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Retreat Sites Shared by Two *Liolaemus* Lizard Species: Exploring the Potential Role of Scents

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Abstract. Some lizard species modulate the use of a retreat site based on the presence of scents from other individuals, behavior that requires scent recognition. Here, we investigated if two congeneric and syntopic lizards (adults of *Liolaemus coeruleus* and juveniles of *L. ceii*, which can be found sharing retreat sites in the wild) discriminate scents from each other during the pre-hibernation period. If the presence of heterospecific scents modulates sharing retreat sites, species would discriminate each other's scents. Lizards were individually exposed to four treatments, which were enclosures with scents of (1) from the focal individual (own); (2) a conspecific of the same sex as the focal lizard; (3) a heterospecific of the same sex as the focal lizard; and (4) a control (i.e., enclosure with a clean substrate). Our results suggest that there is no heterospecific recognition. This finding was not due to an inability to chemo-assess scents, since both species exhibited self-recognition (recognition of their own scents) and juveniles of *L. ceii* also showed conspecific recognition. Although it might be advantageous for species to share retreat sites, chemical recognition of heterospecific scents does not modulate this behavior in these syntopic species.

Keywords. Conspecific recognition; *Liolaemus ceii; Liolaemus coeruleus*; Self-recognition; Tongue flicks.

Resumen. Algunas especies de lagartos modulan el uso de un sitio de retiro basados en la presencia de rastros de olores de otros individuos presentes en el sitio, comportamiento que requiere reconocimiento de estos rastros de olores. En este trabajo, investigamos dos lagartos cogenéricos y sintópicos (adultos de *Liolaemus coeruleus* y juveniles de *L. ceii*, los cuales pueden ser encontrados en la naturaleza compartiendo sitios de retiro), si discriminan los rastros de olores de cada uno de ellos durante el periodo pre-invernal. Si la presencia de rastros de olores heteroespecíficos modula el compartir sitios de retiro, ambas especies discriminarían los rastros de olores entre sí. Los lagartos fueron expuestos individualmente a cuatro tratamientos, los cuales fueron los recintos con diferentes olores de: (1) propio, del individuo focal; (2) conespecífico del mismo sexo que el lagarto focal; (3) heteroespecífico del mismo sexo que el focal y (4) control, i.e., un recinto con substrato limpio. Nuestros resultados sugieren que no habría presencia de reconocimiento químico heteroespecífico, lo cual, no es consecuencia de una incapacidad para evaluar rastros químicos, puesto que ambas especies exhibieron auto reconocimiento (reconocimiento de sus rastros de olores propios) y los juveniles de *L. ceii* además exhibieron reconocimiento de sus conespecíficos. A pesar de que estas especies puedan beneficiarse de compartir sitios de refugio, aparentemente, el reconocimiento químico de los rastros de heterospecíficos, no modularía el sitio de refugio compartido por estas especies sintópicas.

INTRODUCTION

Most animal species need to use retreat sites to protect themselves and/or their offspring (Mateo and Cuadrado, 2012) against different factors such as predation (Downes and Shine, 1998) or environmental conditions, such as extreme temperatures (Aguilar and Cruz, 2010; van den Berg et al., 2015). Evidence indicates that retreat sites are not chosen randomly, as they need to fulfill some basic requirements (e.g., Croak et al., 2008; van den Berg et al., 2015) such as an adequate size (Kroon et al., 2000; Caruso, 2016) or a proper three-dimensional structure (Croak et al., 2008). Besides these basic constraints, retreat site selection can also be modulated by intrinsic characteristics of the individuals, such as age, sex, or/and reproductive condition (Rutherford and Gregory, 2003;

Vasconcelos et al., 2017). All these requirements determine that retreat sites can be a scarce resource for which animals need to compete (Marvin, 1998; Penado et al., 2015). There are, however, cases in which animals share these sites (Mouton, 2011; Mori et al., 2015), as occurs, for example, in communal nesting, a behavior observed across diverse taxa (e.g., mammals, Auclair et al., 2014; birds, Bertram, 2014; reptiles, Dayananda et al., 2016).

For several lizard species, retreat site selection is modulated by the presence of scents from other individuals (e.g., Hayward and Mouton, 2007; Lewis et al., 2007), and the selection process requires scent recognition. In fact, there is clear evidence that sites that contain scents associated with a threat, from conspecifics or heterospecifics, are avoided (e.g., lizards reject sites with predator scents; Stapey, 2003; Amo et al., 2004; Lloyd et al., 2009).

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The threat imposed by conspecifics is exemplified by Podarcis hispanica (Steindachner, 1870), in which females prefer retreat sites with scents from a small male rather than those from a large male, which should reduce the possibilities of sexual harassment (Carazo et al., 2011). Similarly, after agonistic interactions, loser males of Oedura lesueuri (Duméril and Bibron, 1836) prefer retreat sites with scents of unknown males over those with winners' scents (Kondo et al., 2007). In the case of Egernia stokesii Gray, 1845, individuals select retreat sites with scents of familiar rather than unfamiliar conspecifics (Bull et al., 2000). In contrast, for some species retreat site selection is neither modulated by conspecific nor heterospecific scents (e.g., Cooper et al., 1999; Hibbitts and Whiting, 2005; Paulissen, 2006), and the physical presence of an individual seems to be the key element for individuals to choose or reject a retreat site (Schlesinger and Shine, 1994; Langkilde and Shine, 2005).

In Argentinean Patagonia, we observed adults of Liolaemus coeruleus Cei and Ortiz-Zapata, 1983 and juveniles of L. ceii (Donoso-Barros, 1971) in shared retreat sites formed by small rocks in the soil. To understand the mechanism involved in retreat site sharing, and specifically to determine if this behavior is modulated by the presence of heterospecific scents, we tested the ability of these two lizards to discriminate between each other's scents. Since studies have shown that species of Liolaemus Wiegmann, 1834 exhibit chemical discrimination between conspecific and congeneric scents (e.g., Labra, 2011), we postulate that both species may show discrimination of heterospecifics scents. We performed the study during the pre-hibernation season, when lizards are more engaged in acquiring good retreat sites, since the hibernation period is arriving. This increases the possibility of aggregation (e.g., Weintraub, 1968; Elfström and Zucker, 1999; Bishop and Echternacht, 2004).

MATERIALS AND METHODS

Collection and maintenance of animals

As part of an ongoing project, in February 2014 during a field trip near Alumine, Neuquén (Route 13 between Kilka and Primeros Pinos: $38^{\circ}54'14.70''S$, $70^{\circ}43'59.50''W$), we found adults of *Liolaemus coeruleus* sharing retreat sites with juveniles of *L. ceii*. We collected 16 *L. coeruleus* (nine females, seven males) and 11 *L. ceii* (five females, six males). Lizards were captured by hand and kept in individual cloth bags with their identification until arriving at the laboratory, where we measured and weighted them (Table 1). Thereafter, we placed lizards individually in plastic enclosures ($36 \times 27 \times 19$ cm) covered with a plastic mesh lid. Enclosures had 3 cm of sandy substrate, a rock that served as shelter and basking site, and a

Table 1. Descriptive statistics showing $X \pm SE$ (minimum–maximum) of snout–vent length (SVL) and weight (W) of *Liolaemus coeruleus* and L. ceii. Values of t-tests between species are included. In bold, the statistically significant P values (P < 0.05).

Variables	L. coeruleus	L. ceii	t-test (P)
SVL (mm)	54.31 ± 0.77 (50.11-61.36)	53.48 ± 0.47 (50.64–55.45)	0.87 (0.39)
W (g)	6.05 ± 0.22 (4.55–7.45)	4.92 ± 0.16 (4.15–5.70)	4.10 (< 0.001)

small bowl of water. Lizards were maintained in an isolated room with a summer photoperiod (13:11 h, light:dark) using halogen lamps, which also allowed maintaining a mean \pm SD ambient temperature of 30°C \pm 2°C during the light phase. Every other day, we fed each lizard with two *Tenebrio mollitor* Linnaeus, 1758 larvae, dusted with vitamins. Before running the experiments, lizards remained undisturbed in their enclosures for one week. This allowed lizards to habituate to the experimental conditions and to release scents, because enclosures were used as the substrate-borne scents (for more details see Labra, 2011).

At the end of all experiments, and because individuals were primarily collected for systematic studies, they were euthanized via a pericardial injection of pentothal, following standard procedures (Scrocchi and Kretzschmar, 1996). Lizards were fixed in 10% formol and conserved in 70% ethanol to be deposited in the Herpetological collection of Instituto de Bio y Geociencias del NOA (IBIGEO). We dissected the individuals to confirm that adults of *Liolaemus coeruleus* were in post-reproductive condition and that all individuals of *L. ceii* were juveniles, as well as to confirm their sex.

Experimental design

Using a counterbalanced design, each lizard was submitted individually only once to each of the four treatments (henceforth: "scents"). Following established protocols (Labra et al., 2003; Labra, 2011), we used as a source of scents enclosures previously used by: (1) the focal individual (own), (2) a conspecific of the same sex of the focal individual, (3) a heterospecific of the same sex of the focal lizard, and (4) an odorless control (i.e., an unused enclosure with clean sand). Before starting an experiment, we removed the occupant ("donor") of the enclosure that would be used for the experiment ("experimental enclosure"), together with the water container and the refuge. We also removed the focal lizard from its enclosure and placed it in its individual cloth bag for 10 min to minimize the stress associated with handling (Labra, 2011). Next, we placed the bag on the sand of the experimental enclosure, allowing the lizard to exit freely. Then, we removed the bag and, once we were out of the lizard's field of vision, began recording with a digital stopwatch the time to the first tongue flick. This is the period from when the lizard came into contact with the enclosure substrate (without our perturbation) until it's first tongue flick, which represents the beginning of chemical exploration (e.g., Labra and Niemeyer, 1999). Thereafter, we recorded the lizard's behavior for 8 min using a digital video camera (Sony DCR-SR67) installed 50 cm above the enclosure and connected to a monitor. We accepted as maximum time to the first tongue flick 7 min; otherwise, we canceled the trial and repeated it another day (n = 1). At the end of a trial, we verified that lizards (focal and donor) were healthy and returned them to their respective enclosures, remaining undisturbed for at least 3 d before a new trial. We used clean gloves for each trial to avoid scent cross-contamination and potentially bias responses, and appropriate actions were taken to minimize the stress of lizards during the whole process.

Digital videos were subsequently analyzed using VLC Media Player 2.2 (VideoLan, 2006). From videos, we recorded the following behaviors:

- (1) Time in motion (s): index of behavioral exploration, which includes the total time that the lizard moved and changed its position, either the whole body or part of it. Data for *Liolaemus* species show that lizards increase their time in motion when they are exposed to scents that require getting more information (e.g., new scents; Labra, 2008a). A reduced exploration of scents may indicate that they are associated with potential risk (e.g., Labra and Hoare, 2015) or are very familiar (e.g., own scents) and do not require further exploration (e.g., Troncoso-Palacios and Labra, 2012). This variable excluded motions associated with the behaviors described below.
- (2) Time escaping (s): total time spent excavating or standing up against the walls of the enclosure. This behavior may indicate a potential threat detected by the lizard (Font and Desfilis, 2002).
- (3) Number of tongue flicks: index of chemical exploration (Font and Desfilis, 2002) that considers the number of times the lizard protrudes and rapidly retracts its tongue, regardless of whether the tongue touches the substrate or wall or is waved in the air (e.g., Labra, 2008b).
- (4) Marking behavior: the lizard drags its cloaca, which may help to release scents (Alberts, 1992).
- (5) Head-bob displays: stereotyped up and down head movements, usually exhibited in social interactions, involving demonstration of fighting or defense abilities, toward a competitor (Labra et al., 2007) or a predator (Ortega et al., 2017).
- (6) Forelimb displays: forearm waving or circular motions of the forelimbs. Its function in *Liolaemus* is not completely clear, but it is possibly associated with intraspecific communication denoting challenging or

- relative arousal, conflict, or appeasement behavior (Halloy and Castillo, 2002). Alternatively, this can be an antidepredator behavior (e.g., Font et al., 2012).
- (7) Tail waving: rapid side-to-side movement of the entire or the posterior portion of the tail, displayed in agonistic contexts, probably as a demonstration of strength (Vitt et al., 1974). This display can also be exhibited during predation risk (Ortega et al., 2017).

Statistical analysis

We compared the body measurements (snout-vent length and weight) between the species using paired t-tests for independent groups (Liolaemus coeruleus, L. ceii). To determine whether there was an effect of the species (L. coeruleus, L. ceii), scent (own, conspecific, heterospecific, control), and their interactions upon four variables (latency to first tongue flick, time in motion, time escaping, and number of tongue flicks), we used two-way ANOVAs for repeated measurements (scents). Analyses were followed by post-hoc Fisher LSD tests. The residuals of these four variables exhibited normality, except those of time in motion, which was square-roottransformed to fulfill the assumptions of normality and homoscedasticity. Preliminary analyses indicated that sex did not modulate any of these four variables; therefore, we did not include this factor. The other variables recorded (marking behavior, head-bobs displays, forelimb displays, and tail waving) were exhibited in low frequency, and therefore, we pooled them in a new variable named "displays" (for details see Labra, 2006). We tested the effect of scents over displays with Friedman nonparametric tests. For this variable, preliminary analyses showed an effect of the sex, and we analyzed this factor for each species using Friedman tests followed by Wilcoxon matched tests. We present data as $\bar{x} \pm SE$.

RESULTS

Adults of *Liolaemus coeruleus* and juveniles of *L. ceii* had similar body size, although *L. ceii* was lighter (Table 1). Both the latency to the first tongue flick and time escaping were unaffected by the studied factors (species and scents) or their interactions (Table 2). The mean values of latency were $\bar{x}=47.27\pm5.06$ s for *L. coeruleus* and $\bar{x}=36.26\pm6.10$ s for *L. ceii*, while the mean time escaping was $\bar{x}=85.90\pm10.92$ s and $\bar{x}=64.70\pm13.03$ s for each species, respectively.

Time in motion differed between species, with *Liolaemus coeruleus* moving less than *L. ceii* (Table 2; Fig. 1A). Further, this variable was also affected by the interaction between the factors species and scents (Table 2). Figure 1A shows that the species behaved differently when

Table 2. Results of the two-way ANOVA for repeated measurements to determine the effect of species (*Liolaemus coeruleus* vs. *L. ceii*), scents (conspecific, heterospecific, control, and own), and their interactions (species * scents) over: latency to first tongue flick, number of tongue flicks, motion time (square–root-transformed), and time escaping. df, degrees of freedom, and F statistics (P value); in bold, the statistically significant results (P < 0.05).

	Df	Latency first TF	Time motion	Time escaping	№ tongue flicks
Species	1.25	1.59 (0.21)	68.43 (< 0.001)	1.56 (0.22)	15.50 (< 0.001)
Scents	3.75	0.89 (0.45)	0.49 (0.691)	0.74 (0.52)	3.23 (0.027)
Species * Scents	3.75	0.67 (0.57)	8.85 (< 0.001)	0.73 (0.53)	2.87 (0.04)

confronted with their own scents. *Liolaemus coeruleus* moved less when exposed to its own scent than to scents of conspecifics (P = 0.030), heterospecifics (P = 0.041), and the control (P = 0.047). In contrast, *L. ceii* moved more when exposed to its own scent than to conspecific (P < 0.01), heterospecific (P < 0.01), or control scents (P < 0.01, see Fig. 1A).

The number of tongue flicks was affected by the two factors analyzed and their interaction (Table 2). First, Liolaemus coeruleus exhibited significantly fewer tongue flicks than L. ceii (Fig. 1B). Second, lizards, independent of the species, made more tongue flicks to conspecific scents than to any other scents. Third, the interaction between species and scents showed that individuals of

 $L.\ coeruleus$ made fewer tongue flicks when exposed to their own scents than to conspecific (P=0.012), heterospecific (P=0.037), or control scents (P=0.048). In contrast, individuals of $L.\ ceii$ made more tongue flicks when they were exposed to scents of conspecifics than to heterospecifics (P=0.009) or to control scents (P=0.006), but made a similar number of tongue flicks when exposed to scents of conspecifics and their own scents (P=0.161; Fig. 1B).

The frequency of displays was similar across scents in *Liolaemus coeruleus* ($\chi^2_{16(3)} = 3.83$; P = 0.282; Fig. 2A) and L. ceii ($\chi^2_{11(3)} = 6.38$; P = 0.095; Fig. 2B). However, the analysis by sex showed that males of L. coeruleus (Fig. 3) displayed differently with the diverse scents ($\chi^2_{7(3)} = 7.96$; P = 0.047); males exposed to heterospecific scents displayed more than when exposed to their own scents (P = 0.043; Fig. 3); specifically, they exhibited tail waving displays. No other comparison was statistically significant (P > 0.05). Females did not exhibit behavioral differences across scents ($\chi^2_{9(3)} = 7.094$; P = 0.068). In the case of L. ceii, males displayed similarly across the different scents ($\chi^2_{6(3)} = 5.09$; P = 0.173), as well as females ($\chi^2_{5(3)} = 4.00$; P = 0.260).

DISCUSSION

In the Patagonian steppe of Neuquén, we found that adults of *Liolaemus coeruleus* and juveniles of *L. ceii* shared

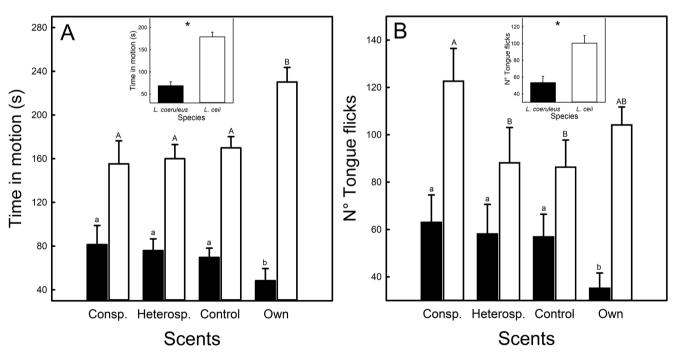


Figure 1. Mean + SE of **(A)** time in motion and **(B)** tongue flicks recorded in the four treatments (scents: conspecific, heterospecific, control, and own) for *Liolaemus coeruleus* (black bars) and *L. ceii* (white bars). Insert shows the mean value (+ SE) of time in motion and tongue flicks exhibited by each species in the different treatments. Different letters and asterisks indicate significant differences (*P* < 0.05); lower and upper case letters indicate intraspecific comparison for *L. ceii*, respectively. Untransformed data of time in motion are presented.

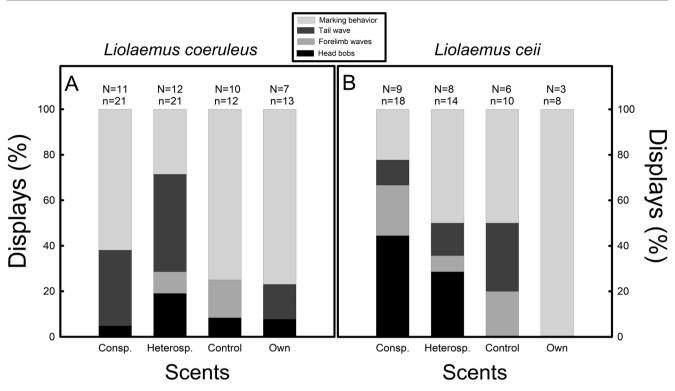


Figure 2. Frequency of the four behaviors included in the "displays" variable (head-bob displays, forelimb waves, tail wave, and marking behavior) exhibited in the four treatments (scents: conspecific, heterospecific, control, and own). **(A)** *Liolaemus coeruleus.* **(B)** *L. ceii.* N = number of individuals that displayed; n = total number of displays exhibited during treatment.

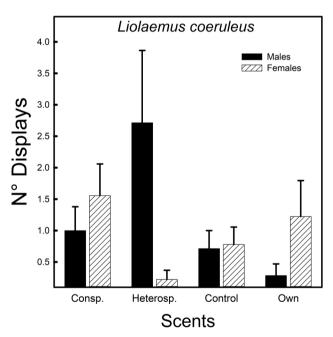


Figure 3. Mean + SE of displays observed in the four treatments (scents: conspecific, heterospecific, control, and own) by both sexes of Liolaemus coeruleus.

retreat sites. Our experiments suggest that this sharing is not modulated by the presence of heterospecific scents; lizards did not exhibit behavioral evidence of chemical recognition of heterospecific scents. There was only very weak evidence that males of *L. coeruleus* discriminate

between heterospecific and own scents, given that they exhibited more tail waving when confronting scents of males of L. ceii. Under the scenario of an interspecific confrontation, the tail movement might signal dominance (Fox et al., 1990), as has been observed during agonistic interactions involving aggressive displays using tail movements (Peters et al., 2016).

In Liolaemus, chemical recognition has usually been established on the basis of variations in time in motion and number of tongue flicks in the presence of different scents, and thus a behavioral variation among scents suggests scent discrimination (e.g., Labra, 2008b). Under this paradigm, it has been proposed that Liolaemus species discriminate heterospecific scents, prey (Mora and Labra, 2017; Ruiz-Monachesi and Valdecantos, 2017), predators (Troncoso-Palacios and Labra, 2012; Labra and Hoare, 2015), and congenerics (Labra, 2011). In contrast, L. coeruleus and L. ceii exhibited similar exploratory behaviors (time in motion and tongue flicks) when confront scents of heterospecifics and the control. This apparent lack of heterospecific (congeneric) scent recognition could be a consequence of two, non-mutually exclusive factors: 1) Heterospecifics scents constitute an irrelevant stimulus and do not trigger any major response (Paulissen, 2006), even if scents are perceived. 2) There are seasonal changes that modulate the discrimination behaviors. Such seasonal changes can entail different compounds and amounts of secretions (Alberts et al., 1992; Martins et al., 2006),

which can be used as cues by heterospecific individuals to assess the presence of the other species. Further, these two species might experience seasonal changes in chemical recognition, as reported in other *Liolaemus* species that exhibit less discrimination after reproduction or before hibernation (Labra et al., 2001; Labra et al., 2003; Labra, 2008b; Vicente and Halloy, 2016). Therefore, considering that our study was carried out during the post-reproductive and pre-hibernation season, seasonal changes in recognition might explain the lack of heterospecific recognition, as occurs, for example, in snake scent detectability, which varies across seasons (Hayes et al., 2006).

The lack of a clear evidence of heterospecific recognition by these two Patagonian lizard species do not imply the absence of chemo-recognition, as both species exhibited self-recognition, such as other *Liolaemus* species do (Labra, 2008a,b; Troncoso-Palacios and Labra, 2012; Labra and Hoare, 2015; Vicente and Halloy, 2018). Self-recognition is considered the simplest and most basal type of chemical recognition (Alberts, 1992), and in the studied species, this ability might allow individuals to recognize their own space or the areas normally visited by them, potentially the retreat sites.

We found that Liolaemus coeruleus showed lower exploration towards own scents (i.e., less time in motion and fewer tongue flicks) than other scents, which indicates that this species exhibits self-recognition (e.g., Labra, 2008a). This is remarkable, because L. coeruleus lacks precloacal glands (Cei and Ortiz-Zapata, 1983), and it has been proposed that the lack of pheromonal glands would be associated with a low ability to use scents (e.g., Phrynosomatidae Fitzinger, 1843, Hews and Benard, 2001; Lacertidae Gray, 1825, Baeckens et al., 2015). Although precloacal glands produce chemical secretions (Valdecantos et al., 2014) with pheromonal properties (Labra et al., 2005; Valdecantos and Labra, 2017), there are other sources of scents in *Liolaemus* species, including feces (Labra et al., 2002), skin derivates (Labra, 2008a) and, presumably, substances produced by proctodeal glands in males (Valdecantos et al., 2015) and urodeal glands in females (Sánchez-Martinez et al., 2007).

Juveniles of *Liolaemus ceii* also show evidence of self-recognition, but in contrast to the low level of exploration with their own scents exhibited by adults of *L. coeru-leus* and other *Liolaemus* species (e.g., Labra, 2008a), they were more active (motion time) when exposed to their own scents. Additionally, juveniles exhibited more tongue flicks in the presence of conspecific and own scents than in the presence of unknown scents (heterospecific and control). This suggests that species-specific scents (i.e., conspecific and own) are a relevant stimulus for the juveniles of *L. ceii. Liolaemus bellii* Gray, 1845 undergoes ontogenetic changes in response to own and control scents, whereby neonates, juveniles, and adults differed in the pattern of self-chemical recognition, which was proposed

as a learning process to the own scents (Labra et al., 2003). Potentially, the greater environmental exploration performed by the juveniles of L. ceii (i.e., longer time in motion and more tongue flicks to own and conspecific scents) might also be part of a learning process that helps juveniles to consolidate the memory of the "species" and its own scents. It is necessary, however, to clarify whether adults of L. ceii exhibit the same behavioral pattern as juveniles or if, in fact, the behavior of these juveniles denotes ontogenetic changes in chemical recognition.

Regardless of whether or not sharing of retreat sites by these Patagonian species is modulated by the presence of heterospecific scents, individuals of both species might benefit from this behavior. The Patagonian steppe is characterized by extremely cold temperatures with high winds (Paruelo et al., 1998), and suitable thermal refuges are limited (Aguilar and Cruz, 2010). Sharing retreat sites might provide lizards with the thermal benefits of huddling behavior, which would help them maintain a stable body temperature, as Shah et al., (2003) postulated to explain aggregation in the gecko Nephrurus milii (Bory de Saint-Vincent, 1825). Moreover, considering that adult Liolaemus coeruleus and juvenile L. ceii are similar in size, sharing retreat sites might reduce predation risk via the dilution effect (Mouton, 2011). In the area where these Patagonian species occur, some predators use the same type of retreat sites as these two lizard species, such as Diplolaemus Bell, 1843 lizards (Garcia et al., 2015) and Brachistosternum Pocock, 1893 scorpions (e.g., Pérez et al., 2010). Potentially, the retreat site selection by these species of Liolaemus might be more affected by predator scents (e.g., Stapey, 2003; Amo et al., 2004; Lloyd et al., 2009), particularly considering that other Liolaemus species are known to respond to predator scents (Labra and Niemeyer, 2004; Troncoso-Palacios and Labra, 2012; Labra and Hoare, 2015). We postulate that for these two Liolaemus species, predator scents might be a more relevant stimulus to select a retreat site than those from nonrisky congeners.

In summary, the evidence gathered here does not support the hypothesis that sharing of retreat sites by adult *Liolaemus coeruleus* and juvenile *L. ceii* is mediated by the scent of these congeneric species during the prehibernation season. The absence of heterospecific recognition, however, does not imply an inability to chemo-assess scents, as *L. ceii* showed conspecific recognition and both species showed self-recognition.

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