions did not change in the presence of visual cues (Kabir et al., 2019a). Also, females did not respond to visual cues alone, indicating that chemical secretions from the femoral and

precloacal glands were solely responsible for male-to-female sexual communication. Males of the species required both chemical and visual stimuli. In the same study, chemical components of male femoral and precloacal gland secretions were identified and two components, squalene and cholesterol, were found to be present exclusively in males (Kabir et al., 2019a).

The sexual dimorphism in the expression of squalene and cholesterol makes it an excellent system to understand roles of individual components in complex signals. Thus, I aimed to elucidate the function of squalene and cholesterol as either sex recognition signals or mate quality assessment signals or both. Since the behavioral response in females can be elicited by male secretions alone (Kabir et al., 2019a), I examined the roles of squalene and cholesterol in sexual communication, specifically as sex recognition signals, when present separately and together. To understand the function of male secretion components in mate assessment and mate choice, I also carried out a female choice experiment in a Y-tube choice maze.

Overall, I conducted four main experiments that examined the following objectives:

1) To determine the female and male behavioral response towards squalene as single-chemical stimuli,

2) To determine the female and male behavioral response towards squalene and cholesterol as single-chemical and multi-chemical stimuli,

3) To determine the behavioral response of females and males towards a concentration gradient of squalene and cholesterol as multi-chemical stimuli and

4) To determine female choice between femoral and precloacal gland secretions of different males.

These objectives allowed me to determine: i) The role of squalene and cholesterol in male-female and male-male interaction ii) whether females differentiate between males based on squalene and cholesterol concentrations and iii) whether male secretion components function as mate assessment signals resulting in female mate choice.

Methods:

Capturing and housing procedure

Mysorean day geckos *Cnemaspis mysoriensis* are small diurnal geckos (snout-to-vent length ca. 29 mm) endemic to Bangalore and Mysore regions of India. The study individuals of *Cnemaspis mysoriensis* were caught by hand from the forested campus of Indian Institute of Science (13.02ºN, 77.57ºE, Bangalore, India), where they are found in crevices on trees, rocks and old human constructions. I captured 30 individuals in a cloth bag and transferred them to a plastic box (30cm X 20cm X 10cm) within 2 hours of capture before each experiment. I washed all the boxes with hydrogen peroxide (H_2O_2) to remove any chemical traces and lined them with damp tissue paper before introducing the geckos. Each experiment used different individuals, and all the captive individuals (focal and stimulus animals) were housed alone in containers under the same conditions. As soon as they were brought to the lab, I took morphometric measurements such as snout-to-vent length (SVL), total length, ectoparasite load and mass. The geckos were acclimatized for a day prior to the experiments in a designated lizard experiment room, which allows for natural conditions including light and dark cycle as well as temperature. Individuals were fed with three *Drosophila melanogaster* flies each day in captivity. I carried out the behavioral trials on the following two days. All the experiments were done while wearing gloves to avoid any contamination and were carried out between 9 AM to 1 PM to minimize the effect of the fluctuations in circadian rhythm of these geckos. The geckos were released at their respective sites of capture immediately after the behavioral trials. To ensure that same individuals were not caught more than once, the individuals were marked on the dorsal side before releasing them to their respective sites of capture.

This species is not covered under the Schedules of the Indian Wildlife (Protection) Act; therefore, collection permits were not required. All capture, handling and experimental protocols were approved by the Animal Ethics Committee of the Indian Institute of Science (CAF/Ethics/489/2016). Experimental protocols were designed to minimize stress and disturbance to animals.

Experiment 1: Receiver response to squalene as a single-chemical component stimulus

Receiver response towards squalene was studied using pure isolated squalene (Sigma Aldrich, GC grade). Due to its non-polar nature, squalene was dissolved in the organic solvent Dichloromethane (DCM). In an earlier experiment in the lab (Unpublished: Bronte Ellsworth, 2019), the optimal concentration for cholesterol (60 mg/ml) was fixed based on the behavioral response of receivers. This was used to determine a working concentration of squalene as 120 mg/ml for all the experiments based on the relative concentrations of squalene and cholesterol (roughly 2:1) in the male femoral pore and pre-cloacal gland secretions. The squalene solution was freshly prepared before every experimental trial by dissolving 143 µl of pure squalene in DCM to make up 500 µl squalene solution. Thorough mixing was ensured by using the vortex machine (IKA Vortex Genius 3). Each behavioral trial consisted of three treatments: 1) Water control, 2) DCM control and 3) Squalene solution. Water was a control for the baseline behavioral response, while DCM was a control for response towards the solvent. All the three treatments were presented to 30 males and 30 females to measure their behavioral response to avoid any effect of individual differences in behavioral response.

Each individual was first cleaned with 70% ethanol to remove all the secretions from the ventral surface before introducing it in the experimental container (30cm X 20cm X 10cm) lined with a damp tissue. The tissue was replaced before every trial. The gecko was covered with an opaque cover for 2 minutes to allow acclimation to the experimental conditions. One of the treatments was then introduced inside the experimental container in a petri dish after which, the opaque cover over the gecko was removed. I recorded all occurrences of: number of tongue flicks, latency to the first tongue flick, number of movement bouts and latency to the first movement bout, as measures of behavioral response towards the chemical stimulus, for the trial length of 5 minutes (similar to Kabir et al., 2019). Tongue flicks with a separation time of less than 3 seconds were considered a single tongue flick bout. Similarly, a movement bout was recorded as a separate bout only when the animal moved more than 3 steps and when

it was separated by the next movement by more than 3 seconds. The latency to the first tongue flick and the first movement bout was recorded as a measure of response time towards each chemical stimulus. A rest period of 45-60 minutes was given to every individual between successive stimuli to avoid sensory overload.

The results were analyzed for their significance by repeated measures ANOVA with ‘Animal ID’ and ‘order of treatment’ as random factors. The pairwise comparisons between the treatments for each measure of behavioral response were done using Tukey’s multiple comparison test in R Studio. The graphs were plotted in SigmaPlot 12.5.

Experiment 2: Receiver response to squalene and cholesterol as single-chemical and multi-chemical component stimuli

To determine whether cholesterol and squalene have an additive or synergistic effect when present together, geckos were presented with the combination of both cholesterol and squalene along with the single chemical treatments and natural male and female secretions. Specifically, each gecko (N=30) was exposed to: 1) Water control, 2) DCM control, 3) Cholesterol solution, 4) Squalene solution, 5) Combination (Squalene + cholesterol), 6) Female secretions and 7) Male secretions. All the treatments were presented over two days, to avoid sensory saturation of individuals. Each individual was given a water treatment on both days as a control for the individual's baseline behavioral response across both days. All the treatments were presented to each individual in a random order to avoid any order effects. The cholesterol and squalene concentrations (60mg/ml and 120mg/ml respectively) were kept constant for the cholesterol, squalene and combination treatments. The final volume of chemical stimuli (50µl) was also kept the same. The chemical solutions were freshly prepared before each trial following the same protocol as the single-chemical stimulus behavioral assays. The combination solution was prepared by dissolving 30mg cholesterol and 143µl squalene in DCM to make 500µl of final solution. All the solutions were thoroughly mixed on vortex after preparing. The treatments with female and male secretions utilized natural secretions from the femoral pores and pre-cloacal glands of males and ventral body secretions of females. For this, tissues lined on the floors of housing containers were used for the trials. The tissues contained chemical secretions of males and females housed for 5 days. The behavioral trials were carried out following the exact same protocol as the single-chemical stimulus behavioral trials, except for the female and male secretion treatments, for which the entire housing container tissue was introduced in the experiment container. The number of tongue flicks, latency to first tongue flick, number of movement bouts and the latency to first movement bout were measured. The results were analyzed for significance by repeated measures ANOVA with 'Animal ID' and 'order of treatment' as random factors in R studio. The post hoc Tukey's multiple comparison test was used to analyze pairwise differences for each measure. SigmaPlot 12.5 was used for plotting the results.

Experiment 3: Receiver response to a concentration gradient of multi-chemical stimuli with both squalene and cholesterol

If individuals are able to distinguish conspecifics based on the femoral pore chemical secretions, I expect that the intensity of behavioral response towards different concentrations of chemical stimuli should be different. To test this hypothesis, behavioral responses of 30 male and 30 female individuals towards different concentrations of chemical stimuli was recorded. For this, the multi-chemical stimulus with cholesterol and squalene was presented to individuals along with the water and DCM controls. The multi-chemical stimulus comprised of four cholesterol-squalene concentrations (45mg/ml-90mg/ml, 60mg/ml-120mg/ml, 75mg/ml-150mg/ml and 90mg/ml-180mg/ml). Cholesterol: squalene ratio was kept constant throughout to match the ratio of these chemicals in male femoral gland secretions. All the treatments were presented to individuals over two days. The water control was presented on both days to check for any change in individual baseline behavior. I presented 50µl of each treatment in a petri dish inside the experimental container, which was lined with a damp tissue that was replaced before each trial. The behavioral trial was carried out following the same protocol as previous experiments. The treatments were presented in a random order to avoid any order effects. A rest period of about 45-60 minutes was given to each individual between two successive treatments.

The analysis of results was done similarly as previous experiments, using repeated measures ANOVA with ‘Animal ID’ and ‘order of treatment’ as random factors followed by Tukey’s multiple comparison test in R Studio. Similarly, SigmaPlot 12.5 was used for plotting the results.

Experiment 4:

A) Characterization of male traits

To determine the role of male secretion components in mate choice, I carried out a female choice experiment (see next section for details), where females would be given only male secretions to make a choice. The two choice males needed to be distinct enough for a female to be able to distinguish between them. For this, I characterized three male traits: SVL, ectoparasite load and sprint speed in order to further carry out female choice experiments for these male traits. SVL and ectoparasite load were measured within 2 hours of capture. Ectoparasites were mostly located on dorsal body surface of individuals near the neck and appendages. For the next 5 consecutive days from capture, sprint speed was calculated as a measure of motor performance. The individuals were chased to run on a 1 meter long (and 10 cm wide) straight running track that was constructed in the lab with a foam board base and walls. The geckos were made to run twice on the track every day with a rest period of 30 minutes between successive runs. The sprint video was recorded using a GoPro Hero 3 (frame rate = 120 frames/sec) and was analyzed using the software Tracker 5.1.3. The track was segmented and highest sprint speed over a 20 cm segment was calculated for each individual across days.

The housing container tissues, on which the males were kept for a week, were frozen and stored for female choice experiments after sprint speed trials. Spearman's rank correlation coefficient was used in Past 4.0 to determine the correlation between traits. Trait value distribution of these traits and linear regression analysis was then used to select male pairs with distinct trait values for the female choice experiments.

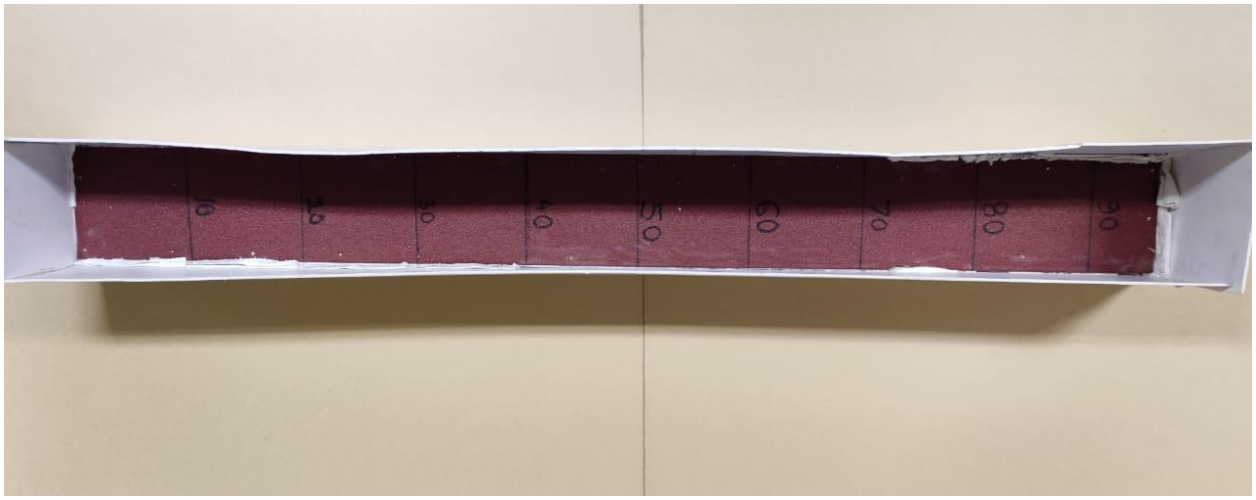

Plate 1: Running track (100cm X 10cm) used to measure sprint speed of males

B) Female choice experiment

To determine the role of male secretion components as a mate quality assessment signal, a female choice experiment was carried out using the male housing container tissues previously stored. The entire tissue paper was cut into elongated strips to be used for the trials, with each strip used for a maximum of 2 trials. The trials were carried out in a Y-tube maze constructed in the lab using foam board base and walls. The maze was cleaned with H_2O_2 and water before each choice trial. The females were introduced to the Y-tube maze and covered with an opaque cover for 2 minutes, which allowed them to acclimatize to the experimental conditions. While females were under the cover, two strips were placed in two arms of the maze. The trial started as soon as the cover was removed, and was recorded for 20 minutes. The trials in which females made a choice before 20 minutes ended as soon as the choice was made, whereas trials in which females did not choose a side ended after 20 minutes and females were considered to have made no choice.

Based on the trait distribution and trait correlations, males were categorized as being either high quality males (higher sprint speed and lower ectoparasite load) or low

quality males (lower sprint speed and higher ectoparasite load). Three high quality males were selected and paired with three low quality males. Each female was presented with tissues belonging to any of the three male pairs. Each choice belonged to one of these three types: 1) High quality male vs Low quality male (High vs Low), 2) High quality male vs High quality male (High vs High) and 3) Low quality male vs Low quality male (Low vs Low). These three choices were presented to all the females (N=30), where High vs High and Low vs Low choices were controls for the inherent bias of females to choose either right or left side more than 50% of times. The order in which females were presented was randomized to avoid any order effects. Similarly, the side on which the high quality male got presented was randomized to avoid any bias. Furthermore, to avert any bias affecting the choice, the tissue samples were relabeled and coded by a person not involved in the experiment, making me blind to the choices presented.

The choice results were analyzed by z-test for single proportion in Past 4.0. The proportions of female choices in all the treatments were compared against the null hypothesis of 50% separately. SigmaPlot 12.5 was used for plotting the results.

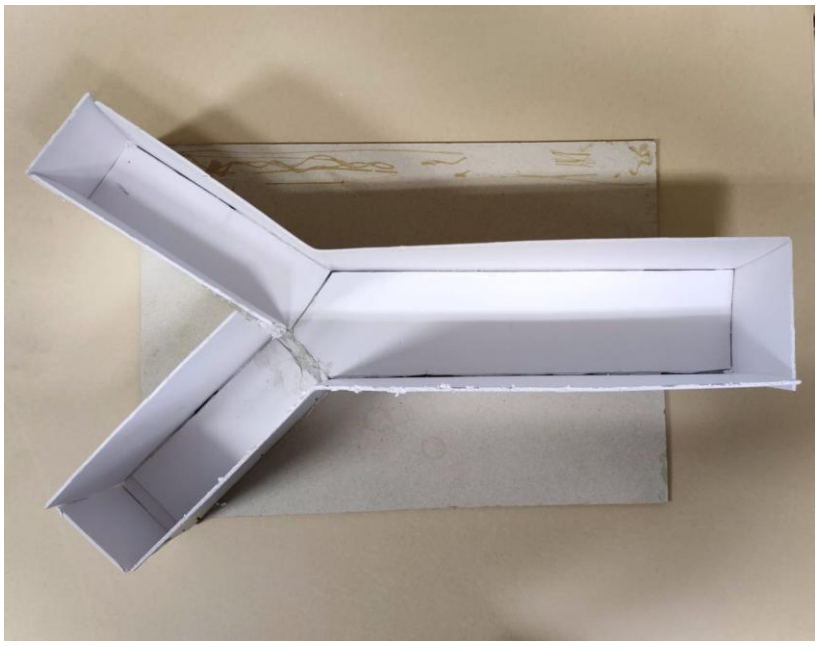

Plate 2: The Y-tube maze used for female choice experiments.

Results

Experiment 1: Receiver response to squalene as a single-chemical component stimulus

Repeated measures ANOVA conclusively showed that the number of tongue flicks, as a measure of behavioral response towards single chemical stimulus, was significantly higher towards squalene compared to both the water ($z = 8.791$, $p < 0.0001$) and DCM ($z = 6.286$, $p < 0.0001$) treatments in females (Figure 1). In females, the number of tongue flicks towards the DCM treatment was also significantly higher compared to water ($z = 3.926$, $p = 0.0003$). In males, however, the number of tongue flicks towards squalene was not significantly different from DCM ($z = 0.667$, $p = 0.7829$). The number of tongue flicks by males towards both DCM ($z= 5.775$, $p < 0.0001$) and squalene ($z = 6.248$, $p < 0.0001$) though, was significantly higher than water. This reveals that squalene elicits a strong tongue flick response in females, but not in males. The latency of first tongue flick (Figure 2), in females, was significantly lower in squalene compared to DCM ($z = 3.521$, $p = 0.0013$) and water ($z = 6.435$, $p < 0.0001$). For females, the latency of first tongue flick, was greater towards water compared to DCM ($z = 5.945$, $p < 0.0001$). In males, the latency of first tongue flick was significantly lower in squalene ($z = 7.66$, $p < 0.0001$) an DCM ($z = 9.133$, $p <0.0001$) treatments as compared to water, whereas, there was no significant difference between the squalene and DCM treatments ($z = 1.954$, $p = 0.1237$)

The number of movement bouts (Figure 3) in females, based on repeated measures ANOVA, were not significantly different towards squalene compared to DCM ($z = 1.660$, $p = 0.2206$). But, both squalene ($z = 5.537$, $p < 0.0001$) and DCM ($z = 6.093$, $p < 0.0001$) elicited significantly more number of movement bouts compared to water in females. This suggests that females do not move significantly more in response to squalene. In males, however, the number of movement bouts towards DCM was significantly higher than both squalene ($z = 3.037$, $p = 0.0068$) and water ($z = 5.538$, $p < 0.0001$). The number of movement bouts towards squalene were significantly higher compared to water ($z = 4.501$, $p < 0.0001$). The latency of first movement bout (Figure

4) was significantly higher for water as compared to squalene (z = 3.124, p = 0.0051) and DCM (z = 6.397, p < 0.0001) in females. Females also showed a higher latency of first movement bout towards squalene compared to DCM (z = 2.079, p = 0.0306). Males showed a significantly greater latency of first movement towards water compared to squalene (z = 8.689, p < 0.0001) and DCM (z = 5.237, p < 0.0001), while the latency towards squalene did not differ significantly compared to DCM (z = 2.53, p = 0.0943).

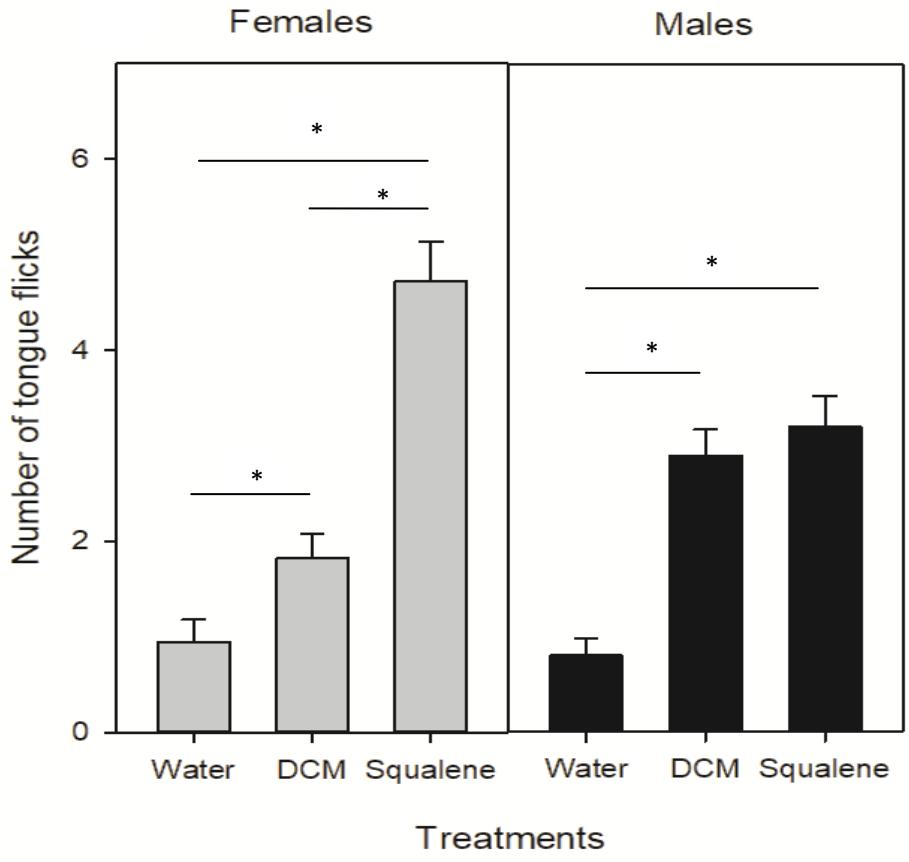

Figure 1: Number of tongue flicks as a measure of behavioral response towards single-chemical stimuli in female and male individuals (mean ± SE) (N=30). Squalene elicited significantly higher behavioral response as compared to water and DCM controls in females, but not in males.

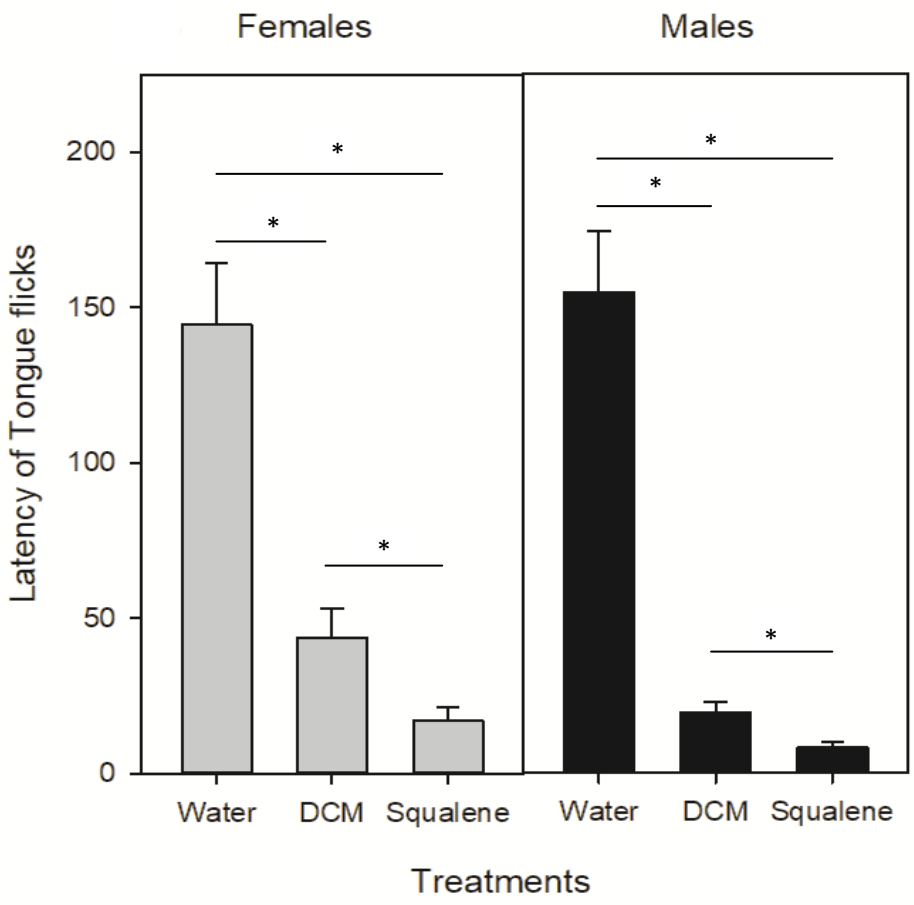

Figure 2: Latency of first tongue flicks in behavioral response towards single-chemical stimuli in female and male individuals (mean ± SE) (N=30). In both females and males, tongue flicks in response to squalene were significantly faster than DCM and water.

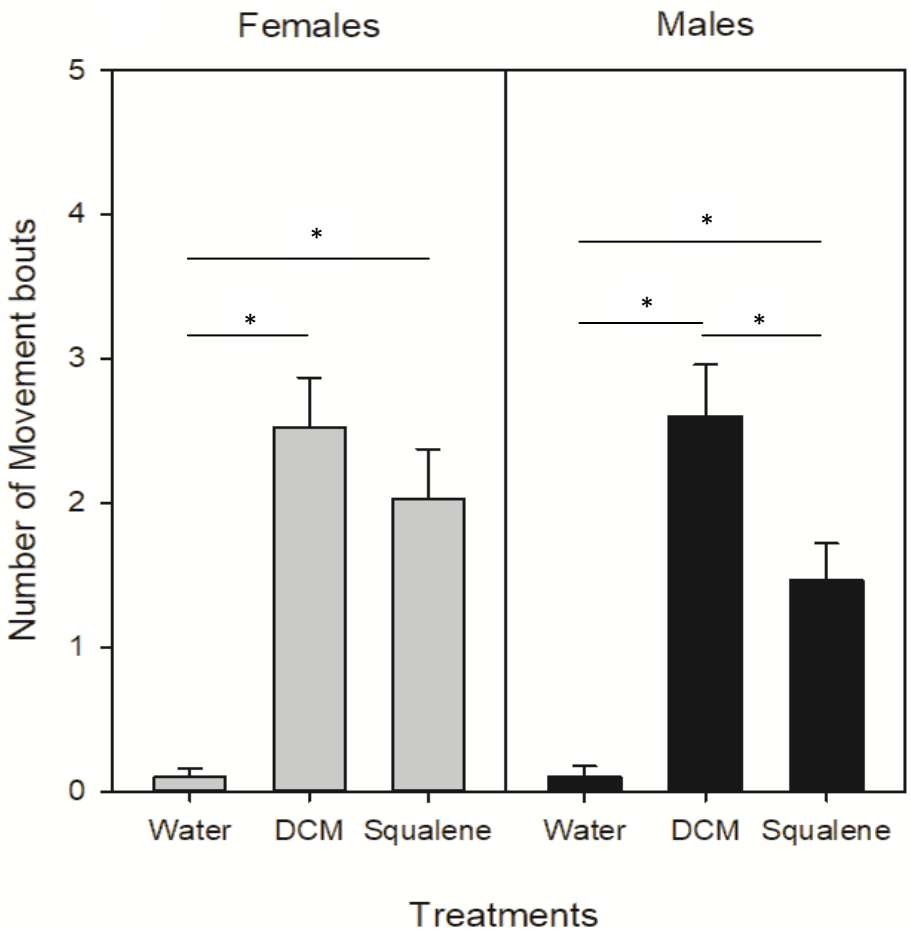

Figure 3: Number of movement bouts as a measure of behavioral response towards single-chemical stimuli in female and male individuals (mean ± SE)(N=30). The number of movement bouts reduced in squalene treatment compared to DCM significantly in males, but not in females.

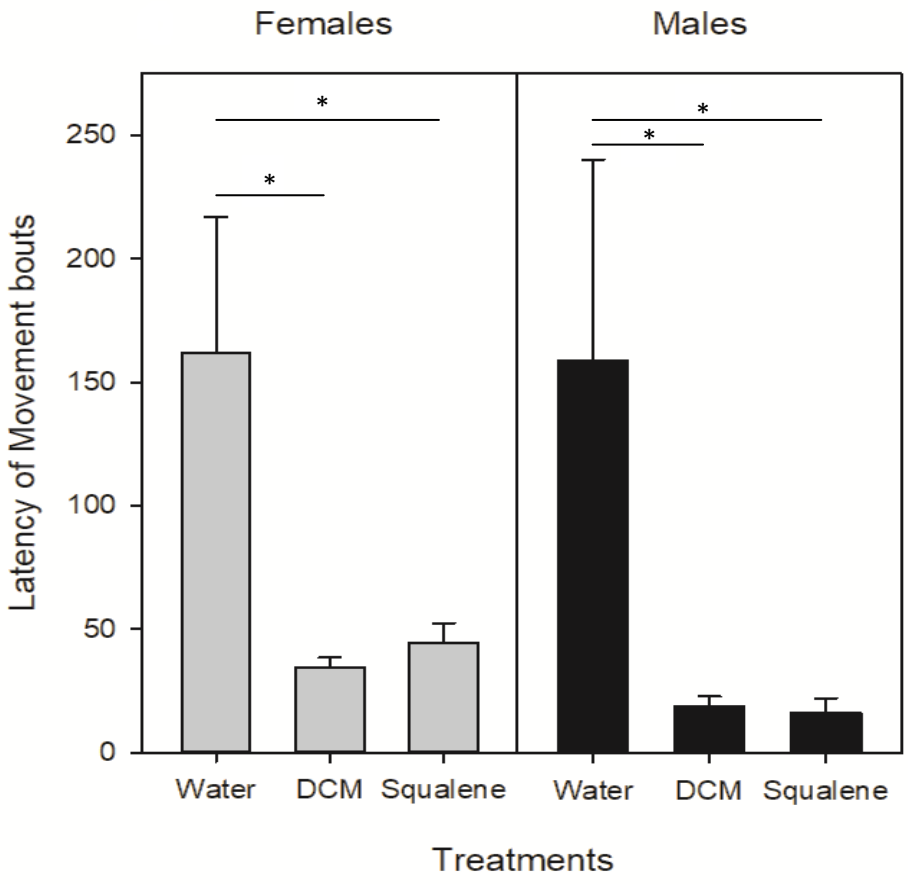

Figure 4: Latency of first movement bout in behavioral response towards single-chemical component stimulus in female and male individuals (mean ± SE) (N=30). The latency of first movement bout was greater in squalene treatment than DCM in females, but did not differ in males. It was significantly lower compared to water in both females and males.

Experiment 2: Receiver response to cholesterol and squalene as single-chemical and multi-chemical components

The baseline behavior of females, as measured by the number of tongue flicks ($t = 0.2567$, $p = 0.7983$) (Figure 5) and the latency of first tongue flick ($t = 0.1641$, $p = 0.871$) (Figure 7) to water, did not differ across days. Males, also, did not show any significant difference in either the number of tongue flicks ($t = 0.2766$, $p = 0.7831$) (Figure 6) or the latency of first tongue flick ($t = 0.2347$, $p = 0.8164$) (Figure 8) to water. The repeated measures ANOVA followed by Tukey's multiple comparison test (Table 1) performed on rest of the treatments in females, revealed that the number of tongue flicks towards the single-chemical squalene ($z = 6.501$, $p < 0.0001$), single-chemical cholesterol ($z = 5.901$, $p < 0.0001$) and multi-chemical cholesterol + squalene ($z = 7.9850$, $p < 0.0001$) treatments was significantly higher compared to the natural female secretion. The number of tongue flicks towards just squalene ($z = 0.4870$, $p = 0.9990$), cholesterol ($z = 0.2490$, $p = 1$) and squalene + cholesterol ($z = 2.401$, $p = 0.1979$) treatments was not significantly different compared to the natural male secretions. This suggests that females exhibited a significantly elevated tongue flick response when presented with male secretion components in comparison with the female secretion and control treatments (refer table 1 for all pairwise comparison results). In case of males, the number of tongue flicks towards cholesterol ($z = 4.810$, $p < 0.0001$) and squalene + cholesterol ($z = 3.079$, $p = 0.0339$) treatments but not towards squalene alone ($z = 2.449$, $p = 0.1785$), was significantly higher as compared to female secretion. Additionally, as confirmed by Tukey's multiple comparison test (Table 2), squalene ($z = 0.296$, $p = 0.9999$), cholesterol ($z = 2.221$, $p = 0.2838$) or squalene + cholesterol ($z = 0.3620$, $p = 0.9998$) did not elicit a greater tongue flick response as compared to DCM in males (refer table 2 for all paiwise comparisons).

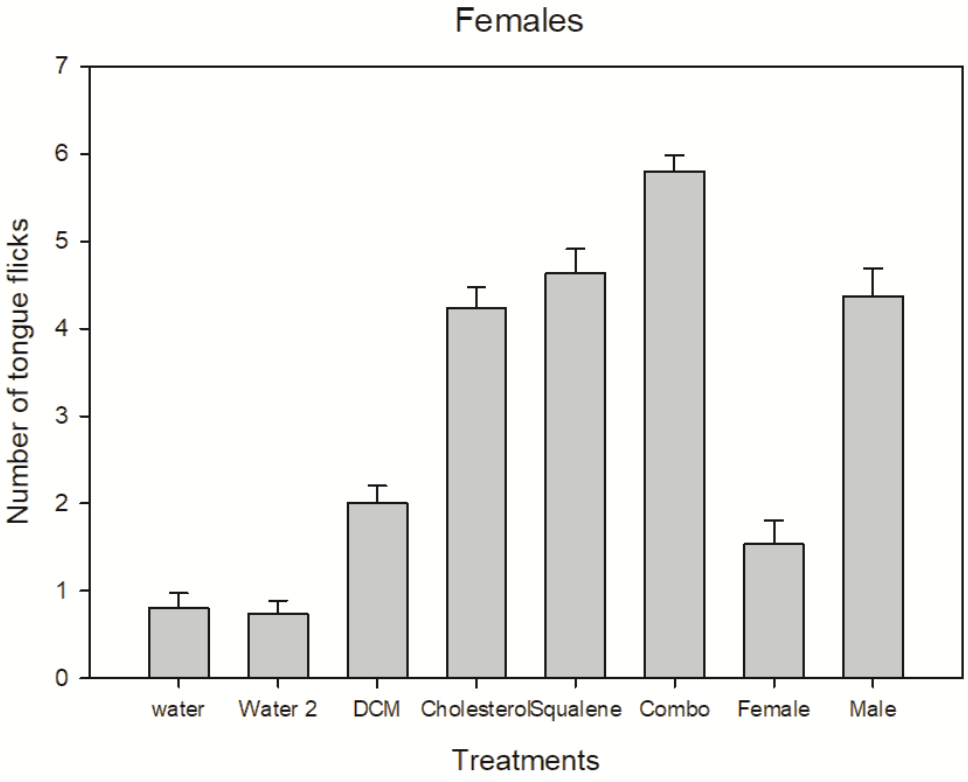

Figure 5: Number of tongue flicks as a measure of behavioral response towards single-chemical and multi-chemical components in females (mean ± SE) (N=30). Squalene and cholesterol elicit a greater behavioral response in females when presented both separately and together.

	Water	DCM	Cholesterol	Squalene	Combo	Female	Male
Water		**0.0028**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.1309	**< 0.0001**
DCM	3.7940		**< 0.0001**	**< 0.0001**	**< 0.0001**	0.8252	**< 0.0001**
Cholesterol	7.4860	4.7870		0.9904	0.1127	**< 0.0001**	1.0000
Squalene	7.9460	5.4390	0.7360		0.4662	**< 0.0001**	0.9990
Combo	9.0680	7.0680	2.6450	1.9210		**< 0.0001**	0.1979
Female	2.5840	1.3560	5.9010	6.5010	7.9850		**< 0.0001**
Male	7.6440	5.0090	0.2490	0.4870	2.4010	6.1060	

Table 1: Pairwise comparisons of all the treatments calculated for the number of tongue flicks in females. The Z-ratios from the Tukey's multiple comparison test are written below the diagonal, whereas the p-values are written above the diagonal. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

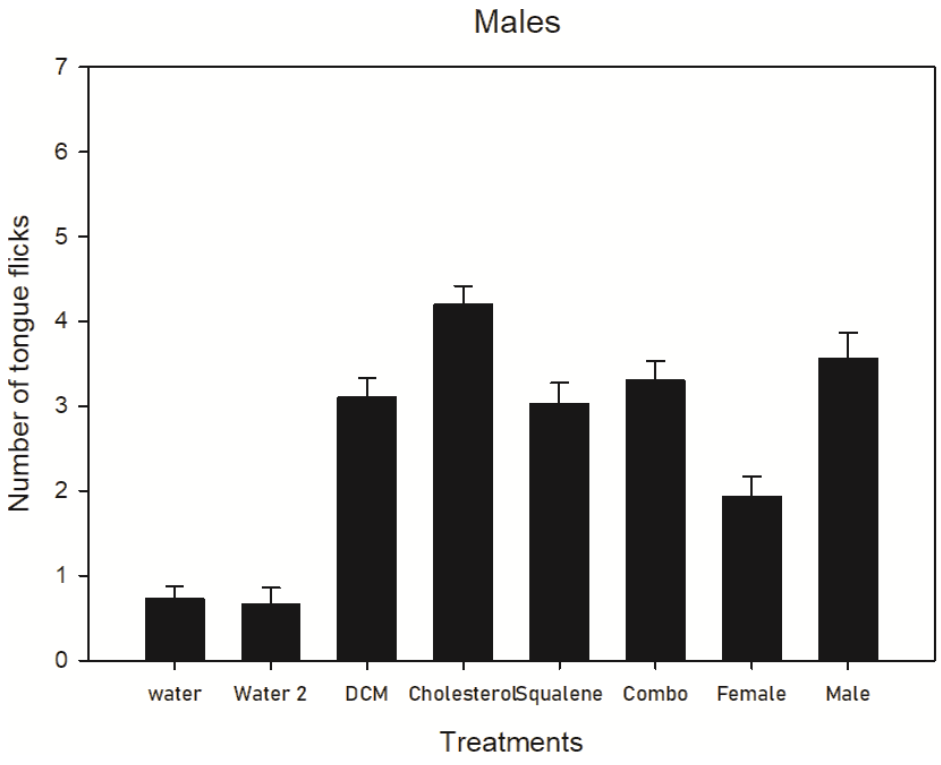

Figure 6: Number of tongue flicks as a measure of behavioral response in males towards single-chemical and multi-chemical components (mean ± SE) (N=30). Males do not show a greater behavioral response towards squalene and cholesterol, even when presented together.

	Water	DCM	Cholesterol	Squalene	Combo	Female	Male
Water		**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.0015**	**< 0.0001**
DCM	6.0800		0.2838	0.9999	0.9998	0.0899	0.9866
Cholesterol	7.5530	2.2210		0.1553	0.5032	**< 0.0001**	0.7753
Squalene	5.8700	0.2960	2.5110		0.9947	0.1785	0.9345
Combo	6.3320	0.3620	1.8660	0.6580		**0.0339**	0.9996
Female	3.9490	2.7340	4.8100	2.4490	3.0790		**0.0091**
Male	6.6190	0.7830	1.4480	1.0790	0.4220	3.4780	

Table 2: Pairwise comparisons of all the treatments calculated for the number of tongue flicks in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

For females, the latency of the first tongue flick, when compared to DCM, did not differ significantly for squalene ($z = 2.024$, $p = 0.3992$), cholesterol ($z = 2.293$, $p = 0.2427$), squalene + cholesterol ($z = 2.304$, $p = 0.2421$), female secretion ($z = 1.295$, $p = 0.8545$) and male secretion ($z = 0.695$, $p = 0.9929$) treatments. This suggests that the time required to detect the chemical did not differ between the treatments for females. Latency of first tongue flick for squalene ($z = 3.323$, $p = 0.0156$), cholesterol ($z = 4.948$, $p < 0.0001$) and squalene + cholesterol ($z = 3.587$, $p = 0.0062$) differed significantly compared to female secretions, but not when compared to male secretion treatment (refer Table 3 for all the pairwise comparisons). Even in males, the latency of the first tongue flick did not differ significantly in squalene ($z = 1.335$, $p = 0.8358$), cholesterol ($z = 2.197$, $p = 0.297$), squalene + cholesterol ($z = 1.007$, $p = 0.9527$), female secretion ($z = 1.454$, $p = 0.7718$) and male secretion treatments ($z = 0.664$, $p = 0.9945$) treatments compared to DCM. As confirmed by Tukey's multiple comparison test (Table 4), latency of first tongue flick also did not differ significantly in squalene, cholesterol and squalene + cholesterol treatments when compared to female and male secretion treatments (refer Table 4 for all pairwise comparisons)

	Water	DCM	Cholesterol	Squalene	Combo	Female	Male
Water		**0.0061**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.0291**	**0.0004**
DCM	3.5910		0.2427	0.3992	0.2421	0.8545	0.9929
Cholesterol	8.2410	2.2930		1.0000	1.0000	**< 0.0001**	0.3186
Squalene	5.6250	2.0240	0.2620		1.0000	**0.0156**	0.8385
Combo	5.8900	2.3040	0.1070	0.2610		**0.0062**	0.6840
Female	3.1290	1.2950	4.9480	3.3230	3.5870		0.4236
Male	4.2900	0.6950	2.1580	1.3290	1.5970	1.9860	

Table 3: Pairwise comparisons of all the treatments calculated for the latency of first tongue flick in females. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

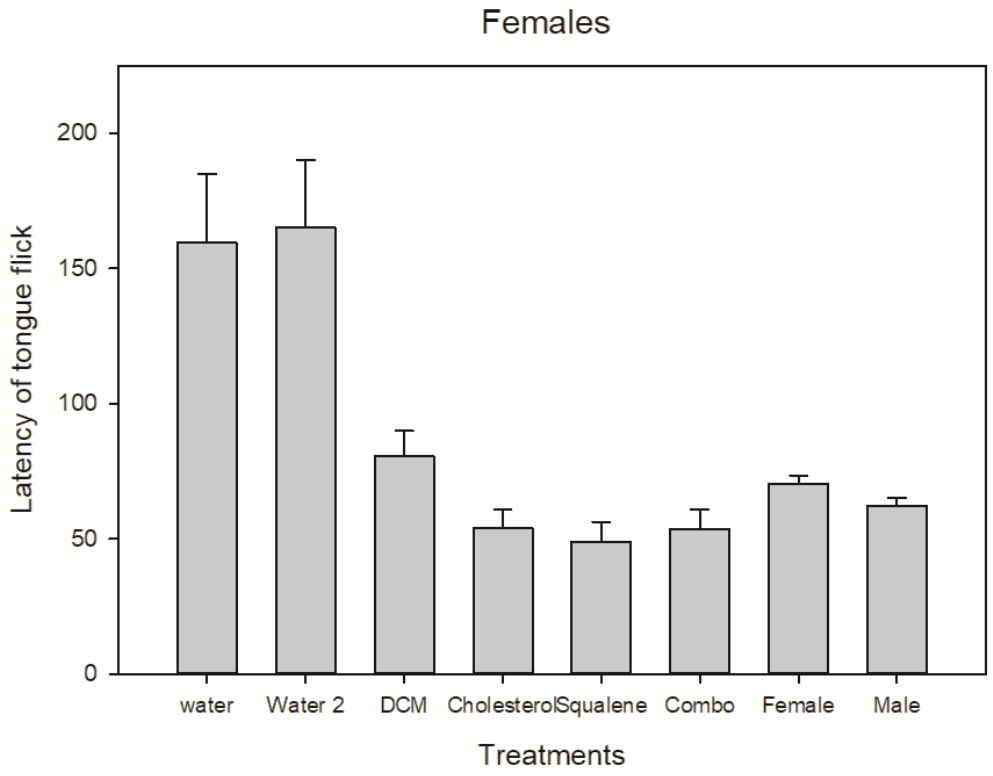

Figure 7: Latency of first tongue flick in behavioral response towards cholesterol and squalene as single-chemical and multi-chemical components (mean ± SE) (N=30). Female response towards male secretion components was not significantly faster compared to female secretion treatment.

	Water	DCM	Cholesterol	Squalene	Combo	Female	Male
Water		0.0895	**< 0.0001**	**0.0009**	**0.0035**	0.8608	**0.0120**
DCM	2.7360		0.2970	0.8358	0.9527	0.7718	0.9945
Cholesterol	6.0660	2.1970		0.9999	0.9875	**0.0004**	0.8708
Squalene	4.0700	1.3350	0.3090		0.9999	0.0778	0.9941
Combo	3.7420	1.0070	0.7730	0.3280		0.1737	0.9999
Female	1.2810	1.4540	4.2540	2.7890	2.4610		0.3418
Male	3.3990	0.6640	1.2580	0.6710	0.3430	2.1180	

Table 4: Pairwise comparisons of all the treatments calculated for the latency of first tongue flick in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

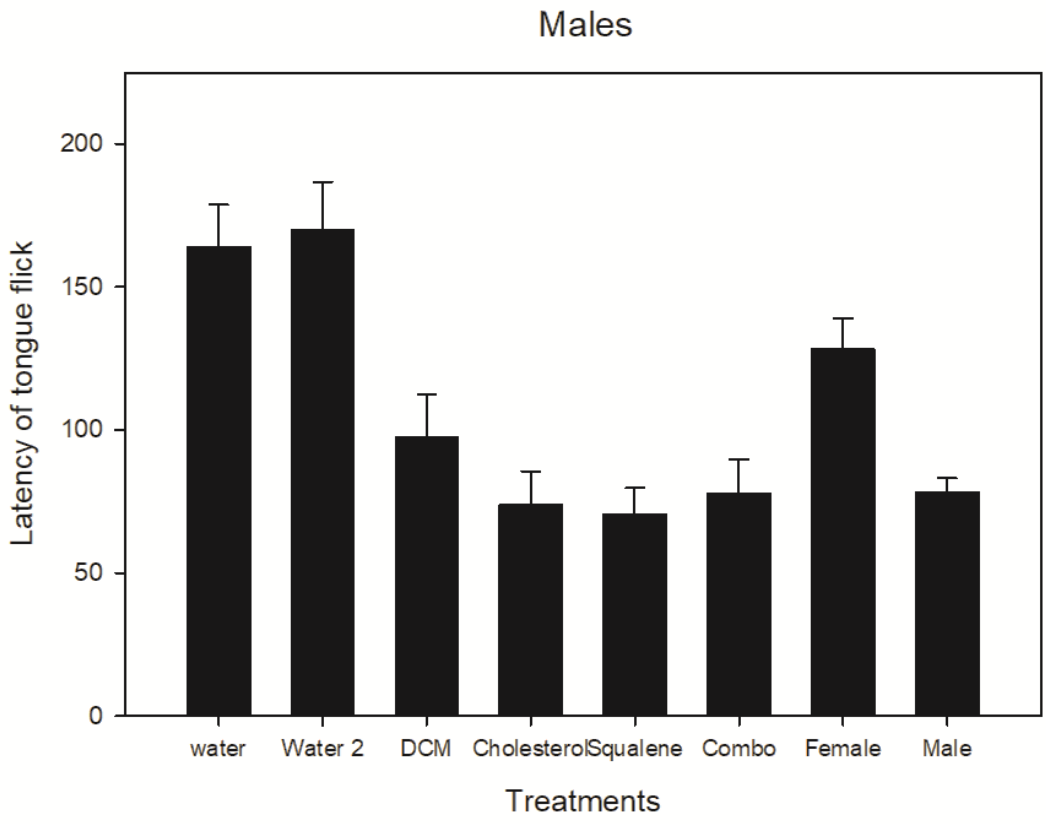

Figure 8: Latency of first tongue flick in behavioral response towards squalene and cholesterol as single-chemical and multi-chemical components (mean ± SE) (N=30). Female response towards male secretion components did not differ significantly compared to female secretions or DCM.

Two-sampled t-tests confirmed that the number of movement bouts ($t = 0.1468$, $p = 0.8838$) (Figure 9) and the latency of first movement bout ($t = 0.1073$, $p = 0.9157$) (Figure 11) did not differ between two water treatments in females. Similarly, even males did not exhibit a significant difference in the number of movement bouts ($t = 0.4616$, $p = 0.6461$) (Figure 10) or latency of first movement bout ($t = 0.0672$, $p = 0.9471$) (Figure 12) between the two water treatments. This conclusively shows that the baseline behavioral response, measured as number of movement bouts and latency of first movement bout, of both females and males did not change across days. Repeated measures ANOVA performed on rest of the treatments in females, revealed that the number of movement bouts in squalene ($z = 0.327$, $p = 0.9999$), cholesterol ($z = 0.722$, $p = 0.9913$) and squalene + cholesterol ($z = 0.681$, $p = 0.9937$) treatments did not differ significantly compared to DCM. When compared to female and male natural secretion treatments, however, the number of movement bouts in females was significantly higher towards squalene, cholesterol and squalene + cholesterol treatments (refer Table 5 for all the pairwise comparisons). In males, the number of movement bouts towards squalene ($z = 4.724$, $p = 0.0002$), cholesterol ($z = 4.228$, $p < 0.0001$) and squalene + cholesterol ($z = 4.146$, $p < 0.0001$) was significantly lower compared to DCM. The movement bouts towards cholesterol and squalene + cholesterol were significantly higher compared to female and male secretion treatments (refer Table 6 for all pairwise comparisons). Squalene, though, did not elicit a greater movement bout response in males as compared to female ($z = 2.731$, $p = 0.0906$) and male ($z = 2.491$, $p = 0.1625$).

	Water	DCM	Cholesterol	Squalene	Combo	Female	Male
Water		**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.1641	0.0570
DCM	5.4990		0.9913	0.9999	0.9937	**0.0044**	**0.0189**
Cholesterol	5.9790	0.7220		0.9997	0.8027	**0.0003**	**0.0017**
Squalene	5.7190	0.3270	0.3960		0.9529	**0.0014**	**0.0068**
Combo	5.0230	0.6810	1.3990	1.0060		**0.0358**	0.1173
Female	2.4860	3.6800	4.3160	3.9700	3.0620		0.9992
Male	2.9020	3.2640	3.9190	3.5620	2.6290	0.4710	

Table 5: Pairwise comparisons of all the treatments calculated for the number of movement bouts in females. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

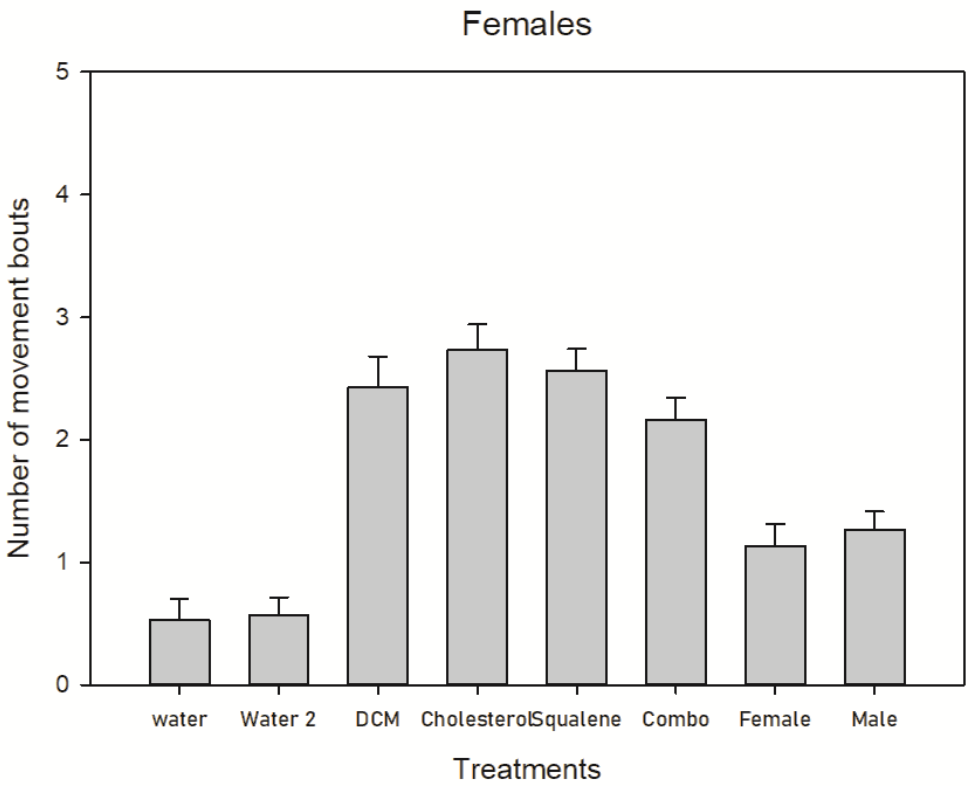

Figure 9: The number of movement bouts as a measure of behavioral response of females to squalene and cholesterol as single-chemical and multi-chemical components (mean ± SE) (N=30). Male secretion components do not elicit a significantly greater movement bout response in females.

	Water	DCM	Cholesterol	Squalene	Combo	Female	Male
Water		**< 0.0001**	**< 0.0001**	**0.0002**	**< 0.0001**	0.3421	0.2182
DCM	7.4740		**0.0005**	**< 0.0001**	**0.0007**	**< 0.0001**	**< 0.0001**
Cholesterol	4.8390	4.2280		0.9977	1.0000	**0.0199**	**0.0410**
Squalene	4.4300	4.7240	0.5670		0.9947	0.0906	0.1625
Combo	4.9030	4.1460	0.0920	0.6580		**0.0152**	**0.0319**
Female	2.1180	6.7510	3.2480	2.7310	3.3300		1.0000
Male	2.3550	6.6030	3.0160	2.4910	3.1000	0.2630	

Table 6: Pairwise comparisons of all the treatments calculated for the number of movement bouts in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

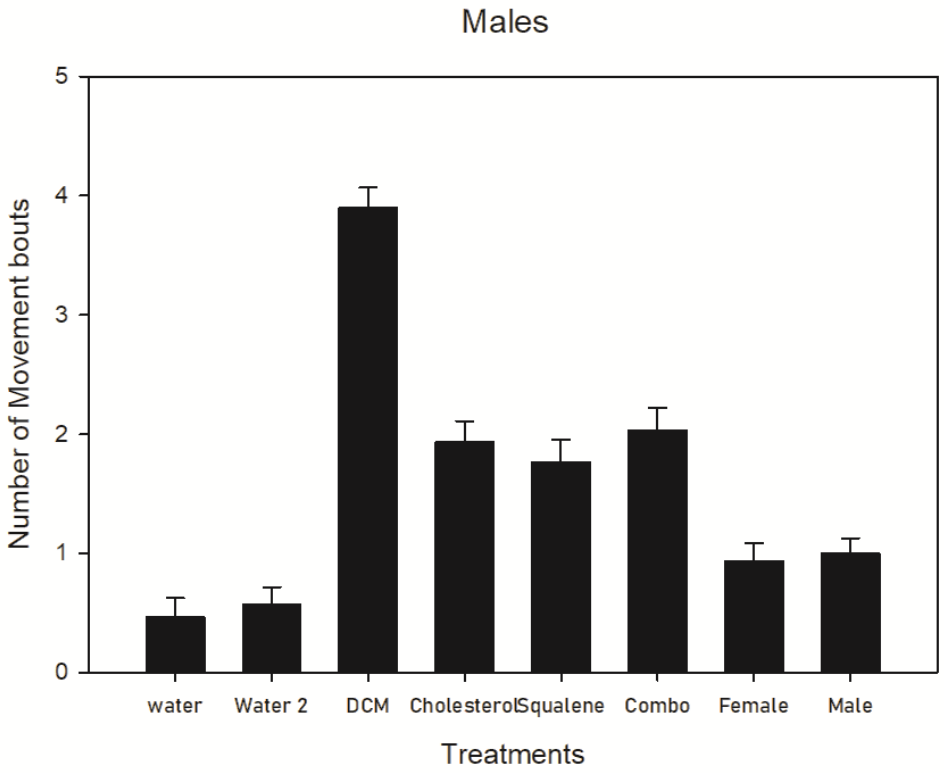

Figure 10: The number of movement bouts as a measure of behavioral response of males to squalene and cholesterol as single-chemical and multi-chemical components (mean ± SE) (N=30). Male secretion components elicited a movement bout response significantly higher than female and male secretions, but significantly lower compared to DCM.

The latency of first movement bout, as revealed by Tukey's multiple comparison test, was not significantly different in squalene ($z = 2.064$, $p = 0.828$), cholesterol ($z = 2.499$, $p = 0.643$) and squalene + cholesterol ($z = 1.235$, $p = 0.9881$) treatments compared to DCM in females. The latency of movement bouts for females was lower in squalene, cholesterol and squalene + cholesterol as compared to female and male secretion treatments (refer Table 7 for all pairwise comparisons). In males, squalene ($z = 2.968$, $p = 0.0472$) and cholesterol ($z = 3.601$, $p = 0.0059$), but not squalene + cholesterol ($z = 2.421$, $p = 0.1897$), showed a significantly greater latency of first movement bout compared to DCM. The latency of first movement bout for squalene and squalene + cholesterol treatments did not significantly differ compared to female and male secretions, whereas it was significantly lesser for cholesterol (refer Table 8 for pairwise comparisons).

	Water	**DCM**	**Cholesterol**	**Squalene**	**Combo**	**Female**	**Male**
Water		**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
DCM	18.1100		0.8280	0.6430	0.9881	**< 0.0001**	**< 0.0001**
Cholesterol	16.0500	2.0640		1.0000	0.9990	**< 0.0001**	**0.0069**
Squalene	15.6100	2.4990	0.4353		0.9864	**< 0.0001**	**0.0194**
Combo	16.8700	1.2350	0.8287	1.2640		**< 0.0001**	**0.0008**
Female	7.3910	10.7200	8.6550	8.2200	9.4840		0.2313
Male	10.8300	7.2780	5.2140	4.7790	6.0430	3.4410	

Table 7: Pairwise comparisons of all the treatments calculated for the latency of first movement bout in females. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

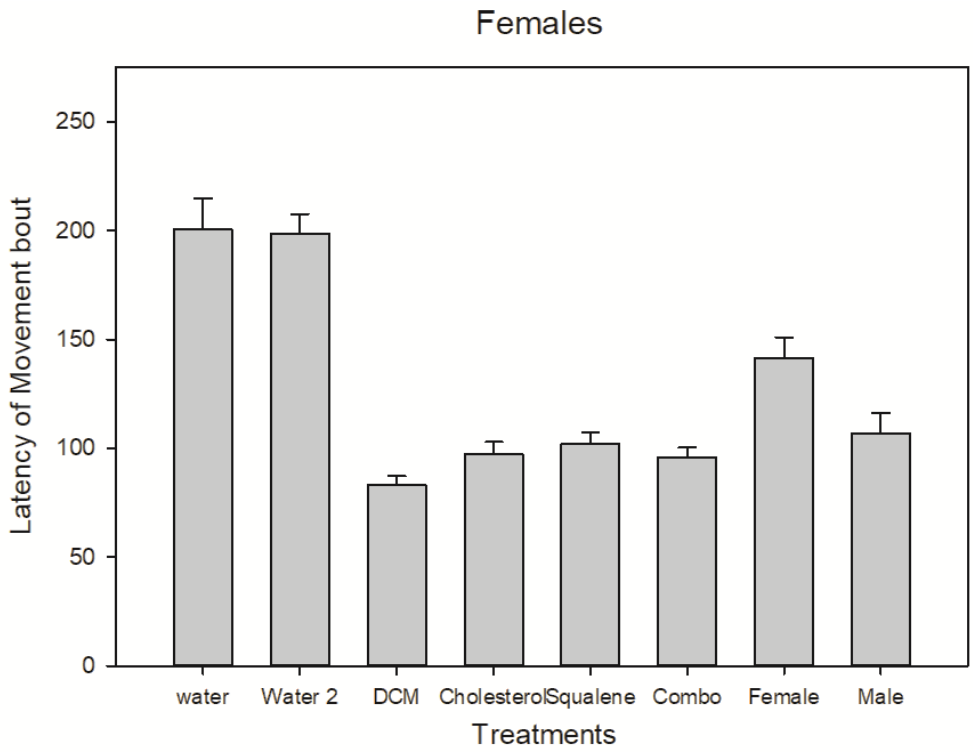

Figure 11: The latency of first movement bout in behavioral response of females to squalene and cholesterol as single-chemical and multi-chemical components (mean ± SE) (N=30). Females did not move significantly faster in response to male secretion components compared to DCM.

	Water	DCM	Cholesterol	Squalene	Combo	Female	Male
Water		**< 0.0001**	**< 0.0001**	**0.0088**	**0.0011**	0.6572	0.5296
DCM	6.4580		**0.0059**	**0.0472**	0.1897	**< 0.0001**	**< 0.0001**
Cholesterol	5.5330	3.6010		0.9969	1.0000	**0.0221**	**0.0499**
Squalene	3.4900	2.9680	0.5970		0.9981	0.5129	0.6409
Combo	4.0380	2.4210	0.1770	0.5480		0.1987	0.2895
Female	1.6380	4.8200	3.2160	1.8520	2.3990		1.0000
Male	1.8270	4.6310	2.9490	1.6630	2.2110	0.1890	

Table 8: Pairwise comparisons of all the treatments for the latency of first movement bout in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

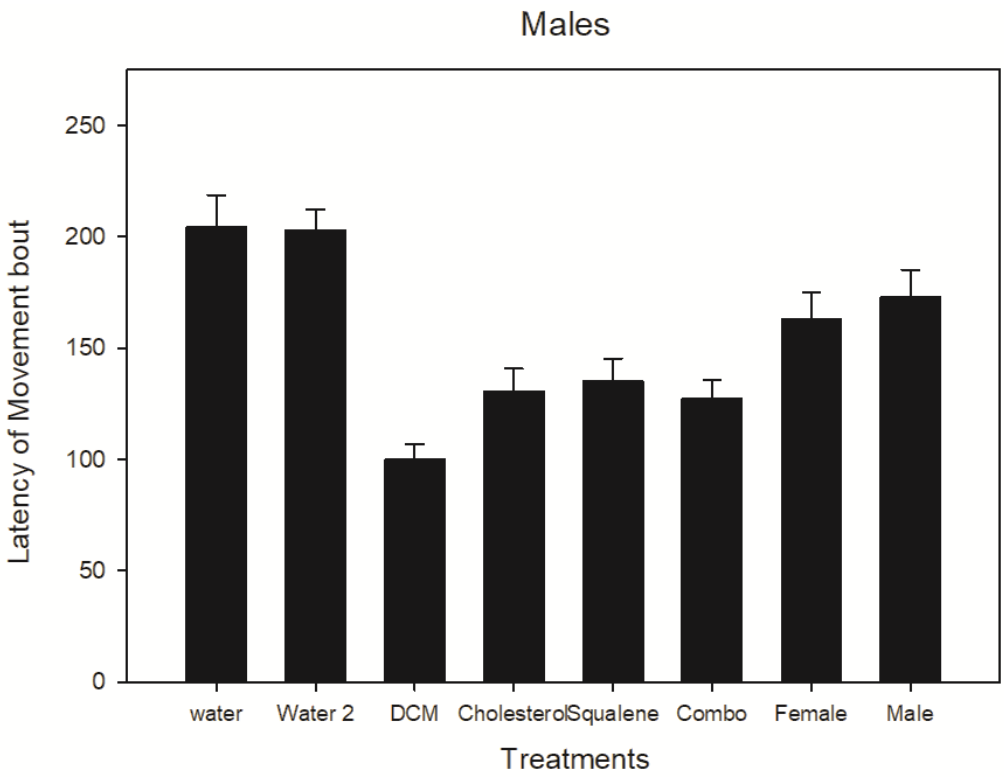

Figure 12: The latency of first movement bout in behavioral response of males to squalene and cholesterol as single-chemical and multi-chemical components (mean ± SE) (N=30). Males moved significantly slower in response to squalene and cholesterol when presented separately, but not together, as compared to DCM.

Experiment 3: Receiver responses to a concentration gradient of multi-chemical component stimuli with both squalene and cholesterol

The baseline behavioral response, as measured by the number of tongue flicks towards water, did not differ significantly across two days for females ($t = 0.1233$, $p = 0.9023$)(Figure 13) or males ($t = 0.3097$, $p = 0.7579$)(Figure 14). Based on the repeated measures ANOVA and Tukey's multiple comparison test performed on rest of the treatments in females, it was seen that the number of tongue flicks towards the 60-120 mg/ml ($z = 3.686$, $p = 0.0031$), 75-150 mg/ml ($z = 5.503$, $p < 0.0001$) and 90-180 mg/ml ($z = 6.043$, $p < 0.0001$) treatments was significantly higher compared to the 45-90 mg/ml treatment. Although not statistically significant, the 75-150 mg/ml ($z = 1.973$, $p = 0.3577$) and 90-180 mg/ml ($z = 2.577$, $p = 0.1029$) treatments elicited an increased tongue flick response in females compared to the 60-120 mg/ml treatment (refer Table 9 for all pairwise comparisons between treatments). In males, however, the number of tongue flicks for any treatment did not differ in comparison to any other treatment (refer Table 10 for all pairwise comparisons between treatments). Moreover, the number of tongue flicks elicited by the 60-120 mg/ml ($z = 0.441$, $p = 0.9979$), 75-150 mg/ml ($z = 0.342$, $p = 0.9994$) and 90-180 mg/ml ($z = 0.258$, $p = 0.9998$) treatments were not significantly different compared to DCM. These results suggest that while females might be able to detect and differentiate between different concentrations of squalene + cholesterol and respond accordingly, males do not respond to these treatments.

	Water	**DCM**	**45-90**	**60-120**	**75-150**	**90-180**
Water		**0.0014**	**0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
DCM	3.8910		0.9832	**0.0002**	**< 0.0001**	**< 0.0001**
45-90	4.4730	0.6890		**0.0031**	**< 0.0001**	**< 0.0001**
60-120	7.2760	4.3170	3.6860		0.3577	0.1029
75-150	8.5320	6.0790	5.5030	1.9730		0.9901
90-180	8.8950	6.6010	6.0430	2.5770	0.6130	

Table 9: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the number of tongue flicks in females. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

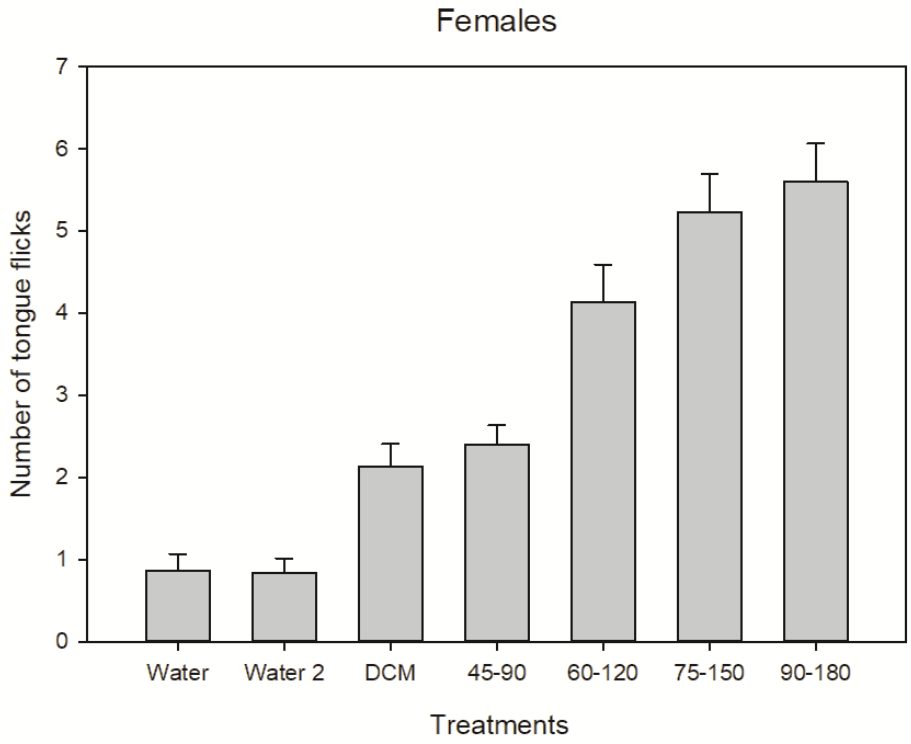

Figure 13: The number of tongue flicks as a measure of behavioral response of females to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). Females show an increasing tongue flick response with increasing squalene + cholesterol concentration.

	Water	**DCM**	**45-90**	**60-120**	**75-150**	**90-180**
Water		**0.0009**	**0.0001**	**0.0040**	**0.0003**	**0.0004**
DCM	4.0000		0.9959	0.9979	0.9994	0.9998
45-90	4.4300	0.5090		0.9337	1.0000	0.9999
60-120	3.6170	0.4410	0.9490		0.9706	0.9822
75-150	4.2900	0.3420	0.1670	0.7820		1.0000
90-180	4.2190	0.2580	0.2510	0.6980	0.0840	

Table 10: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the number of tongue flicks in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

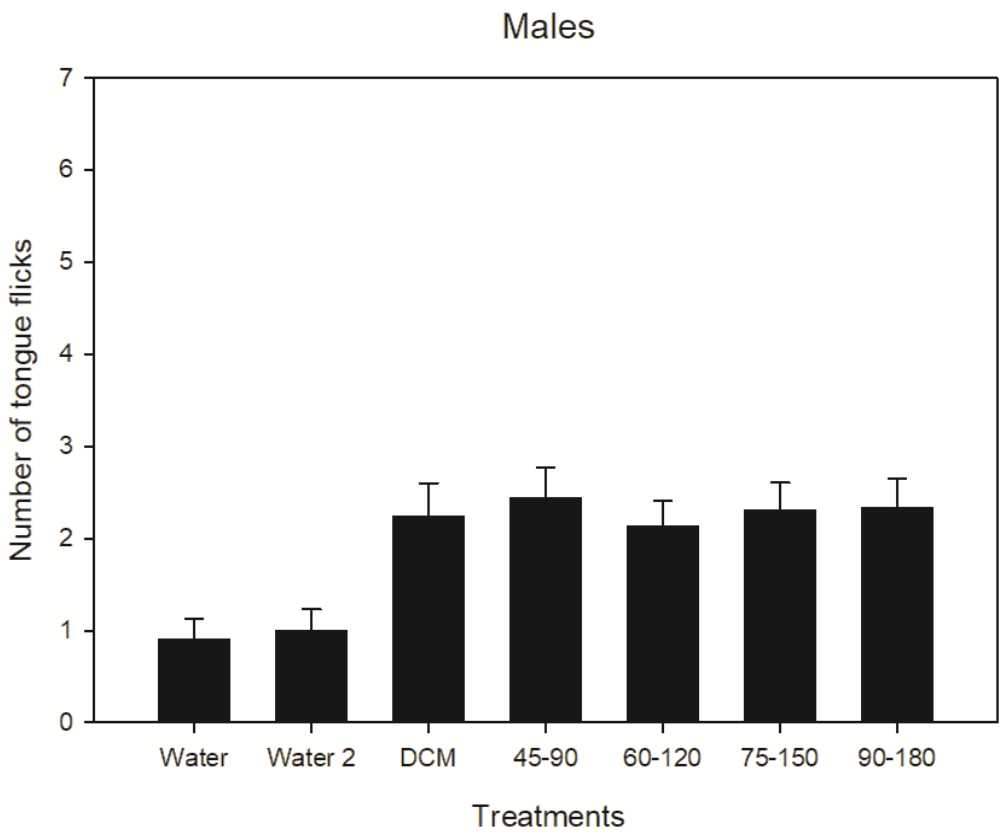

Figure 14: The number of tongue flicks as a measure of behavioral response of males to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). Males do not show a significantly different tongue flick response towards any stimulus concentration.

The two-sampled t-test confirmed that the baseline behavioral response of females (t = 0.1702, p = 0.8661) (Figure 15) and males (t = 0.2045, p = 0.8395) (Figure 16), measured as the latency of first tongue flick, did not differ significantly across days. Repeated measures ANOVA, followed by Tukey's multiple comparison test revealed that the latency of first tongue flick in 45-90 mg/ml (z = 1.02, p = 0.9115), 60-120 mg/ml (z = 1.622, p = 0.584), 75-150 mg/ml (z = 1.812, p = 0.458) and 90-180 mg/ml (z = 2.711, p = 0.073) treatments did not differ significantly as compared to DCM in females. The latency of tongue flicks also did not differ significantly between any two pair of treatments (refer Table 11 for all comparisons between treatment pairs). In males, the

latency of first tongue flick towards 45-90 mg/ml (z = 2.395, p = 0.1579), 60-120 mg/ml (z = 2.016, p = 0.333) and 75-150 mg/ml (z = 1.469, p = 0.684) treatments did not significantly differ from DCM. The latency was significantly lower for the 90-180 mg/ml (z = 2.921, p = 0.0408) treatment compared to DCM. There was no significant difference between any other treatment pair (Table 12).

	Water	**DCM**	**45-90**	**60-120**	**75-150**	**90-180**
Water		**0.0111**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
DCM	3.3330		0.9115	0.5840	0.4580	0.0730
45-90	5.7470	1.0200		0.7949	0.6374	0.0546
60-120	4.9580	1.6220	1.2820		1.0000	0.8832
75-150	5.1560	1.8120	1.5410	0.1930		0.9469
90-180	6.0590	2.7110	2.8180	1.0960	0.8990	

Table 11: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the latency of first tongue flick in females. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

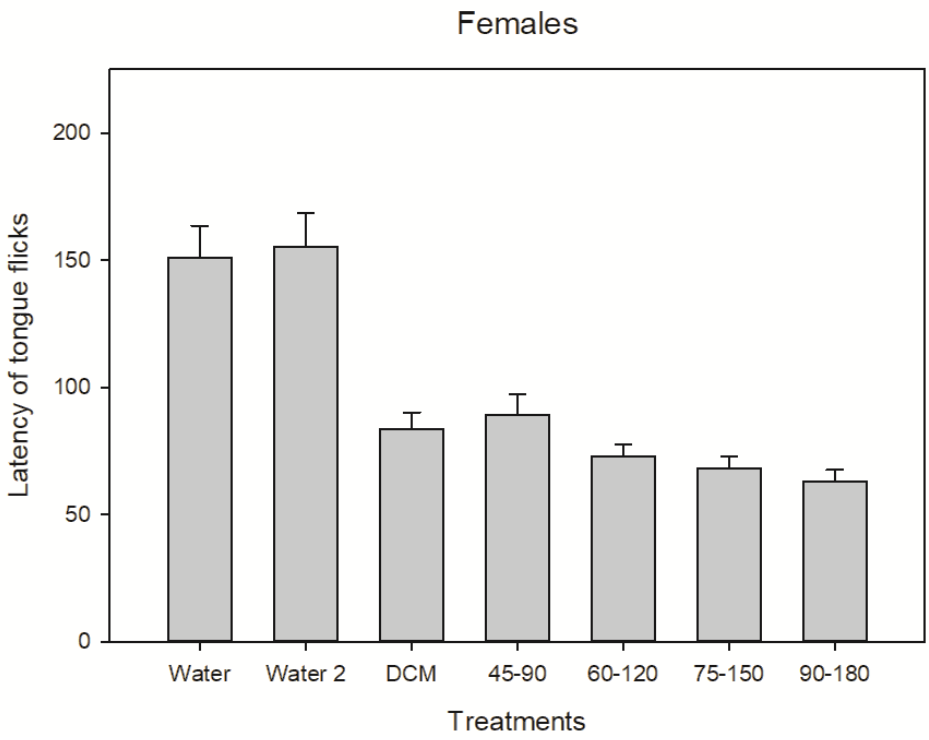

Figure 15: The latency of first tongue flick in behavioral response of females to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). By this measure, females did not respond significantly different to any of the stimulus concentrations.

	Water	**DCM**	**45-90**	**60-120**	**75-150**	**90-180**
Water		0.2168	**0.0002**	**0.0011**	**0.0060**	**< 0.0001**
DCM	2.2460		0.1579	0.3330	0.6840	**0.0408**
45-90	4.3490	2.3950		0.9996	0.9450	0.9799
60-120	3.9490	2.0160	0.3150		0.9926	0.9160
75-150	3.5110	1.4690	0.9060	0.5770		0.6201
90-180	4.6450	2.9210	0.7180	1.0060	1.5680	

Table 12: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the latency of first tongue flick in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

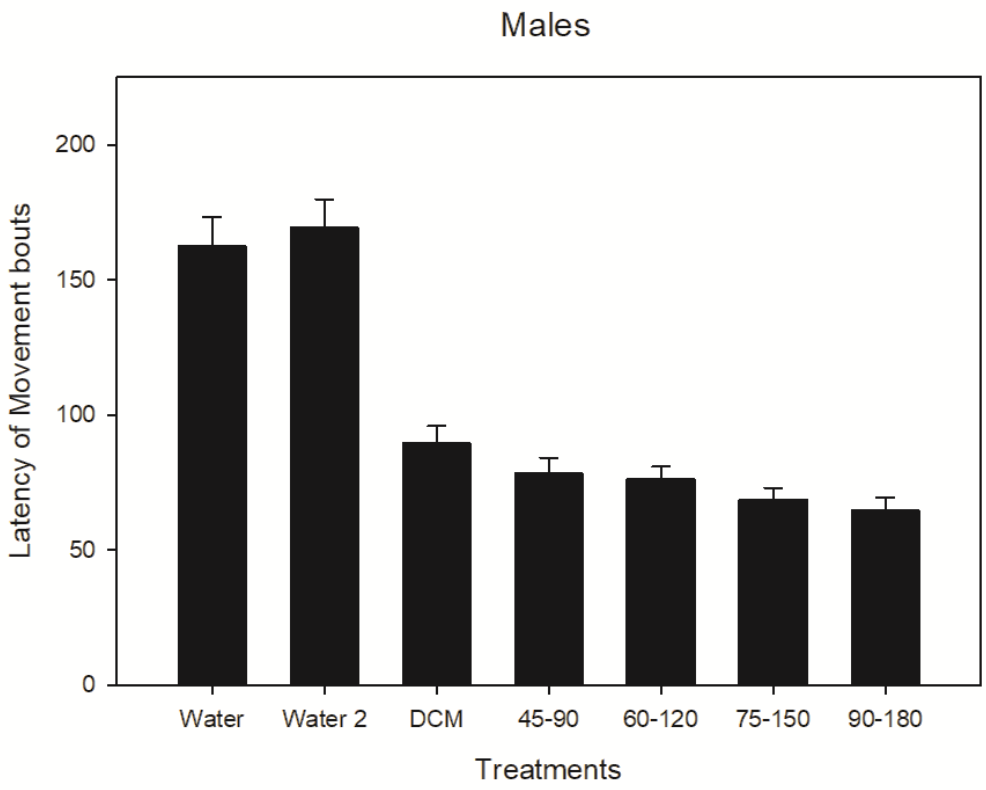

Figure 16: The latency of first tongue flick in behavioral response of males to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). Males did not respond significantly different towards any of the stimulus concentrations.

The number of movement bouts towards, as a measure of individual's baseline behavior, did not change across days for both the females ($t = 0.4692$, $p = 0.6407$) (Figure 17) and males ($t = 0.4347$, $p = 0.6653$) (Figure 18). Based on repeated measures ANOVA and Tukey's multiple comparison test, it can be concluded that the number of movement bouts did not differ significantly for the 60-120 mg/ml ($z = 0.086$, $p = 1$), 75-150 mg/ml ($z = 0.707$, $p = 0.9812$) and 90-180 mg/ml ($z = 0.616$, $p = 0.9899$) treatments compared to the 45-90 mg/ml treatment in females. Females also did not show a significant difference in the number of movement bouts between any other

treatment pair except water (refer Table 13 for all pairwise comparisons). In males too, the number of movement bouts towards the 60-120 mg/ml (z = 0.798, p = 0.9679), 75-150 mg/ml (z = 1.215, p = 0.8298) and 90-180 mg/ml (z = 0.798, p = 0.9679) treatments did not differ significantly compared to the 45-90 mg/ml treatment. None of the other treatment pairs differed in the number of movement bouts in males except when compared with water (refer Table 14).

	Water	**DCM**	**45-90**	**60-120**	**75-150**	**90-180**
Water		**0.0070**	**< 0.0001**	**0.0056**	**0.0222**	**0.0234**
DCM	3.4660		0.9999	0.9998	0.9754	0.9823
45-90	4.7070	0.2540		1.0000	0.9812	0.9899
60-120	3.5260	0.2590	0.0860		0.9974	0.9987
75-150	3.1210	0.7510	0.7070	0.4620		1.0000
90-180	3.1050	0.6980	0.6160	0.3970	0.0700	

Table 13: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the number of movement bouts in females. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

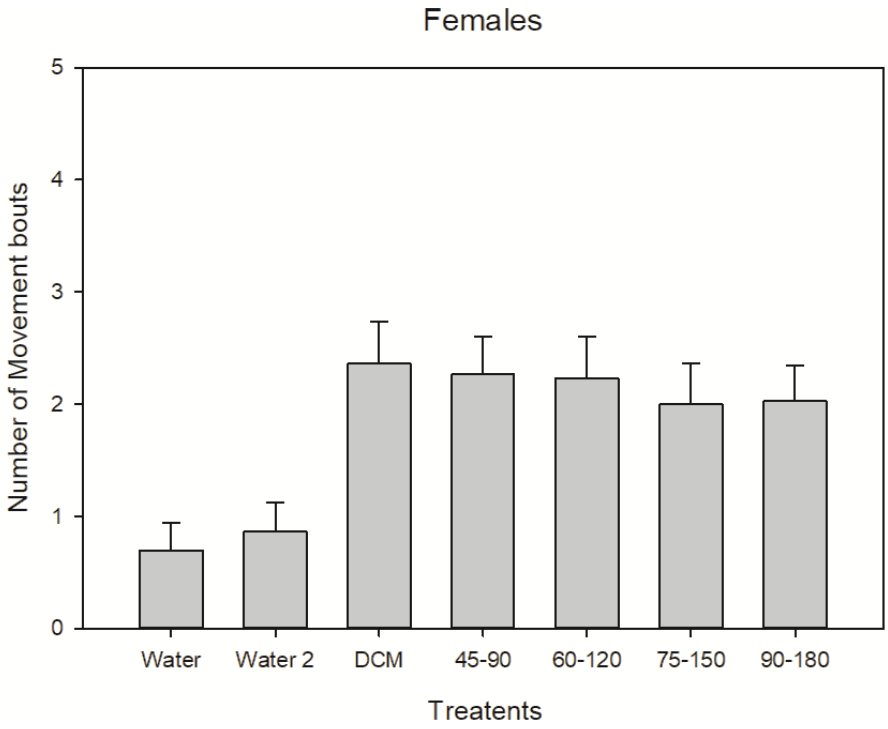

Figure 17: The number of movement bouts as a measure of behavioral response of females to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). Different stimulus concentrations did not elicit significantly different behavioral response as measure by the number of movement bouts in females.

	Water	**DCM**	**45-90**	**60-120**	**75-150**	**90-180**
Water		**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
DCM	6.2270		0.9279	0.4921	0.2518	0.4921
45-90	5.5230	0.9690		0.9679	0.8298	0.9679
60-120	4.9120	1.7600	0.7980		0.9984	1.0000
75-150	4.5790	2.1700	1.2150	0.4190		0.9984
90-180	4.9120	1.7600	0.7980	0.0000	0.4190	

Table 14: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the number of movement bouts in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

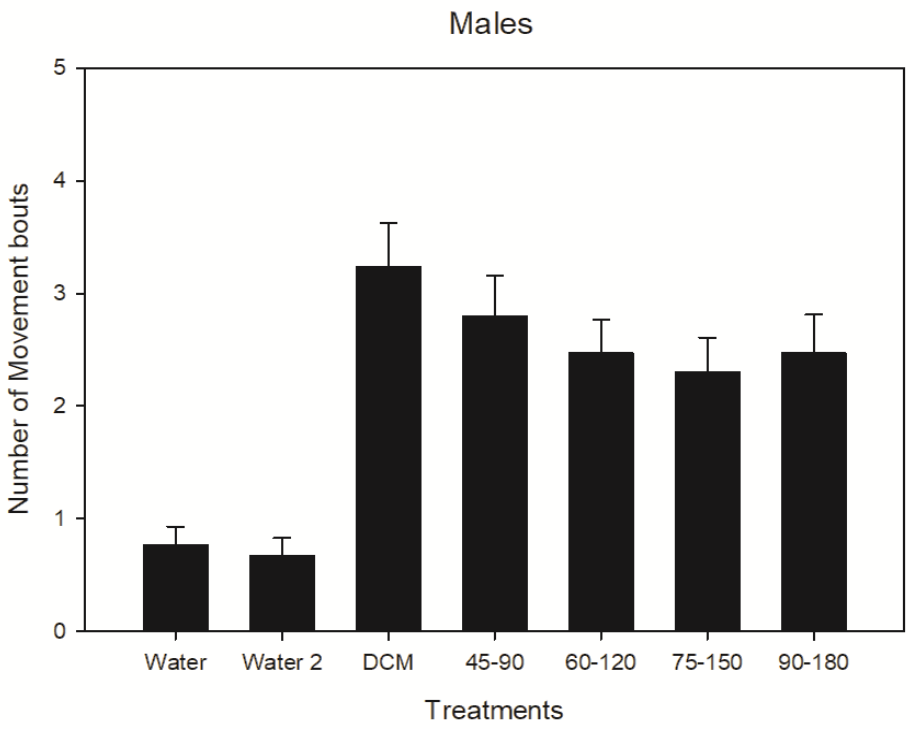

Figure 18: The number of movement bouts as a measure of behavioral response of males to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). Males did not respond differently to different stimulus concentrations as measured by the number of movement bouts.

Two-sampled t-test confirmed that there was no change in the baseline behavioral response of female (t = 0.3768, p = 0.7105) (Figure 19) and male (t = 0.1414, p = 0.8886) (Figure 20) individuals as measured by the latency of first movement bout. In females, the latency of first movement bout did not differ significantly in the 60-120 mg/ml (z = 0.163, p = 1), 75-150 mg/ml (z = 0.204, p = 1) and 90-180 mg/ml (z = 0.263, p = 0.9998) treatments compared to the 45-90 mg/ml treatment. There was no significant difference in the latency of first movement bout between any other treatment pair except when compared with water (refer Table 15). Males too did not exhibit any significant difference in the latency of first movement bout in the 60-120 mg/ml (z = 0.01, p = 1), 75-150 mg/ml (z = 0.568, p = 0.9931) and 90-180 mg/ml (z = 0.993, p = 0.9204) treatments when compared to the 45-90 mg/ml treatment (refer Table 16 for all pairwise comparisons of treatments).

	Water	DCM	45-90	60-120	75-150	90-180
Water		**0.0054**	**0.0162**	**0.0240**	**0.0267**	**0.0075**
DCM	3.5410		0.9990	0.9944	0.9922	1.0000
45-90	3.2190	0.3790		1.0000	1.0000	0.9998
60-120	3.0970	0.5420	0.1630		1.0000	0.9982
75-150	3.0620	0.5830	0.2040	0.0410		0.9972
90-180	3.4480	0.1150	0.2630	0.4270	0.4680	

Table 15: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the latency of first movement bout in females. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted.

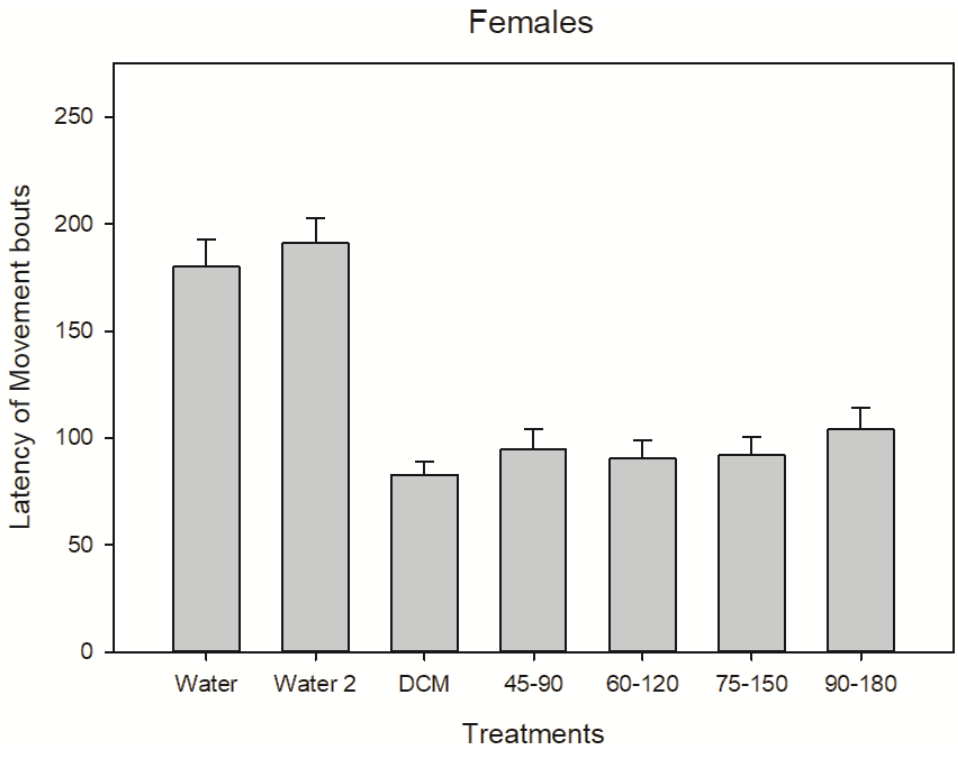

Figure 19: The latency of first movement bout in behavioral response of females to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). Females did not show a significant difference in the latency of first movement bout in response to the stimulus concentration.

	Water	DCM	45-90	60-120	75-150	90-180
Water		**0.0002**	**< 0.0001**	**0.0015**	**0.0064**	**0.0184**
DCM	4.3760		0.9789	0.9953	0.9436	0.8285
45-90	5.4810	0.7250		1.0000	0.9931	0.9204
60-120	3.8660	0.5220	0.0100		0.9988	0.9824
75-150	3.4900	0.9120	0.5680	0.3940		0.9997
90-180	3.1800	1.2170	0.9930	0.6960	0.3010	

Table 16: Pairwise comparisons of different concentrations of squalene + cholesterol calculated for the latency of first movement bout in males. P-values of the pair of treatments showing significant difference at $p < 0.05$ are highlighted in.

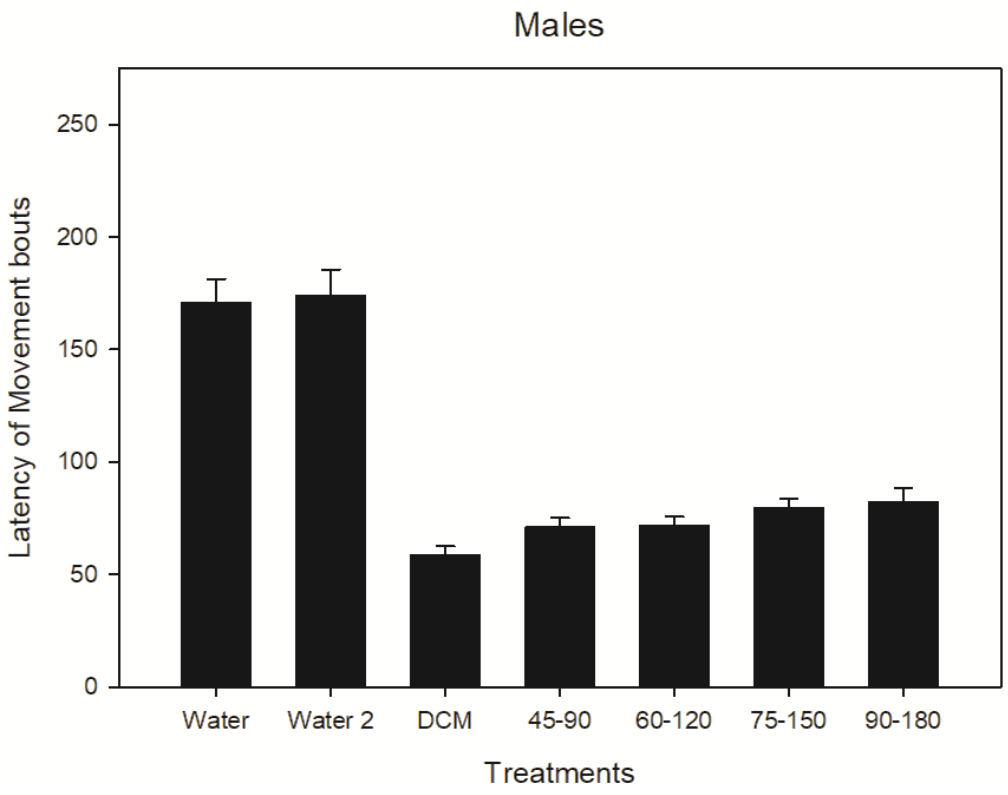

Figure 20: The latency of first movement bout in behavioral response of males to different concentrations of squalene + cholesterol as a multi-chemical component stimuli (mean ± SE) (N=30). Males did not show a significant difference in the latency of first movement bout in response to the stimulus concentration.

Experiment 4: Female choice based on natural male secretions

Males were first characterized based on the size (SVL), ectoparasite load and performance (sprint speed). We found that, amongst the three male traits characterized, SVL correlated positively with the number of visible ectoparasites (r = 0.4924, p = 0.0066) (Figure 21). Sprint speed and the number of visible ectoparasites showed a negative correlation (r = -0.4253, p = 0.0214) (Figure 22). For the female choice experiments, we selected male pairs with animal IDs 22 and 10 (Blue), 17 and 9 (Pink) and 7 and 21 (yellow) that had trait values clearly distinct from each other on at least two axes.

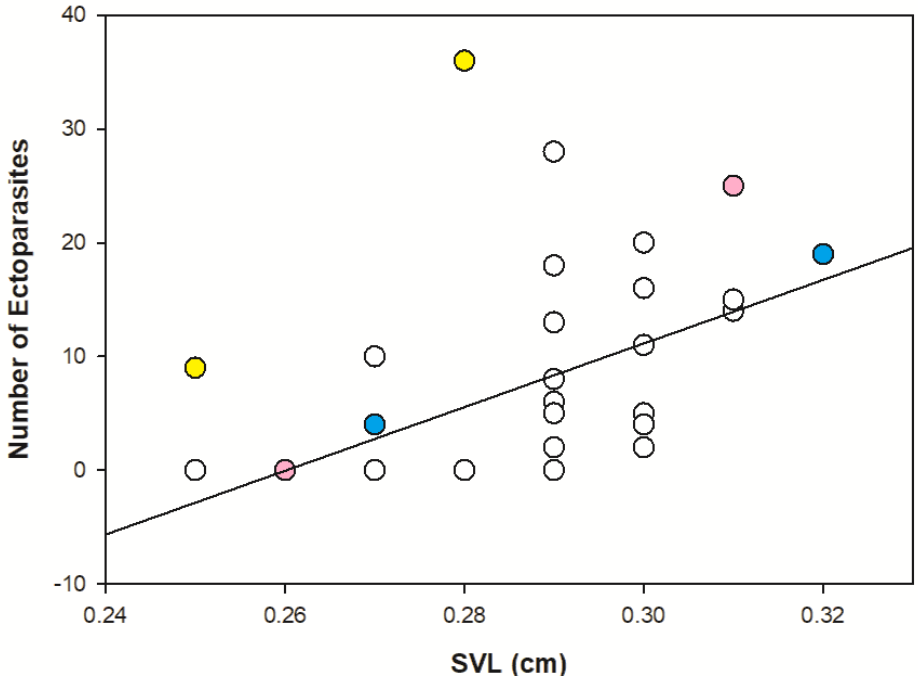

Figure 21: Correlation between male traits SVL and number of Ectoparasites in males (N=29).

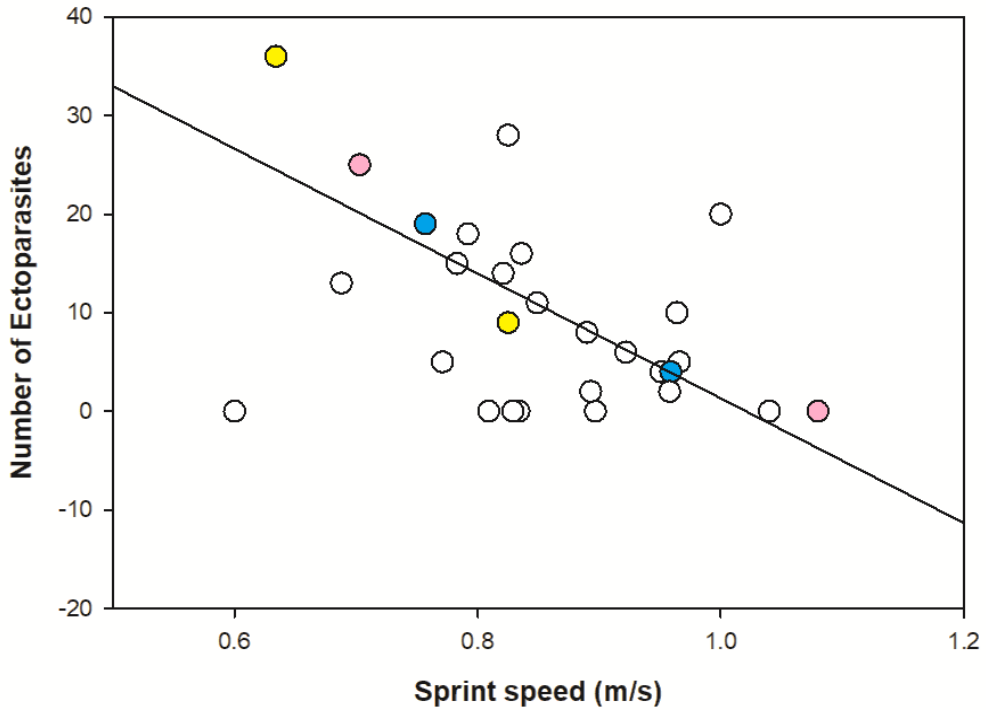

Figure 22: Correlation between sprint speed and number of Ectoparasites in males (N=29)

Out of 30 females used for each treatment, the number of females that made a clear choice in High vs Low, High vs High and Low vs Low treatments was 29, 26 and 25 respectively. When given a choice between two low quality male secretions ($z = 0.6$, $p = 0.5485$) or two high quality male secretions ($z = 1.177$, $p = 0.2393$), females did not choose any one side significantly more than what was predicted by chance alone. This suggested that females did not have any inherent bias towards either right or left side of the experimental setup. Z-test for single proportion conclusively showed that, when presented with one high quality and one low quality male secretion as the two choices, females were able to detect and select the high quality male secretions significantly more than 50% ($z = 3.5282$, $p = 0.0004$). This suggested that females can use male femoral gland secretions to detect male quality and make a choice of males (Figure 23).

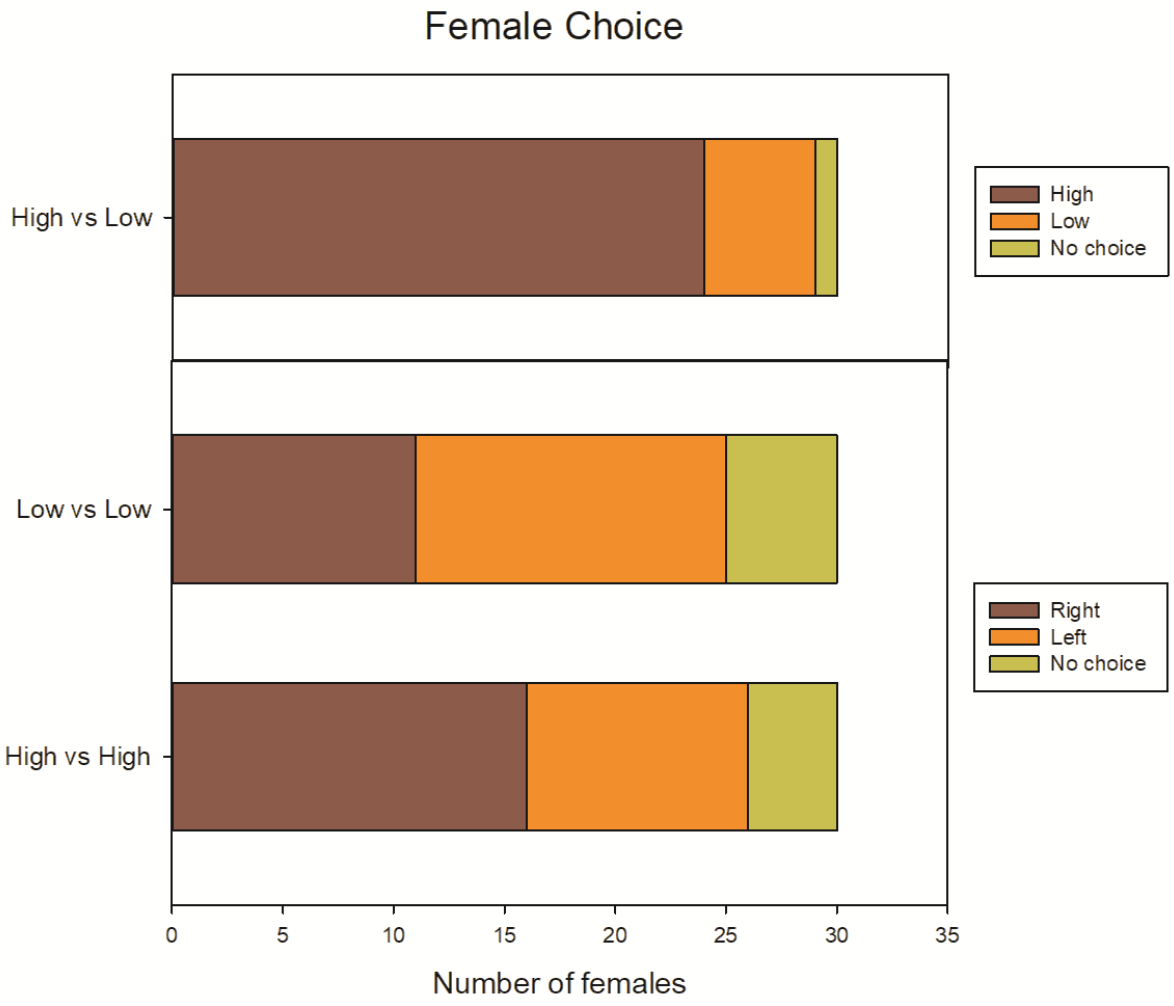

Figure 23: Female choice between male pairs based on the male femoral gland secretion components (N=30). Females were able to detect and select high quality males from the low quality males based on male secretion components.

Discussion

The chemical secretions in *Cnemaspis mysoriensis* contain two chemical components, squalene and cholesterol, found only in the males of this species (Kabir et al., 2019a). This study was aimed to understand the roles of squalene and cholesterol as multicomponent chemical signal and their function in sex recognition and mate choice. I found that squalene and cholesterol elicited similar behavioral responses in females, but not males, when present separately and elicited slightly higher behavioral responses when present together. This behavioral response by females was similar to the behavioral response towards natural male secretions, suggesting that squalene and cholesterol can act as a sex recognition signal of males of this species. My study also demonstrated that females, but not males, were able to differentiate between individuals based on squalene and cholesterol concentrations. Further, I showed that male secretion components also acted as mate quality assessment signals as females preferred the secretions of males with lower ectoparasite load and higher sprint speed.

Understanding the measures of behavioral response in receivers

An animal signal has two equally important aspects: 1) its information content and 2) the behavioral response it elicits in the receiver, although most of the studies on animal signals focus on either of the two (Smith and Harper, 2003, 1995). In this study, we recorded the number of tongue flicks and the number of movement bouts as measures of these two aspects of receiver response respectively. For sensing chemicals in the surroundings, lizards and snakes have a specialized chemosensory organ called vomeronasal organ (Keverne, 1999). Since vomeronasal organs are a major part of chemosensory mechanisms in lizards, tongue flicks have been used commonly as an assay for quantifying their chemosensory and chemical discriminatory abilities (Cooper and Burghardt, 1990; Schwenk, 1995). Movement bouts represent the exploratory behavior of individuals in response to the detected chemical stimuli.

Unlike tongue flicks, I found that the number of movement bouts by individuals did not change significantly in response to any chemical stimuli. This was not surprising

as not all potential signals induce a behavioral response in all receivers (Smith and Harper, 1995). I speculate that another reason for the lack of movement in response to chemical stimuli could also be the size of the experimental container. Chemical stimuli indicate the presence of another individual to an animal. Movement bouts are the measures of exploratory movement, most likely in search of the source of chemical stimuli. Since the container is small and devoid of any obstacles, the clear visibility of the available space could have discouraged the individual from exploratory movement. The reason behind lack of exploratory movement in individuals of this species remains unclear as I did not gather evidence to test it in this study. Hence, I focus on the tongue flick response of individuals towards the chemical stimuli for the remainder of the discussion.

Receiver responses to single-chemical and multi-chemical stimuli

The tongue flick response of individuals towards squalene in the single-chemical stimuli behavioral assays (Figure 1,5,6) showed that females were highly receptive towards individual squalene stimuli, whereas males did not respond to squalene stimuli. These results were similar to an experiment carried out to study behavioral responses towards cholesterol as single-chemical stimuli (unpublished: Bronte Ellsworth, 2019). The tongue flick response by females towards squalene and cholesterol, when present together, was slightly greater, but not additive or synergistic, compared to the responses to separate chemical stimuli.

Multiple components of a complex signal either evolve to encode multiple messages or to increase the effectiveness of signal perception by receivers (Hebets and Papaj, 2005). For example, in peacock blenny *Salaria pavo*, head crest size indicates the general state of health of males, while its colour intensity indicates current health status (Locatello et al., 2012). Receivers of these multiple messages could be the same intended target (Schultz and Fincke, 2009) or different intended targets (Zambre and Thaker, 2017). For example, pipevine swallowtails *Battus philenor* have blue and orange colour patches which are warning signals and are equally effective in averting

predation by birds (Pegram et al., 2013). Multiple components of some signals can also be conditionally redundant. In *Parasemia pantaginis* moth larvae, for example, hairiness is a redundant warning signal when the orange colour patch is prominent, while it reduces predation risk of larvae with no orange patch (Lindstedt et al., 2008). In this study, behavioral responses of females and males were not significantly different towards squalene compared to cholesterol. I did not examine the relative importance of these components. Hence, even if the results suggest that these might be redundant signals, behavioral response of individuals towards squalene and cholesterol in different conditions must be studied to confirm this possibility. It is also possible that these two components encode different information that elicits the response at the same intensity. Correlating signal intensity with different traits is also necessary to further confirm redundancy of these signal components.

I also showed that the behavioral responses of females towards squalene and cholesterol, separately and together, were similar to the response towards natural male secretions. This provides strong evidence for squalene and cholesterol being sex recognition signals of males in this species. The sex recognition signal seems to also be directed towards females, as males do not show highly elevated behavioral response towards these specific compounds. The absolute difference between female and male responses towards these stimuli was not large, but males did not show a response greater than the DCM control stimuli in any of the trials. This lack of response towards chemical stimuli in males could be explained by previous evidence that behavioral response in males is elicited only when chemical and visual stimuli are presented together (Kabir et al., 2019a). Although squalene and cholesterol elicited behavioral responses that were similar in intensity, I cannot comment on whether the information content of these two components is similar. Studies correlating signal concentration to signaller traits are necessary to reveal the information content of signal components and understand how each component is perceived by the receivers.

Individual quality assessment and female mate choice

For a signal to carry information about individual quality, it should vary between individuals and receivers should be able to perceive this variation (Endler, 1993). I found that females of this species are able to differentiate between different squalene and cholesterol concentrations when presented together (Figure 13). The ability to perceive the variation in squalene and cholesterol concentrations makes these compounds eligible to be used as a signal indicating individual variation. I suggest that the relative importance of squalene and cholesterol in differentiating individuals can be determined by varying the concentration of each component at a time.

Females, to increase their reproductive fitness and to get genetic benefits for their offspring, are expected to select males based on their secondary sexual characters that signal male quality (Zahavi, 1975). Hamilton and Zuk (1982) proposed a mechanism in which secondary sexual characters of males signal their resistance towards parasites. A large body of evidence has supported this hypothesis in multiple taxa, in which male secondary sexual traits correlate with immunity related traits and influence female mate choice (Doucet and Montgomerie, 2003; Locatello et al., 2012; Martin and Lopez, 2006; Møller, 1990; Molnár et al., 2013). For my study, I characterized three male traits: SVL, ectoparasite load and sprint speed to examine female choice for these traits based on femoral and precloacal gland secretions.

The female mate choice experiment (Figure 23) showed that females preferred secretions of males with low ectoparasite load and high sprint speed. Female choice confirms that male secretion components act as mate assessment signals. The variation in squalene and cholesterol concentrations in natural femoral and precloacal gland secretions of males needs to be measured to ascertain their role in this mate assessment.

Here, one should be cautious while drawing conclusions related to male quality traits that females select for, as all male traits were not measured in this study. I characterized three male traits, but females could be interested in something unmeasured in this study but was correlated to one of the measured traits. Studies

focused on information content of components in multicomponent signals have provided evidence for male quality traits encoded in signal components. For example, in satin bowerbird *Ptilonorhynchus violaceus*, bower quality predicts ectoparasite load, while plumage colouration signals blood parasite load and feather growth rate and females use both these traits to choose high quality males (Doucet and Montgomerie, 2003). Similarly, females of treefrog *Hyla arborea* use multiple call components such as call duration and peak frequencies to select larger sized males (Plénet et al., 2010). I suggest that a similar study aimed to reveal information content in squalene and cholesterol is necessary to understand male quality traits of importance for females of *C. mysoriensis.*

Signal type and signal reliability

Sex recognition and mate quality assessment signals encode the information about sex and individual quality of the signaling individual (Hasson, 1997). Since our study shows that squalene and cholesterol are sex recognition and mate assessment signals, these are self-reporting signals. Femoral and precloacal gland chemicals are secreted on the ventral body surface for their hypothesized primary function of preventing water loss from the skin (Martin and Lopez, 2014). If this is true and the components of these secretions are secondarily used in signaling, they could be considered cost-free. Based on the evolutionary mechanisms, these could then be categorized as minimal cost signals (Smith and Harper, 1995). This is surprising, as most of the animal signals that communicate individual quality are cost-added to ensure reliability of the signal (Zahavi, 1975, 1987). The added cost ensures that high quality cannot be signaled cheaply and prevents cheating (Grafen, 1990). Hence, whether these signals are actually cost-free, needs to be examined.

Cholesterol is one of the most important cell membrane components and functions in maintaining the membrane fluidity and membrane protein stability in all cells (Simons and Ikonen, 2000). Squalene is also a key physiological chemical and functions as an anti-oxidant, in stabilizing plasma membranes and as a precursor in

biosynthesis of all steroid hormones, including cholesterol (Bhilwade et al., 2010). I speculate that due to this heavy demand of squalene and cholesterol in all the cellular membranes of the body, it is costly for an animal to allocate large amounts of these compounds to femoral and precloacal gland secretions. Hence, this additional cost could ensure reliability in signaling male quality. This can be tested by measuring squalene and cholesterol concentrations in femoral and precloacal gland secretions of high and low quality males that I have collected during the female choice experiment.

Multi-component chemical signal in sex recognition and mate choice

Mate assessment signals are characterized by variation in signal which reliably represents individual quality, whereas recognition signals are selected to have no variation in order to accurately represent a group of individuals (Johansson and Jones, 2007). Hence, a complex signal should either have separate components that bring about recognition and quality assessment, or have variable and constant properties of the same signal. There are some studies which show that different properties of acoustic signals in insects and frogs act as species recognition and male quality signals (Gerhardt and Huber, 2002). In pygmy swordtail fish, *Xiphophorus pygmaeus,* different components of a multimodal signal act as species recognition and mate assessment signals (Hankison and Morris, 2002).

The multi-component chemical signal in *C. mysoriensis* examined in this study is the only known example of a signal which is a sex recognition as well as a quality assessment signal. In this case, both signal components, squalene and cholesterol, are involved in both the sex recognition and mate quality assessment. Here, just the presence of signal components, squalene and cholesterol, acts as a sex recognition signal, while their concentrations in the femoral and precloacal gland secretions contribute in mate quality assessment and female choice. In future, this can be confirmed by externally manipulating squalene and cholesterol concentrations in a female choice experiment.

Summary

In sum, the behavioral responses of females and males of *C. mysoriensis* to single-chemical and multi-chemical stimuli indicate that squalene and cholesterol, together, function as a sex recognition signal for males. Lack of significant response in males of this species also point towards females as the target recipients of this multi-component signal. Since females were able to perceive the variation in squalene and cholesterol concentrations and make a choice, we conclude that squalene and cholesterol also mediate mate quality assessment and female mate choice in this species. A cost-added signal is required to communicate individual quality reliably. Here, the cost of signal production and expression might not be the only cost incurred, but there could be an additional allocation cost for allocating squalene and cholesterol to the femoral and precloacal gland secretions in this species that ensures reliability. Further, squalene and cholesterol do not mediate sex recognition and mate assessment separately. This can be explained if the presence alone of squalene and cholesterol contributes to sex recognition, whereas squalene and cholesterol concentrations mediate mate assessment and female mate choice.

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