Size variation and cell proliferation in chemosensory brain areas of a lizard (*Podarcis hispanica*): effects of sex and season

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Abstract

Many lizards rely on chemoreception for crucial aspects of their biology, including exploration, prey and predator detection, and intraspecific communication. Here we investigate sex and seasonal variation in size and proliferative activity in chemosensory areas of the lizard brain. We captured adult Iberian wall lizards (*Podarcis hispanica*) of either sex in the breeding (April) and non-breeding (November) season, injected them with 5-bromo-2′-deoxyuridine (BrdU) and killed them 3 weeks later. We removed the brains, measured the length of the olfactory bulbs, and counted BrdU-labelled cells in the main and accessory olfactory bulbs (MOB, AOB), lateral cortex (LC) and nucleus sphericus (NS). Our results show that, relative to body size, males have larger MOBs and AOBs than females; however, relative to brain size, males have larger AOBs, but not larger MOBs than females. Additionally, males produce more new cells than females in the olfactory bulbs, LC and NS. We failed to detect significant seasonal changes or sex × season interaction in size or proliferative activity in these areas. Sex differences in the addition of newly generated cells – mainly neurons – may be partly responsible for the size differences in chemosensory brain areas. The presence of sexual dimorphism in AOB is expected given the available behavioural evidence, which suggests that males of *P. hispanica* are more responsive than females to socially relevant chemical stimuli. This is the first demonstration of sexual dimorphism in size and proliferative activity in chemosensory areas of a non-mammalian species.

Introduction

Seasonal variation and sexual dimorphism are common features of the brain of many vertebrates, particularly seasonal breeders. From changes in the morphology and physiology of neurons to gross volumetric differences, sex and seasonal variation have been described in many brain regions of birds and mammals, usually linked to seasonal and/or sexual differences in behaviour (reviewed in Cooke *et al.*, 1998; Tramontin & Brenowitz, 2000). Other vertebrates such as reptiles have received comparatively less attention. Whole brain size is sexually dimorphic in some reptile species (Platel, 1975), and sexual dimorphism has also been described in motor nuclei of the brainstem and spinal cord (Wade, 1998; O'Bryant & Wade, 1999; Ruiz & Wade, 2002), preoptic area and ventromedial hypothalamus (Crews *et al.*, 1990, 1993; Salom *et al.*, 1994; O'Bryant & Wade, 2002; Kabelik *et al.*, 2006), lateral septum (Stoll & Voorn, 1985; Salom *et al.*, 1994) and medial cortex (Roth *et al.*, 2006). In some of these dimorphic areas, seasonal variation has also been reported (Wade & Crews, 1991; Crews *et al.*, 1993; Salom *et al.*, 1994; O'Bryant & Wade, 1999, 2002; Kabelik *et al.*, 2006; Holmes *et al.*, 2007).

To date, sexual dimorphism in chemosensory brain areas of vertebrates has been documented mainly in the vomeronasal system of some mammals (e.g. rats; Segovia & Guillamón, 1993; Simerly,

2002). In reptiles, however, our knowledge is restricted to sex differences in the nucleus sphericus (NS) of geckos (Stoll & Voorn, 1985) and snakes (Crews *et al.*, 1993), and in the amygdala of tree lizards (Kabelik *et al.*, 2006). There is no evidence of seasonal variation in chemosensory brain areas of mammals or birds and, in reptiles, it has been described only in the NS and amygdala of tree lizards (Kabelik *et al.*, 2006).

The aim of the present study is to evaluate the existence of sex and seasonal variation in the size of chemosensory brain areas of the Iberian wall lizard, *Podarcis hispanica* (Squamata: Lacertidae), and in the proliferative activity destined to these areas. On the eastern coast of Spain, *P. hispanica* lizards are active year round, though their activity in winter is greatly reduced. These lizards are seasonal breeders and have well-developed olfactory and vomeronasal systems, with large peripheral sensory organs and olfactory bulbs (Pérez-Mellado, 1982, 1997; Schwenk, 1993; Cooper, 1996). *P. hispanica* lizards use their chemical senses in environmental exploration, detection of prey and predators, and social contexts (Gómez *et al.*, 1993; Van Damme & Castilla, 1996; Cooper & Pérez-Mellado, 2002; Font & Desfilis, 2002; Desfilis *et al.*, 2003; Carazo *et al.*, 2007). Furthermore, chemosensory brain areas of *P. hispanica* such as the olfactory bulbs and their main efferents [the lateral cortex (LC) and NS] add neurons at a high rate throughout adulthood (Font *et al.*, 2001). We hypothesized that, in *P. hispanica*, the sensory demands of the reproductive cycle may correlate with changes in size of chemosensory brain areas, or in the intensity of the proliferative activity destined to these brain areas.

Materials and methods

We captured 20 adult (10 males, 10 females) Iberian wall lizards (*P. hispanica*) in early November (fall, non-breeding season) and again in early April (spring, breeding season) in stonewalls, orange tree orchards and abandoned fields in Burjassot, Valencia. Males ranged 51–61 mm in snout to vent length (SVL), whereas females ranged 48–60 mm SVL. Lizards were transported to the laboratory, where they received a single intraperitoneal injection of 5-bromo-2′-deoxyuridine (BrdU; Sigma, 100 µg/g body weight). We housed animals for 3 weeks in groups of five (three females and two males, or vice versa) in large outdoor terraria (70 × 30 × 40 cm) with a soil substrate, water dishes, and large bricks for basking and shelter. During maintenance, lizards were fed with an insectivorous diet (crickets, mealworms and waxworms), and water *ad libitum*. Animals were maintained outdoors to ensure that weather conditions during survival were as similar as possible to those in their natural environment. Environmental temperatures ranged from 11.0 to 17.7°C (monthly average of 14.1°C) in November, and from 10.8 to 19.9°C (monthly average of 15.1°C) in April. The average photoperiod was approximately 10 h in November and over 13 h in April, and animals had more than 5 h of direct sunlight in both months.

After this 3-week survival time, animals were deeply anaesthetized with ketamine hydrochloride (12 µL/g body weight; Ketolar, 50 mg/mL, Pfizer, NY, USA; Font & Schwartz, 1989) and perfused transcardially with saline followed by fixative solution (paraformaldehyde 4% 0.1 M phosphate buffer). The brains and gonads were then removed, postfixed in fixative solution for 4 h and thereafter kept in 0.01 M phosphate buffer. The brains were kept in phosphate buffer 4–7 days before inclusion in paraffin, and during this time we took measurements of the overall size of the brain and the olfactory bulbs. This duration was consistent across groups, and there was no relationship between time in phosphate buffer and sex or season. We also verified the breeding state of lizards by *post mortem* gonadal inspection.

Animal care and experimentation were conducted according to guidelines established by the Association for the Study of Animal Behaviour, the Animal Behavior Society, and the American Society of Ichthyologists and Herpetologists, and were approved by the University of Valencia's Ethics Committee. The lizards used in this study were captured and killed under permit from the Generalitat Valenciana to E.F.

One male captured in November was discarded from the analyses of whole brain size because its left cerebral hemisphere was slightly damaged during extraction, and one male captured in April died during maintenance.

Morphometrics

Measurements were taken of the body, brain and olfactory bulbs in all the experimental animals. Body size was measured immediately after capture as SVL (±1 mm). In lizards, SVL provides a better estimate of overall body size than body weight, as the latter shows large variation depending on recent feeding, hydration level and, particularly in females, reproductive status. Whole brain and olfactory bulb sizes were measured immediately after extraction. After removing the excess of fixative, we weighted brains (BRAINWT, ±0.1 mg) using a precision balance (Sartørius 121S), and measured the length of the main and accessory olfactory bulbs (MOBL, AOBL, ±0.05 mm) using a stereomicroscope (Leica MZ8) provided with an ocular micrometer. In *P. hispanica*, the olfactory bulbs run parallel to the main longitudinal axis of the brain and are attached to the cerebral

hemispheres by very short olfactory peduncles. A constriction in the cross-sectional area divides the olfactory bulbs into a rostral, ellipsoid-shaped portion (main olfactory bulbs; MOB) and a caudal portion (accessory olfactory bulbs; AOB). AOBs, in turn, are delimited caudally by a marked transversal constriction at the beginning of the olfactory peduncles. Transversal constrictions allow a macroscopical differentiation of MOB and AOB in fixed brains (Fig. 1). Both MOBs and AOBs are elongated structures and their length is a good indicator of their overall size. We also defined brain length (BRAINL, ±0.1 mm) as the distance comprised between the caudal boundary of AOB and the posterior edge of the cerebellum. This measurement embraced most of the length of the brain, excluding only the most caudal part of the brainstem and the olfactory bulbs, and correlated strongly with BRAINWT ($R = 0.914$, $P < 0.01$). Similar measurements to BRAINL and MOBL have already been used as proxies of brain and olfactory bulb size in previous studies (e.g. Healy & Guilford, 1990).

BrdU-immunocytochemistry and quantitative analysis

For BrdU-immunostaining, brains were dehydrated and embedded in paraffin. Transversal slices of the telencephalon were cut at 7 µm and mounted in three series on gelatine-coated slides. Serial 7-µm paraffin sections were deparaffinized in xylene and hydrated in graded ethanol concentrations to distilled water. We processed slides for BrdU-immunocytochemistry in sets of 20 slides, which included
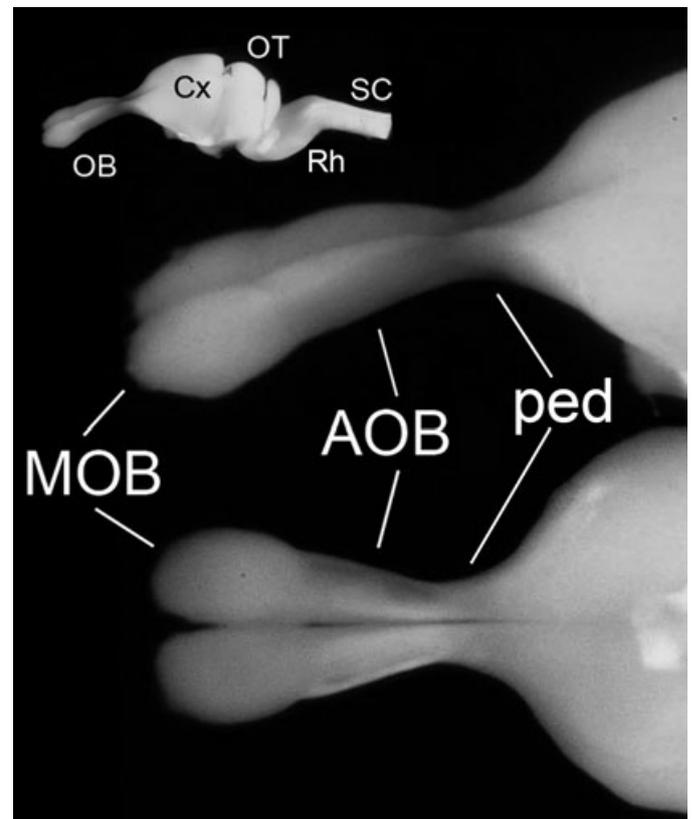

FIG. 1. Lateral view of the brain of the Iberian wall lizard *P. hispanica*, and detail of the olfactory bulbs. Note that in the ventral view of the accessory olfactory bulb (AOB) we can distinguish the lateralized ventricle by transparency. Cx, cortex; MOB, main olfactory bulb; OB, olfactory bulb; OT, optic tectum; ped, olfactory peduncles; Rh, rhombencephalon; SC, spinal cord.

representatives of both sexes and seasons. Every set was first treated with H_2O_2, then with 2 N HCl and, subsequently, neutralized with 0.1 M borate buffer pH 8.4. Slides were then incubated in a blocking solution containing 10% normal goat serum (Sternberger, Baltimore, MD, USA) followed by incubation in mouse monoclonal antibody against BrdU (Dako, Denmark) overnight. The following day, slides were incubated in goat anti-mouse biotinylated antibody (Vector, Burlingame, CA, USA), followed by incubation in ABC complex (Vector). Peroxidase activity was detected using 3-3′-diaminobenzidine (DAB; Sigma). DAB produces a dark reaction product in the nuclei of cells that incorporated BrdU at the time of injection and in their progeny. Finally, slides were rinsed in distilled water, dehydrated and coverslipped with Permount (Fisher, Fair Lawn, NY, USA).

We performed counts of BrdU-labelled cells in the right hemisphere of the MOB and AOB, LC and NS. We began counts in the most rostral MOB hemisection in which the lateral ventricle was visible (the lateral ventricle extends into MOB in adult reptiles). In every 12th section from this first hemisection to the caudal telencephalon, we counted all BrdU-labelled cells found in chemosensory brain areas. Contiguous hemisections were thus separated more than 80 μm, which prevented counting the same BrdU-labelled cells in different sections. We identified endothelial cells and glia associated to blood vessels by their elongate, semicircular nucleus and their proximity to blood vessels, and these were not included in our BrdU-labelled cell counts. Because of the relative low number of labelled cells per hemisection, we did not use a stereological probe to count the number of BrdU-labelled cells. Rather, direct counting was performed with a Leica DM LB microscope under bright-field illumination at 400× magnification. Examining the shape and position of the ventricle, we were able to identify and delineate reliably major structures such as MOB, AOB, LC or NS. We used differential interference contrast microscopy to verify the boundaries of cell layers in these brain areas.

We also estimated the total number of BrdU-labelled cells in chemosensory brain areas from BrdU-labelled cell counts, multiplying the number of BrdU-labelled cells counted in the sampled sections by the inverse of the section sampling fraction (1/12). Similar counts and estimations have been performed in other studies of cell proliferation (e.g. Lavenex et al., 2000b).

Double-fluorescence immunolabelling

To determine the phenotype of the BrdU-positive cells, one series of 7-μm paraffin sections was processed for BrdU and doublecortin fluorescence double-labelling. Doublecortin is a microtubule-associated protein, which is expressed almost exclusively by immature neurons (Gleeson et al., 1999; Brown et al., 2003). To double label, sections were first deparaffinized in xylene and hydrated in graded ethanol concentrations to distilled water. Sections were rinsed in 0.1 M phosphate-buffered saline, followed by a pre-treatment in 0.1 M citrate buffer at 90°C. After slides had cooled down to room temperature, they were treated with 2 N HCl and, subsequently, neutralized with 0.1 M borate buffer pH 8.4. Slides were then incubated in a blocking solution containing 0.1% bovine serum albumin (Sigma) and 5% bovine non-fat dry milk (Sveltesse, Nestle). Finally, sections were incubated overnight in a mouse monoclonal antibody against BrdU (Dako) and a goat polyclonal antibody against doublecortin (Santa Cruz Labs, CA, USA). The following day, the primary antibodies were washed and slides were incubated in Alexa Fluor 488 goat anti-mouse antibody and Alexa Fluor 555 donkey anti-goat antibody (Molecular Probes, Invitrogen, Eugene, OR, USA). After several washes in 0.1 M phosphate buffer, sections were mounted with Fluorsave (Calbiochem, San Diego, CA, USA). Photomicrographs of immunofluorescent stainings were generated by

using a Leica confocal microscope TCS/SP2 equipped with a 40× APO oil objective. To exclude false double-labelling due to an overlay of signals from different cells, BrdU-positive cells were analysed by moving the entire z-axis of each cell.

Statistical analyses

Whole brain size

Complementary to our studies of olfactory bulb size and cell proliferation, we investigated variation in the size of the entire brain. As a preliminary analysis, we first examined sex and seasonal variation in raw data of BRAINWT with a crossed two-way analysis of variance (ANOVA). Allometric scaling between brain and body size has been widely reported (Platel, 1974; Striedter, 2005), so we additionally performed a crossed two-way analysis of covariance (ANCOVA) with SVL as a covariate.

MOB and AOB size

We first examined sex and seasonal variation in raw data with crossed two-way ANOVAs. Subsequently, we examined scaling between olfactory bulb size and body size via standard major axis regression (Sokal & Rohlf, 1995; Legendre & Legendre, 1998), and performed the same procedure for olfactory bulb size and brain size. In these regressions, we used MOBL, AOBL, SVL and BRAINWT as measures of MOB, AOB, body and brain size, respectively. The regressions were performed separately for MOB and AOB, and because all of them were significant (see Results), we studied sex and seasonal variation in olfactory bulb length via crossed two-way ANCOVAs with sex and season as fixed factors. In these ANCOVAs, we used alternatively SVL or BRAINL as covariates. ANCOVAs remove the effects of covariates better than other statistical methods (e.g. the analysis of size-specific indices) and provide a sensitive test of the adjusted data (Packard & Boardman, 1999).

BrdU-labelled cell counts

We studied sex and seasonal variation in cell proliferation separately for each brain area via crossed two-way ANOVAs with sex and season as fixed factors. In order to account for the broad range of body and brain sizes in our sample, for each brain area we provide the mean number of BrdU-labelled cells per hemisection (calculated over all the hemisections in which we counted BrdU-labelled cells in a particular brain area). The olfactory bulbs, cortex and NS in lizards have a laminar organization, and 3 weeks after BrdU administration, BrdU-labelled cells could be found in all cytoarchitectonic layers (see Results). However, due to the low number of labelled cells per hemisection in some layers, we pooled labelled cells in all the layers of the same brain area in our statistical analyses to have more power to detect sex and seasonal variation.

In all the statistical analyses raw data were previously log-transformed to fit normality and homocedasticity assumptions (Quinn & Keough, 2002). Standard major axis regressions were performed using Legendre's Model II Regression program (provided online by P. Legendre and University of Montreal). The remaining analyses were performed using statistics software SPSS 14.0.

Results

The morphology of the gonads of captured lizards allowed us to determine their reproductive status (Pérez-Mellado, 1982, 1997). April males had large and turgid testes, with an opaque, milky appearance indicative of an active spermiogenesis. November males, in contrast,

had smaller and translucid testes. April females had one or two vitellogenic follicles in their ovaries, and some of them had luteinic bodies too. April females had eggs in the abdomen at the time of the capture (detected by abdomen palpation and later verified by *post mortem* examination), and some of these females laid eggs in the holding terraria during maintenance. No luteinic bodies or eggs were found in the abdomen of November females. Moreover, the reproductive status was confirmed by behavioural observations: only in April were social behaviours typical of the breeding season observed (e.g. courtship and male–male aggression), first in the wild and later during maintenance in the holding terraria.

Whole brain size (BRAINWT)

Male BRAINWT ranged 34.8–56.5 mg, whereas female BRAINWT ranged 24.1–44.1 mg. Crossed two-way ANOVAs on raw data revealed that males had larger bodies (SVL, males 55.76 ± 0.69 mm, females 52.95 ± 0.62 mm, ANOVA sex, $F_{1,35} = 11.18$, $P < 0.01$) and heavier brains (Fig. 2A; ANOVA sex, $F_{1,35} = 56.08$, $P < 0.001$) than females. Lizards in April had larger bodies than those in November (ANOVA season, $F_{1,35} = 5.581$, $P < 0.05$), and also had heavier brains (Fig. 2A; ANOVA season, $F_{1,35} = 12.56$, $P < 0.01$). Nevertheless, BRAINWT scaled positively with body size (SVL; standard major axis regression slope = 3.90, $R = 0.87$, $P < 0.01$), therefore, we examined sex and seasonal variation in BRAINWT after removing the effect of body size. ANCOVAs using SVL as a covariate showed that, after removing the effect of body size, males still had heavier brains than females (Fig. 2B; ANCOVA sex, $F_{1,33} = 47.51$, $P < 0.001$), and April lizards had heavier brains than those of November (Fig. 2B; ANCOVA season, $F_{1,33} = 6.62$, $P < 0.05$). We did not detect a sex $\times$ season interaction effect (ANCOVA interaction, $F_{1,33} = 0.82$, $P = 0.37$). Thus, there is both sex and seasonal variation in whole brain size in this species.

MOBL and AOBL

Crossed two-way ANOVAs on raw data showed that the olfactory bulbs were longer in males than in females (Fig. 2C and E; MOBL, ANOVA sex, $F_{1,35} = 18.92$, $P < 0.001$; AOBL, ANOVA sex, $F_{1,35} = 31.07$, $P < 0.001$), but they were not different across seasons (Fig. 2C and E; MOBL, ANOVA season, $F_{1,35} = 0.67$, $P = 0.42$; AOBL, ANOVA season, $F_{1,35} = 0.05$, $P = 0.83$). Nevertheless, because olfactory bulb length showed a positive scaling with body size (MOBL vs. SVL: standard major axis regression slope = 1.54, $R = 0.65$, $P < 0.01$; AOBL vs. SVL: slope = 2.74, $R = 0.40$, $P < 0.01$), we also performed ANCOVAs. ANCOVAs accounting for body size (SVL) detected a significant male-biased sexual dimorphism in the length of the olfactory bulbs (Table 1). However, ANCOVAs did not detect seasonal variation or sex $\times$ season interaction in the length of olfactory bulbs (Table 1).

Olfactory bulb length also scaled positively with BRAINL (MOBL vs. BRAINL: standard major axis slope = 0.45, $R = 0.75$, $P < 0.01$; AOBL vs. BRAINL: slope = 0.76, $R = 0.44$, $P < 0.01$). Therefore, as expected, MOB and AOB were longer in lizards with longer brains. Correcting for BRAINL, males again had longer AOBs than females, but not longer MOBs (Fig. 2D and F; Table 1 ANCOVAs). ANCOVAs did not detect significant seasonal variation or sex $\times$ season interaction either in MOB or in AOB length (Fig. 2D and F; Table 1).

In short, we found slightly different results using body and brain size as a covariate. Relative to body size, MOB and AOB were sexually dimorphic in this species. Nevertheless, relative to brain size, AOB was still sexually dimorphic, but MOB apparently not. We did not find seasonal variation in the length of MOB or AOB in any case.

BrdU-labelled cell counts

Three weeks after BrdU administration, BrdU-labelled cells were found in all the major subdivisions of the telencephalon, including MOB and AOB, rostral forebrain, LC, septum, anterior dorsal ventricular ridge, striatum and NS (Figs 3 and 4A). In MOB and AOB, on average 79 and 96% of all BrdU-labelled cells were in the granular cell layer, whereas the rest were in more external layers (e.g. periglomerular layer; Table 2 and Fig. 3). In the LC, 85% of BrdU-labelled cells were found in the ventricular zone, 11% in the inner plexiform layer and 4% in the cell layer. In the NS, 88% of BrdU-labelled cells were found in the ventricular zone, 4% in the marginal layer and 8% in the mural layer (Table 2 and Fig. 3).

Pooling all chemosensory brain areas (i.e. MOB, AOB, LC and NS) of the right hemisphere, we counted on average 180–220 BrdU-labelled cells in males and 110–125 in females (Table 2). Because we performed counts in every 12th hemisection, we estimate that 3 weeks after BrdU administration, males had 2200–2600 BrdU-labelled cells in their chemosensory brain areas, whereas females had 1300–1500 (figures for the right hemisphere only; Table 2). Our figures do not take into account the problems of oversampling and lost caps, and may thus slightly overestimate the number of BrdU-labelled cells in chemosensory brain areas. Nonetheless, we feel it is safe to conclude that, 3 weeks after a single pulse of BrdU (100 µg/g body weight), the numbers of BrdU-labelled cells in chemosensory brain areas range from hundreds in the NS and LC to over 1000 labelled cells in male AOBs (figures for the right hemisphere; Table 2 and Fig. 5A).

The telencephalon of *P. hispanica* spanned on average about 700 sections in males and 600 sections in females. Three weeks after a single pulse of BrdU (100 µg/g body weight), the number of BrdU-labelled cells per hemisection in chemosensory brain areas of *P. hispanica* ranged from about two labelled cells per hemisection in the LC of females to seven to eight in the NS of males (Fig. 5B). Crossed two-way ANOVAs confirmed the presence of more BrdU-labelled cells per hemisection in males than in females in all the selected brain areas (Fig. 5B and Table 3). However, we did not detect statistically significant seasonal variation or sex $\times$ season interaction in the number of BrdU-labelled cells per hemisection (Fig. 5B and Table 3).

Double-labelling BrdU-doublecortin

Immunofluorescence labelling of doublecortin and BrdU was examined in chemosensory brain areas, such as the olfactory bulbs, LC and NS. In these areas, doublecortin labelling was observed mainly in the granular cell layer of MOB and AOB, and in the cell layer of the LC and NS. In the granular layer of olfactory bulbs, where more BrdU-labelled cells per hemisection were found, the vast majority of BrdU-labelled cells colabelled with doublecortin (Fig. 4B and C).

Discussion

In *P. hispanica*, whole brain size varies across sexes and seasons. Males have brains that are heavier both in absolute and relative terms than those of females, and lizards of either sex have heavier brains in April than in November. These results agree with those reported in other reptiles in which relative brain size varies across sexes or reproductive states (Platel, 1975; Wade & Crews, 1991). Seasonal changes in whole brain size have been shown in some mammals and birds too (Pucek, 1965; Nottebohm, 1981; Weiler, 1992; Yaskin, 1994). Numerous studies in various vertebrate groups have correlated variation in whole brain size with ecological, life-history and
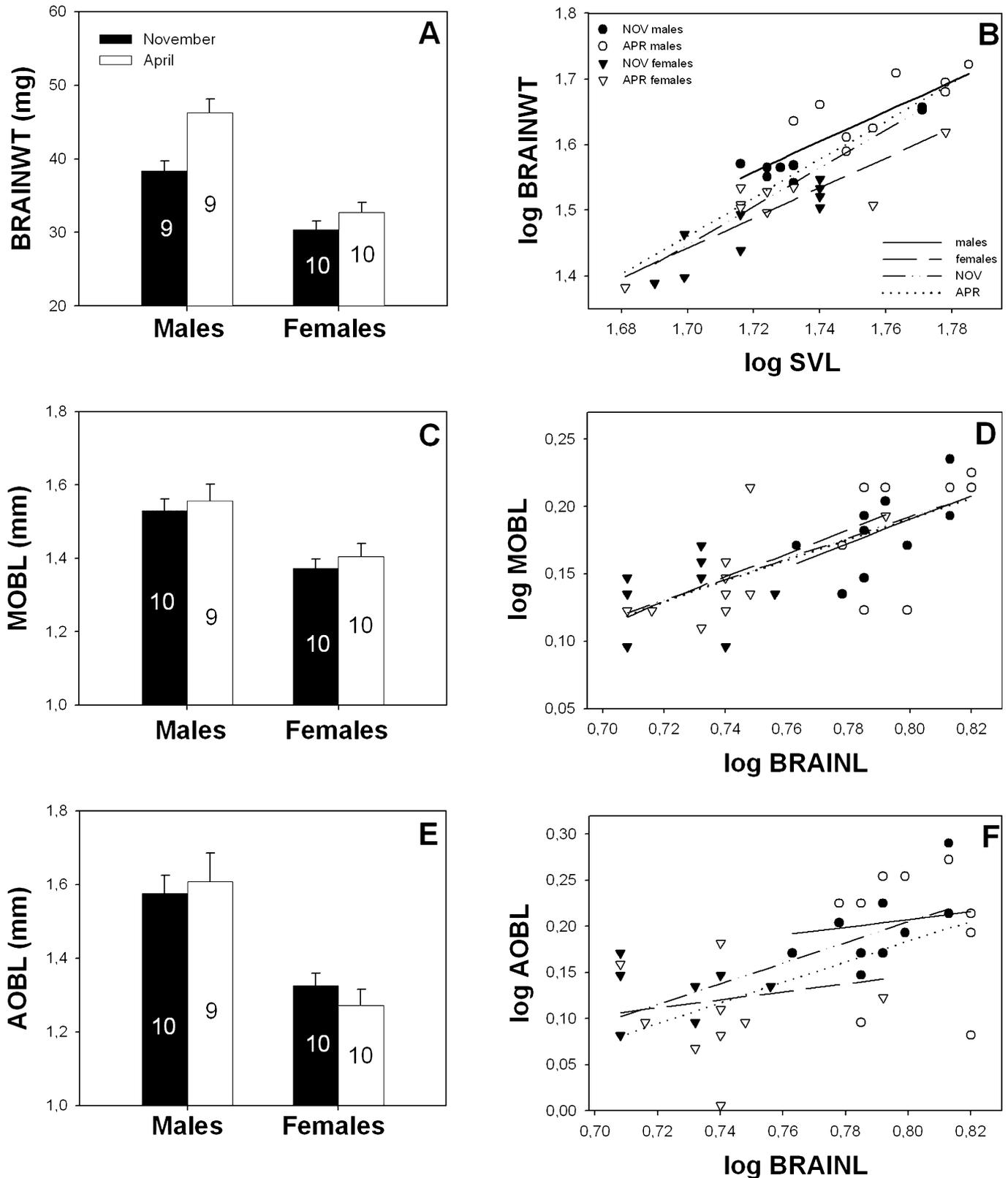
FIG. 2. Sex and seasonal variation in whole brain and olfactory bulb size. In the left column, bar charts of means ± SEM of (A) brain weight (BRAINWT), (C) main olfactory bulb length (MOBL) and (E) accessory olfactory bulb length (AOBL). Black bars are November lizards, and white bars April lizards. Sample sizes inside bars. In the right column, regression plots of log-transformed values of (B) BRAINWT vs. snout to vent length (SVL), (D) MOBL vs. BRAINL, and (F) AOBL vs. BRAINL. Circles are males, inverted triangles females. Dark symbols are November lizards, white symbols April lizards. Regression lines are derived from ANCOVAs pooling lizards of same sex or same season. Note that November and April lizards correspond to lizards captured in the first 2 weeks of November or April, and killed 3 weeks after.

TABLE 1. Effects of sex and season on the MOBL and AOBL using different reference variables

		Sex			Season		Interaction	
	Covariate	$F_{1,34}$	*P*-value	M vs. F	$F_{1,34}$	*P*-value	$F_{1,34}$	*P*-value
MOBL	SVL	6.49	<0.01	M > F	0.32	0.58	0.91	0.35
	BRAINL	0.43	0.52	–	0.13	0.72	0.002	0.96
AOBL	SVL	19.77	<0.001	M > F	0.40	0.53	0.41	0.53
	BRAINL	4.79	<0.05	M > F	0.33	0.57	0.68	0.42

Covariates in crossed two-factor ANCOVAs, SVL and BRAINL. AOB, accessory olfactory bulb; BRAINL, brain length; MOBL, main olfactory bulb length; SVL, snout to vent length.
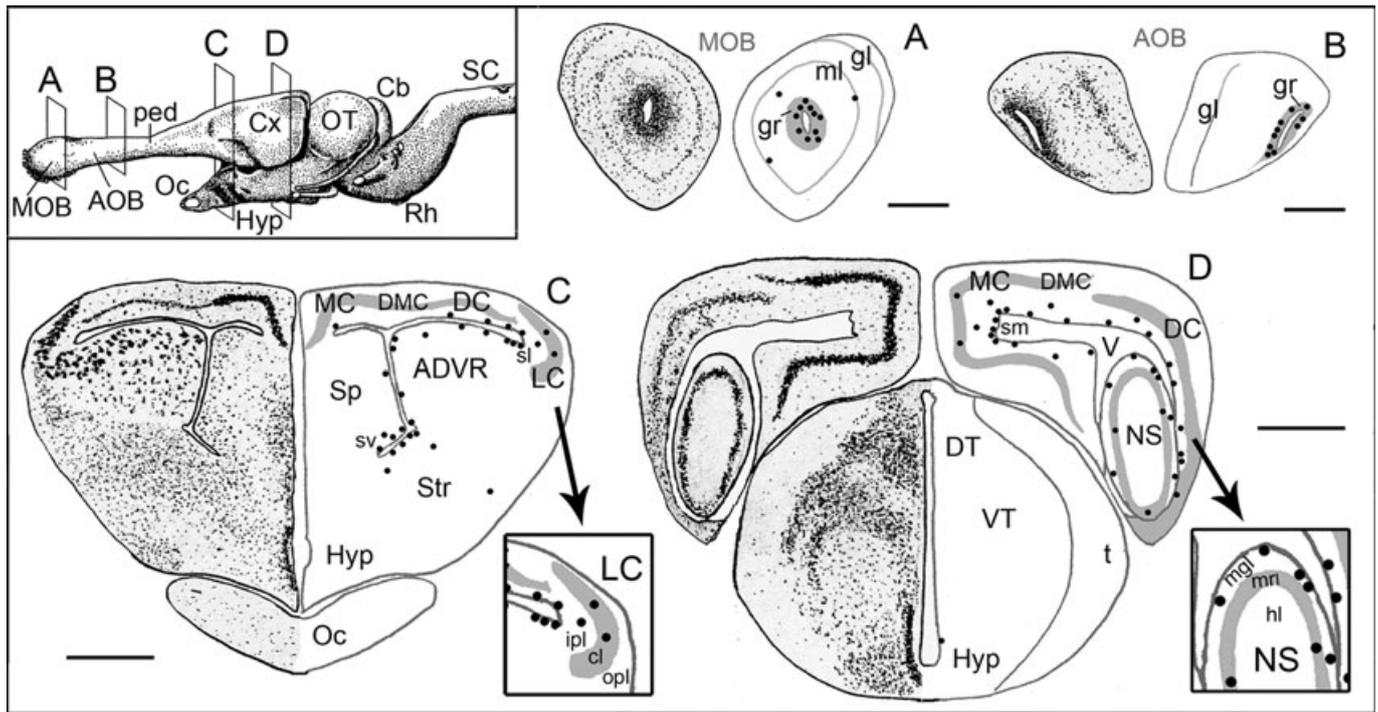

FIG. 3. Distribution of BrdU-labelled cells in four representative sections from the telencephalon of *P. hispanica* 3 weeks after administering BrdU. (A) Main olfactory bulb (MOB). (B) Accessory olfactory bulb (AOB). (C) Precommissural telencephalon. (D) Postcommissural telencephalon. Left half of images are camera lucida drawings derived from Nissl-stained 7-μm-thick transversal sections; right half of images are line drawings in which BrdU labelling is represented as dark dots. The number of dark dots corresponds to the mean BrdU-labelled cell count averaged over three non-consecutive 7-μm sections from the lizard with the most intense cell proliferation. Scale bars: 200 μm. ADVR, anterior dorsal ventricular ridge; Cb, cerebellum; cl, cell layer; Cx, cortex; DC, dorsal cortex; DMC, dorsomedial cortex; DT, dorsal thalamus; gl, glomerular layer; gr, granular cell layer; hl, hilar layer; Hyp, hypothalamus; ipl, inner plexiform layer; LC, lateral cortex; MC, medial cortex; mgl, marginal layer; ml, mitral cell layer; mrl, mural layer; NS, nucleus sphericus; Oc, optic chiasma; opl, outer plexiform layer; OT, optic tectum; ot, optic tract; ped, olfactory peduncles; Rh, rhombencephalon; SC, spinal cord; sl, sulcus lateralis; sm, sulcus medialis; Sp, septum; Str, striatum; sv, sulcus ventralis; V, ventricle; VT, ventral thalamus.
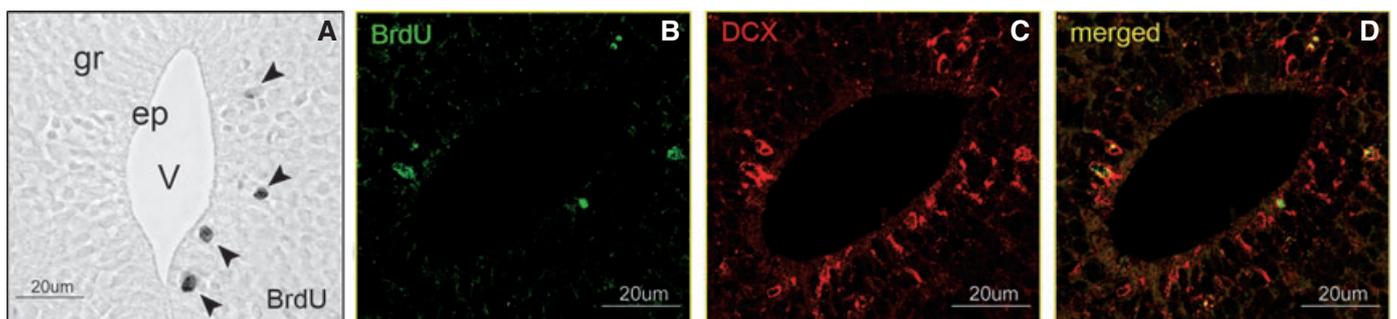

FIG. 4. (A) Photomicrograph illustrating the presence of 5-bromo-2′-deoxyuridine (BrdU)-labelled cells (DAB-immunostained) in a 7-μm-thick transverse hemisection of the MOB. Labelled cells are indicated with arrowheads. (B–D) Confocal fluorescence photomicrographs illustrating colocalization of BrdU (green) with doublecortin (DCX) (red) in granular cells of the MOB. ep, ependyma; gr, granular cell layer; V, ventricle.

TABLE 2. BrdU-labelled cells counts (mean number ± SEM) in different layers of the MOB and AOB, LC and NS, and estimated total number of BrdU-labelled cells per area (in the right hemisphere)

Brain structure	NOV males (n = 10)			NOV females (n = 10)			APR males (n = 9)			APR females (n = 10)		
	Hemisects counted (range)	BrdU + cells counted	Total BrdU + cells* (estimated)	Hemisects counted (range)	BrdU + cells counted	Total BrdU + cells* (estimated)	Hemisects counted (range)	BrdU + cells counted	Total BrdU + cells* (estimated)	Hemisects counted (range)	BrdU + cells counted	Total BrdU + cells* (estimated)
MOB	12.2 ± 0.5 (10–15)	44.2 ± 7.3	530 ± 87	11.3 ± 0.3 (10–13)	17.8 ± 5.6	214 ± 68	12.4 ± 0.3 (10–14)	33.0 ± 6.0	396 ± 72	11.5 ± 0.4 (9–13)	23.1 ± 4.7	277 ± 56
gl		34.0 ± 6.9			12.8 ± 5.0			27.3 ± 5.6			18.9 ± 3.6	
mel		10.2 ± 2.5			5.0 ± 1.6			5.7 ± 1.1			4.2 ± 2.0	
AOB	12.6 ± 0.5 (10–15)	86.1 ± 8.2	1033 ± 98	11.4 ± 0.4 (10–15)	52.8 ± 10.9	634 ± 131	12.7 ± 0.6 (11–17)	69.0 ± 9.6	828 ± 115	11.1 ± 0.6 (8–14)	41.5 ± 5.6	498 ± 67
gl		82.3 ± 8.1			51.3 ± 8.1			67.1 ± 8.1			39.4 ± 8.1	
mel		3.8 ± 0.9			1.5 ± 0.9			1.9 ± 0.9			2.1 ± 0.9	
LC	15.2 ± 0.2 (14–16)	45.8 ± 4.9	550 ± 59	13.0 ± 0.4 (11–15)	23.8 ± 4.3	286 ± 51	16.0 ± 0.5 (14–18)	42.7 ± 6.5	512 ± 78	13.2 ± 0.5 (11–16)	29.3 ± 4.0	352 ± 48
VZ		39.2 ± 4.6			22.7 ± 3.6			34.8 ± 5.2			23.4 ± 3.1	
ipl		4.1 ± 1.7			1.0 ± 0.9			7.0 ± 2.0			4.1 ± 1.1	
cl + opl		2.5 ± 0.9			0.1 ± 0.1			0.9 ± 0.4			1.8 ± 0.6	
NS	5.4 ± 0.2 (4–6)	45.7 ± 7.9	548 ± 95	4.8 ± 0.2 (4–6)	19.9 ± 3.2	239 ± 39	5.7 ± 0.2 (5–7)	35.6 ± 6.2	427 ± 74	5.1 ± 0.3 (4–6)	29.8 ± 3.9	358 ± 56
VZ		41.1 ± 7.8			19.1 ± 3.2			31.4 ± 5.9			23.7 ± 3.2	
mgl		1.3 ± 0.5			0.2 ± 0.1			2.2 ± 0.5			2.0 ± 1.0	
mrl + hl		3.3 ± 0.7			0.7 ± 0.4			1.9 ± 0.9			4.1 ± 0.5	
TOTAL	45.4 ± 1.0	221.8 ± 22.6	2652 ± 271	40.1 ± 0.6	114.3 ± 22.2	1371 ± 266	47.3 ± 0.8	186.7 ± 23.4	2240 ± 281	40.6 ± 1.1	123.7 ± 13.4	1484 ± 161

*Estimated = BrdU-labelled cells counted multiplied by the inverse of the section sampling fraction (1/12). Note that Nov, Apr corresponds to lizards captured in the first 2 weeks of November or April, injected immediately with BrdU after capture, and killed 3 weeks later. AOB, accessory olfactory bulb; BrdU, 5-bromo-2′-deoxyuridine; cl, cell layer; gl, glomerular layer; hl, hilar layer; ipl, inner plexiform layer; LC, lateral cortex; mel, more external layers (e.g. mitral, periglomerular layer); mgl, marginal layer; MOB, main olfactory bulb; mrl, mural layer; NS, nucleus sphericus; opl, outer plexiform layer; VZ, ventricular zone.
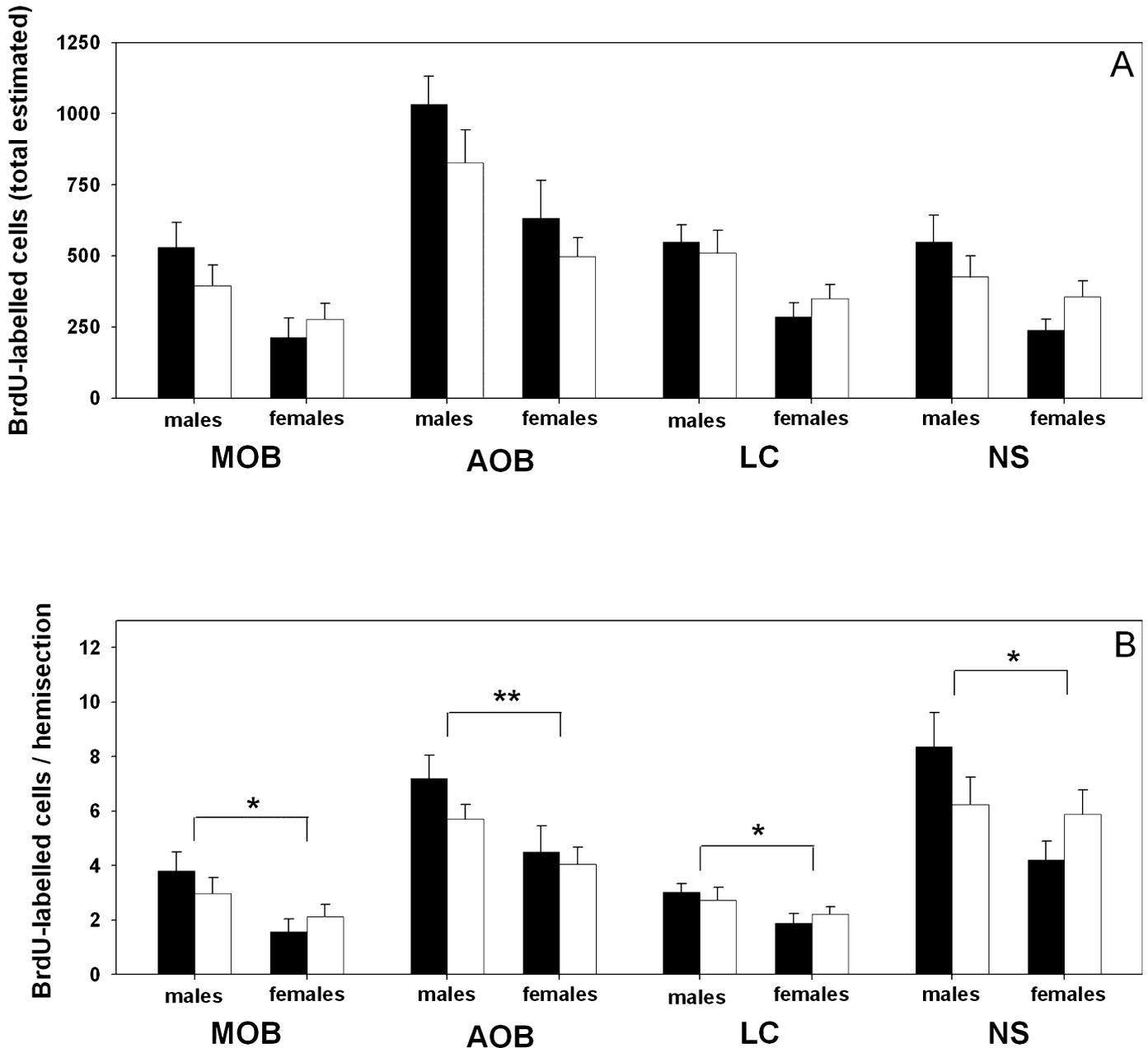
FIG. 5. Sex and seasonal variation in cell proliferation of chemosensory brain areas. (A) Mean ± SEM of the estimated number of 5-bromo-2′-deoxyuridine (BrdU)-labelled cells in the right main (MOB) and accessory olfactory bulbs (AOB), lateral cortex (LC) and nucleus sphericus (NS). (B) Mean ± SEM of the number of BrdU-labelled cells per hemisection in the right MOB and AOB, LC and NS. The black bars are November lizards, and the white bars are April lizards. Note that November and April lizards correspond to lizards captured in the first 2 weeks of November or April, injected immediately with BrdU after capture, and killed 3 weeks later. Asterisks show significance of the sex differences: $*P < 0.05$, $**P < 0.01$. April males, $n = 9$; females and November males, $n = 10$.

behavioural variables (reviewed in Striedter, 2005; Healy & Rowe, 2007). However, brains are highly heterogeneous structures, and several brain regions could contribute differentially to overall brain size (Healy & Rowe, 2007). It is thus more revealing to focus on well-defined regions with known functions such as the olfactory bulbs.

Olfactory bulb size in *P. hispanica* scales positively with body and brain size, represented, respectively, by SVL and BRAINL. In relationship to SVL, male *P. hispanica* have larger MOBs and AOBs than females. Nevertheless, males could have larger olfactory bulbs than females simply because they have also larger brains. Sexual dimorphism would thus not be specific of olfactory bulbs but a general

characteristic of the whole brain. The use of BRAINL as a reference variable allowed us to examine sex differences in olfactory bulb size after removing the effect of brain size. Correcting for BRAINL, males still have larger AOBs, but not larger MOBs, than females. This result reveals that sex differences in MOB are as large as we should expect considering the sex differences in whole brain size, whereas sex differences in AOB overcome sex differences in brain size. These results represent the first demonstration of sexual dimorphism in the olfactory bulbs of a non-mammalian species, and show that the use of different measures as reference variables can provide different allometries.

TABLE 3. Effects of sex and season on the number of BrdU-labelled cells per hemisection in the MOB and AOB, LC and NS 3 weeks after administering BrdU

	Sex			Season		Interaction	
	$F_{1,35}$	P-value	M vs. F	$F_{1,35}$	P-value	$F_{1,35}$	P-value
MOB	7.42	<0.05	M > F	0.06	0.81	1.51	0.23
AOB	7.59	<0.01	M > F	1.55	0.22	0.44	0.51
LC	5.59	<0.05	M > F	0.01	0.94	0.72	0.40
NS	5.26	<0.05	M > F	0.05	0.83	3.74	0.06

AOB, accessory olfactory bulb; LC, lateral cortex; MOB, main olfactory bulb; NS, nucleus sphericus.

Sexual dimorphism in the olfactory bulbs has been so far described only in mammals: reed voles (Tai *et al.*, 2004), mice (Weruaga *et al.*, 2001), rats (Segovia *et al.*, 1984), rabbits (Segovia *et al.*, 2006), ferrets (Waters *et al.*, 2005) and opossums (Mansfield *et al.*, 2005). In most of these species, as in *P. hispanica*, males have larger AOBs than females. Often sexual dimorphism affects not only AOBs but also other structures of the vomeronasal system. In rats, for example, the vomeronasal system comprises a network of sexually dimorphic structures that begins in the vomeronasal organ and ends in the diencephalon and brain stem (Segovia & Guillamón, 1993; Simerly, 2002). In reptiles, some of the primary and secondary efferents of AOB, such as the NS, amygdala, anterior hypothalamus-preoptic area or ventromedial hypothalamus are sexually dimorphic as well (Stoll & Voorn, 1985; Crews *et al.*, 1990, 1993; Kabelik *et al.*, 2006). The available data are scant and come from studies of merely a handful of lizard species, but they suggest that the vomeronasal system may be, at least in part, sexually dimorphic in lizards and possibly in other reptiles.

Three weeks after BrdU administration, large numbers of BrdU-labelled cells were found in the telencephalon of *P. hispanica*. Hundreds of BrdU-labelled cells were found in all the chemosensory brain areas studied, most of them located in the granular cell layer of the olfactory bulbs and in the ventricular zone of the LC and NS. Previous studies have investigated the origin and phenotype of these newly generated cells. New neurons in the adult lizard brain are generated in the ventricular zone of the lateral ventricles. From this germinative zone, some neurons migrate locally to overlying layers (e.g. from ventricular zone to cell layer in LC and NS), whereas others migrate long distances (from rostral forebrain to the olfactory bulbs, in a way analogous to the rostral migratory stream described in mammals; reviewed in Font *et al.*, 2001). During their migration, newly generated cells express transiently the neuron-specific markers doublecortin and polysialylated form of the neural cell adhesion molecule (PSA-NCAM), and acquire ultrastructural characteristics of neurons once they have reached their final destination (Ramírez-Castillejo *et al.*, 2002; González-Granero *et al.*, 2006). These studies essentially have shown that cell proliferation in the ventricular zone of lizards produces new neurons and no free astroglial cells (Font *et al.*, 2001). This conclusion is supported by reports indicating that free astroglial cells are very scarce in the telencephalon of lizards (Lazzari & Franceschini, 2001).

Confocal microscopic analysis of double-immunostained newly generated cells (BrdU-doublecortin) in our study confirmed previous results obtained in lizards. Most BrdU-labelled cells in the olfactory bulbs and outside the ventricular zone in the LC and NS were immunoreactive to doublecortin, which suggests that they were neuroblasts or immature neurons. It seems thus reasonable to suggest that, at least in the olfactory bulbs of *P. hispanica*, sex differences in the number of BrdU-labelled cells are the result of differences in the production/migration of neurons. This is the first demonstration of sexual dimorphism in the proliferative activity of chemosensory areas in non-mammals, which at least in the olfactory bulbs most likely represents a sex-related difference in neuronal production. Thus far, evidence of sexually dimorphic cell proliferation/adult neurogenesis in chemosensory brain areas of other vertebrates has only been reported in rats, in which, 20 days after administration of BrdU, more migrating neuroblasts were detected in AOB of males than in those of females (Peretto *et al.*, 2001). Sex differences in cell proliferation or survival have been found elsewhere in the vertebrate brain, such as in the avian song control nuclei (Rasika *et al.*, 1994), the hippocampus of rodents (Galea & McEwen, 1999; Tanapat *et al.*, 1999), and the hypothalamus and cerebellum of some teleost fishes (Zikopoulos *et al.*, 2001; Ampatzis & Dermon, 2007).

Altogether, our results reveal a male-biased dimorphism in the size of the brain and olfactory bulbs, as well as a male-biased dimorphism in cell proliferation. In zebra finches, sex differences in the song control system have been related to differences in the incorporation of neurons (Nordeen & Nordeen, 1988). Between 20- and 55-day old, the number of neurons and the volume of the HVC increase in males, whereas they remain roughly constant in females. During this period, both neurogenesis and cell death occur in males and females, but the higher addition of neurons in males contributes to sex differences in volume and neuron number (Nordeen & Nordeen, 1988). In lizards, the brain shows ongoing growth throughout adulthood (Platel, 1974), and during this period the size of the entire brain diverges considerably between the sexes (Platel, 1975). This adult brain growth is in part the result from the continued addition of neurons and their long-term survival throughout adulthood, apparently in absence of neuronal replacement (Font *et al.*, 2001). In this developmental scenario, sex differences in the addition of neurons in *P. hispanica* may contribute, like in songbirds, to the development of sexually dimorphic brains.

In vertebrates with neural sexual dimorphisms, sex steroid hormones play a crucial role in generating sex differences (Cooke *et al.*, 1998). Particularly in lizards, there is ample evidence that demonstrates that sex steroid hormones are involved in the sexual differentiation of preoptic-hypothalamic areas (Godwin & Crews, 2002) and some neuromuscular systems (Lovern *et al.*, 2004). Although scarcer, some studies have also found a relationship between sex steroids and sexual dimorphism in some chemosensory brain areas (Crews *et al.*, 1993; Kabelik *et al.*, 2006, 2008). Sex steroid receptor-containing cells have been found in the NS and amygdala of some reptiles, which suggests that sex steroids may act directly on these areas (e.g. Halpern *et al.*, 1982; Rhen & Crews, 2001). Further studies should clarify the role that sex steroids exert on the development of sexual dimorphism in the brain of *P. hispanica*.

Some studies have shown a correlation between larger structures, higher rates of neurogenesis or neuronal recruitment, and enhanced behaviours. In black-capped chickadees, the volume and neuronal incorporation in the hippocampus increase during the fall, when food-storing is more intense and the requirements for spatial memory are

higher (Barnea & Nottebohm, 1994; Smulders *et al.*, 2000; Hoshooley *et al.*, 2007). In many songbirds, the volume and neuronal recruitment of song control nuclei increase in anticipation of the breeding season, coinciding with the enhancement of song production and learning (reviewed in Tramontin & Brenowitz, 2000). To date there is no evidence indicating similar changes in chemosensory brain areas, although seasonal growth has been reported in the olfactory epithelium of crucian carps (*Carassius carassius*; Hamdani *et al.*, 2008) and the vomeronasal organ of red-backed salamanders (*Plethodon cinereus*). In salamanders, a peak of cell proliferation in late spring has been suggested as the basis for the growth of the vomeronasal epithelial volume in the summer. This larger epithelium has been related to the salamander's extensive territoriality depending upon chemoreception during the summer (Dawley *et al.*, 2000, 2006).

Despite clear-cut seasonal variation in whole brain size and a positive scaling between olfactory bulb and whole brain size, we did not detect seasonal changes in the size of the olfactory bulbs. This lack of seasonality was also present in the number of BrdU-labelled cells: BrdU-labelled cells were more abundant in November males than in April males, but the seasonal difference was not large enough to be detected by our statistical analyses. The absence of seasonality in *P. hispanica* is unexpected considering that reproductive status, via sex steroid hormones, has been shown to affect brain size, cell proliferation and cell survival in other vertebrates (Wade & Crews, 1991; Galea & McEwen, 1999; Tramontin & Brenowitz, 2000; Ormerod & Galea, 2001, 2003). One possible explanation for the lack of seasonality in our study may be that seasonal changes in cell proliferation or survival do exist, but they cannot be detected because of high intra-group variability and small sample sizes. Alternatively, cell proliferation or survival may vary more conspicuously during seasons of the year other than those sampled for the present study. Seasonal changes in behaviour may also occur without significant changes in brain size or neurogenesis, as has been reported in multiple studies (O'Bryant & Wade, 1999; Weiler *et al.*, 1999; Lavenex *et al.*, 2000a,b; Leitner *et al.*, 2001; Hoshooley & Sherry, 2004).

Frequently, size differences in brain nuclei involved in the control of certain behaviours are causally linked to differences in the frequency or amplitude with which those behaviours are displayed (Godwin & Crews, 1997). Nevertheless, whether larger sensory structures (such as larger olfactory bulbs) confer enhanced sensation is currently unknown. In rodents, more receptor cells in the olfactory epithelium have been shown to improve chemical sensing acuity (Meisami, 1989; Hildebrand & Shepherd, 1997), whereas a higher neuronal recruitment in the olfactory bulbs can modify odour discrimination (Gheusi *et al.*, 2000) and olfactory memory (Rochefort *et al.*, 2002). Assuming that a larger AOB confers enhanced chemosensation (vomerolfaction), sex differences in the size of AOB should correlate with differences in chemosensory abilities mediated by the vomeronasal system. The available behavioural evidence in lizards supports this hypothesis, as some of the chemosensory abilities described in males are apparently absent from females (reviewed in Barbosa *et al.*, 2006). For example, male *P. hispanica* discriminate chemically between conspecifics and sympatric heterospecifics (*Podarcis bocagei*) of the opposite sex, whereas females are not capable of this type of discrimination (Barbosa *et al.*, 2006).

It is striking that, while sexual size dimorphism has been reported in more than 800 species of reptiles (Cox *et al.*, 2007), very few studies have described sexually dimorphic sensory brain areas or behaviours in these species. With respect to chemoreception, there is a vast literature describing the use of chemical senses by reptiles but, in most cases, they come from studies carried on a single sex (usually males).

Progress in the study of sexual dimorphisms and seasonality in the brain has benefited from the adoption of a comparative approach (e.g. in songbirds; Ball & MacDougall-Shackleton, 2001). The comparative approach has also been very useful for studying the role that reproductive status and hormones exert on neurogenesis (e.g. in rodents; Galea, 2008). Further studies with different reptile species are thus necessary to clarify how sexually dimorphic sensory brain areas control behaviour, and to elucidate how sex steroid hormones influence size and neurogenesis in these areas.

Acknowledgements

Abbreviations

ANCOVA, analysis of covariance; ANOVA, analysis of variance; AOB, accessory olfactory bulb; AOBL, accessory olfactory bulb length; BRAINL, brain length; BRAINWT, brain weight; BrdU, 5-bromo-2′-deoxyuridine; DAB, 3-3′-diaminobenzidine; LC, lateral cortex; MOB, main olfactory bulb; MOBL, main olfactory bulb length; NS, nucleus sphericus; SVL, snout to vent length.

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