# Tantalising tongues: male carpet pythons use chemoreception to differentiate among females

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## Abstract

For animals sparsely distributed across a landscape, finding and identifying a receptive female during a short breeding period can be a challenge for males. Many snakes appear to rely on the production of sex-specific pheromones to synchronise the timing of reproductive behaviour. The rare Australian south-west carpet python (*Morelia spilota imbricata*) displays non-aggressive mating aggregations of up to six males around a receptive female, suggesting that males are responding to some chemical signal that enables multiple males simultaneously to identify and locate the female. We investigated chemoreceptive response (tongue-flicking) of 10 male pythons under laboratory conditions to 12 (randomly ordered) treatments each presented for three minutes. Cutaneous chemicals (dissolved in hexane solvent) were collected on cotton buds from the skin of six female pythons and male responses to these were compared with six control treatments. Male pythons produced a greater number of tongue flicks during the first minute of each trial, with fewer in minutes 2 and 3. Male chemoreceptive response in the third minute varied significantly between treatments and was only maintained for trials presenting cutaneous chemicals collected from the three relatively largest female pythons. This experiment suggests that male carpet pythons can use chemoreception to obtain information about

their social environment, identifying pheromone cues from large, potentially fecund females. This ability would be adaptive for male mate-selection behaviour and is likely to also reduce costs of searching behaviour.

**Additional keywords:** mate searching, reproductive behaviour, snakes, tongue-flicking.

## Introduction

Finding a receptive female during a short breeding period can be a challenge for males of species or populations that are distributed thinly across a landscape. In order to synchronise the timing of reproduction, many snakes rely on the production and accurate interpretation of sex-specific pheromones (Mason 1992). Pheromones are semiochemicals released by individuals that may elicit changes in the physiology and behaviour of the receiving individual (Karlson and Luscher 1959; Ford 1986). Squamates have a well developed nasal olfactory system and vomeronasal system (mediated by vomeronasal sensory epithelia within paired vomeronasal organs) that enables individuals to acquire chemosensory information (including pheromones) via their forked tongue (tongue-flicking behaviour: Schwenk 1995; Le Master *et al*. 2001). Snakes of many taxa rely on chemoreception for multiple roles: for instance, prey location (e.g. Chiszar *et al*. 1986; Webb and Shine 1992; Kardong and Smith 2002; Stark *et al*. 2002; Greenbaum 2004) or location of suitable ambush sites (e.g. Downes 1999; Clark 2007), avoidance of predators (e.g. Burger 1989) and chemoreception even plays a role in kin selection and social organisation (e.g. *Pernetta et al*. 2009).

A North American colubrid, the red-sided garter snake (*Thamnophis sirtalis parietalis*) has been the primary model species in the study of snake olfaction in relation to mate searching, recognition and choice. These snakes emerge from communal hibernacula in spring and breed in large, multiple-mating masses. Males of this species show pheromone trailing behaviour (Heller and Halpern 1981; Mason *et al*. 1990; Le Master and Mason 2001; Le Master *et al*. 2001; Shine et al. 2005*a* , 2005*b* ), and are able to differentiate between male and female trails, following only the latter (Le Master *et al*. 2001). In addition, males not only differentiate between the sexes but are able to

identify both how long the female is and her body condition, courting longer and heavier-bodied females more vigorously (Shine *et al*. 2003). The males' preference for larger females is based on qualities of the pheromonal cues rather than merely higher quantities produced by larger females (Le Master and Mason 2002; Shine *et al*. 2003). This ability to identify the sex and quality of individuals through olfactory means would be particularly advantageous in the large mixed-sex masses of breeding garter snakes adjacent to hibernacula, where visual differentiation is difficult (Shine *et al*. 2003).

Unlike the garter snake, however, breeding individuals of most snake species do not occur in huge, concentrated masses but are sparsely distributed across their habitat. Males therefore need to both find and identify suitable potential mates. Male timber rattlesnakes (*Crotalus horridus*), for example, appear to search for females by straight-line movements in an attempt to encounter a female's pheromone trail (Coupe 2005). Once male sidewinder rattlesnakes (*Crotalus cerastes*) have found a trail they can follow it for more than 100 m and have a 40% success rate in locating females (Coupe 2005), whereas male copperhead pitvipers (*Agkistrodon contortrix*) appear to travel long distances to receptive females by following airborne odour plumes (Smith *et al*. 2008).

In Australia, pythons of the genus *Morelia* show substantial differences in their mating systems. Males of all *Morelia* species are capable of breeding annually. Females, however, are capital breeders, requiring accumulated body reserves to produce and incubate a clutch of eggs; females, therefore, do not breed every year (Pearson 2002; Shine 2005), resulting in receptive females being a scarce resource. Five *Morelia spilota* subspecies (*bredli*, *cheynei*, *mcdowelli*, *metcalfei* and *variegata*) demonstrate male combat for access to females, whereas two (*imbricata* and *spilota*) subspecies apparently do not (Shine and Fitzgerald 1995; Pearson *et al*. 2002). Mating systems appear largely to have driven sexual size dimorphism; where males are larger than females, males compete for access to mates, but where males are smaller than females, no combat between males is evident (Pearson *et al*. 2002).

The south-west carpet python (*Morelia spilota imbricata*) is distributed patchily across a variety of habitats in south-west Western Australia. Female *M. s. imbricata* grow considerably larger than

males: over twice the length and more than 10 times the mass of adult males (Pearson *et al*. 2002). This significant sex difference in size at maturity translates into a difference in age at first reproduction, and a consequent skew in the adult sex ratio (4 : 1 male : female; Pearson *et al*. 2002). Additionally, female *M. s. imbricata* only breed every 2–3 years (Pearson *et al*. 2002), further skewing the operational sex ratio. This subspecies forms apparently non-combative (i.e. no aggression witnessed or bite marks noted) aggregations of up to six males around a single female during the austral spring (September to November), each male waiting for the chance to copulate with the receptive female (Pearson *et al*. 2002).

As part of a study on the biology of *M. s. imbricata*, we set out to examine the role of male chemosensory ability in mate choice and identification. First we investigated whether adult male *M. s. imbricata* can differentiate between female python skin chemicals and control olfactory stimuli. Second, we investigated whether male *M. s. imbricata* demonstrate greater interest in the olfactory stimuli collected from larger and better-condition females, assuming that larger females will be more fecund (Seigel and Ford 1987; Duvall *et al*. 1993; Shine and Fitzgerald 1995; Rivas and Burghardt 2001; Shine *et al*. 2003). We predicted that, despite the mating system of this python being so different from that of the garter snake, male *M. s. imbricata* would show a similar level of chemical cue recognition, an ability that would be adaptive in the face of the operational sex ratio and search costs facing males of this sparsely distributed python species.

## Materials and methods

Eleven male and six female *M. s. imbricata* were collected to remove radio-transmitters that had been previously surgically implanted into the coelomic cavities at least seven months before (Bryant *et al*. 2010). Opportunistic collection sites in south-west Western Australia included Leschenault Peninsula Conservation Park (33°26′S, 115°41′E), Martin's Tank campsite, Yalgorup National Park (32°51′S, 115°40′E) and reserves and State Forest surrounding Dwellingup township (32°43′S, 116°4′E); the maximum distance between capture sites was 170 km. It was beyond the scope of this study to test for

the effects of geographic origin upon chemosensory responses, since the pythons were collected opportunistically from several locations that would not allow a balanced experimental design.

Because we had to wait for the opportunity to hand capture animals when pythons were in an accessible location, they were held in captivity at the Department of Environment and Conservation Dwellingup Research Centre for varying lengths of time before our experiment (maximum of six weeks). The following chemoreception experiment was carried out while the snakes were in captivity before surgery and their subsequent release at point of capture, and was carried out under approval of the Murdoch University (W2028/07) and Department of Environment and Conservation (DEC AEC/55/2006 and DEC AEC 54 / 2006) Animal Ethics Committees.

Each male was kept individually in a 40 × 70 × 40 cm (112 L) plastic box with a recycled newspaper pellet substrate (Old News Cat Litter, Cyber Cycles Pty Ltd, Toowoomba, Qld), a tray of water with a bark and cardboard refugium. The room containing these boxes was maintained at 29.6 ± 0.4°C and pythons were exposed to natural lighting (through three large windows) and fluorescent lights under a ~12 : 12 L : D photocycle. The females were kept in a separate room and housed in purpose-built ventilated enclosures and presented with similar refugia, water and pellet substrate. The females' enclosures each had an external heat pad positioned underneath the plywood flooring (heating the cage floor above the heat pad to ~30°C) and a large window provided natural light supplemented with fluorescent light under a 12 : 12 L : D photocycle. Pythons were fed (dead laboratory mice and rats that were stored frozen and thawed before feeding) if they were held for longer than three weeks.

During experimental trials, males were presented with olfactory cues that had been collected and stored (<48 h) before the commencement of the trials. Odours were collected from individual prey and pythons by rubbing the surface of the animal with two cotton buds dipped in hexane for a total of six minutes (3 min for each cotton bud). The tip of each cotton bud was then clipped off and both were deposited in a vial containing 10 mL of hexane. We elected to use this standard time collection protocol to attempt to collect the scents in a consistent manner. For example Shine *et al*. (2000, 2003) have shown that body size influences the amount of skin lipids that can be collected through rubbing the skin. Rubbing the entire ventral surface would therefore result in varying amounts of chemicals

collected from each individual, according to body size. To ensure that our results reflected quality rather than quantity of skin lipids, we therefore collected our scents by a set time to prevent body size alone biasing the quantity of lipids collected. A separate 200-mm-long cotton bud was used to present the scent to the pythons – this was dipped into the storage vial and dried for 3 min (to allow the hexane to evaporate off) immediately before each trial.

Ten males were tested for their chemosensory response to control and olfactory cues over trials running over a total of two days. During experiments, each of the 10 males were presented with a randomly assigned experimental or control stimulus on a cotton bud held 3 cm from the tip of their snout by one of two observers while the second observer recorded the snake's behaviour on a camcorder for a duration of 3 min. The scent presented for each trial was prepared by the third experimenter (presented in random order to each randomly assigned male), and therefore both observers were blind to the nature of the stimuli presented on cotton buds. Although we randomly selected the male to test each stimulus, we ensured that each male had a minimum of 30 min between each successive trial. Tongue flick rate (per minute) was counted during the course of the trial and was confirmed by later watching the hand-held camcorder footage for each minute over the 3-min period. During replay of each trial, the intensity of each tongue flick was also scored (on a scale of 1–3, where 1 = forked tip of tongue visible, 2 = forked tip and shaft of tongue visible, 3 = forked tip and shaft of tongue visible and swung down and up).

The stimuli presented on a cotton bud (with the exception of the first non-odoriferous control) to male snakes were as follows.

*Three non-odoriferous controls*

1. Staring – the observer leant over the snake's container as in the following stimulus trials but did not present a cotton bud (this was the only stimulus that the observers could not be 'blind' to);

2. Cotton bud – an unscented cotton bud;

3. Solvent – a cotton bud dipped in hexane and then dried for 3 min.

*Three odoriferous controls*

1. Cologne (Jean Paul Gaultier) at a concentration of 1 part to 3 with hexane;
2. Prey scent – collected from a dead laboratory rat by rubbing the surface of the rat for a total of 6 min and stored in hexane;
3. Male python – cutaneous chemicals collected from the randomly selected eleventh male python (not included in the tongue flick experiment) by rubbing the python's ventral scales for a total of 6 min and stored in hexane.

*Stimuli collected from six female pythons*

1. Cutaneous chemicals were collected from the ventral surface of each of the females for a total of 6 min and stored in hexane.

***Analysis***

The number of tongue flicks recorded each minute for each of the 12 treatments was compared by repeated-measures ANOVA log(number of tongue flicks + 1). *Post hoc* analyses were carried out by LSD test (Statistica ver. 7.1., StatSoft Inc., Tulsa, OK).

Body condition scores (BCS) of the females were calculated as the residual scores of body mass ($M_b$, g) relative to snout–vent length (SVL, cm), expressed as a percentage of predicted $M_b$ (Madsen and Shine 2002):

$$\mathrm{BCS}(\%) = \frac{(\text{observed } M_b - \text{predicted } M_b)}{\text{predicted } M_b} \times 100$$

where predicted $M_b$ was calculated using the following equation derived from average data collected for 46 individuals (excluding their first capture to reduce bias as several pythons were initially caught because they had consumed a radio-collared prey) from the long-term ecological study that these animals were part of (Bryant *et al*. in press):

$$\text{Predicted } \mathrm{Log_{10}} M_b\,(\mathrm{g}) = 2.90 \bullet \mathrm{Log_{10}}\ \mathrm{SVL}\,(\mathrm{cm}) - 3.34$$
$$R^2 = 0.876$$

To test for possible effects of female python body size (log SVL and log$M_b$), and body condition (BCS) upon sustained male python tongue flick responses, these measures were included as covariates in a mixed-model ANOVA (Statistica ver. 7.1) with female ID (fixed factor) and male ID (random factor) and the number of tongue flicks in minute 3 log(number of tongue flicks + 1) as the dependent measure.

The proportion of each of the three levels of tongue flick intensity score were compared between trials where controls or python cutaneous chemicals were presented to male pythons by Chi-square analysis, with expected numbers of tongue flicks calculated assuming an equal distribution of each of the tongue-flick intensity categories between control items and python stimuli.

## Results

A significantly greater number of tongue flicks was recorded during the first minute of trials, with fewer in minutes 2 and 3 (RM-ANOVA time: $F_{2,18} = 35.01$, $P < 0.001$) (Fig. 1). *Post hoc* analysis revealed a significant difference in average number of tongue flicks between minute 1 and minute 3 ($P < 0.05$).

In addition to time effects, there were also significant treatment effects upon tongue flick number (RM-ANOVA treatment: $F_{11,99} = 3.09$, $P = 0.001$) (Fig. 2*a* ). Male *M. s. imbricata* rarely tongue-flicked during the staring treatment. When males were presented with a cotton bud within 3 cm of the tip of their snout, however, most individuals would tongue-flick to examine the item. Although the snakes showed some initial response to presentation of the plain cotton bud and solvent (hexane), the tongue-flick rate declined markedly over minutes 2 and 3. The odoriferous controls (cologne, prey and male python) stimulated a response; however, the number of tongue flicks also fell by minutes 2 and 3. By contrast, most male pythons showed sustained, or even demonstrated increased, response to the female python stimuli in minutes 2 and 3.

This difference in response for control and female scents was supported by a significant time × treatment interaction (RM-ANOVA interaction term: $F_{22,198} = 1.69$, $P = 0.033$) and is most clearly demonstrated in Fig. 2*b* , where only the data for minute 3 are shown. During minute 3, male pythons maintained a significantly higher tongue-flick response to female pythons than any other treatment (Fig. 2*b*). There was no significant effect of female python body mass ($P = 0.278$) or SVL ($P = 0.939$) upon the number of tongue flicks in minute 3 (mixed-model ANOVA); however, there was a significant effect of female body condition ($P = 0.012$), with the greatest numbers of tongue flicks directed towards the females that were relatively heaviest (Table 1). We note that there was also a significant effect of male python ID (random factor, $P = 0.013$), with some males not responsive to any scents.

Most (81%) tongue flicks directed towards controls (i.e. staring, cotton bud, solvent, cologne and prey) were scored as Intensity 1 (i.e. they were simple projections of the tongue beyond the labial scales); 18% were scored as Intensity 2 and only 1% as Intensity 3 (Fig. 3). Significantly more Intensity 3 tongue flicks (10%; $\chi^2_1 = 17.09$, $P < 0.001$) were directed towards python scents (both the male and the six females); only 67% of tongue flicks directed towards python scents were Intensity 1 ($\chi^2_1 = 4.70$, $P < 0.05$) and 23% were intensity 2 ($\chi^2_1 = 2.49$, $P > 0.05$) (Fig. 3).

## Discussion

Our results suggest that male *Morelia spilota imbricata* can not only discriminate between different olfactory stimuli presented on cotton buds under laboratory conditions, but can also differentiate differences in female pythons, showing more interest in the cutaneous chemicals collected from relatively heavier females. These observations, coupled with the field observation of mating aggregations, suggest that male *M. s. imbricata* are likely to use chemosensory detection to identify a receptive female.

Although not all females elicited a sustained tongue-flick response from males, the highest body condition females certainly did, and tongue-flick response in minute 3 (i.e. a sustained response by

males) was significantly correlated with female body condition score. In many species, female size reflects fertility and/or fecundity as large female snakes can potentially either lay more eggs than small females or invest more energy into each egg produced (e.g. Shine and Fitzgerald 1995; Rivas and Burghardt 2001). It would, therefore, be adaptive for male *M. s. imbricata* to differentiate between females of different sizes. Shine *et al*. (2003) found that male garter snakes were able to differentiate females from males in mating masses and even in these masses, males courted longer, heavier-bodied females more vigorously than they did smaller females. Being able to distinguish the scent of a larger female could also presumably lead to more efficient mate searching, where better-condition females would be the target of males' investment in searching. In the present study, even though female python Msi53 was in relatively good body condition, she was the smallest female in terms of body mass and length (Table 1). Males lost interest in her scent and showed minimal tongue flicking during minute 3. Under field conditions, this may translate to males not showing sustained pursuit of such a small female.

In addition to tongue-flick rate, it appears that tongue-flick intensity (type of tongue-flick) also reflects level of interest to different cues. We recorded, on average, more tongue flicks of Intensity 2 and 3 occurring in response to python cues presented. We found little interpretation in the literature of the role of different lingual responses in snakes, and this is an area that warrants further investigation.

Even when mating occurs in smaller groups than those observed in garter snakes, the ability to differentiate between females and other males would be adaptive in other snake species. Rivas and Burghardt (2001) report that large male green anacondas (*Eunectes murinus*) often become the recipients of courting and mating behaviour from other males in mating balls around large females. Mating aggregations last several weeks in this highly sexually size-dimorphic species, and although pheromones are presumably involved in both trailing and mating behaviour, it is likely that males become marked with female lipids containing pheromones during mating sessions and larger males become mistaken for females (Rivas and Burghardt 2001). Although they do not form mating balls, aggregations of male south-west carpet pythons have been reported where males presumably associate

with females for long periods, some mating with the female (Pearson *et al*. 2002). It would therefore be advantageous for males to differentiate female olfactory stimuli from those of nearby males.

The male *M. s. imbricata* in our experiment responded to stimuli presented on cotton buds through tongue flicking, showing a higher tongue-flick rate in minute 1 than in subsequent minutes. Most studies of snake and lizard chemosensory responses generally record for only one minute commencing only after the first tongue flick. The benefit of recording tongue flicks over three minutes after stimulus presentation allows examination of the maintenance of interest in an olfactory cue, and also allows data to be collected where no response towards a particular cue (e.g. staring) was shown. Maintenance of tongue-flick response into the third minute occurred for cutaneous chemicals collected from the largest female pythons, suggesting generally sustained interest in these scents. We note, however, that not all males maintained an interest into the third minute (a significant effect of male ID was noted for minute 3 tongue-flick response), with several individuals showing little sustained interest towards any scent presented to them. Although none were at the 'blue-eye' stage of pre-ecdysis, some of these unresponsive males were observed to moult within the following weeks of the trials. Their physiological state may have contributed to lower tongue-flick response in these males: behavioural changes during pre-ecdysis (when eyes are at the 'blue' stage) have been recorded in common garter snakes (*Thamnophis sirtalis*), including reduction in feeding frequency and strike rate (at a moving stimulus) and an increase in movement latency following presentation of a stimulus (King and Turmo 1997).

The potential to detect scents may be different between the sexes. The copperhead pitviper is similar to *M. s. imbricata* in that females are widely dispersed and males must find them over large distances (Fitch 1960; Smith *et al*. 2008) and there is sexual dimorphism in the tongue tine length, with males having significantly longer tines (Smith *et al*. 2008). This increased tongue bifurcation is likely to result in increased tropotactic ability in males, reflecting their mate-searching activities. It is possible that there is similar sexual dimorphism in the tongues of *M. s. imbricata*.

Our data support the hypothesis that male snakes, whether *M. s. imbricata* that mate in small aggregations or garter snakes that mate in large mixed-sex groups, are able to accurately identify

potential partners through olfactory stimuli. This ability may be important during the mating season if male pythons, as found for other snakes (e.g. garter snakes: Le Master and Mason 2001), follow scent trails (either airborne or substrate-based) leading them to a receptive female. Under these circumstances, an ability that minimises the time required in mate searching would be highly advantageous and is therefore likely to be under strong selection.

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**Fig. 1.** Male south-west carpet pythons showed a greater tongue-flick response over all 12 treatments (pooled for this graph) for the first minute of trials, with decreasing numbers of tongue flicks in minutes 2 and 3. Letters link data that are not significantly different from each other at $P > 0.05$.

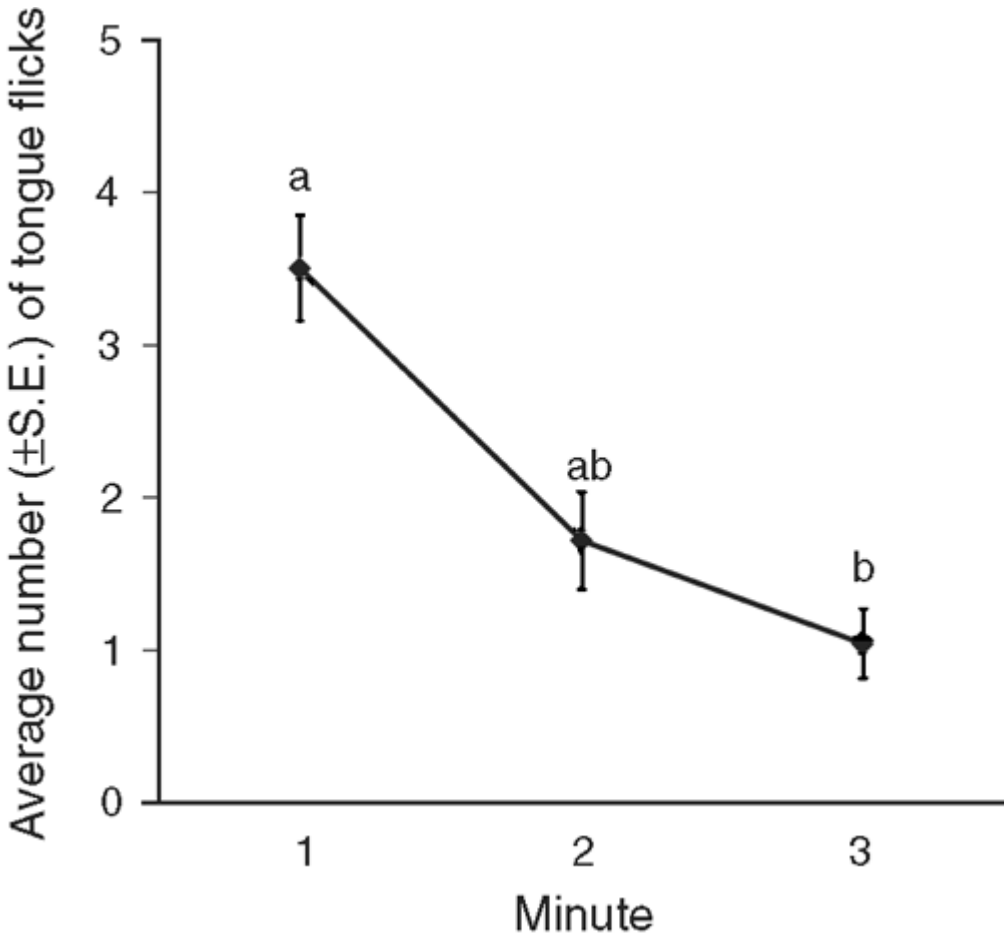

**Fig. 2.** Although male pythons ($n = 10$) tongue-flicked towards all 12 treatments tested (*a*), their activity was sustained towards the female stimuli; this is particularly evident for the data for minute 3 (*b*). Numbers inside each treatment column indicate tongue-flick activity between minutes. Asterisks represent *post hoc* analysis between minutes for each treatment: asterisks above numbers indicate a significant difference between minutes 1 and 2, or between minutes 1 and 3 (asterisks placed above the signs separating each number); asterisks below the numbers indicate significant difference between minutes 2 and 3. $*P < 0.05$; $**P < 0.001$. Letters in (*b*) link data that are not significantly different from each other at $P > 0.05$. Females (bracket) are ordered from left to right in increasing body condition score; note that female Msi53 was the smallest individual (Table 1).

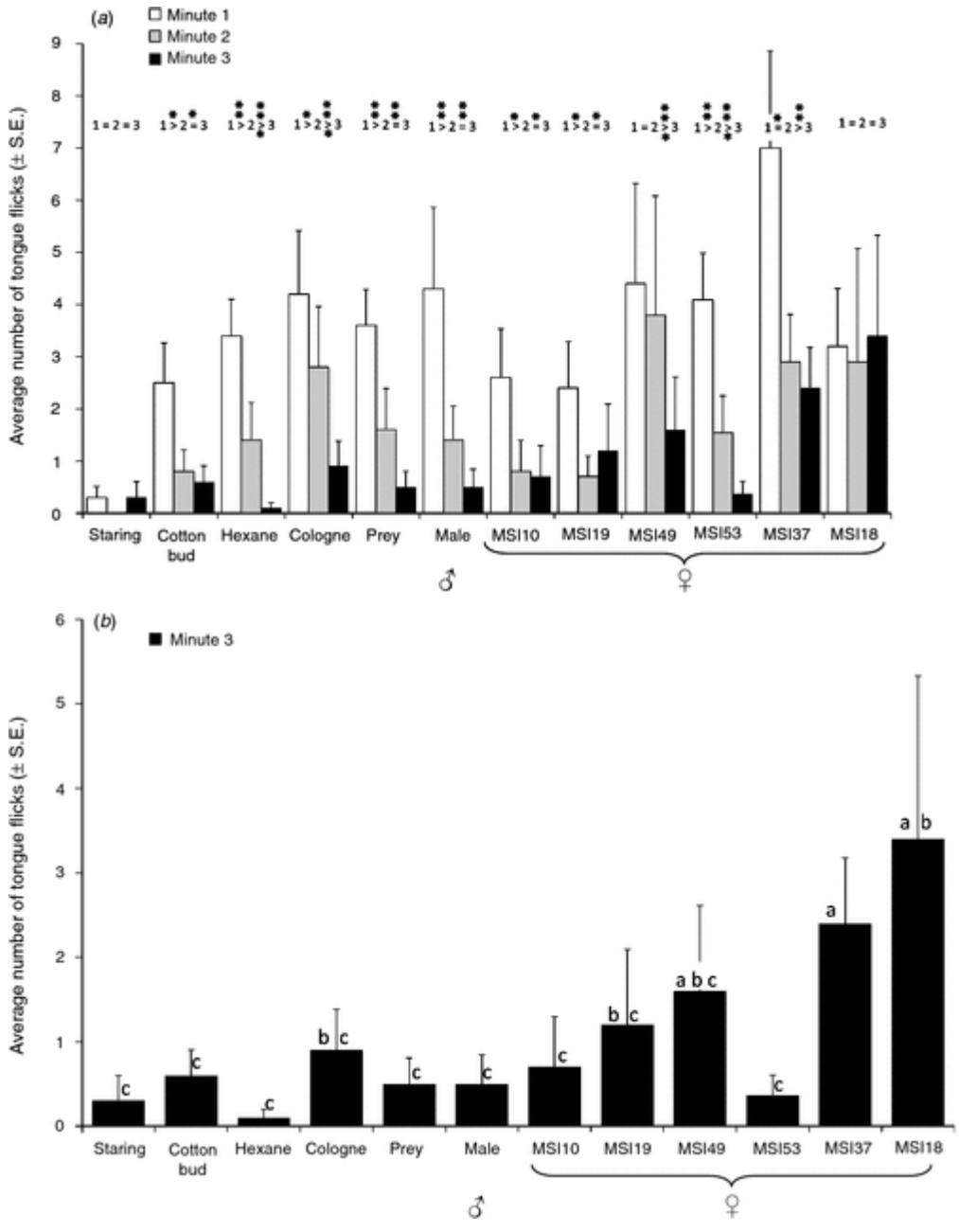

**Fig. 3.** A greater proportion of intensive tongue-flicks (Intensity Score 3) were directed towards python scents (male and the six females) compared with the controls tested (the five categories on the left-hand side of the graph). A greater proportion of tongue-flicks directed towards 'control' items were Intensity Score 1; there was no significant difference for the numbers of tongue-flick Intensity 2. Females (bracket) are ordered from left to right in increasing body condition score.

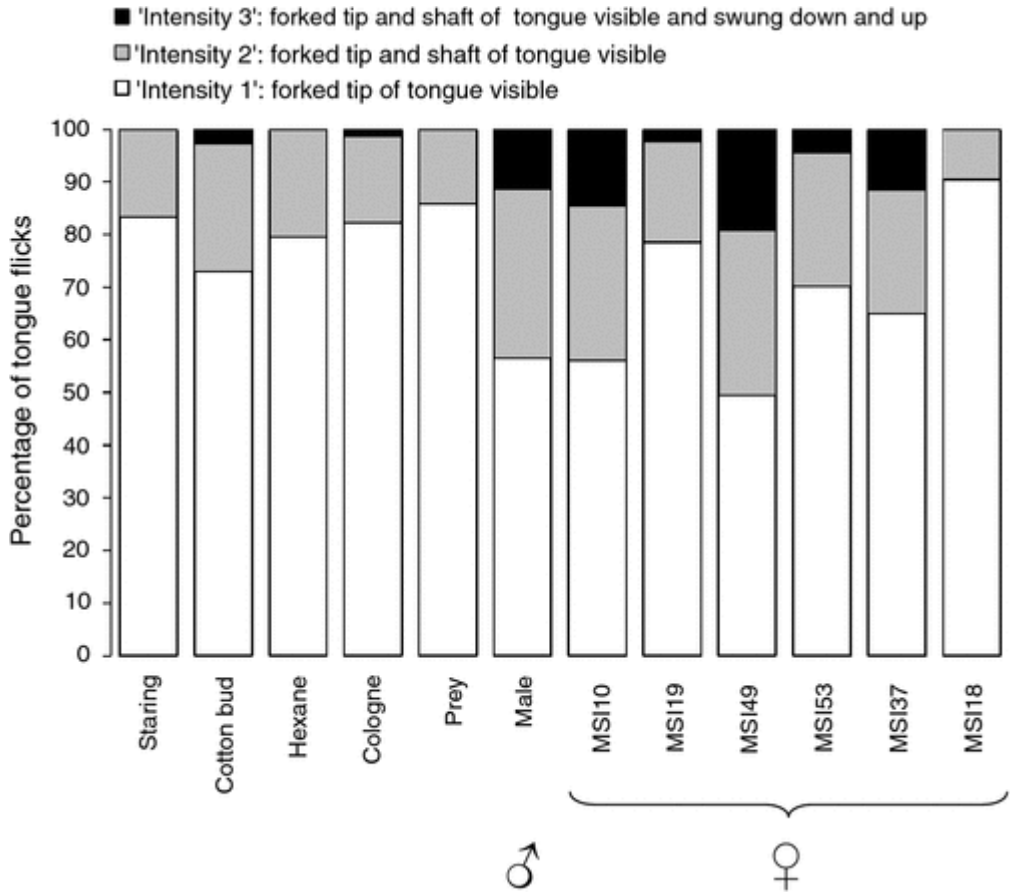

**Table 1.** Body mass, snout–vent length and body condition score (% above or below average population values) of the six female carpet pythons from which skin lipids were collected for presentation to male pythons
Females are ordered by increasing body condition score

| Female ID | Body mass (g) | Snout–vent length (cm) | Body condition score (%) |
|---|---|---|---|
| Msi10 | 1108 | 181 | –32.22 |
| Msi19 | 1416 | 191 | –25.89 |
| Msi49 | 1318 | 179 | –16.73 |
| Msi53 | 695 | 139 | –8.58 |
| Msi37 | 2414 | 192 | 23.68 |
| Msi18 | 3731 | 223 | 24.62 |