

GROUP LIVING, PARENTAL CARE, AGE
STRUCTURE, AND GENETIC RELATEDNESS IN
LIOLAEMUS LEOPARDINUS, A HIGH-ELEVATION
LIZARD FROM THE ANDES OF CHILE

By

ENRIQUE SANTOYO BRITO

Licenciatura en Biología
Universidad Veracruzana
Córdoba, Veracruz, México
2006

Master of Science in Wildlife Management and
Conservation
Colegio de Postgraduados
Texcoco de Mora, Estado de México, México
2009

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Dissertation Approved:

Dr. Stanley F. Fox

Dissertation Adviser

Dr. Jennifer Grindstaff

Dr. Matthew Lovern

Dr. Timothy O'Connell

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Abstract: Social behavior refers to any interactions between two or more conspecifics. A relatively common social behavior in the animal kingdom is group living, which provides benefits to group members (e.g., enhanced vigilance and predation avoidance). Predation risk is an important force that selects for behavioral traits, and promotes the evolution of sociality favored by kin selection. Most studies of sociality have focused on animal groups in which interactions are overtly obvious. Although interactions differ among the various vertebrate lineages, taxa are often categorized as social or non-social, ignoring the diversity and complexity of social behavior. Reptiles have been usually labeled as non-social; however, the degree of sociality can vary among species as shown in the genus *Egernia*.

Liolaemus leopardinus is a high-elevation lizard species endemic to the Chilean Andes. It is viviparous, lives in large colonies, and adults and juveniles are highly social, but little is known about the natural history of the species. Our findings over two field seasons suggest that bird predation causes mothers of *L. leopardinus* to engage in parental behavior, and that predation by birds forces newborns of the species to behave secretively and to seek solitary refuge underneath rocks partially covered by bushes. Skeletochronology revealed the formation of annual growth rings in the phalangeal bones of subjects of *L. leopardinus*; however, the method was poorly suited for aging individuals. However, when body size of various free-ranging subjects repeatedly captured and measured was plotted against activity periods of three years, we could assign individuals to four age groups, and estimated the age when female lizards became sexually mature. We provide spatial, behavioral, and genetic evidence that supports the conclusion that *L. leopardinus* forms social groups of closely related individuals with non-relatives mixed in. Genetically related juveniles and adults spend time in close association, share home ranges and communal refuges during the day and night, and overwinter together in deep rock crevices. As a whole, our study revealed fine details regarding the gregarious behavior of *L. leopardinus*, a social but highly secretive species. We suggest that predation, kin recognition, and roosting drive the evolution of sociality in *L. leopardinus*, and not thermoregulation, refuge availability, or food.

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CHAPTER I

PREDATION PRESSURE SHAPES BEHAVIOR OF MOTHERS AND NEONATES OF THE LIZARD *LIOLAEMUS LEOPARDINUS* IN THE HIGH ANDES OF CHILE

The following chapter is formatted for *Behavioral Ecology*:

Abstract

Parental care is any form of parental behavior that increases survival of the offspring; this benefit outweighs the cost of parental care. It is well documented that predation is an ecological pressure that triggers such behavior. In this study we documented the behavior of pregnant adult females and neonates of *Liolaemus leopardinus*, a social, high-elevation, saxicolous, viviparous lizard. The species is endemic to central Chile and its distribution is limited to the presence of rock outcrops above timberline. During March-April, when females give birth, birds are abundant at our study site--El Colorado--where it is very common to see foraging *Geositta* and *Agrionis* spp., known predators of neonate *L. leopardinus*. Our results from two field seasons during austral late-spring and summer of 2011-12 and 2012-13 suggest that bird predation is the ecological pressure that has caused pregnant females to leave their social groups to seek out protected habitats to give birth underneath flat rocks and protect their frail newborns from potential predators. Additionally, newborns receive indirect benefits (i.e., thermoregulation) from the habitat selected by the mother. The flat rocks are surrounded by dense spiny bushes located in open terrain. Our results on predation pressure measured from clay models suggest that lizards' vulnerability to bird predation decreases with body size and use of shrubby habitat. The neonates' secretive behavior contrasts to that exhibited by juveniles and adults, who form groups on open rock outcrops and are rarely attacked by birds.

Key words: Aves, predation, squamate, *Liolaemus*, neonate

Parental care is any form of care expected to increase the successful production of offspring; it is a form of parental behavior (Clutton-Brock 1991; Smiseth et al. 2012) that has evolved independently in a broad range of taxa to cope with ecological pressures that could affect offspring survival (Clutton-Brock 1991; Balshine 2012; Trumbo 2012). Like any trait, parental care will evolve only if costs outweigh benefits (Clutton-Brock 1991; Davies et al. 2012). Parents give benefits to offspring by building them a nest or burrow, brooding them, ensuring adequate conditions for growth and survival, and providing food, physical protection from harsh temperatures, protection against predation and cannibalism, and access to a high quality habitat or territory (Clutton-Brock 1991; Alonso-Alvarez and Velando 2012). Costs (to the parents) can be energetic, as in traveling long distances looking for optimal places to build nests or burrows, constructing the nest or burrow, or in making many trips to feed young; parental care may restrict parents from other concomitant activities like additional breeding opportunities, feeding, territory defense, predation avoidance, etc., (Clutton-Brock 1991; van den Berghe 1992; Alonso-Alvarez and Velando 2012; Santos and Nakagawa 2012). Parental care is favored by natural selection when the benefits of producing viable offspring--and lifetime reproductive success--outweigh the costs of the care provided (Clutton-Brock 1991; Reguera and Gomendio 1999; Klug and Bunsall 2014).

It has been well documented that predation is a selective force that molds the evolution of antipredator traits; for example, direct repulsion, mimicry, startle response, aposematic coloration, crypsis, etc. (Krebs and Davies 1993; Davies et al. 2012). Among those traits, cryptic behavior (i.e., remaining still) and cryptic coloration (i.e., background matching) (Webster et al. 2009; Vignieri et al. 2010) are two very effective techniques animals use to avoid visually oriented predators (Diaz et al. 1983; Lima and Dill 1990; Capodeanu-Nägler et al. 2016). Animals may also reduce predation pressure through a shift to safer habitats (Pierce 1998; Dickman 1992; Winandy et al. 2016), especially for juveniles since small conspecifics--in relation to the adults' size--tend to have more predators than their adult counterparts (Reznick 1996; Costelloe and Rubenstein 2015). Even so, this

disproportionate predation pressure decreases over time as small juveniles achieve a certain size threshold (Caldwell et al. 1980; Smith 1983; Reznick 1996; Costelloe and Rubenstein 2015).

Liolaemus leopardinus is a high-elevation, viviparous lizard endemic to central Chile and faces low temperatures, long periods of reduced activities, and seasonal scarceness of resources. Fox and Shipman (2003) confirmed that the species lives in colonies, and forms social groups on bare, rock outcrops above timberline. Social groups vary in number, age, sex, and reproductive status of their members (SF Fox, H Núñez, E Santoyo-Brito, personal observation). In 2005, SF Fox and H Núñez observed that neonates were not found among these social groups (and we observed the same thing in 2013), perhaps due to strong size-specific predation combined with the risks of living in an open habitat. At the high elevations where *L. leopardinus* lives, the visually oriented predators that likely prey on *Liolaemus* lizards are two abundant species of shrike-tyrants (*Agrionis montana* and *A. livida*) and the less abundant American Kestrel (*Falco sparverius*) (Ridgely and Tudor 1994). In 2013 we confirmed *A. montana* as a predator of neonate *L. leopardinus*, and also discovered that the very common Rufous-banded Miner (*Geositta rufipennis*) preys on them (Santoyo-Brito et al. 2014). Thus, bird predation on small neonate *L. leopardinus* is intense.

To learn more about the effect of strong avian predation on the behavior of adult and neonate *L. leopardinus*, we conducted a thorough study during the austral summer and fall of 2011-12 and 2012-13 at our study site at El Colorado, Chile, at an elevation of 2760 m. We hypothesized that: 1) Avian predation will be stronger on smaller than bigger lizards, and weaker on those under cover than in the open. 2) Parental care will be in evidence, and mothers will give birth in habitats that offer benefits to neonates. 3) Mothers, because they are there at birth, may protect just-born neonates and directly repel predators if neonates are threatened. 4) Newborns will show cryptic behavior to avoid bird predation. We tested these hypotheses to obtain a deeper understanding of the behavior of neonates and adults in an environment with strong predation and to help comprehend the evolution of social behavior in *L. leopardinus*. At the same time, results from this species can be extrapolated to other high-altitude lizard species that face comparable ecological pressures and present similar behavior.

Materials and methods

Our study site was located in the Andean cordillera of central Chile at El Colorado (Fox and Shipman 2003), 35 km northeast of Santiago at 2760 m (33° 14' S, 70° 16' W). Lizards were captured via noosing and permanently marked by both a unique toe-clip and a dorsal color code of non-toxic latex paint. For color code combinations we used Blue (B), Orange (O), Red (R), White (W), and Yellow (Y).

In mid-March of 2012 and 2013, we estimated predation pressure from placement of 108 clay models of three sizes ($n = 36$ models/size) in three habitats (open rock faces, open soil, and under sparse ground cover; $n = 36$ models/habitat) for eight days for a total of 864 model days. To match snout-vent length (SVL) of each age class (40, 60, and 90 mm SVL, respectively) models were made of one hatchling *Crotaphytus collaris* (very similar body morphology to *L. leopardinus* at this young age), and one juvenile and one adult *L. leopardinus* deposited at the Collection of Vertebrates (COV) at Oklahoma State University. Lizard specimens were arranged in a basking position and covered with silicone (Dragon Skin® Series). Once hardened, the silicone was cut open and the lizard removed, leaving a detailed inverse mold. Molds were filled with custom mixed clay matching the background color of the lizards, and after removal of the models, black dots and a line resembling the dorsal vertebral line were painted over the body to match the color pattern of live animals. To prevent predators from carrying away the models, models were tied to a branch or rock in the appropriate habitat using monofilament fishing line. Models were placed 3 m or more from each other. We also used six camera-traps, each one placed at an approximate distance of 1 m from six different clay models located in either open soil, on open rock, or under vegetation. The camera (set to high sensitivity for motion triggering) was positioned horizontally at substrate level or above the model using a tripod. The camera was used to document the time and species that made attacks.

We tested for possible predation on *L. leopardinus* by a sympatric snake and lizard species. On 10 December 2012, we conducted two laboratory trials with two adult *L. leopardinus* (one male and one pregnant female) and an adult snake, the sympatric but quite rare saurophagous *Tachymenis chilensis*. Trials lasted 35 minutes each. The first interaction took place at 11:45 am; we

placed all three subjects in an inflatable wading pool (1.5 m diameter x 30.5 cm deep) for one hour at room temperature. The second interaction took place an hour later at 12:45 pm, but this time we placed all three subjects into a 48-quart cooler set at room temperature. In April 2013, we conducted one laboratory trial with a single *L. leopardinus* neonate and one adult male of the sympatric lizard, *Liolaemus nigroviridis*. The next day we repeated the experiment but this time with an adult male of the sympatric lizard *Liolaemus belli*. Interactions took place in mid-April, 2013, from 1:00 to 3:00 pm. Subjects were placed into a 48-quart cooler at room temperature and video-recorded so as to impose minimal disturbance. All subjects were released at their place of capture after trials.

During the austral summer and fall 2012-13, we attached radio-transmitters (model: BD-2H, 0.9 to 1.04 g, internal antenna; Holohil Systems, Ltd., Carp, Ontario, Canada) to the tail base of each of nine adult pregnant females. Subjects were radio-located from late December to early April at least two times a day from approximately 10:00 am to 6:00 pm. In late April of 2013 we glued a radio-transmitter (model BD-2x, 11.5 by 5.3 by 2.8 mm; 0.25 g, external antenna; Holohil Systems) to the dorsum of each of two neonates, and in November 2013 (early austral spring) we revisited our field site to capture and radio-tag the now 7-8 month-old juveniles (model BD-2x). To locate all radio-tagged subjects, we used a hand-held, three-element Yagi antenna and a radio-receiver (Model R-1000; Advanced Telemetry Systems, Inc., Isanti, Minnesota). When the subject was radio-located underneath a rock or within a rock crevice, we removed the co-axial cable from the antenna and used that end of the cable to pin-point the exact location. To observe and video-record any behavior of refuged lizards (adults or neonates), we used a Rigel digital video borescope (Medit Inc. Winnipeg MB, Canada) with a 360-degree, two-way articulating probe (4 mm in diameter and 2 m long).

From late March through all of April 2015--once we discovered the nature of the birthing sites--we measured temperatures of the microhabitat located underneath flat rocks similar to natal chambers during night and day hours (night hours: 8:00 pm to 6:00 am) day hours: (10:00 am to 6:00 pm). We placed 16 paired iButtons (iButtonLink, Whitewater MI, US); one underneath a flat rock and its pair underneath a nearby bush.

Results

Hypothesis 1, Avian predation will be stronger on smaller than bigger lizards, and weaker on those under cover than in the open—Small clay models (40.0 mm SVL) were attacked more often and large clay models (90.0 mm SVL) less often than expected by chance (Chi-square = 8.57, df = 2, P = 0.012). Models in the open (rock and soil) were attacked more often and those under bushes less often than expected by chance (Chi-square = 26.72, df = 1, P < 0.001). Marks left on the models (Fig. 1), indicated bill strikes from predatory birds. We observed many of the most suspected species, shrike-tyrants and miners (*Agriornis* and *Geositta* spp., respectively) at the study site. We did not capture any images of those species attacking models, but 12 pictures show *Geositta* sp. in proximity to the models (Fig. 2). During our complementary predation trials, *Tachymenis chilensis* did not attempt predation on adult *Liolaemus leopardinus*. The snake tongue flicked the air most of the time during the trials, and on three separate occasions crawled onto the dorsum of each lizard while the lizard stayed still. On two occasions the male tongue flicked the dorsum of the snake. In separate instances, both the male and the female undulated its tail, possibly as a deterrent signal against the snake. However, the lizards did not show any agonistic behavior against the snake even when all three subjects were confined in the 48-quart cooler. Neither adult of both sympatric lizard species--*L. nigroviridis* and *L. bellii*--harassed in any way (i.e., attack or chase) the neonate, and on one occasion the rostrum of *L. nigroviridis* and the neonate were in contact but the neonate did not flee nor react aggressively to the *L. nigroviridis*.

Hypothesis 2, Mothers will show parental care, and will give birth in habitats that offer benefits to neonates—During both field seasons—2011-12 and 2012-13--we captured and permanently marked 179 free-ranging adults, yearlings and neonates on our study site, and made a total of 1162 detections (visual or radio locations). In 2013 after radio-tracking females for almost four months, we discovered that although pregnant females spent most of their time with their social groups, in late term they occasionally took solitary refuge beneath large, flat rocks in open areas situated 10-70 meters from the rock outcrops. On 11 March 2013 we located individual WOWW underneath such a rock and observed her through the borescope. She gave birth to three neonates, two of which we observed still inside their embryonic sac. In the following two days we discovered BRBR and YYYW giving birth. Both females were located in a natal chamber, each underneath a

different flat rock largely covered by dense low vegetation (*Berberis* sp.; Fig. 3). In all three cases, mothers stayed with their 3-4 newborns for about 24 hours after parturition. During this time, the neonates (mean SVL 40.7 mm) did not leave the natal chamber. The neonates were born covered with a transparent embryonic sac through which it was possible to observe the newborn moving, and the immediate substrate in contact with the embryo was visibly moist. The enveloped neonate was held in a tight ball inside the embryonic sac. We do not know if the mother helped the neonates escape from their tight embryonic sacs. These are the first records of neonate *L. leopardinus* in nature. We found no dead neonates in natal chambers or underneath rocks that resembled the chambers, but we did find an embryo in an early-development stage on top of a rock and a dead newborn with its yolk still attached to it, also out in the open.

Our field observations suggested that the mother closed the entrances to the natal chamber with soil when she left, sealing the fragile neonates inside. On three different occasions, we observed natal chambers with packed soil heaped up against the entrances. After we discovered the natal chambers, we placed a motion-sensitive camera pointing to the entrance of each chamber to document any parental behavior by the mother and the behavior of neonates. Some 20 h post-parturition, we video-recorded mother WOWW, who was located facing outward partially in an entrance of the natal chamber (Fig. 4). With all four limbs in contact with the ground, she vigorously moved both front and hind legs to move surrounding soil into the entrance, piling up soil at the entrance (Fig. 4). Via borescope observations, we confirmed the presence of the neonates inside WOWW's chamber 24 h after parturition. In a different instance, the entrance of YYYW's natal chamber was closed with soil and pebbles; we did not video record the female moving the soil and pebbles to close the entrance, but the marks on the substrate suggested a behavior similar to that shown by WOWW. Neonates occupied the chambers for 2-5 days, and the mothers were radio-located and observed close to their natal chambers for the next few days. On one occasion three days after parturition, we photographed mother BBRB and three neonates basking in close proximity at the entrance of their natal chamber (Fig. 3).

Hypothesis 3, Mothers may directly repel predators—While giving birth in her natal chamber, mother WOWW bit the tip of the borescope a few times, an agonistic behavior never observed in any other context (including 40-50 observations of adults in group refuges with the borescope).

Hypothesis 4, Newborns will show cryptic behavior to avoid bird predation—Newborns are solitary and very secretive. They can be found--rarely--in the open, but mostly they remain concealed under flat rocks covered by dense bushes (*Berberis* sp.). Neonates with radio-transmitters--GOOG and OOOY--were radio-located for the next five to eight days and found mainly underneath flat rocks similar to their natal chambers. Only occasionally did they move inside a bush. Up to the end of our field season in late April, neonates spent a couple of days in a torpid state under a flat rock, and then moved to another flat rock usually < 1 m distant and remained under that rock for a day or two even under good weather conditions (clear and sunny days). We also observed four non-radio-tagged neonates in the open, who subsequently remained solitary under flat rocks in the same habitat. It appears probable that the neonates overwinter solitarily underneath these flat rocks in open habitat. Temperatures underneath flat rocks with microhabitat similar to that of the natal chambers offered thermoregulatory advantages to the neonates, especially at night. The mean overnight temperature (8:00 pm to 6:00 am) underneath the flat rocks was warmer than that under a nearby bush ($t = 3.740$, $df = 7$, $P = 0.007$). Daytime temperatures under the bush were often higher than those under the rock, however.

Neonates were not found with larger social groups until the next active season when they became yearlings. In November (early austral spring) of 2013, we captured 4 and radio-tagged and followed three 7-month-old lizards. These were bigger (mean SVL = 48.75 mm) and more agile than the neonates from the fall before and located in transitional habitat: mostly hiding under dense bushes (*Berberis* sp.) growing at the base of the large rocky outcrops, sometimes for several days at a time. Seven-month-old yearlings were sometimes found solitary and sometimes with older juveniles and adults. When located under bushes, they remained immobile and were very hard to spot. They occasionally made brief forays up into the big rocks or back to their open natal habitat. One 7-month-

old yearling (ORRO) was found in a small crevice where it stayed overnight with two adult males (BBBW and WWYY) and one adult female (RBRB).

Discussion

In this study, we tested four different hypotheses to obtain a deeper understanding of the antipredator behavior, parental care, and secretive behavior of *L. leopardinus* in the high Andes of central Chile—an environment with strong predation—and to help comprehend the evolution of social behavior in *L. leopardinus*.

Hypothesis 1, Avian predation will be stronger on smaller than bigger lizards, and weaker on those under cover than in the open—Our results support this hypothesis. During March-April, when females give birth, birds are abundant at our study site and it was very common to see foraging *Geositta* and *Agriornis* spp., known predators of neonate *L. leopardinus* (Ridgely and Tudor 1994; Santoyo-Brito et al. 2014). Our analysis of attack frequencies confirmed our hypotheses that neonate-sized models, simulating newborn *L. leopardinus*, are more vulnerable to avian predators than larger models. Other studies have shown that small individuals in relation to conspecific adult-sized ones tend to suffer greater predation (Rivas et al. 1988; Janzen 1993; Kacoliris et al. 2013). Also, models of *L. leopardinus* located on open rock or soil were more vulnerable than models under sparse ground vegetation. A similar predation pattern has been described using models of hatchling broad-headed snakes (*Hoplocephalus bungaroides*); exposed models were attacked by birds significantly more than those under rocks (Webb and Whiting 2005). It is likely that neonates move little and mostly remain hidden under flat rocks in the habitat of the natal chambers through the fall because of the heavy avian predation pressure at this time. Neonates of *L. leopardinus* tend to take refuge in places surrounded by vegetation with long, sharp spines, making it nearly impossible to capture them. A similar behavior has been reported in the green iguana (Henderson 1974). In Chile, predation has been considered an important ecological pressure that determines habitat use of several lizard species (Jaksić and Simonetti 1987). In our predation experiment, the number of models of each size was identical for all three sizes in all three different microhabitats. Thus, we can discard the possibility that the abundance of individuals of different age classes (i.e., size) could have biased our results

showing significantly more attacks on the smallest models (Downes 2006). *Liolaemus leopardinus* neonates do not show striking differences in color pattern when compared to juveniles or adults. Thus, we discard the hypothesis that the coloration of *L. leopardinus* neonates makes them more visually attractive to predators (Martín and López 1999; Stuart-Fox et al. 2003).

Our complementary laboratory trials of possible predation by other species demonstrated no evidence of predation on neonate *L. leopardinus* by the sympatric lizard *L. bellii*, or by the sympatric snake *Tachymenis chilensis*, despite that *T. chilensis* is considered primarily saurophagous (Green and Jaksic 1992). Neither is there any evidence of infanticide by conspecifics. In 2005, SF Fox and H Nuñez (personal communication) conducted laboratory trials with captive-born neonates and unrelated adult male *L. leopardinus* collected off the study site. They left an adult male with a litter of three neonates under a basking lamp in an inflatable wading pool (1.5 m diameter x 30.5 cm deep) for 30 minutes and conducted three such trials. In no case was any aggressive behavior shown by the male and in several instances, the neonates approached the male and climbed up on its back, a behavior observed between neonates and their mother on other occasions in captivity. The male tolerated this behavior with no negative reaction. Likewise, we observed no infanticide or aggression by various conspecific females toward newborns when they were held in captivity in groups in inflatable wading pools (Santoyo-Brito et al. in Review). In trials in the field in 2005, SF Fox and H Nuñez (personal communication) also observed no infanticide or aggressive behavior of adult males toward yearling juveniles. In each of three trials, Fox and Nuñez tethered a yearling juvenile at the entrance of a crevice into which an adult male had just taken refuge. They stood back 20 m and observed the crevice entrance through binoculars. In each case, the male poked its head out the entrance and partially emerged after some 10-15 minutes of waiting. The yearling male was visible to the adult male, but the adult male simply ignored the juvenile, until after some 5-10 minutes, left the vicinity.

Hypothesis 2, Mothers will show parental care, and will give birth in habitats that offer benefits to neonates—Our findings support this hypothesis. In 2013, we learned that free-ranging pregnant females leave their social groups on bare rock open habitat to give birth underneath natal

chambers located in brushy habitat away from conspecifics. It has been suggested that environmental conditions play a significant role in the evolution of parental care and the willingness of a parent to engage in parental care (Halloy et al. 2007; Huang and Wang 2008; Pike et al. 2015). Female parturition occurs about two months before hibernation, when night-time temperatures grow colder. Our results indicate that natal chambers offer thermoregulatory advantages to the neonates, especially during the cool nights at our high-elevation site. We suggest that the thermoregulatory advantage is an indirect benefit to the neonates from their mothers' nest-site selection. We also learned that the mother stayed with her neonates in the natal chamber for at least the first 24 h after parturition, probably protecting them. Borescope videos of very young newborns in their natural natal chambers in the field and our manipulations with other captive-born neonates (unpublished data) showed that neonates are frail, inept, and very vulnerable to harm. Newborn guarding is considered the most primitive form of parental care (Wesolowski 2004), and is seen in *L. leopardinus*. Field observations and videos suggest that when the mother leaves her natal chamber, she packs the entrances with soil, sealing the fragile neonates inside, probably to protect them from bird predation. A similar behavior was reported in pregnant *Lioalemus elongatus*, another viviparous, high-elevation Andean lizard. Four *L. elongatus* females in captivity constructed and occupied a burrow in which they gave birth. After giving birth, the females covered the underground shelter, apparently with their newborns inside. Females also were observed scratching at the surface of the burrow where the young emerged, and when a neonate re-entered the burrow, the females covered the entrance and sat down on top of it (Halloy et al. 2007).

Hypothesis 3, Mothers may directly repel predators—Our field observations support this hypothesis. The aggressive behavior shown by one mother as she was giving birth in the field might be a response to protect her altricial neonates. Other squamate species show aggression against predators when their offspring are present (Green et al. 2002; Sinn et al. 2008; Huang et al. 2013), and we have observed adult *L. leopardinus* showing protective guarding behavior against us in the field (but not biting) when they were with juveniles. Mortality of young represents a significant loss of fitness to the female (Clutton-Brock 1991); thus we suggest guarding neonates for—at least—their first 24 h outweighs this potential cost by increasing newborn success (Huang et al. 2013). Rattlesnakes

Crotalus viridis and *Sistrurus miliarius*, two species that show parental care, increase their defensive behavior after parturition during the period that neonates are present (Greene et al. 2002). Long-tailed skinks (*Eutropis longicaudata*) actively deter egg-eating snakes (*Oligodon formosanus*) from entering the nest (Huang 2006). Interestingly, this antipredator behavior is expressed only when the female is gravid, immediately prior to oviposition (Huang and Pike 2011). A comparable behavior to that of *L. leopardinus* (i.e., defensive mother and her offspring occupying the same refuge) was reported in the viviparous lizard *L. elongatus*. The female stays with her neonates for at least six days, and in the presence of a predator covers the main entrance of the burrow with her body, shielding the neonates inside (Halloy et al. 2007).

We suggest that pregnant females give birth in other habitat away from the conspecific social groups not only to keep their neonates safe from bird predation, but to keep them away from groups of larger conspecifics (i.e., juveniles and adults), which might accidentally trample neonates if these helpless, altricial newborns were inside the larger refuges used by older individuals. Adults and juveniles precipitously stampede *en masse* and fling themselves into the big refuges when a group is approached.

Hypothesis 4, Newborns will show cryptic behavior to avoid bird predation—Our observations of free-ranging neonates support this hypothesis. None of the radio-tracked newborns were observed or located in proximity to other neonates, juveniles or adults. Neonates moved very short distances over the day, and were located mainly underneath flat rocks similar to the natal chambers, and only occasionally moved to ground cover. We believe this secretive behavior is a strategy to decrease predation risk by birds (Sih 1987) at a life stage when they are very vulnerable. After overwintering underneath these flat rocks, and once they become bigger and more agile, they emerge as yearlings the next spring, but still behave very cryptically. In early spring, neonates move toward the rock outcrops where the larger social groups are located, but stay mostly in bushes. Only one 7-month-old yearling was found in a small crevice where it stayed overnight with two adult males and one adult female, suggesting that once the juveniles are stronger, bigger, and less susceptible to predation, they join groups of older conspecifics (Kullberg and Lind 2002; Mathis et al. 2003).

In summary, neonate solitary behavior during the first months of life contrasts to that exhibited by juveniles and adults, which interact in social groups and are rarely attacked by birds while in groups (Santoyo-Brito and Fox, personal observ.), a strategy considered to decrease predation (Sih 1987). Our findings show that predation risk in *L. leopardinus* decreases with use of protective microhabitat, and increased age (size) and experience (Cooper 2011; Cooper 2015). We conclude that the increased vulnerability of small neonates in open habitat to bird predation is the primary selection pressure that has caused mothers to seek out protected habitat away from their normal habitat and to give birth under flat rocks in open habitat with abundant ground cover, and that has caused neonates to remain in that protected habitat in a spaced-out, solitary pattern until they grow large and agile enough to be less susceptible to predation, wherein they join the social groups of older juveniles and adults.

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Figure 1. Distinctive marks (circled) of bird attacks left on clay models of neonate *Liolaemus leopardinus* located at El Colorado, Chile, during austral summer 2012.

Figure 2. A Rufous banded-Miner (*Geositta rufipennis*) near a clay model of a neonate *Liolaemus leopardinus* (circled) located on an open rock face at El Colorado, Chile, during austral summer 2012.

Figure 3. In A, female *Liolaemus leopardinus* (BBRB) and her 24-h-old neonate in proximity to the entrance of their natal chamber. In B, two 24-h-old neonate *L. leopardinus* at El Colorado, Chile, during austral summer 2013. Subjects are indicated by black arrows

Figure 4. Female *Liolaemus leopardinus* (WOWW) leaving its natal chamber ~ 20 h after parturition. In A, WOWW is located in the entrance of its natal chamber. In B, WOWW is in proximity of the entrance after pushing surrounding soil to seal neonates inside at El Colorado, Chile, during austral summer 2013.

Figure 1



Figure 2



Figure 3

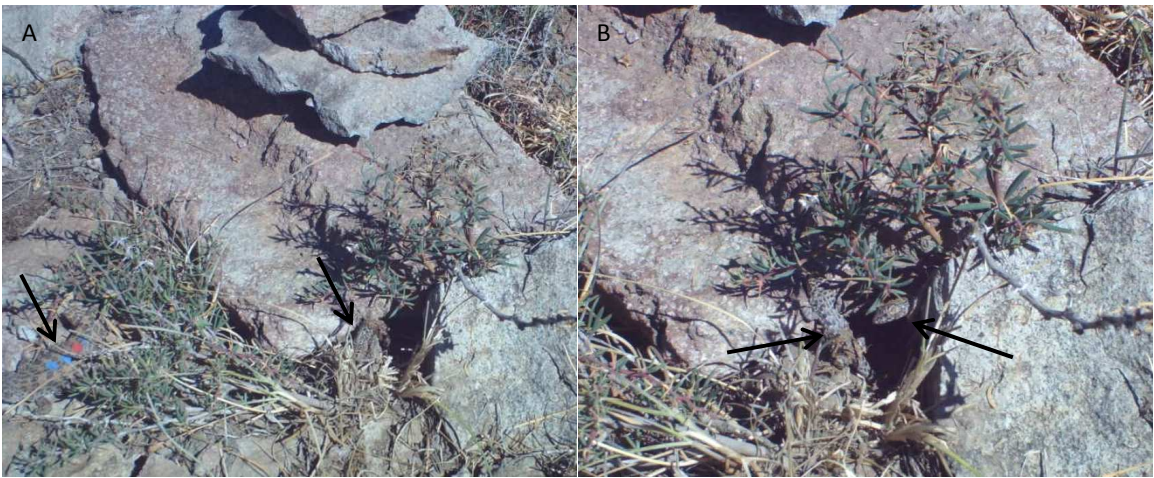
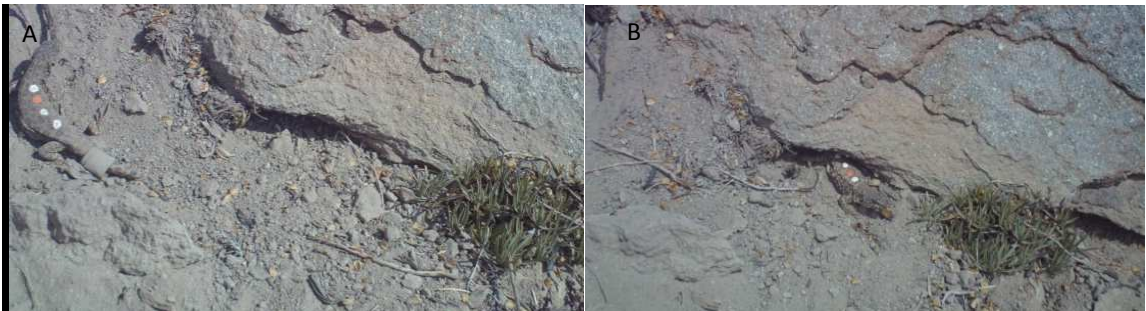


Figure 4



CHAPTER II

AGE ESTIMATION THROUGH SKELETOCHRONOLOGY AND MARK-RECAPTURE OF FREE-LIVING INDIVIDUALS IN A POPULATION OF A HIGH-ELEVATION, VIVIPAROUS LIZARDS FROM CHILE, *LIOLAEMUS LEOPARDINUS*

The following chapter is formatted for submission to *Phyllomedusa*:

Abstract

Age determination is a crucial component of ecological studies. Researchers have relied on different methods and techniques, for example mark-recapture, body size, and skeletochronology, to assess the age of free-ranging individuals. In this study we aimed to estimate the age structure of a population of *Liolaemus leopardinus*, a highly social, diurnal, and saxicolous lizard species endemic to the temperate region of central Chile. This high-elevation and secretive species is considered endangered; and although efforts have been made to understand and know more about the species' natural history, crucial details of its biology are still unknown. For the skeletochronology analyses, toe-clips were collected at the moment when each subject was first captured. Snout-vent length (SVL) data were collected during two different field seasons (austral spring to fall of 2011-12 and 2012-13), when subjects were active. We aimed to associate the numbers of Lines of Arrested Growth (LAGs) to the SVL measurements and use LAGs as an age estimation proxy on free-ranging individuals of the species. Our skeletochronology

results revealed the formation of LAGs in subjects of *L. leopardinus*; however, deficient staining and a high degree of bone remodeling and resorption were observed in most of the slices. These results combined with the high rapprochement in peripheral LAGs made the method poorly suited for aging free-ranging individuals of *L. leopardinus*. On the other hand, our mark-recapture results allowed us to assign individuals to four different age groups when a subject's SVL was associated with activity periods and recaptures. Our data also indicated the age when female lizards become sexually mature.

Keywords: bone, body-length, Squamata, Liolaemidae, lizards

Accurate age determination is a very important aspect in studies of population ecology (Borczyk and Paško 2011; Comas et al. 2016), but it is of particular relevance on those conducted on endangered species (Lindquist et al. 2012) or on those for which little is known about their demography (Comas et al. 2016). The most direct and reliable method to assess the age of free-ranging subjects is to gather life history and morphometric data through the lifespan of a cohort of newborns (Halliday and Verrel 1988; Castanet 1994). This is achieved by marking and recapturing the animals systematically, measuring them, and building growth curves or displaying the data in charts showing the lifetime relationship between age and growth (Halliday and Verrel 1988; Forester and Lykens 1991; Arankelyan et al. 2013). However, following individuals of a cohort over time can be a challenge if the species of interest is long-lived or elusive (Castanet 1994; Lemos-Espinal et al. 2005; Mills 2006). Therefore, researchers have relied on the size-frequency method and used body size as a proxy for age of free-ranging animals as both traits are positively correlated (Castanet 1994; Chen et al. 2011; Comas et al. 2016). To utilize the size-frequency method, a large number of animals are

captured and measured during a short period of time, the data are displayed in a histogram, and age classes are assigned based on the size-frequency distribution (Halliday and Verrell 1998). The size-frequency method requires a clear-cut knowledge of age-specific variation in body size of the species studied to avoid overlapping age classes (Gibbons 1976; Halliday and Verrell 1988; Castanet 1994). But, in order to minimize overlaps, researchers tend to group subjects into only two, three, or sometimes four age classes (Halliday and Verrell 1988; Castanet 1994; Borczyk and Paško 2011).

In addition to the methods mentioned above, researchers have focused their efforts on the study of natural cyclic growth marks recorded in large bones--skeletalochronology (Kleinenberg and Smirina 1969; Castilla and Castanet 1986; Castanet 1994; Guarino et al. 2010). In many species of reptiles, bone grows by an appositional process of surface deposition (Enlow 1963; Piantoni et al. 2006; Arakeylan et al. 2013; Çiçek et al. 2015). Bone growth occurs when the subject is active and resources are available for its development (Adolph and Porter 1993; Castanet 1994; Valdecantos et al. 2007; Comas et al. 2016). The appositional surface deposition can be observed as concentric rings through cross sections of long bone diaphyses--such as the femur or humerus (Castanet et al. 1993; Comas et al. 2016). Bone growth appears as a broad zone followed by a fine, but well-defined dark line (Halliday and Verrell 1988; Valdecantos et al. 2007; Prieto et al. 2012). The line in the shape of a ring is known as a Line of Arrested Growth (LAG) and indicates a period of drastically constrained growth--e.g., brumation (Halliday and Verrell 1988; Vitt and Caldwell 2009; Guarino et al. 2015). Lines of Arrested growth are formed yearly in species that inhabit well defined seasons, for example, temperate regions (Ortega-Rubio et al. 1993; Piantoni et al. 2006).

Skeletochronology has been considered for more than 30 years a rigorous and trustworthy technique to age free-ranging reptiles (Castanet 1982; Comas et al. 2016); its results are more accurate than that of any other known criterion of age estimation (Castanet et al. 1988). Skeletochronology studies often involve large numbers of animals being sacrificed when long bones are used, which can be of concern when the species under study is critically endangered. As an alternative to long bones, researchers have used phalanges since those offer similar results when compared to results obtained from long bones, and most importantly, removing a toe is non-lethal (Ledentsov and Melkunyan 1987; Yakin and Tok 2015; Comas et al. 2016). Skeletochronology has been applied successfully in studies of different animal groups, for example, mammals (Frylestam and Achantz 1977; Castanet et al. 2004), and amphibians and reptiles (Halliday and Verrel 1988; Castanet 1994; Guarino et al. 2005; Comas et al. 2016).

Liolaemus leopardinus is a medium-to-large, high-elevation, non-territorial, viviparous lizard endemic to temperate central Chile (Pincheira-Donoso and Núñez 2005). The species is limited to a narrow altitudinal band (2100–2800 m), and considered Endangered by IUCN (Díaz et al. 2016). It is active approximately seven months during the austral spring to fall and inactive during the cool and snowy austral winter. Little is known about the species' population demography, but different studies have assigned subjects to two or three age-classes—neonates, juveniles, and adults--solely on their snout-vent length (SVL) (Carothers et al. 1997; Fox and Shipman 2003; Santoyo-Brito et al. In prep.), or categorized individuals as mature based on the body size at sexual maturity: SVL 68.0 mm (Leyton and Valencia 1992). However, information on the age structure or lifespan of the species obtained through a more thorough or accurate method

has never been gathered. Information on both traits is critical when so little is known about this endangered endemic species. Our main objective in this study was to determine whether skeletochronology is a useful method to estimate age in *L. leopardinus*. We also aimed to determine the relation between SVL and the number of LAGs so as to use SVL as a proxy to estimate the age of free-ranging *L. leopardinus*.

Materials and Methods

We collected free-ranging *Liolaemus leopardinus* of different SVLs during their active season (austral spring to fall; December–April) in 2011-12 and 2012-13 and right after emerging from brumation in November and December, 2013, at El Colorado in the Andean cordillera of central Chile (Fox and Shipman 2003; Santoyo-Brito et al. In prep.), 35 km northeast of Santiago at 2760 m (33°14' S, 70°16' W). All lizards were caught via noosing, their SVL measured with a ruler, and sex determined by the presence (males) or absence (females) of precloacal pores when a hemipenal bulge was not observed.

Subjects were assigned to one of three groups, neonates (40.0 to 45.0 mm), juveniles (57.0 to 67.0 mm) (Santoyo-Brito et al. In prep.), or adults (> 67.0 mm) based on the body size when females reach sexual maturity (i.e., presence of follicles) (Leyton and Valencia 1992). We evaluated sexual size dimorphism between the five largest adults of both sexes using a Student's t-test. Statistical tests were completed using SPSS version 21.

All captured lizards were permanently marked by a unique toe clip combination and assigned a unique dorsal color combination of painted dots, which they lost at molting. This individual marking allowed recognition of free-ranging individual lizards

since subjects were part of a large behavioral ecology study (Santoyo-Brito et al. In prep.). Marked subjects were recaptured only when the color code was missing or incomplete. Clipped toes from each individual caught in the course of the field season of 2012-13 were collected for subsequent skeletochronology analyses. Excised digits were deposited into a dry Eppendorf centrifuge vial labeled with the unique toe clip combination of the individual. After we obtained the morphometric data and collected the clipped toes, all lizards were released at their site of capture.

The skeletochronology laboratory work was carried out in 2015 at Oklahoma State University. First, we chose the longest of the toe clips collected in the field from each lizard. Toe clips had been stored dry in individual Eppendorf vials. Then we placed all samples in 95% ethanol for fixation for at least 24 hours, ensuring that the individual identity of each toe clip was maintained. Soft tissue was carefully removed manually from most of the toe clip and the bone of the second phalange detached. The length of the bone was measured to the nearest 0.25 mm using an optical micrometer to estimate the number of cuts needed to obtain slices from the mid-section of the bone. All bones were decalcified using Fisher Scientific Cal-Ex® II (Product # MFCD00211744) for 8 h. Each phalange was removed from its cassette and embedded in a block of paraffin for its transversal sectioning with a manual microtome. Slice thickness was set to 10 μm , and a ribbon of serial cross sections of 10 to 12 slices was placed on a microscope slide and stained with Harris' hematoxylin. Slices were obtained from the mid-diaphysis of the phalange, which is considered to yield the best sections for accurate aging. For a detailed description of the skeletochronology method, refer to McCreary et al. 2008.

All stained slides were observed utilizing a compound Olympus microscope CX22LED (200 and 400X-magnification). We took a series of photographs of different slices from each bone utilizing a digital camera (Amscope MU1000 10MP Still and Live Image Microscope Digital Camera) attached to one of the oculars of the microscope. Three to 10 of the best photographs of different sections of each sliced toe clip were selected to estimate the number of LAGs. Each photograph was enlarged using the morphometric software tpsDig (Rohlf 2004) to count the LAGs. Samples with observed double lines were discarded for age determination (Bülbül et al. 2015). It was not possible to estimate the loss of one or more innermost LAGs by means of osteometric analysis because we analyzed phalanges of different toe clips with different diaphyseal diameters, thus they were not comparable (Guarino et al. 2010; Guarino et al. 2015). Any samples showing a total resorption of LAGs were excluded from the study.

Results

Objective 1. Combining both field seasons (2011-12 and 2012-13), we captured and permanently marked a total of 179 free-ranging lizards of different ages (neonates, juveniles, and adults). The range of SVL for all subjects was 40.0 to 102.0 mm. There was no sexual size dimorphism in SVL (t-test: $t_{(8)} = 1.518$; $p = 0.168$) between the five largest males (range = 99.0 to 102.0 mm, mean = 100.8; SD = 1.15), and females (range = 99.0 to 102.0 mm, mean = 99.6; SD = 1.34).

Unfortunately, for the skeletochronology analyses we had to exclude samples of 30 lizards because the bone became detached from the paraffin block during the slicing process in a few cases, or more often due to the inferior quality of the slide (i.e., poor staining or poor tissue slice). The exclusion of those samples reduced our sample size to

43. In all stained cross sections we observed a medullar cavity, endosteal deposition and periosteal bone, and growth zones delimited by thin hematoxylinophilic outer lines corresponding to LAGs. Rapprochement of peripheral LAGs was observed mostly in adults (Fig. 1).

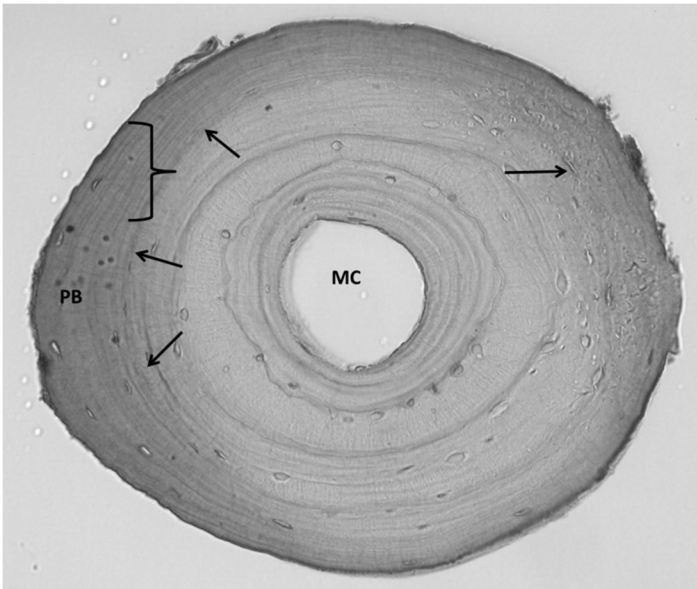


Figure 1. Cross section through the mid-section diaphysis of a phalanx of BBGB (adult male, SVL = 92.0 mm) *Liolaemus leopardinus* lizard caught February 13, 2013, at El Colorado in the Andean cordillera of central Chile. Black arrows point to the limit of the remodeling zone, and bracket indicates Lines of Arrested Growth (LAGs) formed during each brumation period. Marrow Cavity (MC), Periosteal bone (PB).

An unexpected result in our study was that all samples, both juveniles and adults, showed bone remodeling (Fig. 1). We identified scalloped lines (indicative of bone remodeling), and in most cases the remodeling was so extensive that counting LAGs was not feasible, facing the risk of false counts and underestimating age of the lizards. The degree of remodeling and endosteal deposition was such that we could not estimate the

number of lost LAGs, not even via image overlap. Furthermore, because of extensive rapprochement, we could not confidently count peripheral LAGs, either.

Objective 2. We also aimed to assess the degree of association between SVL and the number of LAGs to use length as an age proxy on free-ranging lizards. However, we could not estimate the correlation between both variables due to the problems faced in the skeletochronology analyses. Consequently, we plotted the SVL of all subjects caught in either or both field seasons, which allowed us to assign subjects into one of four different age classes--neonates, yearlings, juveniles, and adults--by associating SVL and active and inactive (brumation) periods of the year (Fig. 2). We know that lizards are active from October to mid-April and that subjects of the population brumate from mid-April to October. Mothers give birth during March and April (neonates' mean SVL = 40.6 mm); thus, the smallest subjects found in the field in November right after brumation are the now 7-8 month-old neonates (mean SVL = 48.8 mm). The complete lack of data points between 49 and 56 mm in Fig. 2 suggest that the yearling cohort is missing in both active periods (AP) depicted in the figure. Those lizards that start the active period at a size of 56-62 mm appear to be second- year juveniles, and all subjects whose SVL is 68.0 or above should be considered adults. Following Leyton and Valencia (1992), we assumed that the species reaches sexual maturity during the third to fourth year of age; when lizards' SVL is ~68.0 mm.

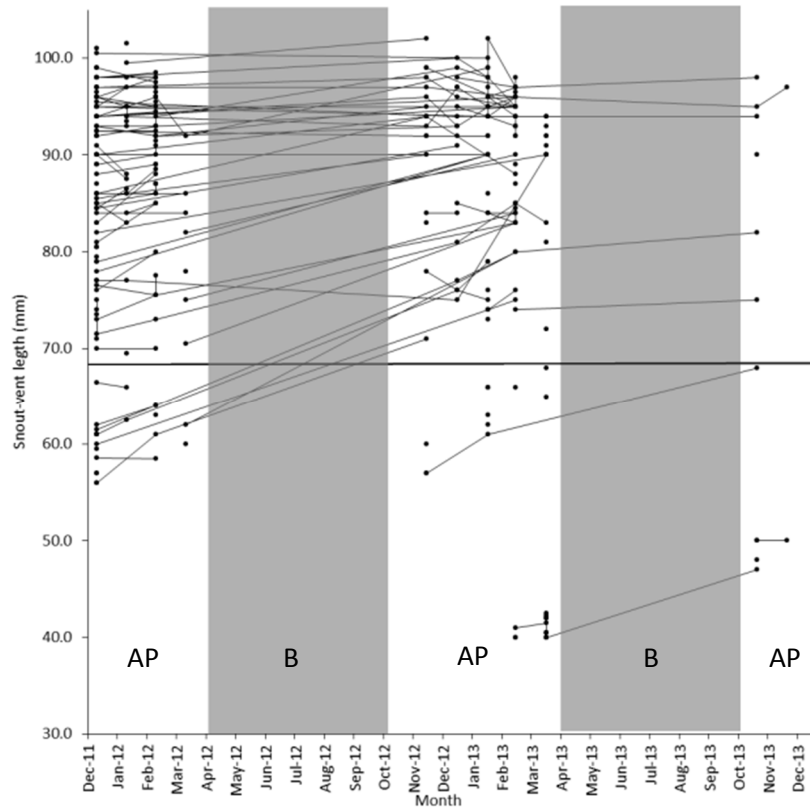


Figure 2. Snout-vent length of 179 different lizards caught during the late austral spring to fall of 2011-12 and 2012-13 and November-December of 2013, at El Colorado, Chile. Solid circles represent each of the lizards caught and lines link their subsequent recaptures. Bold horizontal line at 68 mm represents minimal size at sexual maturity. AP = active period; B = brumation.

Interestingly, during our two field seasons—2011-12 and 2012-13—we captured nine subjects that were first caught and permanently marked during a pilot study at the same site from December 2004 to April 2005 (Table 1).

Table 1. Size (SVL) of nine *Liolaemus leopardinus* lizards first caught during the austral summer and fall (December–April) in 2004-05 and recaptured during the same seasons in 2012-2013 at El Colorado in the Andean cordillera of central Chile. Age group: A = adults, J = juveniles.

		Field season					
		2004-05		2011-12		2012-13	
Toe-clip	Sex	Age	SVL	Age	SVL	Age	SVL
1-7-11-18	♂	A	91.5	A	98.0	--	--
1-8-11-17	♂	A	94.5	A	98.0	--	--
1-6-12-13-16-17-19	♂	A	94	A	98.0	--	--
1-6-11-19	♂	A	94.5	A	99.5	A	102.0
1-9-11-16	♂	A	96	A	101.0	--	--
1-9-20	♀	A	82	A	94.0	--	--
1-7-11-20	♀	J	63	A	98.0	A	100.0
1-9-12-16	♀	A	94	A	98.0	--	--
1-9-14	♀	A	69	A	99.0	--	--

Eight of the subjects caught in 2004-05 fit into the adult age class and one can be assigned to the juvenile age class based on Fig. 2. The average SVL for adult males in 2004-05 was 99.2 mm and for females was 97.3 mm. By associating the SVL at different periods of activity depicted in Fig. 2, we suggest that these adult lizards were at least 4 to 5 years old when first captured. Seven years later at the moment of recapture those subjects should have been at least 11 to 12 years old or more since their SVL is close to

the upper bound of the SVL range (102.0 mm) for all subjects caught in either of our two field seasons (Fig. 2).

Discussion

We provide results on the first attempt to estimate age in *Liolaemus leopardinus* lizards through skeletochronology. Our main goal was to determine the effectiveness of the aging method using various phalanges of individuals. Unfortunately, the objective was not fulfilled since we faced problems during the skeletochronology. First, although the staining method was the same for all samples, the optical sharpness varied among tissue slices, making it sometimes difficult to unmistakably identify the LAGs. Misinterpretation of LAGs is common when they are not strongly expressed (i.e., clearly defined and stained) (Castanet and Smirina 1990) and double lines, probably formed due to temporary, short-term ecological factors (i.e., very high temperatures, very dry conditions, variations in food availability, etc.) in the environment (Jakob et al. 2002), could be interpreted as two LAGs. Second, unexpectedly we observed a high degree of bone remodeling (i.e., erosion, scalloped surfaces, and deposition of endosteal bone) in all samples. It has been shown that bone remodeling increases as individuals get older (Castilla and Castanet 1986) and considerable remodeling and rapprochement of peripheral LAGs has been detected in long-lived species (Wagner et al. 2011; Ergül et al. 2014). Remodeling is the result of bone growth (Enlow 1963) by eroding and reshaping the periosteal bone on the edge of the marrow cavity; this phenomenon can cause the complete loss of one or more LAGs (Ergül et al. 2014). Remodeling could be the result of environmental conditions (Smirina 1972) in a habitat with harsh weather and possibly limited resources as is recognized in high altitude or high latitude regions (Nagy and

Grabherr 2009; Ergül et al. 2014). During bone growth, rebuilding also occurs by endosteal deposition (Enlow 1963; Curtin et al. 2005), which was notably present in our samples. Third, we observed rapprochement of peripheral LAGs, a state that reduces the reliability of age estimates (Wagner et al. 2011; Sinsch 2015). Obviously, reliability of skeletochronology depends on the correct interpretation of the phenomenon (Castanet and Smirina 1990; Guarino et al. 1999), and our samples did not yield sufficient reliability. Thus, a true estimation of age using LAGs was not possible in our study.

Sexual size dimorphism was not evident in *L. leopardinus*, but the largest males were slightly larger than the largest females. We suggest that adult males may live longer than adult females. If so, the longer lifespan in males might have a genetic basis (Badyaev 2002; Cox et al. 2009; Barret and Richardson 2011) since males do not defend territories or females, and do not engage in agonistic behavior against male conspecifics (Fox and Shipman 2003; Santoyo-Brito et al. In prep.), while females face the energetic challenge of viviparous reproduction.

Body-size measurements derived from the mark-recapture data collected during three field seasons aided us in assigning individuals not only to three but to four different age classes. We assigned young specimens to one of three age classes: neonates, yearlings, or juveniles based on active periods. Interestingly, Fig. 2 suggests that the yearling cohort is missing from our dataset; the lack of these data could be the result of our search effort. During both field seasons we mainly searched for lizards located on the rock outcrops since we were not then aware of the secretive behavior of neonates and yearlings (Santoyo-Brito et al. In prep.). However, it is also likely that most of the 7-8 month “new yearlings” do not survive because of heavy bird predation documented at our

field site (Santoyo-Brito et al. In prep.), and so their density might be very low, making it difficult to observe them in the field. It is likely that adults live so long and continue to reproduce throughout their long lives as evolutionary compensation for heavy mortality of neonates and small juveniles.

Nevertheless, for three main reasons our results should be taken with caution and be considered as a mere age class designation and not an absolute age estimator (Halliday and Verrell 1988; Castanet 1994). First, in our study we did not follow a cohort of known-age individuals (i.e., neonates) through their lifespan; our mark and recapture data were limited roughly to three active seasons. Second, we did not continuously recapture known subjects and their recapture was limited to the event of missing color codes, which limited the accumulation of data on SVL. Third, *L. leopardinus* as an ectotherm strongly depends on ecological and physiological conditions (i.e., thermal environments, length of activity period, food availability, predation, etc.) to grow, but most importantly those conditions could differ within a season (Adolph and Porter, 1993; Peacor 2002; Roitberg and Smirina 2006), thus increasing body size variability within age classes (Klauber 1937; Halliday and Verrell 1988; Castanet 1994).

The SVL of the eight adults first captured in 2005 and recaptured in 2012-13 shows that *L. leopardinus* is a long-lived species. Although a life span of up to 12 years or more could sound overstated for a medium-sized lizard, other lizard species located at high elevations are also known to be long-lived. For example, a skeletochronology study suggested that one adult female from a 60-70 mm cohort of the viviparous *L. multicolor* was 19 years of age (Valdecantos et al. 2007), and *Phymaturus patagonicus* (SVL = 88.0-109.0 mm) can live up to 16 years (Piantoni et al. 2006). However, for *L. multicolor* the

authors suggest caution since that specific finding might be an outlier and not accurately represent what occurs within the remaining sample. Even so, they report a mean life span of 12 years for *L. multicolor* (Valdecantos et al. 2007). Longevity in *L. leopardinus* could be explained in part due to the selective predation pressure on smaller body sizes (Reznick 1996; Costelloe and Rubenstein 2015; Santoyo-Brito et al. In prep.). Although several predatory species are located at El Colorado (Fox and Shipman 2003), predation decreases once the subjects reach the size of older juveniles and beyond (Santoyo-Brito et al. In prep.). The survival benefit derived from having achieved a large body size has been shown in other lizard species. For example, larger (and older) *Egernia stokesii* were more prone to recapture nine years later than smaller lizards (Pearson et al. 2016). In addition to body size, the short length of the activity season at high-elevation sites decreases predation opportunities (Wapstra et al. 2001; Roitberg and Smirina 2006), resulting in increased longevity (Piantoni et al. 2006; Cabezas-Cartes et al. 2015).

In summary, our skeletochronology results using phalanges did reveal the formation of LAGs in subjects of *L. leopardinus*. However, the high degree of remodeling and rapprochement make the method poorly suited for aging individuals. Before utterly discarding the method, however, we suggest using a large bone like the femur (and not phalanges), which has been shown to be very suitable for skeletochronology (Castanet et al. 1998; Piantoni et al. 2006; Guarino 2010). We also suggest collecting a larger number of samples corresponding to free-ranging neonates and yearlings to back-calculate the number of LAGs lost if remodeling is present also in the femur. However, collecting free-ranging neonates is not an easy task since they are rarely found in the field due to their secretive and cryptic behavior (Santoyo-Brito et al. In

prep.). Most importantly, and a serious detraction, is that to obtain a femur it is necessary to euthanize individuals, an undesirable option when studying *L. leopardinus*, which is endangered. There is no size dimorphism in the species; although, males are slightly bigger than females. SVL and mark-recapture data suggest that *L. leopardinus* males might live longer than their female counterparts.

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CHAPTER III

GROUP LIVING, KIN RECOGNITION, GENETIC RELATEDNESS, AND SOCIALITY IN THE HIGH-ELEVATION CHILEAN LIZARD, *LIOLAEMUS LEOPARDINUS*

Abstract

Sociality means group living through the mechanism of social attraction; the size of social groups varies within and among species. In many instances groups are aggregations of relatives, suggesting the capability of individuals to discriminate among related and unrelated individuals, allowing for kin selection. Group living is a widespread phenomenon in the animal kingdom and has been widely studied in taxa in which social interactions are overtly obvious. Due to the apparent lack of sociality, squamate reptiles have been usually labeled as non-social. But this has been challenged and complex social systems have been documented in recent years. *Liolaemus leopardinus* is a medium-to-large, high-elevation, viviparous lizard endemic to a narrow altitudinal band in temperate central Chile. The species is non-territorial and the home ranges of individuals of different ages overlap. It is neither aggressive nor cannibalistic, and rarely displays agonistic behavior. We recently discovered that mothers of the species show relatively simple maternal care. During two field seasons, we observed social aggregations that ranged from small groups with a male and a female (sometimes pregnant), at times with one to three juveniles, to larger groups of up to 6–10 (or more) adults and juveniles within rock crevices. Field observations and behavioral studies suggested that the social structure of this population appears to be family

groups embedded within a larger colonial group; however, the genetic relatedness of members of social groups remained unclear. To reveal the possible benefits of social interactions within aggregations of *L. leopardinus* located at El Colorado, Chile, we aimed to document, evaluate, and quantify the grouping behavior, thermoregulatory benefits of refuge use and communal refuging, kin recognition, and genetic relatedness among group members. Our results confirm that individuals of different ages share communal refuges, but we discard the thermoregulatory benefit of communal refuge use. The home ranges of males and females of different ages overlap and there is no evidence of territoriality. Neonates recognize their mother's scent, and may use it when they join groups of adults and older juveniles. Results of DNA microsatellites support the conclusion that groups formed by *L. leopardinus* at El Colorado, Chile, are indeed social groups of closely related individuals mixed in with non-relatives.

Introduction

Sociality means group living (Alexander 1974; Ebensperger and Hayes 2016) through the mechanism of social attraction (Graves and Duvall 1995; Ward and Webster 2016). Conceptually, a social group can be defined as a set of conspecifics in close proximity that interact with each other over a period of time short enough that there are few changes in group membership (Whitehead and Dufault 1999; Krause and Morrell and James 2007). The size of social groups varies within and among species, ranging from individuals that tend to form dyads, to highly social individuals that form complex colonies (Brown and Orians 1970; O'Connor and Shine 2003; McGlynn 2010). Many social groups are aggregations of relatives (Wilson 1975; Krause and Ruxton 2002). This implies the capability of individuals to discriminate among related and unrelated individuals (Bull et al. 2001; O'Connor and Shine 2006; While et al. 2009) and allows for kin selection (Hamilton 1964; Wilson 1975; Klug et al. 2012). Group living is a widespread

phenomenon in the animal kingdom and has been widely studied in birds and mammals (Gomper 1996; Hughes 1998; Silk 2007), in which social interactions are somewhat obvious. Due to the apparent lack of sociality broadly accepted in the past (Doody et al. 2011), squamate reptiles have been usually labeled as non-social (Wilkinson et al. 2010; Wilkinson and Huber 2012). This situation has led researchers to ignore the diverse and often complex social behavior in this large taxon (Doody et al. 2013). Recently, the assumption of “lack” of sociality in reptiles has been challenged and the literature reporting squamate species found in aggregations has skyrocketed during the last years (Brent and Duvall 1995; Mouton et al. 1999; O’Connor and Shine 2003; Davis et al. 2010; Gardner et al. 2016). These aggregations have been reported especially within the order Squamata, in which many species display social systems (Brattstrom 1974; Doody et al. 2013; Gardner et al. 2016). Some of these are simple. For example, adults of the lizard *Liolaemus kingii*, *L. elongatus*, and different species of the genus *Egernia* tolerate juveniles within their home ranges (Ibargüengoytia et al. 2002; Chapple 2003; Halloy et al. 2007). On the other hand, complex social systems have been documented in recent years. Most notably are Australian skinks in the genus *Egernia* and the evidence based on long-term studies of social aggregations in 23 of the 30 described species (reviewed in Chapple 2003; Chapple and Keogh 2005 and references therein). Within the social groups documented in *Egernia*, the degree of genetic relatedness, number of members, age of individuals, proximity to each other, and years of aggregation varied (Gardner et al. 2002; Duffield and Bull 2002; O’Connor and Shine 2003; Chapple 2003; Osterwalder et al. 2004; Gardner et al. 2016). Our study is a comprehensive one that relates group living, sociality, and genetic relatedness in a non-*Egernia* species of lizard, *Liolaemus leopardinus*.

Liolaemus leopardinus is a medium-to-large, high-elevation, viviparous lizard endemic to temperate central Chile (Pincheira-Donoso and Núñez 2005). It is limited to a narrow altitudinal

band (1800–3000 m) (Pincheira-Donoso et al. 2008), and considered Endangered by IUCN (Díaz et al. 2016). It is active during austral spring, summer, and fall and inactive during the cool and snowy austral winter. The species is non-territorial and the home ranges of individuals of different ages overlap (Fox and Shipman 2003). It is not aggressive nor cannibalistic (Santoyo-Brito et al. In prep) and rarely displays head-bobs or pushups, suggesting that conspecifics rely on chemical rather than visual communication (Fox and Shipman 2003) as reported in many other reptile species (Mason and Parker 2010). During a field study in the Andean cordillera of central Chile, Fox and Shipman (2003) observed subjects of *L. leopardinus* in close proximity and reported it was common to see an adult male and female with one or three juveniles--but not neonates--on rock outcrops. This grouping behavior was confirmed by Fox and Núñez in 2005 and by Santoyo-Brito et al. (In prep.) during two field seasons, 2011-12 and 2012-13, when they observed social aggregations that ranged from small groups with a male and a female (sometimes pregnant), at times with one to three juveniles, to larger groups of up to 6–10 (or more) adults and juveniles within rock crevices.

We recently discovered that pregnant *L. leopardinus* females leave their social groups to give birth in solitary underneath flat rocks located 10-40 meters away from the rock outcrops where the majority of social interactions take place. We also discovered that the mothers display relatively simple parental care during the first 24 hours of the neonates' life inside the small natal chamber (Santoyo-Brito et al. In prep.). Field observations and behavioral studies suggested that the social structure of this population appears to be family groups embedded within a larger colonial group (Fox and Shipman 2003; Murphy et al. 2009); however, the genetic relatedness of members of social groups remains unclear. To reveal the possible benefits of social interactions within aggregations of *L. leopardinus* located at El Colorado, Chile, we aimed to document,

evaluate, and quantify a) grouping behavior; b) thermoregulatory benefits of refuge use and communal refuging, c) kin recognition, and d) genetic relatedness among group members.

Materials and methods

Mark-recapture--Our field site was located in the Andean cordillera of central Chile at El Colorado (Fox and Shipman 2003; Santoyo-Brito et al. In prep.), 35 km northeast of Santiago at 2760 m (33° 14' S, 70° 16' W). We collected free-ranging *L. leopardinus* of different snout-vent length (SVL) during their active season (austral spring to fall; December–April) in 2011-12 and 2012-13, and right after emerging from hibernation in November, 2013. All lizards were caught via noosing, their SVL was measured with a ruler, and sex was determined by the presence (males) or absence (females) of pre-cloacal pores. Subjects were assigned to one of four different age groups, neonates (SVL 40.0-45.0 mm), yearlings (SVL 47.0-57.0 mm), juveniles (SVL 58.0-67.0 mm; Santoyo-Brito et al. In prep.), and adults (SVL \geq 68.0 mm, when females reach sexual maturity; Leyton and Valencia 1992). All captured lizards were permanently marked by a unique toe-clip combination, an implanted Passive Integrated Transponder (PIT) tag, and a unique dorsal color code of non-toxic latex paint. For color code combinations we used Blue (B), Orange (O), Red (R), White (W), and Yellow (Y). All subjects were released at the site of capture. Population density for each field season was estimated by dividing the total unique individuals caught per season by the area of the study site.

Grouping behavior--In both field seasons, we made use of radio-transmitters (model: BD-2H, 0.9 to 1.04 g, internal antenna; Holohil Systems, Ltd., Carp, Ontario, Canada) to locate adult males and females. Radios were either attached to the base of the tail with surgical tape, or implanted into the abdominal cavity. Radio-tagged subjects were radio-located from late December to late April at least two times a day from approximately 10:00 am to 6:00 pm. In late

April 2013, we glued a radio-transmitter (model BD-2x, 11.5 by 5.3 by 2.8 mm; 0.25 g, external antenna; Holohil Systems) to the dorsum of each of two neonates. In November 2013, we revisited our field site to capture and radio-tag (model BD-2x) the now 7-8 month-old yearlings. In all cases, radios did not exceed 5% of the body mass of the subject, as recommended by Ferner (2007). We used a hand-held, three-element Yagi antenna and a radio receiver (Model R-1000; Advanced Telemetry Systems, Inc., Isanti, Minnesota) to locate radio-tagged lizards. When the lizard was radio-located underneath a rock or within a rock crevice, we removed the co-axial cable from the antenna and used that end of the cable to pin-point the exact location. To observe and video-record any behavior of refuged lizards, we used a Rigel digital video borescope (Medit Inc. Winnipeg MB, Canada) with a 360-degree, two-way articulating probe (4 mm in diameter and 2 m long) (Santoyo-Brito et al. In prep.).

To register the subsequent geographic location of known subjects, we placed a number of flags at prominent landmarks throughout our study site and registered their coordinates with a handheld GPS. With that information, we created a scale map of our site using ArcGIS 10.5.1. Each sighting via binoculars or radio location was registered on printed hard copies of the scale map using visual triangulation from closest flags. These locations were subsequently digitized. The home range (HR) of each subject with a minimum of three geographic locations was delineated by a Minimum Convex Polygon (MCP) using ArcGIS 10.5.1. We defined the home range as the area of the polygon that encompassed all sightings within 95% of the distance from the geometric center of activity (i.e., discarding points more distant than 1.96 SD of an individual's set of center-to-sighting distances; Fox and Shipman 2003). We chose MCP because it is easy to calculate, accurate, and it is based on real observations of each individual (Hayne 1949; Rocha 1998; Kacolis et al. 2009). MCPs are easy to compare among studies since they are frequently used (Harris et al. 1990; White and Garrott 1990; Fox and Shipman 2003). We used a

Student's t-test to evaluate differences in HR size between sexes. We calculated HR overlap for each year using the intersect tool in ArcGIS 10.5.1.

In both field seasons, we made use of PIT tag antennas to record visitations to deep refuges that were repeatedly occupied by many lizards on the rock outcrops. Following Rehmeir et al., (2006), we employed a custom-made pass-through antenna (3.0-cm inner diameter ring) cemented into the refuge entrance to record the date and time of passage of PIT tagged individuals into or out of the refuge (Santoyo-Brito and Fox 2015). All other openings to the refuge were sealed with modeling clay that hardened in a few hours. As a lizard passed through the ring antenna to enter or exit a refuge, its unique PIT tag ID, date, and time were recorded in the memory of the reader, which was powered by a 12-V automobile battery. The custom-made ring antennas were made with such sensitivity that only lizards exactly inside the ring were recorded, which we tested in the field. We also employed a motion-sensitive waterproof game camera focused on the refuge entrance (ring antenna) to record if a PIT tag reading corresponded to a refuge entrance or exit (Santoyo-Brito and Fox 2015).

Thermoregulatory benefits of refuge use and communal refuging--To measure temperatures of known refuges, we made use of a thermocouple affixed to the tip of the borescope to measure inside air and substrate temperatures. We also measured the temperatures inside and outside of different rock crevices, and of the skin of refuged subjects (Santoyo-Brito et al. 2015). For temperatures inside the refuge, we used the borescope to position the thermocouple and then turned off the light and waited approximately 5 minutes until temperatures stabilized. Since *L. leopardinus* naturally takes refuge and sleeps through the night in groups of variable size within rock crevices, we performed an experiment with captive adults to test if there was any evidence of endothermy in the species. We held six adult females

in an open-top cage (40 x 50-cm floor surface) and each night for five nights we positioned a TidBit temperature logger inside the pile and another one on the substrate away from the pile in a sectioned-off part of the arena and recorded temperatures every hour.

Kin recognition--To expand the preliminary results on kin recognition in *L. leopardinus* (Fox and Núñez unpublished data), we ran T-maze trials with individual neonates to determine if they can discriminate the scent of their own mother (paper towel conditioned by mother for 48 h) from the scent of another recent mother (paper towel conditioned by another mother for 48 h) (Fig 1). To accomplish this, we kept pregnant females in the laboratory and kept track of parturition to match mothers with litters. Newborns were separated from their mothers within the first 10 hours after birth and housed individually in shoeboxes (20.2 cm wide x 34.4 cm long x 11.8 cm high) with mesh lids.



Fig 1. T-maze with choice chambers as dark plastic bags (containing paper towels conditioned by mother) into which the test *L. leopardinus* neonate falls and cannot reverse its decision.

Genetic relatedness analysis--During both field seasons we collected tissue samples of *L. leopardinus* lizards via impregnation of blood onto Whatman FTA Classic Cards for the genetic analysis. Blood was collected at the time toes of each subject were excised. DNA was extracted at

Oklahoma State University where 11 hypervariable microsatellite loci (Gardner et al. 2001; Murphy et al. 2009) were amplified by PCR following the protocol designed by Walsh et al., (1991). The PCR product was analyzed using gel-agarose electrophoresis. Amplified DNA samples were multiplexed, and the sequences scored using GENE Mapper. Four of the 11 microsatellites failed to amplify most of the time, so we conducted the genetic relatedness analysis using seven loci. We tested to see if the genotypes at each locus were in Hardy-Weinberg equilibrium by running a 100,000 Monte Carlo randomizations test (Guo and Thompson 1992) and employing the U test statistic (Rousset and Raymond 1995). Relatedness between pairs of lizards was computed utilizing the computer software ML-Relate (Dakin and Avise 2004; Kalinowski et al. 2006). The probability of identity was estimated utilizing the computer software GIMLET (Valérie 2002). Because null alleles are common in microsatellite data and normally fail to amplify (Dakin and Avise 2004)--a situation that can lead to errors in estimating relatedness or relationship--we tested our data for null allele frequency also with ML-Relate.

Results

Mark-recapture--During both field seasons we captured and permanently marked a total of 179 lizards of four age groups (neonates, yearlings, juveniles, and adults) in our 2.97 ha study area. We made a total of 1162 sightings and/or radio locations and fixed them on a scale map. Adult male SVL range was 68.0-100.5 mm (n = 77, mean = 89.4 mm; 1 SD = 8.47), and adult female SVL range was 69.5-99.0 mm (n = 59, mean = 87.2 mm; 1 SD = 7.73). The SVL of the 10 largest adult males and females was not significantly different (See Chapter 2).

Grouping behavior--There was no difference between the mean HR size of males and females in either field season (2011-12: $t_{(36)} = -1.612$; P = 0.126 and 2012-13: $t_{(13)} = -0.408$; P = 0.690; Table 1). Population density during 2011-12 was 109/2.97 ha = 36.70 individuals per ha,

and for 2012-13 was: $70/0.51 \text{ ha} = 137.25$ individuals per ha. Densities for 2012-13 were substantially higher since in that season we focused our search efforts on a smaller area where more lizards were seen.

Table 1. Descriptive statistics of home ranges of adult males and females of *Liolaemus leopardinus* from a population located at El Colorado, Chile. Data collected during field seasons of 2011-12 and 2012-13.

		Field season 2011-12			Field season 2012-13			
Sex	<i>n</i>	Range (m ²)	Mean (m ²)	1 SD	<i>n</i>	Range (m ²)	Mean (m ²)	1 SD
♂	23	2.00-771.00	205.96	241.49	7	9.00-1509.00	436.00	517.76
♀	15	1.00-2740.00	327.55	713.85	8	6.00-822.00	389.20	301.12

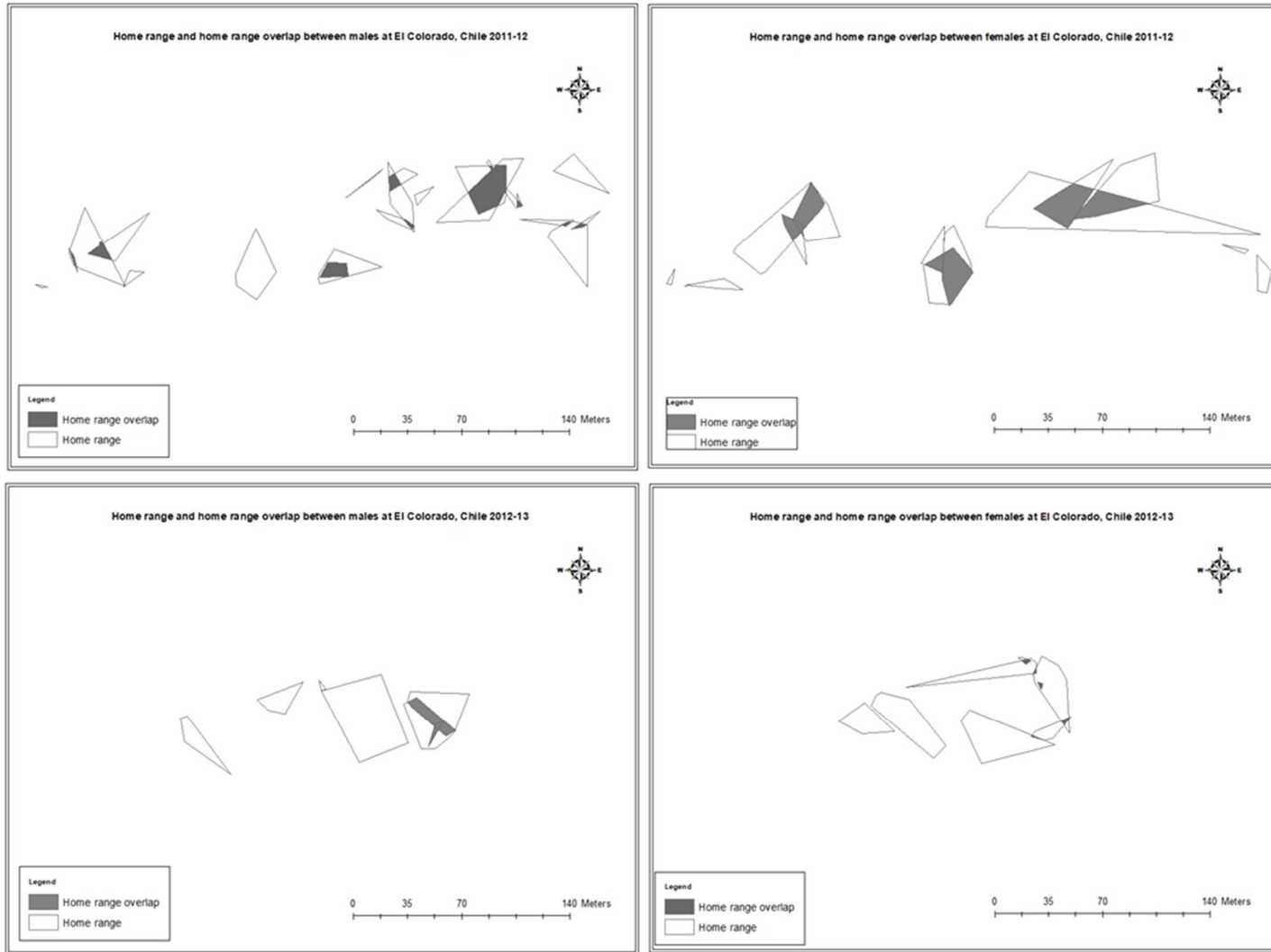


Figure 2. Home ranges and same-sex home range overlaps between adults during field seasons of 2011-12 and 2012-13 at El Colorado, Chile.

In April 2012-13 (austral fall), we discovered that free-ranging neonates ($n = 6$) are very secretive, are found mainly close to flat rocks protected by dense spiny bushes (*Berberis* sp.) located in open habitat away from rocky outcrops and mostly outside of adult HRs, and overwinter in solitary underneath flat rocks. In November 2013 during the next active season, the new yearlings (7-8 months old) moved to be in closer proximity to juveniles and adults; however, they were found mainly at the outcrop bases protected by bushes (Fig. 3).

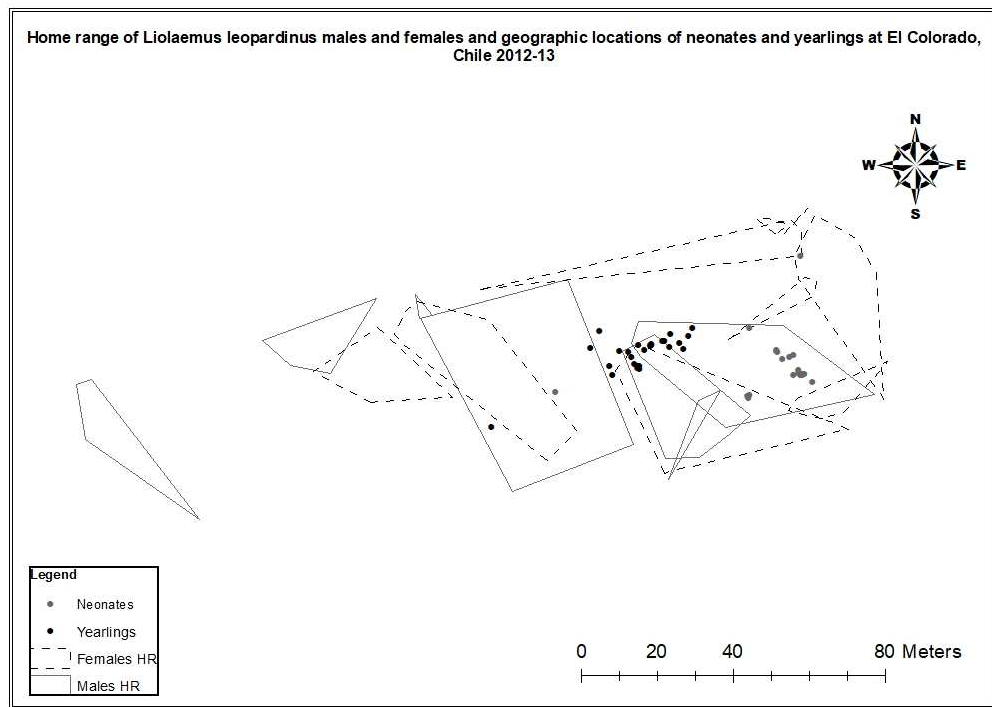


Figure 3. Home ranges of adult males and females, and geographic locations of neonates and new yearlings at El Colorado, Chile. Neonates were located during mid-March to late-April, 2013, and yearlings during November 2013.

In 2011-12, we identified eight communal refuges. These were deep crevices inside massive rock outcroppings. Openings were small, but the interior often opened into a sizeable chamber, up to 130 cm deep. Others were much smaller. In the most used refuge (1a) in two 1-day monitoring

sessions and one 3-day session during mid-April, we recorded PIT tag readings or radio signals of 11 individuals: 7 males and 4 females (Table 2). Observations through the borescope, however, revealed quite a few more cohabiting unidentified lizards as well.

Table 2. Subjects detected in refuge 1a by PIT tag reading and/or radio signal at El Colorado, Chile, during mid-April of field season 2011-12.

Color code	Sex	SVL (mm)	Age group	PIT tag/Radio
WWWG	♂	82.5	A	P
GWWW	♂	70.5	A	P
WGWW	♂	62.0	J	P
YBBB	♂	84.5	A	P
RRBB	♂	94.0	A	P
GWGW	♀	75.0	A	P
WWYY	♂	97.0	A	P/R
WGGW	♀	91.5	A	P/R
WWWY	♂	84.0	A	R
YWWY	♀	94.0	A	R
YYYW	♀	95.0	A	R

Age group: A = adult, J = juvenile

P = PIT tag reading, R = radio signal.

We had planned to use four setups of motion-sensitive cameras at four different refuges, but discovered that the four motion-sensitive game cameras were not triggered to turn on with the very slow gradual exit motion of these lizards leaving a refuge. For that reason, although the PIT tag reader recorded the identity of refuge occupants when they moved through the ring antenna, it was not possible to know with certainty if the subject had left or entered the refuge.

Interestingly, two lizards (adult females YWWY and YYYW) known to be inside from their radio signals (but never recorded via their PIT tags), stayed in the refuge at least 76.4 consecutive hours. Moreover, radio locations suggest YWWY stayed inside this refuge probably for > 7 consecutive days (169.4 h) without leaving, although we did not have 24-h surveillance to confirm our speculation. Groups of radio-tagged adults spent long series of days and nights together in the same deep crevices. We located radio-tagged individuals 73 times and 55.1% of locations were inside the eight known refuges on site.

In 2012-13, we placed a PIT tag-reading antenna for three days at the entrance of one of the known refuges (La Cruz), with all other entrances sealed with cement and modeling clay. This time we recorded activity of 18 different subjects. These were 17 adults (14 males, 2 females, and one individual whose sex was unknown), and one juvenile female (Table 3). During the time the antenna was in service, we placed a motion-sensitive camera that captured images and recorded videos of different individuals in close proximity to each other and near the entrance of the refuge (Fig. 4). Although this time we did get pictures and videos of different subjects in close proximity to the entrance of the refuge, it was not possible to know with certainty when the subjects had left or entered the refuge. We did not detect any radio-signals

of radio-tagged individuals in refuge La Cruz, probably because the refuge was located at the extreme edge of our study site.



Figure 4. Adult male RRYY and two unknown *Liolaemus leopardinus* (one inside the PIT-tag-reading ring antenna) in close proximity to each other and the antenna located in the entrance of refuge La Cruz on 18 April, 2013, at El Colorado, Chile. All entrances were blocked with cement and modeling clay and only the entrance where the antenna was placed remained open.

Table 3. Subjects detected in refuge La Cruz by PIT tag reading at el Colorado, Chile, during austral fall in 2013.

Color code	Sex	SVL (mm)	Age group
BYYY	♂	98.0	A
GGRG	♂	84.0	A
YOOO	♂	90.0	A
YYWW	♂	96.0	A

GGOO	♂	93.0	A
YOYY	♂	94.0	A
RRRG	♂	99.0	A
OGGG	♂	87.0	A
GOOO	♂	68.0	A
OYYO	♂	92.0	A
BBWB	♂	85.0	A
RRYY	♂	90.0	A
WWWO	♂	86.0	A
YOOY	♂	90.0	A
RGGR	♀	92.0	A
GGGR	♀	79.0	A
GOGO	♀	65.0	J
WRRR	-	90.0	A

Age group: A = adult, J = juvenile.

Thermoregulatory benefits of refuge use and communal refuging--Ambient temperatures inside refuges were colder (mean = 15.9 ± 1 SE 1.0°C) than those outside refuges (mean = $20.7 \pm 2.5^{\circ}\text{C}$) and these differences were marginally statistically significant (paired t-test:

$t_{(6)} = 2.25$, $P = 0.059$). Temperatures were measured over several days near the end of the season after we had used radiolocations to demarcate refuges. Thus, the sample size is very low and outside temperatures had begun to drop with the end of the fall season. Temperatures of refuge residents were significantly colder than outside ambient temperatures (paired t-test: $t_{(6)} = 3.08$, $P = 0.02$), and temperatures of inside adult and yearling residents were not significantly different than ambient temperatures inside refuges (paired t-test: $t_{(6)} = 0.83$, $P = 0.44$). We also measured and compared the temperatures inside and outside rock crevices (not known refuges) and related the inside temperature to depth. The temperatures inside 20 crevices were significantly colder than outside air temperatures by a mean of 5.3°C (paired t-test: $t_{(19)} = 8.21$, $P < 0.001$), and inside temperature decreased (but not significantly) with depth of the crevice ($F_{(1,18)} = 6.68$, $P = 0.19$). Thus, refuges were colder than outside, marginally colder the deeper they were, and residents with long-term occupancy took on the ambient temperature of the refuge.

During the five nights we measured temperatures inside the pile of sleeping lizards in the laboratory compared to ambient temperatures away from the pile, piled lizards' temperatures were no different than ambient temperatures (paired t-test: $t_{(71)} = 0.132$, $P = 0.90$), and the regression coefficient was 0.968 ($P < 0.001$).

Kin recognition--We conducted T-maze trials with lab-born neonates to determine if neonates of *L. leopardinus* discriminate and approach their mothers (vs. other recent mothers) from scent alone in the dark. In total we conducted trials on 17 neonates and found that neonates indeed significantly discriminated and approached the scent of their mothers compared to the scent of other mothers (Chi-square = 4.764 , $df = 1$, $P = 0.029$).

Relatedness analysis--We intended to utilize 11 DNA microsatellite loci to conduct the analyses of genetic relatedness, but alleles of four of them consistently failed to amplify, which reduced our number of microsatellite loci to seven. We genotyped the samples and scored the readings of 179 subjects, but excluded 19 because they lacked information on two or more loci. This reduced our sample size to 160, and the total number of alleles analyzed was 42. The results of our test for Hardy-Weinberg equilibria indicated a heterozygote deficiency at three loci, suggesting the possible presence of null alleles (Table 4). An alternative hypothesis, which seems more likely, is the heterozygote deficiency was caused by inbreeding in this rather closed population where relatives sometimes breed with one another. Consequently, we did not include null alleles in our subsequent genetic analyses. The probability of identity using the combined information from the seven loci was 0.00001.

Table 4. Loci and number of alleles used to conduct relatedness analyses from samples collected from a population of *Liolaemus leopardinus* located at El Colorado, Chile. Blood samples were collected during December to April of 2011-12 and 2012-13, and in November, 2013.

Locus	Number of alleles (range of base pairs)	P-value*	Null allele frequency
Lio01**	7 (169-193)	0.000	0.099
Lio06	8 (146-166)	0.154	--
Lio07**	7 (198-210)	0.003	0.103
Lio09	6 (124-142)	0.999	--
Lio36	4 (62-70)	0.500	--
Lio45	5 (136-148)	1.000	--
Lio134**	5 (133-145)	0.000	0.162

*100,000 Monte Carlo randomizations test for deviation from Hardy-Weinberg expectations.

* Loci with low P-values indicated the presence of null alleles

Over both field seasons we observed 76 social interactions. These ranged from pairs of individuals basking very close to each other (often in contact) or located within a rock crevice to groups of more than six subjects sharing a refuge, or various subjects in close geographic proximity. Of the 76 interactions, 33 corresponded to related adult individuals; the coefficient of relatedness (r) was 0.10-0.74. Eighteen of the 33 interactions were between a male and a female; relatedness was $r = 0.10$ -0.74, with an average r of 0.33. We recorded eight interactions of adults and juveniles, of which two corresponded to related adult males and juveniles and six to related adult females and juveniles. The male-juvenile coefficient of relatedness was 0.14 and 0.50. The female-juvenile coefficient of relatedness ranged from 0.10 to 0.54, with a mean of 0.21.

For 2011-12, based on the data derived from HR overlap analyses, we identified 19 pairs of overlapping adult males. Of these, 10 pairs were genetically related, and relatedness ranged from 0.18 to 0.63, with a mean of 0.36. In the same field season, we identified 10 pairs of females whose HRs overlapped, and seven were related; range of relatedness was 0.12 to 0.50 (mean $r = 0.26$). In 2012-13, we identified four pairs of overlapping males, but only two pairs were related, with r of 0.16 and 0.21 (mean $r = 0.18$). For the same field season, we identified five pairs of overlapping females; however only two pairs were related, with r of 0.10 and 0.50 (mean $r = 0.30$) (Table 5).

Table 5. Same-sex relatedness coefficients between pairs with HR overlap during field seasons of 2011-12 and 2012-13, at El Colorado, Chile.

Field season					
2011-12			2012-13		
Male	Male	<i>r</i>	Male	Male	<i>r</i>
RRBB	RRRW	0.18	BBBW	GWGG	0.16
BBYY	RWRW	0.22	GYGG	WWYY	0.21
WWRW	YYWW	0.21	--	--	--
RWRW	WYWW	0.25	--	--	--
BBBR	BBYY	0.24	--	--	--
BBYY	WYWW	0.41	--	--	--
BBBR	RWRW	0.50	--	--	--
BRBB	WWYY	0.50	--	--	--
BRBB	RRBB	0.50	--	--	--
RRBB	WWYY	0.63	--	--	--
Female	Female	<i>r</i>	Female	Female	<i>r</i>
GGGW	WRRW	0.12	WOWO	YWWY	0.50
YWWY	YYYW	0.13	BBRB	RBRB	0.10
WYYW	YYR	0.15	--	--	--
YYWY	YYR	0.20	--	--	--
BWBB	RYYY	0.37	--	--	--
YWWY	YYWY	0.38	--	--	--
WRRW	YWWY	0.50	--	--	--

Based on the data derived from the HR analyses, we identified juveniles within HRs of adults (juveniles located within a 1-m buffer of the adult's HR were included) (Fig. 5).

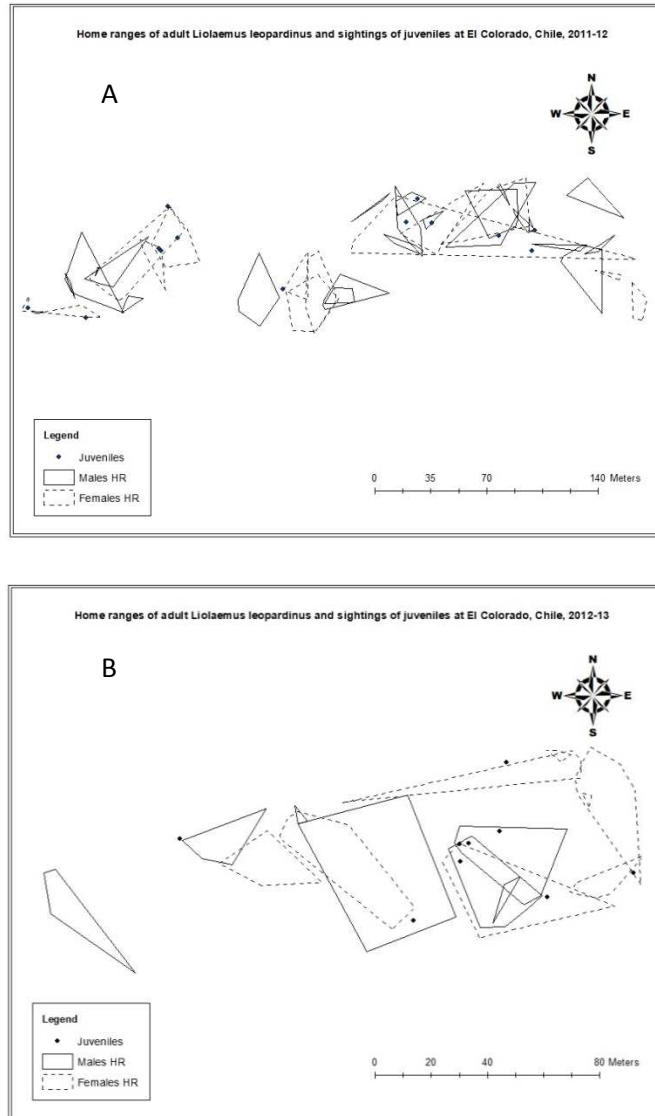


Figure 5. Juveniles found within home ranges of adults (juveniles located within a 1 m buffer of the adult's HR were included) during field seasons of 2011-12 (A) and 2012-13 (B), at El Colorado, Chile.

In the field season of 2011-12, we identified 10 different juveniles within the HR of nine different adults (3 males and 6 females). The mean coefficient of relatedness between juveniles and adult males was 0.36, and between juveniles and adult females was 0.35. In 2012-13, we identified four different juveniles within the HR of three different adults (2 males and 1 female).

The mean coefficient of relatedness between juveniles and adult males was 0.34, and the relatedness between the juvenile and the adult female was 0.10 (Table 6).

Table 6. Juveniles found within adult male or female HRs during field seasons of 2011-12 and 2012-13 at El Colorado, Chile.

Field season					
2011-12			2012-13		
Juveniles	Males	<i>r</i>	Juveniles	Males	<i>r</i>
RRRY	RWRR	0.17	ORRO	WWYY	0.14
WYWY	WWYW	0.41	ORRO	BBBW	0.50
WBBW	YWWW	0.34	RGRG	BBBW	0.38
WWBW	YWWW	0.50	--	--	--
Juvenile	Females	<i>r</i>	Juveniles	Females	<i>r</i>
BYYB	YBYB	0.13	RGRG	RBRB	0.10
WYWY	BWBB	0.19	--	--	--
WYWY	YYR	0.16	--	--	--
RWWW	YYR	0.50	--	--	--
WBBW	BWBW	0.37	--	--	--
RRRR	BWWW	0.54	--	--	--
BYYB	BBWB	0.55	--	--	--

Of the lizards found using refuge 1a in 2011-12, the range of the coefficient of relatedness was 0.10-0.63 (mean $r = 0.26$). Of those lizards in refuge La Cruz, the range of the coefficient of relatedness was 0.10-0.81 (mean $r = 0.30$). In mid-November 2013, we found in a crack below a flat rock shelf two adult males (BBBW and WWYY), adult female RBRB, and a young yearling male (ORRO). All four lizards spent the night together and all were hand captured as they exited the next morning. Adult male BBBW and the yearling ORRO were related as 0.50, likely a father-son combination; the remaining lizards were not related.

Discussion

After years of anecdotal observations, many field and laboratory studies, and various genetic analyses, we can now confirm that sociality in reptiles, especially squamate species, is a fact, and often associated with close relatives (Brent and Duvall 1995; Mouton et al. 1999; O'Connor and Shine 2003; Davis et al. 2010; Doody et al. 2013; Gardner et al. 2016). In our study of *L. leopardinus*, we documented, evaluated, and quantified a) grouping behavior, b) thermoregulatory aspects of refuge use and communal refuging, c) kin recognition, and d) genetic relatedness among group members.

Mark-recapture—Adult *L. leopardinus* were only minimally sexually dimorphic in body size; males were slightly larger than females. Home range size also did not differ statistically between males and females, which makes sense since in lizard species sexually dimorphic in body size and in which males are significantly larger, males have larger home ranges and defend them, i.e., territories (Turner 1969; Perry and Garland 2002). Home range overlap within and between sexes was ongoing and extensive in both field seasons. The behavior and space use by *L. leopardinus* clearly shows that the species does not defend territories or females; females move around the site freely, and males do not engage in intra-sexual agonistic behavior (Fox and Shipman 2003; Santoyo-Brito et al. In prep.). Although we did not measure food availability, apparently it was not a limited resource during the time period of our study. Invertebrates and plant matter were abundant during the lizard active season, and we never observed intraspecific competition over food. We found larger HRs in both sexes in both years than HR size reported by Fox and Shipman (2003) for the same species at the same site some 25 years before. Although it is expected that HR size might differ between years (Tinkle 1967; Ferner 1974; Rose 1982; Ruby and Dunham 1987), this difference could be attributable to differences in lizard density between the two studies. The density of the species during our study was substantially lower than that reported by Fox and Shipman (2003).

Use of communal refuges has been described in various lizard species (Leu et al. 2001; Chappel and Keogh 2006; Gardner et al. 2016), and it is known that lizards make use of many refuges within their home range (Bull 2000; Mounton 2011). During our field work, we discovered various communal refuges used by different adults and older juveniles of both sexes. We did not estimate rock crevice abundance or availability; however, our field observations indicate that suitable crevices for communal refuges are all over our field site. Since crevices are not a limited resource--especially for the lizard density estimated for our site--we suggest that aggregations of *L. leopardinus* and the use of communal refuges is driven mainly by a social component rather than an ecological factor (Vasconcelos et al. 2017).

Thermoregulatory benefits of refuge use and communal refuging--The refuges we discovered do not appear to offer thermoregulatory benefits to *L. leopardinus* during their active season since refuges were cooler than outside and because daily temperatures at this site do not reach such high levels so as lizards cannot be active (Fox and Shipman 2003). We also know that piled *L. leopardinus* do not generate heat. But, we do not discard other benefits that the refuges and aggregations may offer the lizards, for example lowered water loss, decreased metabolism and thus energy savings (Fox 1975), and predator protection (Cooper et al. 1985; Shah et al. 2003). Roosting behavior or association during inactive periods during the active season has been reported in mammals, birds, and reptiles (Anderson 1998; Beauchamp 1999; Willis and Brigham 2004; Rhodes et al. 2006; Leu et al. 2011). Sleeping aggregations are recognized as an integral part of social behavior (Hamilton 1982), especially when a large number of suitable sleeping sites within home ranges are available (Kerr et al. 2003) but not used. Refuge sharing behavior in *L. leopardinus* might be a precursor to more complex social behavior during periods of activity (Shah et al. 2003; Leu et al. 2011).

Kin recognition—Discrimination of kin is a probable and perhaps universal requirement to form stable social groups (O'Connor and Shine 2006). Our results suggest that neonate *L. leopardinus* discriminate between the odor of their mother and a different recent mother. Kin recognition by odor has been documented in other squamate species (Marin and Bull 1996; Leña and de Fraipont 1998; Leña et al. 2000). We discovered that the mother stays with her sequestered neonates for at least the first 24 h after parturition (Santoyo-Brito et al. In prep.). It is possible that during that period of time, neonates imprint on their mother by olfactory cues (Leña and de Fraipont 1998; Bull et al. 2000; Leña et al. 2000; O'Connor and Shine 2006) and later use that cue when joining established social groups of older juveniles and adults. Kin recognition is considered an important trait in many social species and its function is of major importance when it is beneficial to interact with related and unrelated individuals (Bull et al. 2001). Also, kin recognition is the basis for most if not all sociality and may lead to the formation of family groups (Hamilton 1964; Leña and de Fraipont 1998; Bull et al. 2000; Leña et al. 2000; Chapple and Keogh 2006). Living in groups is advantageous especially for young individuals (i.e., yearlings) since groups can reduce predation pressure via increased vigilance (Krebs and Davies 1993; Laham and Bull 2004; Gardner et al. 2016) and if groups are kin, costs to those scanning for predators is reduced due to the positive aspects of kin selection (Hamilton 1964; Lanham and Bull 2004; Sinn et al. 2008). During our study we observed three bird attacks toward grouped lizards and not a single lizard was harmed since the startle reaction of one provoked the others to flee and take refuge within a crevice in close proximity. Nevertheless, newborns are not with the large social groups on the rock outcrops because these small-sized individuals would be extremely vulnerable to bird predation in this open habitat (See Chapter 1). We know that predation pressure by birds is heavy during the first year of life of the solitary neonates (Santoyo-Brito et al. 2014), but predation risk decreases as subjects reach the larger size of an

older juvenile (See Chapter 1). During their next active season, in November, the new yearlings can be found in closer proximity to social groups of related juveniles and adults.

Relatedness analysis—In a pilot study conducted in 2005, it was common to observe groups of *L. leopardinus* (various individuals of different ages, or pairs of an adult male and female with two or three juveniles) on rock outcrops. This behavior was also observed in our two field seasons. Of eight social interactions between adults and juveniles observed in the field during 2012-13, five were between related females and juveniles (mean $r = 0.23$). One of these female-juvenile interactions appeared to be between mother and offspring. Mother-offspring interactions have been reported in different lizard species, suggesting kin recognition and discrimination of nonrelated subjects (Leña and de Fraipont 1998; Bull et al. 2000; Leña et al. 2000; Chapple and Keogh 2006). We also determined that groups of lizards of multiple ages found in communal refuges were often closely related individuals. The occupancy of refuges, membership, and relatedness within groups varied, suggesting that social aggregations in *L. leopardinus* are probably short term, and formed by fusion of family units found within the “related individual’s home range,” and not formed by large and long-term stable aggregations as shown in different species of the highly social genus *Egernia* (Garner et al. 2016). It has been proposed that recognition of kin and close proximity associations are the basis for more complex social behavior, for example, direct and indirect parental care (Waldman 1988; O’Connor and Shine; 2004; Sinn et al. 2008; McAlpin et al. 2011). Interactions between females and juveniles in our study are of special interest since female *L. leopardinus* care for their neonates (Santoyo-Brito et al. In prep.). Nevertheless, the genetic analysis does not indicate which lizards were parents and mothers to specific subjects; it estimated only the degree of genetic relatedness.

In summary, in this study we provide spatial, behavioral, and genetic evidence that supports the conclusion that groups formed by *L. leopardinus* at El Colorado, Chile, are indeed social groups of related individuals with non-relatives mixed in. Our results indicate that genetically related juveniles and adults sometimes spend time in close association on top of rock outcrops, sometimes share home ranges, share communal refuges during the day and night, and overwinter together in deep rock crevices. Aggregations in this species are not resource-based, are not sex-specific, and members of social groups are for the most part genetically related. It is of special importance to highlight that *L. leopardinus* is limited to inhabit rock outcrops, a condition that might have forced conspecifics to coexist in close proximity. It has been proposed that concentrations of individuals may necessitate a different social structure than that of strict territoriality (Ferner 1974), facilitating the evolution of a kin-structured society offering benefits to its members (Hamilton 1964; Davis et al 2010; Leu et al. 2011). At our study site, refuges are not a limited resource and plenty of suitable crevices are available. This rejects the environmental constraint hypothesis to explain the evolution of sharing refuging sites (Leu et al. 2011). Social aggregations (Brown and Orians 1970; O'Connor and Shine 2003; McGlynn 2010) occur when there is mutual attraction among conspecifics because being together increases an individual's fitness (Graves and Duvall 1995; Ward and Webster 2016). It is probable that older adults tolerate yearlings and juveniles because they do not jeopardize their mating success (Gardner et al. 2016 and references therein).

Liolaemus leopardinus can discriminate between kin and non-kin based on olfactory cues, a trait that can help yearlings find and join groups of relatives. However, we do not discard learning of other kin signals during the short period of time that the mother is with her newborns (Clark 2004; O'Connor and Shine 2006) and additionally, even any learning phenomena during embryonic development should not be eliminated (Bull et al. 2001). We

suggest that right after emerging from brumation, the new yearlings are in the process of looking for and joining social groups composed of relatives. Our sightings and geographic locations show that during November yearlings are moving toward the habitat occupied by juveniles and adults, and in some instances they can already be found next to kin within crevices. Once they have reached the body size of a juvenile, they are less susceptible to bird predation and they can be found within social groups on exposed rock outcrops. We suggest that decreased predation is one of the benefits that young individuals obtain from living in groups, since predation decreases in groups (Krebs and Davies 1993). It would not be far-fetched to propose that the new yearlings seek out one social group after another until they find their mother in a group (via scent-matching to their imprinted maternal odor during the neonate's first 24 hours of life) and then stay within the group. However, it is possible that some yearlings and juveniles fail to find their mothers and simply stay with a group of other relatives or non-relatives.

It is important to highlight that *L. leopardinus* lizards are not always found in social groups and at times are solitary. This behavior has been described in other group-living lizard species (Mounton et al. 2014). Our observations suggest that the social structure in the species is more likely to be a fission-fusion society characterized by short-term associations of groups of individuals that allow mixing, resulting in the formation of new groups (Archie et al. 2006; Lusseau et al. 2006; Strickland et al. 2014). As a whole, our study revealed fine details regarding the gregarious behavior of *L. leopardinus*, a social but highly secretive lizard species. We suggest that predation, kin recognition, and roosting (i.e., refuge sharing) are driving the evolution of social groups in *L. leopardinus*, and not thermoregulation, refuge availability, or food.

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VITA

Enrique Santoyo Brito

Candidate for the Degree of

Doctor of Philosophy

Thesis: GROUP LIVING, PARENTAL CARE, AGE STRUCTURE, AND GENETIC RELATEDNESS IN *LIOLAEMUS LEOPARDINUS*, A HIGH-ELEVATION LIZARD FROM THE ANDES OF CHILE

Major Field: Zoology

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy/Zoology at Oklahoma State University, Stillwater, Oklahoma in December, 2017.

Completed the requirements for the Master of Science in Wildlife Management and Conservation. Colegio de Postgraduados. Texcoco de Mora, Estado de México, México, in 2009.

Completed the requirements for the Bachelor of Science Licenciatura en Biología, Universidad Veracruzana. Córdoba, Veracruz, México, in 2006.

Experience:

Graduate Teaching Associate, Department of Integrative Biology at OSU.
Project Coordinator Amigos de Sian Ka'an, Cancún, Quintana Roo, México.

Professional Memberships:

Society for the Preservation of Natural History Collections (SPNCH).

Society for the Study of Amphibians and Reptiles (SSAR).

The Herpetologists' League (HL).

The Southwestern Association of Naturalists (SWAN).

The American Society of Naturalists (ASN).

OSU-Zoology Graduate Student Society (ZoGSS).