Differential Mate Choice Responses to Chemical Cues in Male and Female Rough-skinned Newts (*Taricha granulosa*)

By

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ABSTRACT

Purpose of the Study: To understand male and female mate choice patterns affecting species and gender recognition via long distance chemical cues, this study examined the rough skin newt, *Taricha granulosa*, in both pond and creek habitats. Response to chemical cues of males vs. females was examined for T. granulosa, whereas the California newt (*Taricha torosa*), and the red-bellied newt (*Taricha rivularis*) were used to examine how *T. granulosa* would respond to heterospecific chemical cues as compared to conspecific cues in a region of the species' ranges where all three species occur in sympatry.

Methods: To simulate mate choice decisions in nature as closely as possible, we conducted behavioral experiments in the field using a Y-maze apparatus (olfactometer). All trials were run in the field using unique individuals. Data were combined across multiple years. Mate choice was analyzed using a nominal logistic regression model accounting for variation among year, researcher, Y-maze apparatus, sex, and species.

Findings: *T. granulosa* females chose chemical cues of conspecific males significantly more than heterospecific males, whereas males showed no preference between chemical cues of conspecific and heterospecific females. In addition, *T. granulosa* males chose chemical cues of conspecific males over conspcific females, whereas females showed no preference between chemical cues of conspecific males and females.

Conclusions: The results of this study support long-standing theoretical paradigms that males, who have less parental investment in offspring will adopt a strategy of seeking matings with females regardless of quality, whereas females, with greater levels of parental investment, will engage in a more discriminating mate choice strategy. In this case, females use chemical cues to avoid heterospecific males during breeding season encounters and reduce wasting her singular mating event on an act of hybridization. A result of this mating strategy is that reinforcement of reproductive isolation is maintained in this region of sympatric range overlap. In contrast, will mate with any female across multiple mating events, and use chemical cues to either locate females or to avoid other males (or both).

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INTRODUCTION

Since the age of Darwin, early evolutionary theory has told us that in many mating systems, females tend to be more selective for quality of mate than males, whereas males tend to be less interested in quality of any individual mate and more interested in increasing the amount of mating events (Orians 1969; Trivers 1972; Emlen and Oring 1977). In many species, females contend with a larger parental investment (e.g. egg production) along with the tendency to only mate once per season. In comparison, males, with little parental investment (e.g. in many small sperm), tend to mate with multiple individuals in a season. This asymmetry in reproductive investment can drive the potential for differences in behaviors associated with mate choice (Bateman 1949; Trivers 1972). The idea that females choose carefully and males are more inclined to select for more breeding events makes sense, because females have a greater fitness cost from choosing suboptimal mates (Hayward and Gillooly 2011). Perhaps, the most basic level of mate choice discrimination would generally include an individual ensuring it mates with an individual from its own species. In general, it is expected that sexual isolation and avoidance of mating with heterospecific species will be driven primarily by the “choosy” sex, which, in most cases, are females for the reasons described above (Wirtz 1999). How individuals within a species use cues to discriminate among species and sexes to select an appropriate mate varies greatly across the spectrum of mate choice systems.

Chemical senses are among the oldest, shared by all organisms including bacteria, so animals are pre-adapted to detect chemical signals in the environment (Wilson 1970). For many organisms, chemical information is used to locate potential food sources and to detect predators as well as to receive chemical signals in social interactions (Wyatt 2003). Chemical communication with sex pheromones is ancient, diverse, and widespread and has been a significant factor of sex recognition and courtship in invertebrates and vertebrates alike (Johnson and Li 2010, Rafferty and Bauman 2006, Phelan PL and Baker TC. 1987). Thus, one might expect that differential response to chemical cues between males and females might exist in systems where males and females have differences in reproductive contribution to their offspring and where chemical cues play an important role in species recognition, mate choice and breeding behaviors.

Among vertebrates, salamanders have been shown to make use of chemicals for a variety of functions. For example, Plethodontid salamanders are known to use chemical cues in species recognition, reproductive isolation, territorial displays and female stimulation (Ovaska 1989, Houck & Reagan 1989, Dawley 1992, Ovaska & Davis 1992, Arnold et. al. 1993, Uzendoski & Verrel 1993). In the salamander family Salimandridae, often referred to as newts, chemical communication has also been shown to function in enhancing mate recognition/discovery (Toyoda et al., 1994; Twitty, 1955), repelling competition (Park and Propper 2001), facilitating mate choice (Osikowski, 2012), and synchronizing reproductive behaviors such as the transfer of sperm from males to females (Halliday, 1990).

Pacific newts, a subgroup of newts within the Salamandridae, consist of three species which have overlapping ranges and include the rough-skinned newt (*Taricha granulosa*), the California newt (*Taricha torosa*), and the red-bellied newt (*Taricha rivularis*) (Figure 1). These newts have an aquatic courtship during which a male locates the female, engages in amplexus for a period of time, then deposits a spermatophore in front of the female. The spermatophore is composed of a gelatinous base that supports an apical sperm cap. Sperm transfer occurs when the female moves over the spermatophore, lowers her cloaca, and lifts off after the sperm mass is lodged in her cloaca. These species are examples of lentic and lotic breeders, which could play a role in their reproductive strategies. *T. torosa* and *T. grnulosa* are classified as lentic species, meaning they lay eggs in more stagnant pools and ponds. *T. rivularis* is classified as a lotic species, meaning it will lay eggs in moving water such as streams and rivers (Nussbaum 1987). Egg size is also associated with water source differences in breeding. Lotic species will tend to have larger eggs and lentic species will tend to have smaller eggs (Nussbaum 1987). With different eggs sizes we may see differences in parental investment and reproductive strategy (Hayward and Gillooly 2011, Trivers 1972).

In regions where we see overlap in the ranges of these species, we observe a maintained reproductive isolation in sympatry (Davis and Twitty 1964). It is not uncommon to find that when closely related species come into contact, pre-zygotic reproductive isolating mechanisms prevent hybridization and reinforce reproductive isolation (Phelan and Baker 1987, Butlin and Tregenza 1997, Coyne and Orr 1997, Smadja and Butlin 2009). Courtship behavior and post-zygotic mechanisms have been ruled out as reproductive isolating mechanisms among the Pacific newt species (Davis and Twitty 1964). Temporal and habitat isolation may play a role in reproductive isolation between *T. torosa* and *T. rivularis*. While *T. torosa* breeds primarily in ponds in late winter, *T. rivularis* breeds almost exclusively in creek habitats in early spring. However, *T. granulosa* is a breeding generalist, occupying both creeks and ponds during both seasons thus overlapping in breeding habitat and reproductive timing with both *T. torosa* and *T. rivularis* (Davis and Twitty 1964, Harvey et al. 2011). This is not unexpected given that both *T. rivularis* and *T. torosa* have both evolved from *T. granulosa* (Davis and Twitty 1964).

Previous studies have shown that chemical communication likely plays a role in species recognition and mate choice in Pacific newts and may act as a reproductive isolation mechanism for *T. granulosa* (Harvey 2011, Minarik 2013). Through the use of a Y-maze olfactometer system, these studies showed that female *T. granulosa* could detect, and significantly avoided, heterospecific males relative to conspecific males based only on chemical cues in the water. Harvey's (2011) data suggested that chemical cues from heterospecific males were acting as repellant cues rather than conspecific males producing attractant cues.

In this study, we have adapted a Y-maze system to evaluate mate choice by both *T. granulosa* males and females to determine if they demonstrate any differences in species recognition capability related to mate choice. We evaluated whether males and females in this species show a differential response to chemical cues from conspecific vs. heterospecific individuals of the opposite sex. In addition, we also evaluate whether there is a difference in response between males and females to chemical cues from conspecific males and females in breeding condition. By completing these series of experiments, we seek to further develop a more complete understanding of the roles long-distance chemical cues play in mate choice and reinforcement of species boundaries among Pacific newts. A comprehensive view of how this system of mate selection occurs will further our knowledge of mechanisms of species isolation and reproductive success.

METHODS

Specimens Collected

Newts were collected from pond and creek habitats at Sonoma State University’s Galbreath Preserve (Mendocino County, CA) as well as ponds at the Mitsui Ranch on Sonoma Mountain (Sonoma County, CA) (Figure 1). *T. torosa* and *T. granulosa* were collected together between January through February and *T. rivularis* and *T. granulosa* were collected together from March through April. Specimens were gathered in the morning to be used in behavioral experiments on site, that day and released back to the site of capture the same day. Individuals were captured using dip nets the day of or minnow traps set the night before. Once safely captured, individuals were transported in separate containers based on location of capture to a location where they could be sorted by sex and species. Handling is permitted through California Department of Fish and Wildlife under SCP-13351. Individuals completed a y-maze trial, then were placed in a buffered MS-222 bath of 0.2g/L until anesthetized. Then individuals are measured for snout-vent length. Once measurements were taken, newts were pit tagged at the base of the tail then placed in a recovery bath. Once newts are fully recovered, they were released in the same location they were captured.

Experimental Design

Newts were tested for their responses to chemical cues using an olfactometer Y-maze apparatus (Park and Propper 2001) but with some slight modifications (Figure 2). Aged water from a main reservoir or tap with untreated well water was directed into two side arms where flow meters produce a consistent flow rate of 0.3L/min. Flow rate was determined using previous studies in *T. granulosa* and other newt species (Dawley 1984, Harvey 2011). Water then entered each arm of the Y-maze, flowed into the 'stimulus' newt holding areas, and left the Y-maze via a drain in the floor near the start gate. Stimulus newts were blocked by a perforated gate that allows aqueous chemical cues to pass through, and additionally prevents the test newt from receiving visual cues from the stimulus newts. The chooser or test newt moved up the y-maze either towards a conspecific or heterospecific male/female. Placement of odor source individuals was randomized to either side of the y-maze. Once stimulus newts and test newt were placed in the holding areas of the Y-maze, water then flowed through the maze for 10 minutes to allow equilibration of possible chemical cues, then the gate was lifted for the test newt to be allowed to move. The trial was discontinued when either the test newt moved at least halfway up into one of the Y-arms, or 20 minutes had elapsed. If 20 minutes had elapsed with no movement into an arm, no choice was documented.

Species recognition experiments were performed using male or female T. granulosa as the choosers, and odor source newts consisted of one conspecific and one heterospecific individual of the opposite sex as chooser. Gender based experiments were performed using male or female *T. granulosa* as choosers, with odor source newts consisting of one conspecific male and one conspecific female.

Statistical Analysis

Data from experiments conducted in different studies using the same experimental design were pooled across years. Researcher/year was included as a fixed effect in all analyses to account for any variation between researcher or year. To determine whether males and females differed in their mate choice patterns with respect to chemical cues from different species of potential mates, a nominal logistic regression was performed using Jmp Pro v.14 software with choice of conspecific or heterospecific as the response variable and sex, heterospecific species (*Taricha torosa* or *Taricha rivularis*), side of Y-maze containing conspecific, and researcher/year as fixed effects. The interaction term between heterospecific species and sex was also included. To determine whether males and females differed in their mate choice patterns with respect to chemical cues from different sexes of potential conspecific mates, a nominal logistic regression was conducted with choice of male or female as the response variable and sex, side of Y-maze containing conspecific, and researcher/year as fixed variables. All trials were run independently with unique individuals. The models were reduced by eliminating non-significant variables. Probability was estimated via a log likelihood ratio test.

RESULTS

Species Recognition

In 219 independent experimental trials (137 females and 82 males), the sex of the individual had a significant effect of whether *T. granulosa* moved towards the chemical cues of heterospecific or conspecific individuals in a final model that combined sex, heterospecific species, and their interaction term (LR χ^2=6.51, p=0.01). Heterospecific species, the interaction term, and all other variables did not have a significant effect. Females showed a preference for the chemical cues of conspecific males, whereas males had no preference between the chemical cues of conspecific or heterospecific females (Figure 3).

Choice Among Sexes

After 118 independent experimental trials (52 females and 66 males), we found that the sex of the individual had a significant effect of whether *T. granulosa* moved towards the chemical cues of same sex or opposite sex conspecific individuals in a regression of sex with choice (LR χ^2=6.33, p=0.01). All other variables were insignificant. In this instance, males showed a preference for the chemical cues of conspecific females, whereas females had no preference between the chemical cues of conspecific males or females (Figure 4).

DISCUSSION

Overview

Mate choice in Pacific newts followed predicted patterns of sexual selection. Females were shown to be choosy, preferring conspecific individuals over heterospecific males, whereas males did not show a preference between conspecific and heterospecific females. The differential pattern of species recognition for *T. granulosa* was not affected by the identity of the hetersopecific species, whether the pond-dwelling, winter breeding *T. torosa* or stream-dwelling, spring breeding *T. rivularis*. Male *T. granulosa* did show a preference toward chemical cues from the female sex, which differed from females, suggesting that *T. granulosa* males are responding to a chemical cue associated with gender whereas females may not be using long-distance chemical cues to find mates. The combination of results supports the idea that whereas females rely on long-distance chemical cues to avoid or reduce hybridization with heterospecific males, the primary breeding season drive for males is to find female Pacific newts, regardless of exact species, showing an attractant cue to females and/or potential for a repellent cue from other males.

Differential Mate Choice Patterns and Reproductive Success

In a pioneering study published in 1948, Bateman extrapolated from experimental results in Drosophila melanogaster to propose that aspects of sexual selection are normally more intense in males because, compared with females, males have higher variance in number of mates (i.e. mating success); males also have higher individual variation in the number of offspring produced (i.e. reproductive success) (Bateman, 1948; Collet, et al 2014). Evidence of sexual selection in *T. granulosa* has been demonstrated through the presence of greater standardized variances in reproductive and mating success (Jones et al., 2002). The standardized variance in mating success has been shown to be 19 times higher in males than in females. The use of long-distance chemical cues may be one component of a series of mating behaviors in *T. granulosa* that impact relative fitness through avoidance of hybridization. The use of long-distance chemical cues in species recognition as a component of female breeding behavior may be an important reason that females are able to maintain a more consistent level of reproductive success. In contrast, because of the great level of competition for mates among males, their fitness may be driven by efforts to locate and mate with as many *Taricha* females in the area as possible.

Asymmetry in Parental Investment

The differences in response to chemical cues related to mate choice can be explained by reproductive asymmetry in the species (Trivers 1972). The genus *Taricha* shows evidence of reproductive asymmetry in gamete production (Hayward and Gillooly 2011). A female’s cost to produce few large eggs is much higher than males producing many small sperm. Comparing gamete production in different vertebrates, Hayward and Gillooly (2011) found, in terms of the energetic cost of gamete biomass production, that differences between males and females were substantial. The cost of egg production was roughly 3.5 orders of magnitude higher than the cost of sperm production. This being said, we would expect females to be more selective about who fertilizes her eggs and choose the males that have characteristics that ensure higher fitness, in this case, conspecifics. Males, on the other hand, would be expected to show much less inclination to reserve mating efforts for only ideal mates.

Energetic Costs and Risk of Mating Events

Energy costs may also in play in courtship events and may impact selection for use of long-distance chemical cues by females to allow avoidance of additional fitness risks associated with mating events. Laboratory experiments with red spotted newts have shown that females often flee from groups of males, perhaps because of energetic cost associated with the newt-balls, where multiple males will engage in an attempt to amplex with a single female (Verrell 1985). In numerous cases, newt ball formation among *Taricha* newts can lead to injury or even death of the female (Davis and Twitty 1964). Therefore, engaging in mating events can be associated with potential high fitness consequences associated with each event and should be entered into carefully for females.

Repellant Chemical Cues Affecting Females

Previous research on female response to chemical cues in *T. granulosa* have suggested that female species discrimination is due to a repellent cue effect of heterospecific males rather than attractant cues from conspecific males (Harvey 2011). This suggests that the differential response related to species recognition may be driven by avoidance behaviors rather than attractant behaviors related to long-distance cues. Recent work by Cummings (2015) suggests that *T. granulosa* may produce a different variant of a sex pheromone than that of *T. rivularis* or *T. torosa*. The genomic region associated with sodefrin precursor-like factor (SPF), a known pheromone expressed in male cloacal glands, consists of a Beta 1 region in *T. granulosa* as opposed to a Beta 2 region in *T. rivularis* and *T. torosa* (Kikuyama et al. 1997). Beta 1 and Beta 2 SPF show different patterns of protein evolution and Beta 2, which occurs in the more derived *Taricha* species, may be associated with positive selection.

An alternative chemical candidate may be associated with the production of Tetrodotoxin (TTX) by these species. TTX has long been shown as a key toxin used primarily for predator defense, but has also been shown to have conspecific communication (Buciarelli and Kats 2015). Stokes et al. (2011) examined a population of Pacific newts in nearby Santa Rosa, CA and demonstrated that TTX levels were significantly higher in *T. torosa* than they were in *T. granulosa*. This may explain a repellant response by *T. granulosa* females to long-distance chemical cues of *T. torosa* found in our study. Additional research is necessary to determine if either SPF or TTX play any role in the long-distance chemical-based species discrimination in *T. granulosa* females.

Attractant or Repellant Cues Affecting Males

It is not clear what drives the *T. granulosa* male preference for female chemical cues over male chemical cues. It may be that males have developed a response to female cues that allow them to locate females for further close-encounter courtship behaviors. Alternatively, it may be that males likewise are responding to chemical cues of other males and avoiding males for purposes of effective use of the competitive breeding space. In a study with red-spotted newts, it was discovered that test males, during experiments using a Y-maze, preferred the one female side to the one female plus three male side, indicating that some aspect of a mixed sex group of newts produces a chemical that repels conspecific males (Park and Propper 2001). Male repellent response has been hypothesized as adaptive in that it increases the opportunity for breeding males to find other available breeding females. Similarly, during their breeding season male European pond turtles, exhibit avoidance behavior of larger males via chemical cues alone (Poschadel et al. 2006). However, the frequent occurrence of newt balls consisting of many males attempting to engage in amplexus with a single female may provide counter-evidence to male-male avoidance in Pacific newts (Davis and Twitty 1964). Further research is required to determine whether the repellant effect of male-male chemical cues in red spotted newts translates to mating strategies in Pacific newt species.

Role of Long-distance Chemical Cues

In many salamanders, male chemical cues are transferred directly to females by contact as part of the courtship behaviors (Rollman et al. 1999). Unlike most studies showing close encounter courtship chemical communication, we have demonstrated sexual selection being influenced in a long-distance chemical cue stage. Pheromones typically act as attractants and sexual stimulants in most vertebrates (Hay 2009). For example, in red-spotted newts, female pheromones attract males, and male pheromones increase female receptivity (Park and Propper 2001). By examining lesser known long-distance communicators, we can better understand mating interactions prior to close-encounter courtship behavior. Our understanding of salamander long distance chemical cues stretches primarily to success in homing and migration as well as assessing risk of predation (Twitty 1959; Kats et al. 1994). Our research demonstrates that long distance chemicals are the basis for early mate selection prior to close contact courtship displays and plays a role sexual selection and reproductive isolation in *T. granulosa*.

Future Research

The system of chemical cue utilization in Pacific newts calls for further research in both breadth and depth. Expansion of analyses of species interactions using *T. rivularis* and *T. torosa* as the choosing species is needed to determine if these effects go in both directions among Pacific newt species that regularly encounter each other in zones where ranges overlap. In addition, experimental trials are needed to determine whether the male response to chemical cues is an attractant or repellent cue. Deeper studies to identify the chemical agent associated with the use of chemical cues are also needed to discriminate among potential chemical candidates such as SPF or tetrodotoxin (Cummings 2015; Buciarelli and Katz, 2015). Finally, research is needed to connect the use of long-distance chemical cues to additional use of chemical cues in close-encounter and courtship behaviors in Pacific newts .

Conclusions

The use of long-distance chemical cues for species recognition by females and gender recognition by males found in this study, provides a more complete understanding of the mating system of T. granulosa and reconfirms our theoretical understanding of the roles of parental investment in the evolution of mating strategies associated with sexual selection. In addition, these long-distance chemical cues simultaneously play a role in the evolution of reinforcement of reproductive isolation among Pacific newt species in regions of sympatric occurrence. In order for these species to be successful when encountering each in sympatric populations, the adoption of long-distance chemical communication appears to indeed be an important adaptation as a key component of the collection reproductive behaviors found in these species.

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Figures

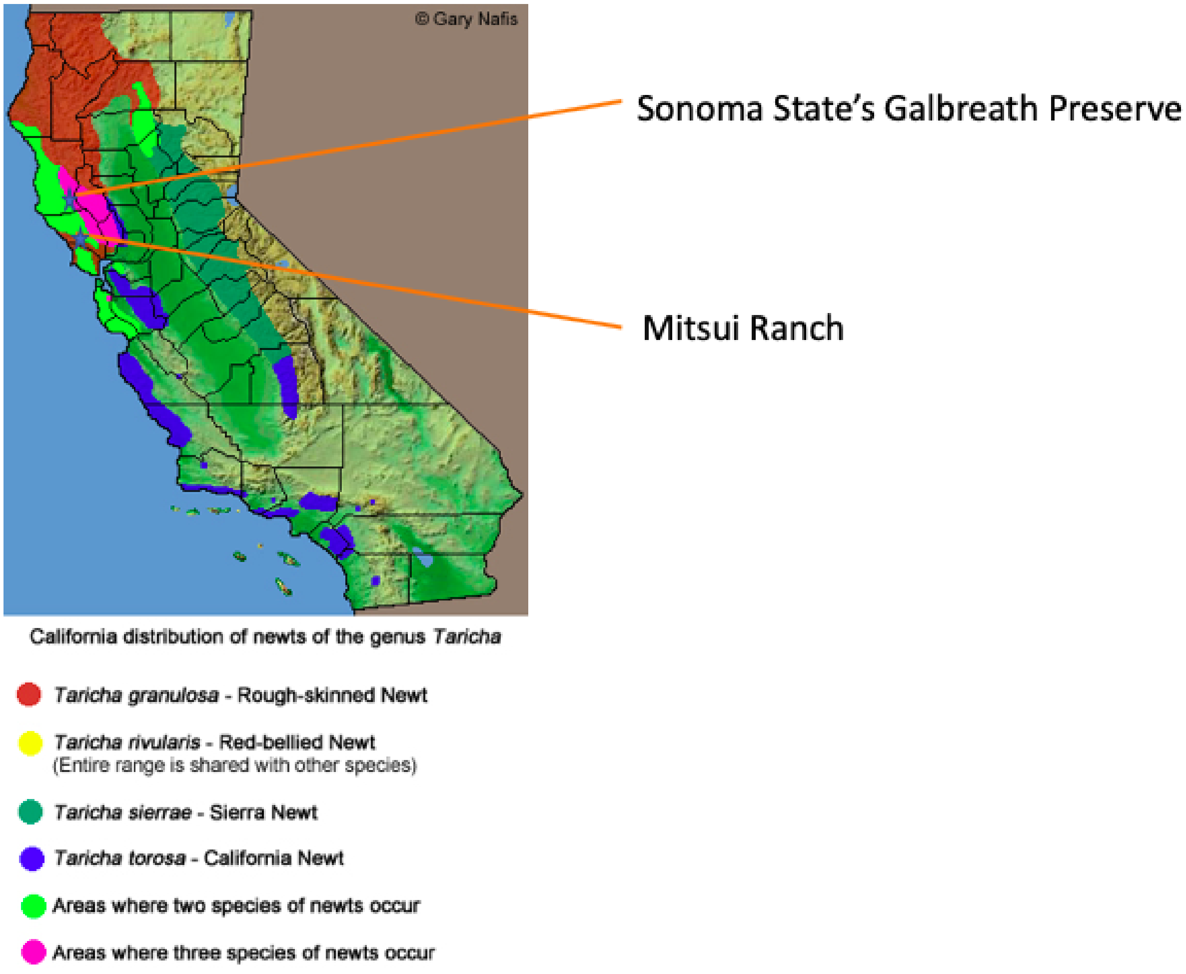

Figure 1 - shows the ranges of the species and their area of overlap [California Herps] with relative locations of research sites

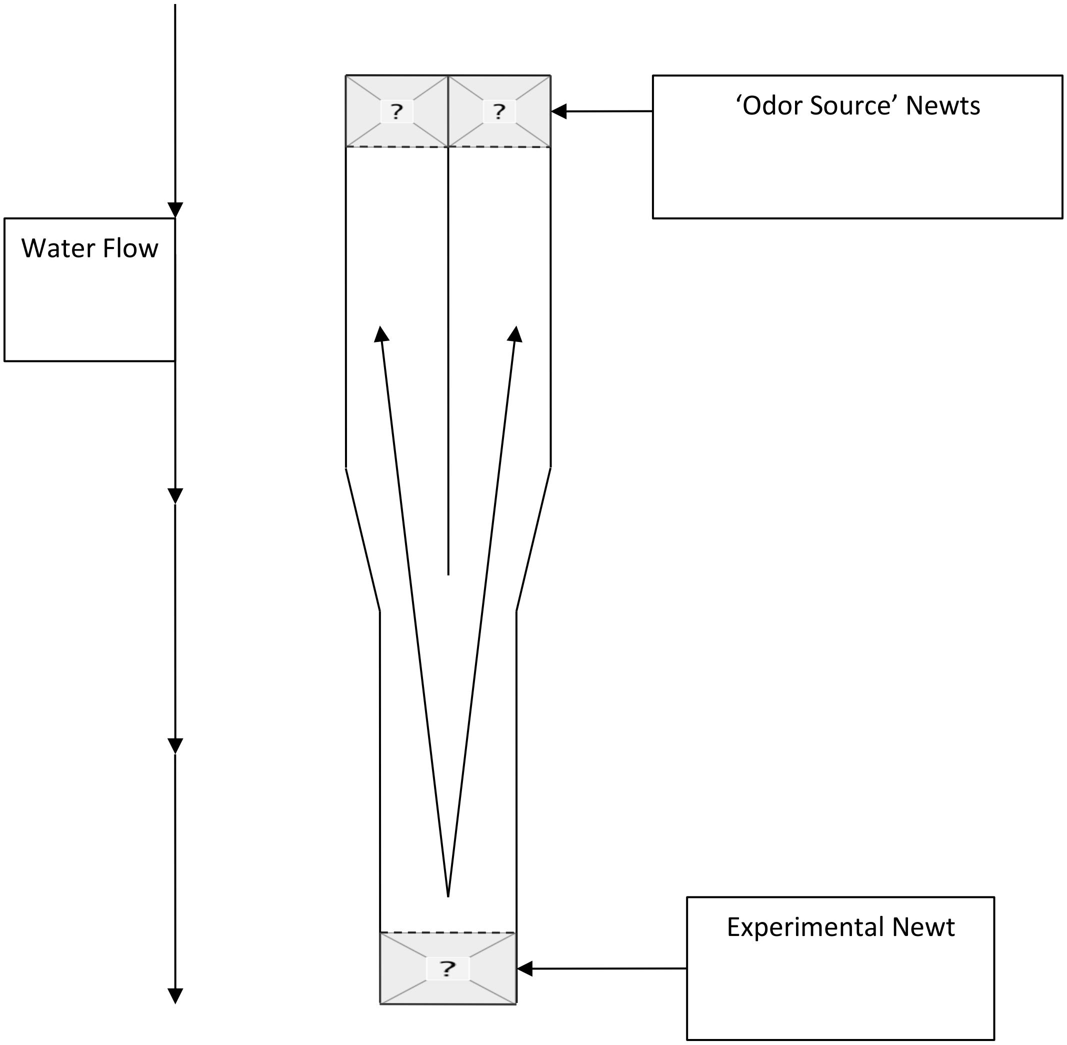

Figure 2 - showing Y-maze set up

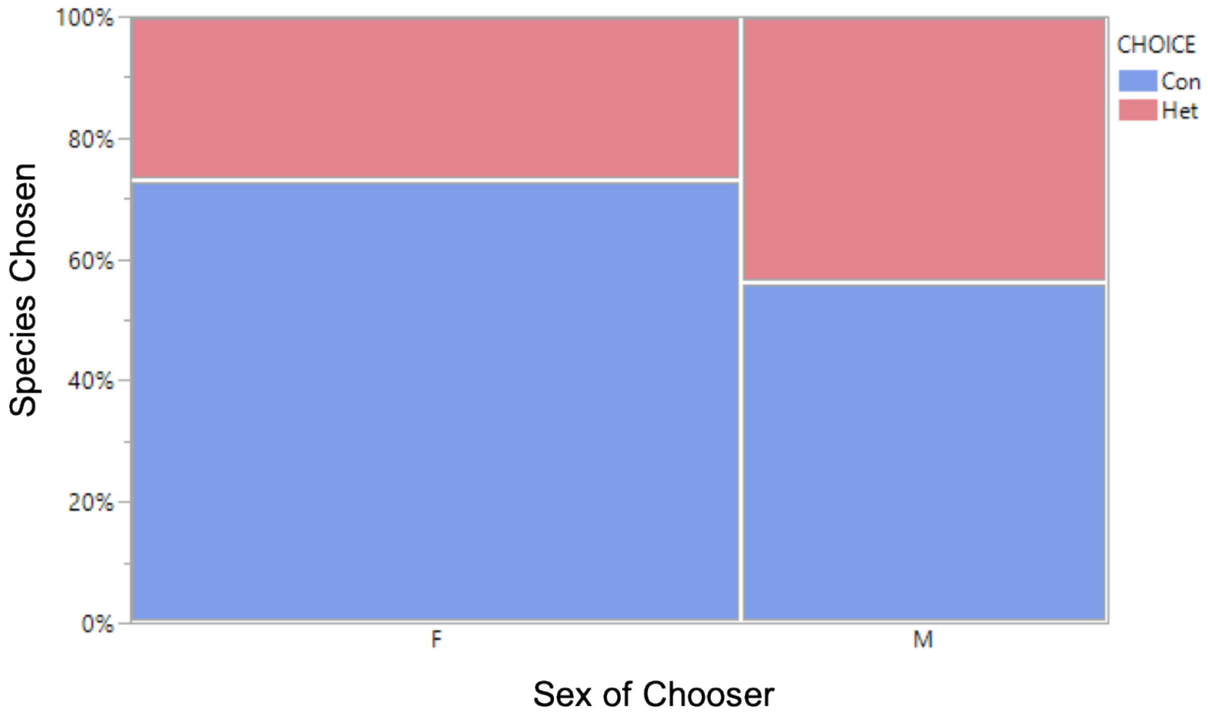

Figure 3 - Graph of results of Y-maze experiments showing differences in patterns of preference for chemical cues from heterospecific or conspecific individuals of the opposite sex by *T. granulosa* males and females. Females show a preference for conspecific male chemical cues whereas males showed no preference between conspecific and heterospecific females.

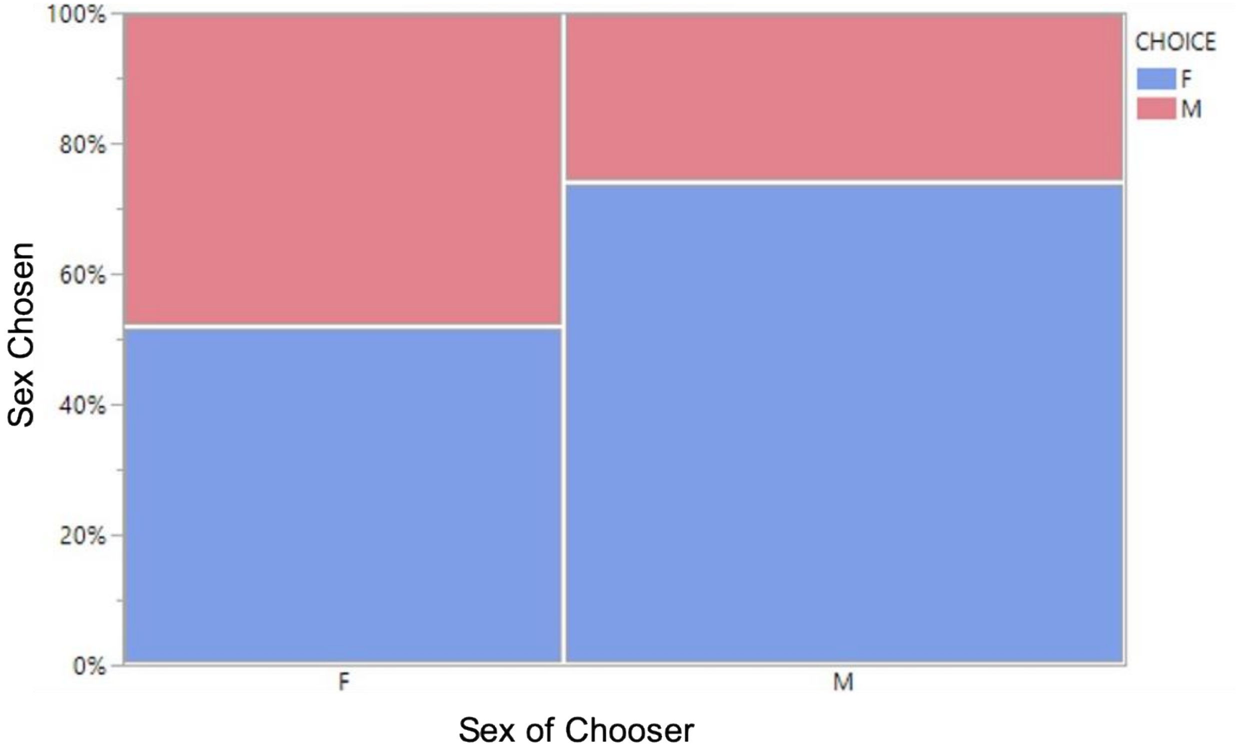

Figure 4 - Graph of results of Y-maze experiments showing differences in patterns of preference for chemical cues by *T. granulosa* males and females. Males show a preference for female chemical cues whereas females showed no preference between the sexes.