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Seasonal Morphometry of the Vomeronasal Organ in the Marsupial Mouse, *Antechinus subtropicus*

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ABSTRACT The vomeronasal system consists of a peripheral organ and the connected central neuronal networks. The central connections are sexually dimorphic in rodents, and in some species, parameters of the vomeronasal organ (VNO) vary with sex, hormonal exposure, body size and seasonality. The VNO of the dasyurid marsupial mouse, *Antechinus subtropicus* is presumed to be functional. The unusual life history (male semelparity) is marked by distinct seasonality with differences in hormonal environments both between males and females, and in males at different time points. Body size parameters (e.g., length, weight) display sexual dimorphism and, in males, a pronounced weight gain before breeding is followed by a rapid decline during the single, short reproductive season. VNO morphometry was investigated in male and female *A. subtropicus* to identify possible life cycle associated activity. The overall length of the VNO is positively correlated with the size of the animal. The amount of sensory epithelium exhibits a negative correlation, decreasing with increasing size of the animal. The effects of sex and breeding condition are not obvious, although they do suggest that sensory vomeronasal epithelium mass declines in the breeding period. The VNO may be more important in *A. subtropicus* before breeding when it may participate in synchronising reproduction and in the development of the male stress response. J. Morphol. 277:1517–1530, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: Pheromone; chemical communication; sex hormones; cortisol; seasonal variation

INTRODUCTION

The olfactory sense is conveyed by two components, the main and accessory olfactory systems. The main olfactory system (MOS) originates as sensory neurons in the olfactory mucosa, whilst the accessory olfactory or vomeronasal system (VNS) originates with sensory neurons in neuroepithelium located inside the vomeronasal organs (VNO). The VNO are paired, blind-ending tubes found in the ventral nasal septum in most tetrapods (Bertmar, 1981; Døving and Trotier, 1998).

The wide variation in VNO structure has led to speculation on how size and structure can be used to infer function. It is probably reasonable to infer that reduction in size or secondary loss of the VNO or its sensory components as is seen in marine mammals, some bats and some primates results in a loss of function, and thus to infer lack of importance of that sense in those animals (Halpern, 1987; Bhatnagar and Meisami, 1998). Conversely, a larger VNO or one containing more prominent sensory components, a bony or cartilaginous capsule, and accompanied by venous sinuses, such as is found in mice and rats, could be interpreted as indicating importance for those species (Dawley and Crowder, 1995).

Dawley (1998) cautioned that comparisons of VNO size between species could be confounded by differences in body size and life histories. The concept of allometry in the VNO and the importance, or not, of scaling to body size in chemosensory structures has been discussed by numerous authors (Dawley, 1998; Dawley et al., 1999; Maico et al., 2003; Smith and Bhatnagar, 2004), but there is not universal agreement as to how or whether it should be done.

Sexual dimorphism in the VNS has been reported in some species (Segovia and Guillamón

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1996), and it was the first multisynaptic neural system demonstrated to be sexually dimorphic (Segovia and Guillamón, 1993). Either male or female animals have been reported as having larger structures in different species. Other factors that have been suggested to affect VNO size are: habitat and seasonality (Dawley 1998) and reproductive strategy (Maico et al., 2003). In contrast, Weiler et al. (1999b) found that differences in VNO size in male and female rats may simply reflect the overall size of the animal.

The reported sexual dimorphism is hypothesized to result from differences in pre- and post-natal hormonal environments. The effects of behaviors associated with hormonal secretion (such as the priming effect of pheromones and behaviors elicited by them) may also contribute (Simerly, 1990).

The mature structure of the VNO depends on the presence of sex steroids. Gonadectomy at three months resulted in significant reductions in nuclear size and neuroepithelial height in adult rats (Segovia and Guillamón, 1986).

The accessory olfactory bulb (AOB) also displays several sexually dimorphic features dependent on the presence of sex steroids shortly after birth (Segovia et al., 1984; Roos et al., 1988). Male rats have larger AOB volumes and numbers of mitral and granule cells than female rats (Segovia et al., 1986; Valencia et al., 1986). Mitral cells are larger, and have increased dendritic branching (Caminero et al., 1991), and the rate of neurogenesis is higher in males than females (Peretto et al., 2001).

A similar pattern is seen in other central vomeronasal connections. Although not a universal pattern, in male mammals, structures are often larger and contain more neurons and synaptic connections than females of the same species; differences which can be abolished by early castration or androgen administration.

With the establishment of the VNS as a sexually dimorphic neural network in rodents, and reports from several differing species that parameters of the VNO may vary with sex, hormonal environment, the size of the animal and seasonality, this current study investigated the morphometry of the VNO in the insectivorous dasyurid marsupial *Antechinus subtropicus* (Braithwaite, 1974; Van Dyck and Crowther, 2000).

Most mammalian data are from eutherians (placentals) rather than metatherians (marsupials) or protherians (monotremes). Differences in anatomy, social communication and reproduction, as well as the reported morphological diversity of the VNO suggest that direct comparison between even closely related species is risky and may not be meaningful (Salazar and Sánchez Quinteiro, 2009). The limited data in marsupials generally supports the presence of a well-developed VNO similar to that reported in Rodentia (see e.g., *Tarsipes rostratus*—Kratzing, 1982; *Monodelphis domestica*—Poran 1998; *Macropus eugenii*—Schneider et al., 2008; *Antechinus*—Toftegaard, 1998).

The family Dasyuridae is one of the largest groups of marsupial carnivores in Australia and New Guinea, with more than 60 species currently recognised within this group. Several small Australian dasyurid species from the genera *Antechinus* and *Phascogale*, and the dasyurine *Dasykaluta rosamundae* display an annual post-mating male mortality, which is linked with signs of stress (Bradley, 2003). Lee et al. (1982) in their work on life history strategies in dasyurids, described a single oestrous in females (monoestrous), a very short and highly synchronized breeding season that is predictable from year to year, and the sudden death of all males following their first breeding season (male semelparity). Males display various pathologies related to a persistent state of stress that may contribute to their death (Barker et al., 1978; Bradley et al., 1980; Lee and Cockburn, 1985). As the males die before the young are born, male cohorts do not overlap.

This unusually short semelparous male life history is accompanied by distinct seasonality and marked differences in hormonal environments both between males and females and in males at different times of their life. Body parameters (e.g., length, weight) display sexual dimorphism. In males, a pronounced weight gain before the breeding period is followed by a rapid decline in weight during breeding. In males, biologically available cortisol and testosterone concentrations in plasma rise dramatically leading up to and during breeding (Bradley et al., 1976, 1980). The pathogenesis of the stressed state is hypothesized to be a loss of the feedback mechanism to the hypothalamus that controls corticosteroid levels (Lee and Cockburn, 1985; McDonald et al., 1986; Bradley, 2003). Superimposed are extrinsic factors such as social interactions and chemosensory cues that may modulate the development and progression of the stressed state.

The presence and general anatomy of the VNO in *A. subtropicus* was reported by Toftegaard (1998). An endocrine response following olfactory stimulation by a urinary chemosignal was also demonstrated in this species (Toftegaard et al., 2002), although the involvement of the VNO in this response was not conclusively demonstrated.

With an extremely unusual life history involving communication using socially relevant chemical cues, and sexually dimorphic body parameters, it was of interest to determine whether VNO parameters varied with size, sex or breeding condition. The parameters measured were those that might be relevant in assessing functionality (e.g., volume and surface area of sensory epithelium) and those that might conceivably change with body size (e.g., total length of the VNO). The volume of the sensory epithelium was chosen as an estimate of the amount (number and/or size) of sensory neuronal cell bodies

(see Dawley, 1998) whilst the surface area was chosen as an estimate of the amount of space available for the parts of the sensory neurons that bear receptors, so the total area available for interaction of sensory epithelium with chemical cues.

Several measures of body size were chosen for possible allometric scaling, to describe possible variation between animals and the sudden changes in body condition in males around the time of breeding. Palatal measurements were also included, as the skeletal elements of the VNO are not completely independent of the palate, and might be influenced by alterations in palatal size.

MATERIALS AND METHODS

Animal Collection

Nasal tissues were obtained from individuals of *Antechinus subtropicus* (Van Dyck and Crowther, 2000) trapped from Mt Glorious, Brisbane State Forest Park, south-east Queensland, in 2003 and 2004. The animals were housed individually in polypropylene boxes in a temperature controlled room ($22 \pm 1^{\circ}C$) with a light/dark cycle of 16:8. Water and food were available *ad libitum*. On observation, animals appeared free from overt respiratory disease or injury to the nasal region.

All trapping was done in accordance with the guidelines of the Queensland National Park and Wildlife Service, Permits to Take and Traverse (TWB/11/2003, SPP:WISP01313403) were obtained from the Department of Forestry and Natural Resources. All animals were held and experimental procedures performed in accordance with The University of Queensland Animal Ethics Committee, (ethics number ANAT/133/03/URG) and adhered to the legal requirements of the state of Queensland, and Australia.

Castrations

Surgical castration was performed on males in the pre-breeding period (May–June) under light halothane anesthesia (2%) by removal of the testes and suturing of the scrotal sac.

Conditions

Male and female animals were captured at various times of the year, representing seven different sex or breeding conditions, to investigate potential changes to the VNO that might occur through their life.

Animals were captured in May and June (pre-breeding) and in September (breeding). For the post-breeding group, males and females were captured in May and June, and kept in captivity for varying periods of time after breeding period. Males were kept in isolation to ensure their survival past September.

Male castrate group were captured and castrated in May and June. They were sacrificed just before or during the breeding period in September. A total of 42 animals were used in this experiment, six per group in the following groups: Pre-Breeding Male; Pre-Breeding Female; Breeding Male; Breeding Female; Post-Breeding Male; Post-Breeding Female; Castrated Male.

Histology

Animals were euthanized by overdose of Lethabarb ® (Sodium pentobarbitone, Virbac, 200 mg/kg) injected intraperitoneally. Before perfusion, the total length, snout-rump length (exclusive of tail) and weight of the animal were recorded. The total length was measured from the tip of the snout to the tip of the tail. The snout-rump length was measured from the tip of the snout to the base of the tail.

They were then perfused intracardially with heparinised saline, followed by 4% neutral buffered formalin in phosphate buffer.

Following perfusion, the external musculature and brain were removed and the nasal cavities were immersed in fresh fixative. Before further processing, each head was dissected and various craniofacial measurements were obtained. The length of the head was measured from the tip of the snout to the base of the skull. The palatal length was measured from the anterior central incisors to the posterior of the palate, in the midline. Palatal width was measured at the maximum, usually found in the caudal half of the palate.

Specimens were decalcified in either ethylenediaminetetraacetic acid or formic acid and washed in several changes of phosphate buffer over several hours. They were then dehydrated in an alcohol series, cleared in xylene, embedded in paraffin and serially sectioned in the coronal plane at 10 μm intervals. Sections were mounted in a serial fashion through the extent of the VNO and then for the remainder of the nasal cavity, every 10th section was mounted on gelatin-subbed slides. Sections were stained with either haematoxylin and eosin or toluidine blue.

Digital photographs were taken of every 10th section (every 100 μm) using a Spot Insight Color digital camera and Zeiss axiophot microscope with SPOT Imaging software (Diagnostic Instruments, Sterling Heights, MI).

Measurements

Measurements of VNO parameters were made using the digital images and the image analysis program NIH image (Version 1.62, NIH, Bethesda, MD). No correction factor was used for shrinkage as all animals were processed in a similar manner. Measurements were made on left and right sides separately for each animal.

The VNOs in *A subtropicus* are paired blind-ending tubes lying either side of the ventral nasal septum and enclosed in osseo-cartilaginous capsules. The organ is crescent shaped on cross-section, with medial sensory epithelium, and lateral non-sensory epithelium. The crescent shape results from large vascular sinuses that lie against the non-sensory epithelia.

Length of the VNO

To determine the length of the VNO (i.e., the rostro-caudal extent) the beginning of the VNO was defined as the first appearance of the VN duct in the most rostral section. Because the VNO terminates as the duct of a series of glands, the posterior extent was defined as the last section on which the large patent lumen was visible in the absence of typical glandular structures. This occurred at about the termination of the vomeronasal sensory epithelia. The length was then calculated by multiplying the thickness of a section (10 μm) by the number of sections in which the VNO was present.

As this study was concerned with the portions of the VNO involved in possible sensory transduction, the extension of the VNO as the duct of the vomeronasal glands was excluded from measurement.

Volume of the Vomeronasal Complex

The vomeronasal complex (VNC) was defined as the VNO and the associated vascular and glandular structures immediately surrounding it within the cartilaginous or bony capsule, extending to the basement membrane of the respiratory epithelium lining the nasal cavity (Meisami and Bhatnagar, 1998)

These structures are presumed to be involved in the functioning of the organ.

The volume of the VNC was calculated by tracing the area occupied by the structures within the complex on every 10th section, then multiplying by the distance between sections (100 μm). These values were summed for the length of the VNO to generate the total volume.

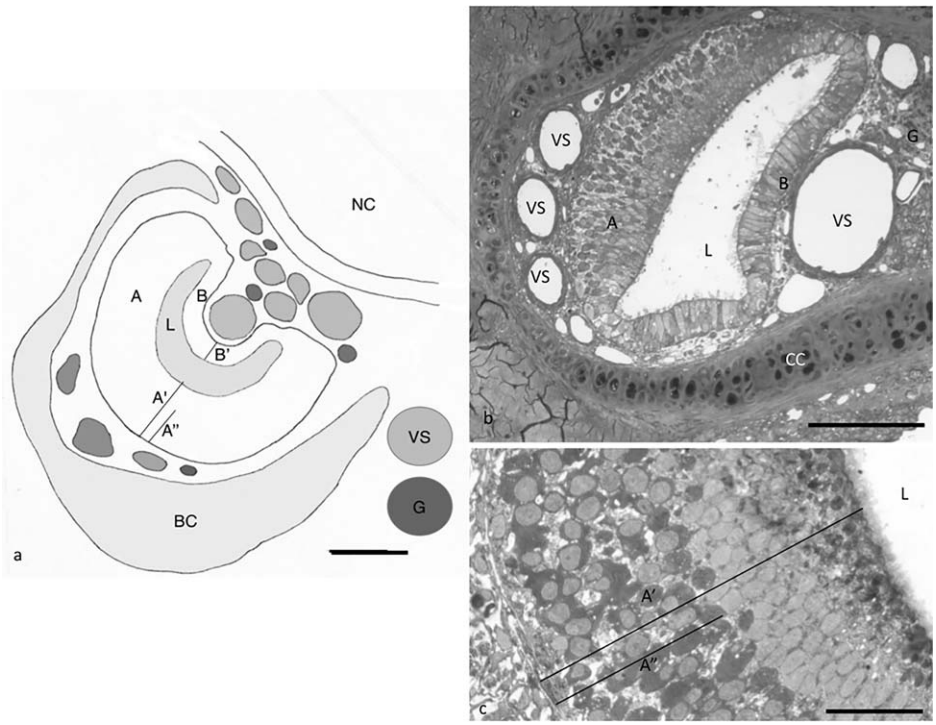

Fig. 1. Cross-section of the vomeronasal complex. **a** Diagram of a typical section taken approximately mid-length, with bony capsule. Scale bar represents 100 μm. **b** Micrograph of vomeronasal complex in ventral extent, with initial cartilaginous capsule. Scale bar represents 100 μm. **c** Micrograph of vomeronasal sensory epithelium. Scale bar represents 25 μm. The midline is to the left, and ventral is inferior. A – vomeronasal sensory epithelium (VSE); A'—total height of the vomeronasal sensory epithelium (VSE); A"—height of the neural component of the vomeronasal sensory epithelium (VSE); B—vomeronasal non-sensory epithelium; B'—total height of the vomeronasal non-sensory epithelium; L—lumen of the VNO; BC—bony or cartilaginous capsule; G – vomeronasal glands; VS—vascular sinuses, NC—lumen of the nasal cavity.

Height of Vomeronasal Sensory and Non-sensory Epithelia

The thickness of the sensory epithelium and the non-sensory epithelium were measured from the basement membrane (basal lamina) to the apical surface in the middle portion of the crescent-shape, for the entire anterior-posterior extent of the VNO. A mean height value for the whole VNO was then calculated. Measurements were taken as close to the center of the sensory epithelia (measured from each end).

The thickness of the neuronal cell layer was measured from the basement membrane to the lower border of the supporting cell nuclei. The nuclei of the neural cells and those of the supporting cells are stratified and easily distinguished. Figure 1 illustrates a typical cross-section of the VNC with the components and some measures indicated. Measurement of all the dependent variables are summarized in the Supporting Information.

Surface Area of Vomeronasal Sensory and Non-sensory Epithelia

The surface area of the sensory epithelium and the epithelium were calculated by tracing the length of the lumenal surface of each epithelium on every 10th section, then multiplying by the distance between sections (100 μm). These values were summed for the length of each epithelium, to generate the total surface area.

Volume of Vomeronasal Sensory and Non-sensory Epithelia

The volume of the sensory epithelium and the non-sensory epithelium were calculated by tracing the area of each epithelium present on every 10th section, then multiplying by the distance between sections (100 μm). These values were summed for the length of each epithelium, to generate the total volume. The volume of the neuronal cell layer was measured in a similar manner.

All original histological material used in this study remains with AJB.

Statistical Analysis

The data generated were used to investigate the following research questions:

1. Is body size influenced by breeding condition?
2. Are there differences between the size of the left and right parts of the VNO?
3. Is the size of the VNO influenced by body size?
4. Is the size of the VNO influenced by breeding condition when adjusted for any effects of body size?
5. Is the amount of sensory tissue contained in the VNO influenced by body size?
6. Is the amount of sensory tissue contained in the VNO influenced by breeding condition when adjusted for any effects of body size?

Statistical analysis was performed using SPSS Version 22 (Armonk, NY: IBM Corp), figures were drawn using the R package ggplot2 version 2.1.0. The statistical analysis is described in detail in the Supporting Information, and summarized here: Tests of normality (Skewness and Kurtosis) of the dependent variables associated with body size were carried out on the whole population, the population divided by sex and on the individual groups. Homogeneity of variance (Levene's test) was tested on the groups of the male and female populations. The majority of

TABLE 1. Descriptive statistics of body size parameters: male animals

Parameter	Group	*N*	Mean	SD	95% C. I. of mean	Skewness	Kurtosis
Weight (g)	Pre-Breeding	6	39.39	6.650	32.41, 46.37	−0.48	−2.40
	Breeding	6	45.11	6.36	38.44, 51.78	−2.21	5.02
	Post-Breeding	6	52.60	6.13	46.16,5 9.03	0.35	−2.08
	Castrated	6	37.23	4.29	32.74, 41.73	−0.11	−1.03
Total Length (cm)	Pre-Breeding	6	20.63	1.08	19.50,21.77	0.19	−2.40
	Breeding	6	21.48	0.74	20.70,22.26	−2.21	5.02
	Post-Breeding	6	21.12	0.46	20.64, 21.60	0.35	−2.08
	Castrated	6	20.58	0.38	20.19, 20.98	−0.11	−1.03
Snout-Rump Length (cm)	Pre-Breeding	6	11.37	0.71	10.63,12.11	0.09	−2.14
	Breeding	6	12.47	0.51	11.93,13.00	2.40	5.81
	Post-Breeding	6	12.65	0.33	12.31,12.99	−0.31	−2.08
	Castrated	6	12.57	0.34	12.21, 12.02	0.71	−0.51
Head Length (cm)	Pre-Breeding	6	3.82	0.28	3.52, 4.11	−1.50	2.90
	Breeding	6	4.10	0.06	4.03, 4.17	0.00	2.50
	Post-Breeding	6	4.12	0.13	3.98, 4.26	−0.44	1.34
	Castrated	6	4.07	0.10	3.96, 4.18	−0.66	0.59
Palatal Width (mm)	Pre-Breeding	6	8.0	0.0	8.0, 8.0	—	—
	Breeding	6	7.0	0.6	6.3, 7.7	0.00	2.50
	Post-Breeding	6	8.0	0.6	7.3, 8.7	0.00	2.50
	Castrated	6	7.8	0.4	7.4, 8.3	−2.45	6.0
Palatal Length (mm)	Pre-Breeding	6	16.7	0.6	16.1, 17.2	−0.97	−1.88
	Breeding	6	17.0	0.9	16.1, 17.9	0.00	−1.88
	Post-Breeding	6	18.7	0.6	18.1, 19.2	−0.97	−1.88
	Castrated	6	18.0	0.9	17.1, 18.9	−1.88	7.0

the parameters showed a normal distribution and homogeneity of variance for all breeding groups but because of a few exceptions and also because the small group sizes ($n = 6$) limited the power of these tests to detect non-normality and heterogeneity of variance, corrections for heterogeneity of variance (Welch's correct) and post hoc testing which did not assume equal variance (Games-Howell), were used in the analysis of variance for these parameters. Possible asymmetry of the VNO and associated structures was investigated using paired comparisons (paired *t* test, left vs. right) and when it was found that no biologically significant asymmetry existed, data was pooled for further investigation. For VNO metrics involving length or height, the mean of left and right were used, for metrics involving areas of volumes, left and right were summed.

The relationship between the length of the VNO and the size of the animal was then investigated using multiple linear regression with length of VNO as dependent variable and the body-size related parameters as predictor variables.

Finally, since a relationship between body size and VNO was demonstrated, the differences in VNO metrics between breeding groups were explored in male and female animals using analysis of covariance (ANCOVA) with the best predictor of VNO length as the covariate.

Because this analysis involved multiple dependent variables, Benjamini–Hochberg correction was used to maintain an overall 5% false discovery rate. In addition, because of the small group sizes, which had limited power to detect non-normality and heterogeneity of variance, the analyses were carried out with 1,000 sample bootstrapping generating bias-corrected accelerated confidence intervals. In all cases, bootstrapping produced results which supported the initial analysis. All of the figures and tables show the results of the initial analyses.

RESULTS

Analysis of Body Size Parameters

The population as a whole ($n = 42$) showed a normal distribution. All body size parameters had skewness and kurtosis within the range −3 to +3, consistent with normality. When divided into male and female ($n = 24$ male and 18 female), distributions again appeared normal with the exception of male head length which had a kurtosis of 7.20. When further divided by breeding group as well as sex, no group showed skewness outside of the −3 to +3 range (see Supporting Information) but a few groups showed kurtosis greater than +3 suggesting possible outliers. However, none were considered sufficiently non-normal to preclude the use of parametric statistics for group comparisons given that the methods used were robust to non-normality and the data was complete.

Descriptive statistics (mean, standard deviation, 95% confidence interval, skewness, kurtosis) divided by breeding group are shown in Tables 1 (males) and 2 (females) a scatterplot of weight versus breeding group is shown in Figure 2 and snout-rump length versus breeding group in Figure 3. Homogeneity of variance was tested using Levene's test and the majority of parameters showed homogeneity of variance (see Supporting Information).

A two-way analysis of variance (ANOVA) showed highly significant differences between breeding groups ($P < 0.001$) and sex ($P < 0.001$). The interaction of sex and breeding condition was also significant ($P < 0.05$) for all parameters except for weight ($P = 0.340$) and palatal width ($P = 0.155$). Given the significance of the interaction between sex and group, the data from each sex were analyzed separately using one-way analysis of variance with post-hoc testing where significance was found.

Body Size Parameters

For both male and female animals, weight increased steadily from the pre-breeding condition

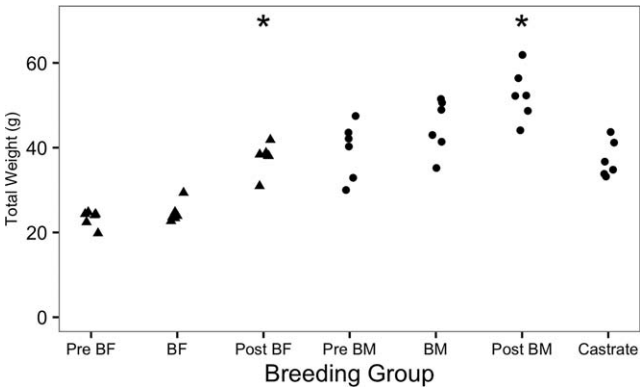

Fig. 2. Scatterplot of weight versus breeding condition. BF= Breeding Female, BM= Breeding Male. Groups that are significantly different from the Pre-Breeding Group for the appropriate sex are indicated by an asterisk.

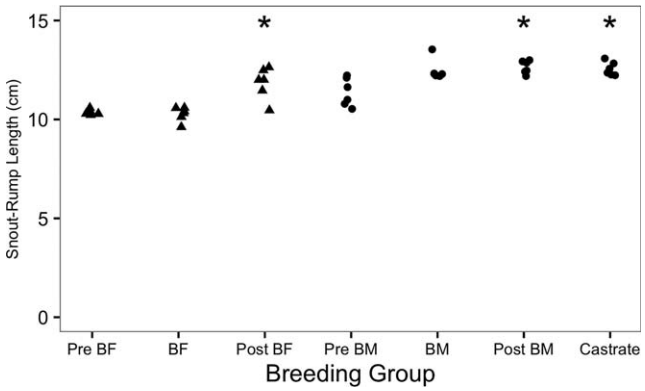

Fig. 3. Scatterplot of snout-rump length versus breeding condition. BF= Breeding Female, BM= Breeding Male. Groups that are significantly different from the Pre-Breeding Group for the appropriate sex are indicated by an asterisk.

but the increase was only statistically significant in the post-breeding groups. The castrated males showed a slight (2 g) decrease in weight which was neither contextually nor statistically significant (Fig. 2). Total length (which included tail) showed small, statistically insignificant increases from the pre-breeding group in females, but no changes in males. Snout-rump length, however, showed an increase in the female post-breeding group, which was statistically significant, but no increase in the breeding group. Male animals showed increased snout-rump length in all groups including castrated animals, but this was only statistically significant in post-breeding and castrated groups (Fig. 3). Female head length showed the same pattern as snout-rump length, that is, increase in the post-breeding group only, but males showed an increase in all groups over pre-breeding which was only statistically significant in breeding and post-breeding groups. However, this finding is probably unduly influenced by one outlying low value in the pre-breeding group. Palatal width showed a decrease in breeding and post-breeding groups which was only statistically significant in the post-breeding group; males showed no differences. Palatal length showed no significant changes in females and a small increase in males, which was only statistically significant in the post-breeding group.

Vomeronasal Metrics, Left versus Right Side

Data from all groups and both sexes were pooled for the comparison of left and right. All the metrics had a normal distribution with skewness and kurtosis between +3 and −3, with the exception of lumenal volume, which had a very high positive kurtosis in both left and right (see Supporting Information). Left versus right for this metric was compared using nonparametric statistics. The left versus right data was intrinsically paired, so paired sample *t*-tests were used for all parameters and the Paired Wilcoxon test was used for lumenal volume.

Evidence for significant asymmetry in the VNO parameters was absent; all parameters were statistically insignificant when Benjamini–Hochberg correction was applied (see Supporting Information).

As there was no evidence of significant asymmetry, left and right data from each individual animal were pooled for further analysis as described earlier, that is, linear measured were averaged, area and volume measures were summed.

Factors Affecting the Mean Length of the VNO

The effect of parameters relating to body size and sex of the animal were analyzed by univariate multiple linear regression to determine their ability to predict the mean VNO length (mean of left and right sides).

Weight, total length, snout-rump length, head length and palatal length were significant predictors of mean VNO length, palatal width was not (See Supporting Information). Snout-rump length had the largest R^2 (0.185) of all of the metrics tested and thus accounted for the largest proportion of the variability in mean VNO length, sex was the next largest (R^2 0.143).

Because of the small sample size, and highly correlated nature of a number of the parameters (particularly those related to body size), it was impractical to include all of them in a single regression analysis. Therefore, Exploratory Factor Analysis was undertaken to determine whether the independent variables could be reduced into fewer latent variables that were better predictors of mean VNO length than the individual variables alone, full alone. Full details are shown in the Supporting Information. Two biologically plausible factors were extracted (one containing all body size related parameters, and one containing palatal parameters), but

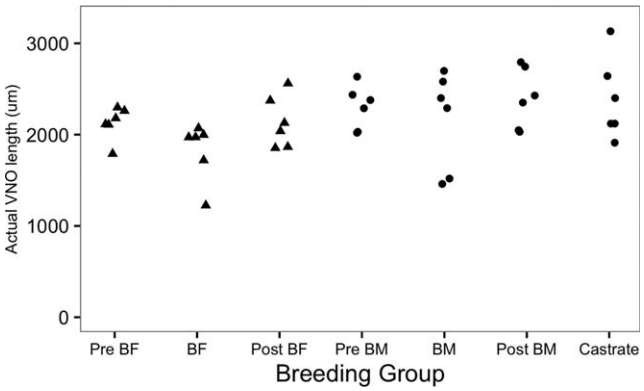

Fig. 4. Scatterplot of vomeronasal organ (VNO) length (raw data) versus breeding condition. BF= Breeding Female, BM= Breeding Male. Groups that are significantly different from the Pre-Breeding Group for the appropriate sex are indicated by an asterisk.

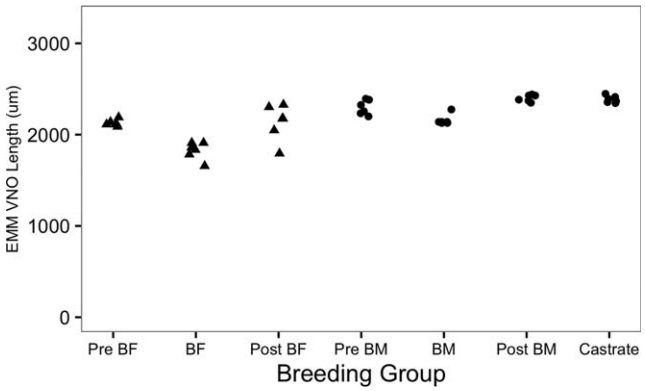

Fig. 5. Scatterplot of VNO length (estimated marginal means) versus breeding condition. BF= Breeding Female, BM= Breeding Male. Groups that are significantly different from the Pre-Breeding Group for the appropriate sex are indicated by an asterisk.

linear regression models showed that neither latent variable was a better predictor than the best individual parameter (snout-rump length).

However, the effect of body size on VNO length was undeniable (see Supporting Information) so the effect of breeding condition on all of the vomeronasal metrics measured was investigated using analysis of covariance, with snout-rump length as a covariate in order to investigate the effects of sex and breeding group which otherwise would have been confounded with body size.

Effect of Breeding Condition on Mean Length of VNO

In both sexes, the pre-breeding group was used as the reference and all other group means were compared against it. Mean VNO length did not significantly differ from the pre-breeding group in either males ($F_{3,19}$ =0.461, $P = 0.713$) or females ($F_{2,14}$ =2.63, P =0.107); when adjusted for snout-rump length; no group mean was significantly different from the corresponding pre-breeding mean. Figure 4 shows the actual values and Figure 5 shows the estimated marginal means generated by the model, there were no significant differences between groups.

Analysis of Other VNO Metrics

Descriptive statistics for all VNO metrics, divided by breeding group are shown in Tables 3 (male) and 4 (female). Scatter plots of the estimated marginal means for the mean neural height (a representative parameter that is related to function) and the mean height of the non-sensory epithelium (a representative parameter that is related to structure, not function) are shown in Figures 6 and 7, respectively. Scatter plots for the other parameters may be found in the Supporting Information. Males and females were again analyzed separately using analysis of covariance with snout-rump length as a covariate and the pre-breeding group for each sex as the

TABLE 2. Descriptive statistics of body size parameters: female animals

Parameter	Group	*N*	Mean	SD	95% C. I. of mean	Skewness	Kurtosis
Weight (g)	Pre-Breeding	6	23.31	1.89	21.32, 25.29	−1.60	2.23
	Breeding	6	24.63	2.42	22.10, 27.17	2.03	4.39
	Post-Breeding	6	37.69	3.60	33.91, 41.47	3.61	18.05
Length (cm)	Pre-Breeding	6	18.05	0.49	17.53, 18.57	−0.81	−1.03
	Breeding	6	18.48	0.87	17.57, 19.39	1.23	1.06
	Post-Breeding	6	19.73	0.71	18.99, 20.47	−1.13	0.63
Snout-Rump Length (cm)	Pre-Breeding	6	10.30	0.14	10.21, 10.50	1.38	2.36
	Breeding	6	10.27	0.38	9.87, 10.66	−1.25	1.43
	Post-Breeding	6	11.85	0.77	11.04, 12.66	−1.18	1.33
Head Length (cm)	Pre-Breeding	6	3.35	0.16	3.18, 3.52	−0.81	−1.03
	Breeding	6	3.25	0.10	3.14, 3.36	0.00	−0.25
	Post-Breeding	6	3.92	0.15	3.76, 4.07	0.71	−2.05
Palatal Width (mm)	Pre-Breeding	6	7.7	0.5	7.1, 8.2	−0.97	−1.88
	Breeding	6	6.7	0.5	6.1, 7.2	−1.88	0.83
	Post-Breeding	6	6.8	1.0	5.8, 7.9	0.46	−2.39
Palatal Length (mm)	Pre-Breeding	6	16.5	0.8	15.6, 17.4	−1.54	1.43
	Breeding	6	16.8	0.41	16.4, 17.3	−2.45	6.0
	Post-Breeding	6	16.3	0.5	15.8, 16.9	0.97	−1.88

TABLE 3. Descriptive statistics for male vomeronasal metrics

		Male			
Parameter (unit)	Breeding Group	Mean	SD	Skewness	Kurtosis
Mean vomeronasal length (μm)	Pre-Breeding	2298.0	240.1	0.01	1.80
	Breeding	2158.3	537.0	1.28	2.26
	Post-Breeding	2400.0	328.0	−0.36	−1.39
	Castrate	2386.7	444.2	−0.60	0.03
Total sensory volume (mm^3)	Pre-Breeding	0.214	0.023	0.92	0.11
	Breeding	0.131	0.036	0.15	−1.03
	Post-Breeding	0.145	0.030	2.04	4.52
	Castrate	0.179	0.033	−0.13	0.00
Total non-sensory volume (mm^3)	Pre-Breeding	0.051	0.033	2.18	5.07
	Breeding	0.034	0.006	1.33	1.83
	Post-Breeding	0.038	0.008	−1.00	1.90
	Castrate	0.040	0.011	0.74	−1.77
Total lumen volume (mm^3)	Pre-Breeding	0.087	0.019	0.75	0.67
	Breeding	0.135	0.096	2.31	5.46
	Post-Breeding	0.169	0.089	0.13	−2.83
	Castrate	0.062	0.021	−0.76	−1.67
Total volume vomeronasal complex (mm^3)	Pre-Breeding	0.915	0.141	0.19	−1.46
	Breeding	0.744	0.134	0.45	−1.42
	Post-Breeding	0.821	0.083	0.05	0.05
	Castrate	0.865	0.139	1.48	2.30
Total sensory area (mm^2)	Pre-Breeding	2.049	0.169	0.86	0.48
	Breeding	1.891	0.601	0.16	−0.42
	Post-Breeding	2.520	0.708	0.82	−0.50
	Castrate	1.954	0.341	−0.12	0.92
Total non-sensory area (mm^2)	Pre-Breeding	1.416	0.130	−1.70	3.90
	Breeding	1.530	0.511	2.11	4.72
	Post-Breeding	1.670	0.247	−1.78	2.96
	Castrate	1.584	0.300	0.32	−1.41
Mean sensory height (μm)	Pre-Breeding	94.82	6.43	−0.33	−0.78
	Breeding	62.10	10.25	−0.03	2.54
	Post-Breeding	60.09	13.20	−0.79	−1.09
	Castrate	70.25	4.41	−0.23	0.65
Mean neural height (μm)	Pre-Breeding	64.07	5.78	0.84	1.80
	Breeding	39.51	8.03	1.28	2.26
	Post-Breeding	37.72	8.62	−0.36	−1.39
	Castrate	43.31	2.69	−0.60	0.03
Mean non-sensory height (μm)	Pre-Breeding	28.17	3.04	0.22	−1.69
	Breeding	27.59	3.58	−1.39	1.47
	Post-Breeding	25.98	3.00	−0.71	−1.63
	Castrate	29.64	3.65	−1.11	2.68

reference. Table 5 shows the analysis of covariance results and Table 6 the post hoc testing where significance was found.

Volumes of tissue, lumen and VNO complex after adjustment for snout-rump length. The volume of sensory tissue in both females and males was decreased in comparison to the pre-breeding group, although for females only the breeding group was statistically significant. For males, all breeding groups were significantly different to the pre-breeding group, including the castrated group.

The volume of non-sensory tissue, conversely, showed no statistically significant differences to the pre-breeding group in either sex, although there was a slight decrease in the breeding female group and an increase in the post-breeding group which was higher than the pre-breeding group; in males the amount of non-sensory tissue was decreased in all groups.

The volume of the VNO lumen showed no statistically significant differences from pre-breeding group in either sex, females followed the same pattern as the non-sensory tissue volume, that is a decrease in the breeding group and an increase in the post-breeding group. Males showed an increase in lumen volume in both breeding and post-breeding groups, this was not statistically significant but the lack of significance is probably related to the presence of a large outlier in the male breeding group and when this was removed, there was significance in the analysis of variance and the post-breeding group was significantly different from the breeding group but the breeding group was not. The castrated group showed no difference in lumen volume from the pre-breeding group.

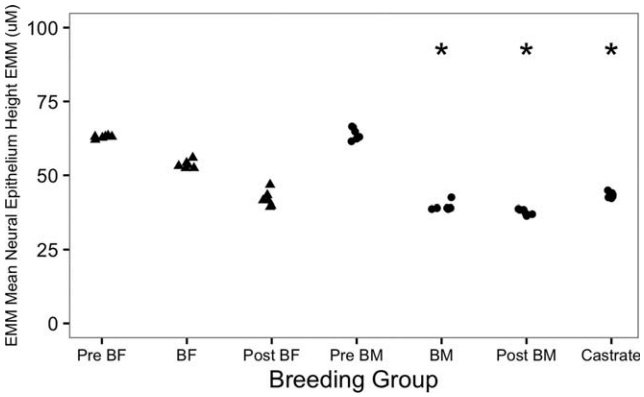

Fig. 6. Scatterplot of mean neural epithelium height (estimated marginal means) versus breeding condition. BF= Breeding Female, BM= Breeding Male. Groups that are significantly different from the Pre-Breeding Group for the appropriate sex are indicated by an asterisk.

The total volume of the VNO complex showed no significant differences in females, but followed the same pattern as the lumen volume and the volume of non-sensory tissue. In males the volumes were decreased for all groups including the castrated group and this was statistically significant.

Epithelial areas and heights. Neither the area of sensory epithelium nor the area of non-sensory epithelium showed any statistically significant differences The mean height of the sensory epithelium showed statistically significant differences in females, where the height decreased in the breeding group and showed a further decrease in the post-breeding group (see Supporting Information). In males, the height also decreased significantly in all three groups, but the castrated group declined less than the other two. The mean height of the neural epithelium showed the same pattern as the mean height of the sensory epithelium just described, but the mean height of the nonsensory epithelium showed no significant differences in either females or males.

DISCUSSION

A visual comparison of the scatterplots of the actual data and the estimated marginal means from the ANCOVA model demonstrate the importance of adjustment for body size in comparing VNO parameters. The variability of the estimated marginal means is smaller, and allows the detection of effects related to sex and breeding condition, which are otherwise confounded by body-size related changes. Snout-rump length proved the best body size metric for allometric scaling of VNO length in this study, that is, it accounted for the greatest amount of variability of all of the body size parameters tested. This may be because it is primarily determined by skeletal size, and so is more resistant to sudden alterations in body condition, particularly weight, seen in males.

The VNO of *A. subtropicus* displays some significant differences related to breeding condition and sex. VNO size itself did not vary between breeding conditions once adjusted for body size. However, the same pattern existed for all sensory components that displayed significant differences: animals sampled before breeding had larger volume, and height (but not area) of the neuroepithelium than animals sampled during or after breeding, or following castration.

This pattern suggests that the differences arise from changes in number and/or size of neurons in the sensory epithelium, rather than non-sensory elements, implying potential alterations in neurogenesis or neuronal survival.

The other significant difference was the decrease of VNC volume in males from pre-breeding. This might be explained by concomitant decreases in sensory components, but also by decreases in other functionally important structures (glands or blood vessels) that were not measured directly.

Palatal width was slightly but significantly greater for pre-breeding males and females than for breeding males or females. However, this parameter showed a very narrow range of values and it is probable that this is an artefact of the small sample size rather than a genuine difference.

Allometric comparisons of chemosensory-associated brain structures or peripheral structures can be poor predictors of chemosensory performance and caution must be used when inferring functionality from size alone. Given that this study used allometric scaling, it is worth exploring this. For MOS and VNS, both sensitivity and discrimination are important elements of performance. Correction for body size has been argued therefore, by Smith and Bhatnagar (2004) to needlessly distort data arising from the MOS in primates, and that the

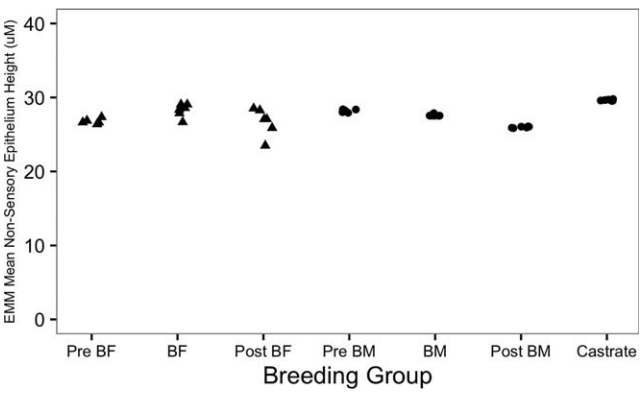

Fig. 7. Scatterplot of mean non-sensory epithelium height (estimated marginal means) versus breeding condition. BF= Breeding Female, BM= Breeding Male. Groups that are significantly different from the Pre-Breeding Group for the appropriate sex are indicated by an asterisk.

TABLE 4. Descriptive statistics for female vomeronasal metrics

Parameter (unit)	Breeding Group	Mean	SD	Skewness	Kurtosis
Mean vomeronasal length (μm)	Pre-Breeding	2125.8	181.3	−1.51	2.80
	Breeding	1825.8	317.40	−1.83	3.11
	Post-Breeding	2137.5	282.3	0.60	−1.07
Total sensory volume (mm^3)	Pre-Breeding	0.190	0.023	−0.49	−1.38
	Breeding	0.126	0.011	−0.99	−0.22
	Post-Breeding	0.152	0.047	1.91	3.83
Total non-sensory volume (mm^3)	Pre-Breeding	0.030	0.004	−0.16	−1.60
	Breeding	0.026	0.002	−1.07	−0.96
	Post-Breeding	0.052	0.014	0.69	1.57
Total lumen volume (mm^3)	Pre-Breeding	0.176	0.267	2.44	5.96
	Breeding	0.072	0.015	1.79	3.62
	Post-Breeding	0.147	0.080	−2.01	0.77
Total volume vomeronsasal complex (mm^3)	Pre-Breeding	0.774	0.088	0.72	0.29
	Breeding	0.579	0.086	0.97	1.04
	Post-Breeding	0.804	0.233	1.73	3.27
Total Sensory Area (mm^2)	Pre-Breeding	1.747	0.259	−0.10	−1.36
	Breeding	1.549	0.165	0.94	0.81
	Post-Breeding	2.001	0.488	0.10	−2.11
Total non-sensory area (mm^2)	Pre-Breeding	1.201	0.141	−0.89	0.64
	Breeding	1.057	0.087	0.18	−1.65
	Post-Breeding	1.880	0.388	0.08	2.00
Mean sensory height (μm)	Pre-Breeding	92.82	13.21	1.94	3.94
	Breeding	76.41	4.84	0.22	−1.62
	Post-Breeding	64.48	10.41	0.46	−0.72
Mean neural height (μm)	Pre-Breeding	62.89	9.45	1.89	3.91
	Breeding	53.61	2.69	−0.56	−1.01
	Post-Breeding	42.09	6.96	0.69	−0.57
Mean non-sensory height (μm)	Pre-Breeding	26.75	2.92	0.09	−2.05
	Breeding	28.26	1.05	−0.39	−1.70
	Post-Breeding	26.72	3.31	−1.08	1.40

relationships with brain or cranial sizes may be more useful in discussions of functionality, as may closer examination of size and number of neurons within chemosensory neuroepithelia.

Pihlström et al. (2005) demonstrates a tendency for main olfactory organ size to be proportional to cribriform plate size in mammals, and that the larger absolute (not relative) size of the main olfactory organ, the higher olfactory sensitivity of that species. Bird et al. (2014) likewise suggest that cribriform plate measures are closely correlated with main olfactory organ size.

The studies quoted cannot be applied directly to the present study—they are discussing interspecific rather than intraspecific comparisons, in the MOS. However, the VNS is linked via development, neural connectivity and functional overlap to the MOS, and findings in the MOS may apply to other chemosensory systems. The concept of absolute separation between the two systems has been overturned; both can detect general odorants and pheromones (Spehr et al., 2006; Keller et al., 2009), and the respective central projections are highly interconnected (Mucignat-Caretta et al., 2012). Martínez-García et al. (2009) suggested that MOS and VNS are complementary, the MOS detecting volatile stimuli related to diet, activity pattern, habitat, navigation and sociosexual signals (Jacobs, 2012), and VNS

TABLE 5. Significance of vomeronasal organ metrics

	Male		Female	
Parameter	$F_{3,19}$	P	$F_{2,14}$	P
Vomeronasal organ length (uM)	0.461	0.713	2.63	0.107
Volume of sensory tissue (mm^3)	10.19	<0.001*	7.517	0.006*
Volume of non-sensory tissue (mm^3)	0.221	0.881	1.524	0.252
Vomeronasal lumen volume (mm^3)	2.574	0.084	0.588	0.569
Vomeronasal complex volume (mm^3)	7.992	<0.001*	2.39	0.128
Sensory epithelium area (mm^2)	1.794	0.182	0.482	0.627
Non-sensory epithelium area (mm^2)	0.236	0.870	1.64	0.229
Mean height of sensory epithelium (μm)	10.12	<0.001*	5.505	0.017*
Mean height of non-sensory epithelium (μm)	1.179	0.344	2.567	0.112
Mean height of neural epithelium (μm)	11.52	<0.001*	4.446	0.032

Significant parameters following Benjamini–Hochberg Correction are marked with an asterisk.

TABLE 6. Comparisons of estimated marginal means of vomeronasal organ metrics (corrected for Snout-Rump length by ANCOVA)

Parameter	Sex	(a) Group	(b) Group	Mean Difference (b–a)*	*P*
Volume of sensory tissue (mm^3)	Female	Pre-Breeding	Breeding	−0.062	0.009
			Post-Breeding	−0.068	0.099
Mean height of sensory epithelium (μm)	Female	Pre-Breeding	Breeding	−16.64	0.044
			Post-Breeding	−24.30	0.084
Volume of sensory tissue (mm^3)	Male	Pre-Breeding	Breeding	−0.113	<0.001
			Post-Breeding	−0.104	0.002
			Castrate	−0.068	0.043
Mean height of sensory epithelium (μm)	Male	Pre-Breeding	Breeding	−36.13	<0.001
			Post-Breeding	−38.70	<0.001
			Castrate	−28.29	0.007
Vomeronasal complex volume (mm^3)	Male	Pre-Breeding	Breeding	−0.358	0.001
			Post-Breeding	−0.312	0.006
			Castrate	−0.254	0.023
Mean neural height (μm)	Male	Pre-Breeding	Breeding	−27.81	<0.001
			Post-Breeding	−30.14	<0.001
			Castrate	−24.30	0.001

*A negative value indicates the group mean is smaller than the pre-breeding group mean.

detecting nonvolatile stimuli for sociosexual behavior and predator avoidance (Døving and Trotier, 1998; Fortes-Marco et al., 2013).

The absolute size of peripheral chemosensory structures alone cannot predict olfactory function; odorant receptors or central olfactory structures may be more important. As an example, olfactory sensitivity in squirrel monkeys was as good or better than rats or dogs for certain odorous substances (Laska et al., 2000). Rat and dog are held to possess an acute olfactory sense, coupled with relatively large olfactory structures. Squirrel monkeys have relatively small olfactory structures, which may, however, be specific for substances with biological relevance, which are not as important for rat or dog, and may not be detected with the same acuity. Discrimination, the ability of the VNS and MOS to detect and discriminate between different odorants, depends upon receptors encoded by several gene families, which are involved in a variety of intraspecific and interspecific behaviors (Ibarra-Soria et al., 2013).

Garret and Steiper (2014) demonstrated that variation in genes that encode receptors and structural variation in both MOS and VNS were highly correlated. Species with well-developed (larger, complex) olfactory structures tended to have well-developed receptor gene families, and better discrimination. Determinants of chemosensory functionality appear more involved than size or complexity.

With these considerations, the overall relative size and development of the VNO of *A. subtropicus*, in comparison with more extensively studied species with proven functionality, suggest that it too is most likely functional and involved in the same social and reproductive processes and behaviors. The VNO of the adult male ferret is a rudimentary structure that does not vary seasonally, despite seasonal changes in physiology, hormone levels, testis size and behavior (Weiler et al., 1999a). The average body weight of the adult male ferret is 1 kg, while the average length of the VNO was 2.7 mm. In comparison, the mean body weight of male *A. subtropicus* in this study was 43.6 g while mean VNO length (measured in a manner similar to Weiler et al., 1999a) was 2.3 mm. The VNO of *A. subtropicus* is relatively larger than that of the adult ferret and is similar to VNO length in a juvenile (10-day old) hamster pup weighing 10 g (Taniguchi et al., 1982). Larger rodents tend to have longer VNOs; 6 mm in the chinchilla (Oikawa et al., 1994) and 7 mm in the albino rat (Addison and Rademaker, 1927; Weiler et al., 1999b).

The colugo, a large arboreal gliding mammal, has one of the relatively longest VNOs at 48% of nasal cavity length and 23% of head length (Bhatnagar and Wible, 1994), exceeded only by the gray mouse lemur *Microcebus murinus* at 53% nasal cavity length (Schilling, 1970). The VNO organ of *A. subtropicus* is approximately 8–10% of the total head length, and about 20% of the nasal cavity length.

Sexual dimorphism raises the possibility of functional differences between male and female animals. The larger VNO in male rodents has been hypothesized to inhibit feminine sexual and reproductive behaviors (Segovia and Guillamón, 1993; Del Cerro, 1998) and to facilitate masculine behaviors, specifically, a decreased level of parental care and increased aggressive behavior (Segovia and Guillamón, 1996). This corresponds to experimental lesioning of the VNO, which eliminates aggressive behavior (Wysocki and Meredith, 1987; Mennella and Moltz, 1988; Wekesa and Lepri, 1994). However, in several wild *Microtus* species with varying levels of male parental involvement, no association between VNO size and type of parental behavior was apparent (Smith et al., 2001). Interpreting sexual dimorphism in the VNO

may not be straightforward, especially as hormonal factors that may contribute to peripheral sexual dimorphism also act on related brain structures.

It is not clear from the data in this study how to interpret the decreases in sensory components from pre-breeding to breeding and post-breeding conditions. What appears to be a decline may be a return to a baseline level, following a temporary, brief increase at the pre-breeding time point that was sampled in this study.

Whether a decrease or a return to baseline, these results may imply that the VNO is more important prior to breeding. For wild animals, this may indicate a role in breeding synchronisation and initiation and exacerbation of the stress response in males.

For females that may live and breed in second and possibly third years, the situation is more complex. In this study, none of the post-breeding females were examined when the pre-breeding group were examined. It would be interesting to discover whether the decline in sensory components in the breeding period one year was followed by an increase prior to breeding the subsequent year, or whether the apparent decline of the first breeding period is permanent.

That significant differences remain with breeding condition once body size has been accounted for suggest hormonal involvement. Hormonal milieu alters as breeding approaches with, in males, large increases in biologically active testosterone and cortisol. A decrease in sensory components might be part of the general decline in condition of the males; neurogenesis and/or neuronal survival decreases or ceases in males as energy and resources are directed elsewhere. However, females display similar changes, without a decline in body condition, and without the extreme changes in biologically active testosterone or cortisol seen in males.

Castrated males and post-breeding males also display a decrease in sensory components, when, presumably, removal of the testes or isolation from conspecifics respectively, have shielded them from the worst ravages of the stress response. The post-breeding males represent an artificial situation rarely encountered in the wild, of intact males surviving the breeding season. It is unclear whether this group contributes to an understanding of the wild situation, except to confirm that a decline in sensory components occurs independently of contact with conspecifics, or the development of the full stress response.

As castrated males were sampled at the breeding period, it is also not clear how much loss of testosterone contributed to the decrease, and how much occurred due to the same factors that cause the decrease in noncastrated breeding males. However, neither castration nor isolation from conspecifics, and the presumed concomitant reductions in testosterone or cortisol, protect against loss of sensory components. The similarity of changes in intact breeding males to females, castrated males and post-breeding males makes it unlikely that this is a simple direct effect of testostone or cortisol on the neuroepithelium, but instead suggests a more complex biological underpinning.

In mammals, seasonal changes in volume and cell proliferation have been reported in the hippocampus, which receives olfactory input (Yaskin, 1984; Galea et al., 1999). These changes are influenced by adrenal and gonadal steroids. Neurogenesis of vomeronasal neurons also increases during pregnancy in female mice (Kaba et al., 1988). In some animals, the neural sensory epithelium of the VNO varies seasonally; proliferation being temporally associated with changes in seasonal behaviors (Dawley and Crowder, 1995; Dawley, 1998; Dawley et al., 2000; Kondoh et al., 2012). Male salamanders have significantly larger VNOs than females at all times. However, before the breeding period, the size of the VNO increases in both males and females. As salamanders use their VNO to locate potential mates, the increase in size might reflect increased neurogenesis, controlled by gonadal or pituitary hormones (Dawley and Crowder, 1995). In Japanese striped snakes, by contrast, morphological and histological features of the VNO sensory epithelium do not change seasonally, but VNO receptor cell proliferation and turnover appears to increase rapidly during hiberation (Kondoh et al., 2012). Whether this seasonality is reflected in central VNS pathways in alterations of cell number or activity has not been determined.

This study has indicated a potential area for research, particularly to investigate whether vomeronasal sensory epithelia increases again in females breeding in second and subsequent years, and the relationship of testosterone and cortisol to the sensory epithelium.

The VNO and its sensory components vary significantly with body size, sex and breeding condition in *A. subtropicus*. Systematic variations in sensory components may affect functionality of the organ differentially between the sexes, and relative to the synchronised breeding period. Sensory components are largest in males and females before breeding, and decrease with and after breeding. This suggests that the VNO in *A. subtropicus* has important functions prior to breeding, perhaps in the synchronisation of reproduction and the initiation and exacerbation of the stress response in the males.

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