1 **Ontogeny of daily activity and circadian rhythm in the Iberian lynx**

2 **(*Lynx pardinus*)**

3
4 Javier Yerga¹ (Corresponding author)

5 ¹Facultad de Ciencias Experimentales, Universidad de Huelva. Avda. Fuerzas Armadas s/n,
6 21003 Huelva, Spain.

7 Tel: +34 959-219894 E-mail: javiyr_86@hotmail.com

8
9 Javier Calzada²

10 ²Facultad de Ciencias Experimentales, Universidad de Huelva. Avda. Fuerzas Armadas s/n,
11 21003 Huelva, Spain.

12
13 Xavier Manteca³

14 ³Facultad de Veterinaria, Universidad Autónoma de Barcelona Bellaterra, 08193, , Barcelona,
15 Spain.

16
17 Astrid Vargas⁴

18 ⁴ Tompkins Conservation. Gerard Doustraat 9A, 1072VJ, Amsterdam, The Netherlands.

19
20 María José Pérez⁵

21 ⁵Centro de Cría del Lince Ibérico “La Olivilla”. Agencia de Medio Ambiente y Agua de
22 Andalucía. Consejería de Medio Ambiente. 23213, Santa Elena, Jaén, Spain

23
24 Francisco Palomares⁶

25 ⁶Department of Conservation Biology, Estación Biológica de Doñana, CSIC, Américo Vespucio
26 s/n, 41092 Sevilla, Spain

⁷Centro de Cría del Lince Ibérico “El Acebuche”. P. N. Doñana. Carretera El Rocío-
Matalascañas, 21760, Matalascañas, Huelva, Spain

Abstract

The aim of this paper is to describe the ontogeny of the circadian activity rhythms in captive-born Iberian lynx (*Lynx pardinus*) from birth to subadult age and to verify if they develop a bimodal circadian pattern similar to the one they show in the wild. The amount of daily activity and the circadian rhythmicity of 61 Iberian lynx (48 mother-raised and 13 hand-reared) cubs were studied in two breeding centers. During the first month of life, the cubs were active 30% of the day. Activity increased up to 50% during the following two weeks, and then it remained constant until the end of the lynx physical development. The location of the breeding center affected the amount of daily activity of the cubs probably as result of different climatic conditions. Once the lynx cubs had completed their development, there was no difference in the time spent active between wild and captive lynx. Newborns had a constant level of activity throughout the day (24 hours) during the first month of life. During the second month, once they had developed sufficiently to leave the den, a crepuscular bimodal circadian rhythm started to emerge. This pattern was completely defined by the third month of life. The overlap in circadian activity was over 0.88 when comparing the sexes, breeding centers and rearing methods. However, there was a significant difference between mother-raised and hand-reared cubs -the latter being more active in the morning-, as well as between breeding centers -again due to climate differences-. Finally, an overlap of 0.85 in circadian activity pattern was observed between wild and captive lynx, although the wild animals were more active at night.

Keywords: circadian rhythm, daily activity, Iberian lynx, ontogeny, reintroduction.

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57 **1. Introduction**

58 Circadian rhythmicity is present in various physiological mechanisms of animals. It affects
59 sleeping and feeding patterns (Borbély and Neuhaus, 1978), as well as body temperature, brain
60 activity and the production of hormones (Albrecht and Eichele, 2013). These rhythms have an
61 endogenous time period, called free-running period, which lasts about 24 hours, but they can be
62 modified by external environmental factors (Aton et al., 2004). Daylight is the main *zeitgeber* or
63 synchronizer of circadian rhythmicity (Kavanau and Ramos, 1975). Within these rhythms, the
64 circadian rhythm of activity is one of the main adaptations of species to their environment
65 (Daan & Aschoff, 1982). In predators, interspecific relationships between them and their prey
66 play a fundamental role in the modeling of the activity rhythms (Halle, 2000; Lima, 2002),
67 given that most predators must adapt their activity to that of their prey to improve their
68 predatory efficiency (Monterroso et al., 2013). However, circadian activity rhythms can be
69 modified by the combination of various environmental factors such as light (Pittendrigh, 1993)
70 and temperature (Kolbe et al, 2007; Palkova et al, 1999).

71 Wild animals living in captivity often suffer disturbances in both their behavior and their
72 activity rhythms (Berger, 2011). The captive environment may be very different to the wild one.
73 The lack of space and natural stimuli, as well as captive husbandry practices, may hamper the
74 development of the normal behavior of the species. A familiar example is provided by the
75 artificially altered endogenous activity rhythms of carnivores as a result of the way they are fed,
76 namely with dead meat (i.e. they cannot develop hunting behaviors) and on a fixed time
77 schedule during the working hours of the keepers (Shepherdson et al. 1993). The difficulties in
78 developing natural behaviors in captivity may lead to a reduction in animal welfare and to an
79 increase in the prevalence of diseases (Munson et al., 2005) and stereotypical behaviors
80 (Carlstead, 1996; Shepherdson et al., 1993), such as pacing (Clubb and Vickery, 2006).

81 Preventing these negative effects is always important for ethical reasons, but even more so when
82 the goal of breeding animals in captivity is to reintroduce them into the wild. Animals that are
83 being breed for reintroduction programs should be housed in well-enriched and spacious

enclosures and husbandry practices must strive to prevent the modification of circadian cycles of activity, as released animals must have their activity pattern synchronized with that of species in the wild. And in the case of predators that actively chase their prey, they have to be able to hunt, and particularly during the time of day that prey are active, i.e. available, in the wild (Eriksen et al., 2011).

The Iberian lynx, *Lynx pardinus*, is a wild felid species that is currently being breed for reintroduction in Spain and Portugal. The objective of the Iberian Lynx Captive Breeding Program (hereafter ILCBP) is to breed physically and behaviorally healthy lynx, which can reinforce wild populations (Vargas et al., 2009). The ILCBP lynx intended for reintroduction are subadults (8-20 months). The Iberian lynx is a specialist predator whose circadian rhythm is synchronized with the activity of its main prey, the rabbit (*Oryctolagus cuniculus*; Delibes, 1980). Both rabbits and lynx are active primarily at night and during twilights (Beltrán, 1988; Jilge and Hudson, 2001).

The Iberian lynx is a semialtricial species whose young are born with poorly developed sensory and locomotor systems (Yerga et al., 2014). The pattern of circadian activity is not usually present in altricial species at birth (Ibuka, 1984, Kowalska et al., 2010). Rhythms typically appear days or weeks after birth, once the cubs leave the den and are exposed to local environmental factors such as the daylight cycle and temperature. Furthermore, mothers can act as synchronizers of the circadian behavioral rhythm of their young (Favreau et al., 2009).

The aim of this paper is to describe the ontogeny of the circadian activity rhythms in Iberian lynx born as part of the ILCBP from birth to subadult age and to verify if they develop a bimodal circadian pattern similar to the one they will need in the wild.

2. Material and methods

The activity of 61 Iberian lynx (36 males and 25 females) born in two specific breeding centers, "El Acebuche" (hereafter EA, 37°03'00" N 6°33'17" W) and "La Olivilla" (hereafter LO, 38°20'37"N 3°33'33"W), both located in Andalusia, Spain, was studied. The cubs were born between 2008 and 2011 and derive from 28 litters from 13 different mothers.

Housing conditions and protocols for handling animals were similar in the two breeding centers. Each mother and its litter were housed in a big and enriched enclosure. The size of the enclosures varied between the breeding centers (550 m² in EA and 1000 m² in LO). In both breeding centers, the facilities included a main outdoor area enriched with the typical vegetation of the Mediterranean habitat, sleeping structures, two or three dens, burrows where rabbits were released (see below), and visual barriers that allowed the lynx to hide from the view of keepers. The husbandry of the animals was planned so as to minimize disturbances to the animals and to avoid imprinting on humans as far as possible. Sometimes cubs were abandoned by their mothers and had to be hand-reared (13 cubs in the study). Those hand-reared cubs were kept in an incubator room during the first month of life. Later these animals were transferred to the enclosures and housed alone (i.e. without their mothers and brothers), where they were bottle fed for one additional month. At two months of age the cubs were weaned, and from that age onwards both mother-reared and hand-reared cubs were fed live wild rabbits. In order to stimulate behaviors similar to those exhibited by lynxes in the wild, the animals were fed, as far as possible, with wild rabbits released in the burrows throughout the day.

2.1 Sampling method

The activity of the cubs was recorded by a remote video surveillance system. It was considered that an animal was active when it was awake. The enclosures had 2-3 mobile cameras and an additional fixed camera in each den. All cameras were equipped with infrared lights that allowed the monitoring of the cubs 24 hours a day. The video surveillance system was continuously operated (24h) by trained observers from a room located 150 meters away from the enclosures. Thus, it was possible to record activity throughout the day without disturbing the lynxes.

The activity of the cubs was tracked between birth and 7 months of age using a point sampling method. The interval of recordings was predefined. The activity of each cub was recorded every 20 minutes in 2008 and 2009, and every 60 in 2010 and 2011, 24 hours a day, three to seven days a week. In total, 139,897 activity records were obtained, and the activity of each cub was

documented between 168-216 times per week. The potential bias due to the amount of activity records obtained by lynx in a time interval was considered for the analysis of the activity. In order to establish whether the amount of time that the captive young lynx remained active, and the activity pattern were similar to that observed in nature, the activity of captive lynx was compared with the activity of young and subadult lynx monitored in a long-term study on the ecology of the Iberian lynx performed in the north of Doñana National Park between 1993 and 1996 (Palomares et al., 2001), 15 km from the EA breeding center. In that study, 26 lynx were caught and radio tracked an average of 274 (± 60) days, of which 18 (10 females and 8 males) were non-adult lynx. All lynx were fitted with 150-151 MHz radio-collars (Wildlife Materials, Inc., Carbondale, Illinois) which contained tip switches to monitor activity. The activity of each lynx was recorded an average of 10 times a week (range 1-59), including day and night locations. In addition, 11 of these animals were followed in 20 intensive 24 h monitoring sessions, where the animals were located and their activity recorded once per hour, beginning at 12:00 h of a given day and ending at 12:00 h of the next day (see details in Palomares et al., 2001).

2.2 Statistical analysis

2.2.1 Daily activity

The daily activity (estimated as the proportion of daily records where the lynxes were active) was calculated and the influence of age (in weeks), sex, rearing method (hand or mother-reared) and breeding center (EA, LO) was evaluated through a generalized linear mixed model (GLMM). This method is suitable for repeated measures data and provides estimates of the parameters for each level of the random effects. Sex, rearing method, breeding center and age were included as fixed factors and the individual lynx and the number of activity records registered by lynx as random factors to account for the non-independence of the data from the same animal and the variations in the number of records obtained by lynx in a time interval. A model for each of the possible combinations of fixed effects was constructed. The set of candidate models were ordered according to the AIC value. Analysis was performed using the

'lme4' package of R software (R Development Core Team, 2008). Finally, an ANOVA-test was performed to assess differences in the daily activity of the captive-born cubs and non-adult wild lynx. Normality was tested before performing the analysis. In all cases, α -value was 0.05.

2.2.2 Circadian rhythm

The circadian activity rhythm of each lynx cub, estimated as the proportion of active records in every hour in each month, was calculated. It was verified that the number of activity records registered per animals did not alter the proportion of activity in this analysis (Mardia-Watson-Wheeler test, $W = 5.0968$, $df = 2$, $p = 0.078$). The effect of age (in months), sex, breeding center, rearing method and birthplace (captivity or wild) on the circadian rhythms of the cubs was evaluated. Pairwise comparisons of the activity rhythms between sexes, breeding centers, rearing methods and birthplace were performed by estimating the overlap coefficient Δ_4 , as suggested by Ridout and Linkie (2009) for large sample sizes. This overlap coefficient, which can have values between 0 (no overlap) and 1 (complete overlap), was obtained by taking the minimum of activity of the two groups at each time of day. The accuracy in the estimation of Δ_4 was obtained by calculating the standard deviation of 500 bootstrap samples. However, the overlap coefficient is solely descriptive, and thus it does not provide a threshold that can determine the existence of significant differences between the compared groups. Thus, a Mardia-Watson-Wheeler test (MWW test; Batschelet, 1981) was performed to compare the distribution of circadian activity. This test combines the activity data and orders them in increasing angles, so that they are distributed interspersed around the daily cycle, and a uniform, or circular rank, score is then calculated. If the distribution of samples is the same, the resulting vector will be short and similar. Significant differences between the lengths of the vectors lead to a high value of the statistic W and the rejection of the null hypothesis that the distributions are equal (Kovach, 2011). All analyzes were performed using R (R Development Core Team, 2008) software, including the package 'circular' for the MWW test.

3 Results

3.1 Daily activity

During the first five weeks of age, while the cubs remain in the den, they were active around 30% of the day (Fig. 1). Afterwards, between the sixth and the ninth week of life, there was a progressive increase in daily activity from 30% to 50%. And finally, daily activity remained at 50% from the ninth week of age to the end of the study (at seven months old). A slight decrease in the amount of activity occurred between weeks 21 and 25 (during the summer, Fig. 1). The model that best explained (i.e. had the lowest AICc) the daily activity of the Iberian lynx cubs included age and breeding center as explanatory variables (Table 1). The daily activity of lynxes changed significantly with the age ($z = 10.926$, $p < 0.01$), and it was different for each breeding center ($z = 5.663$, $p < 0.01$). Cubs born in LO displayed less activity than those born in EA ($5\% \pm 1$; mean $\pm$ SE). No significant differences between mother-reared and hand-reared cubs were found ($z = 1.353$, $p = 0.202$), nor between males and females ($z = 0.594$, $p = 0.745$). Daily activity of wild lynx was $45 \pm 5\%$. Only the daily activity of cubs over 9 weeks was used to assess differences with the daily activity of wild animals. No significant differences were found comparing the daily activity of captive and wild lynx (ANOVA, $F = 0.94$, $p = 0.336$).

3.2 Circadian rhythm

The evolution of the circadian pattern of lynx cubs born in captivity is summarized in Fig. 2. During the first month of life, the activity of cubs was quite uniform throughout the 24 hour day, varying between $19 \pm 3.2\%$ at 20:00h and $39 \pm 6.2\%$ at 12:00h. In the second month of life, a bimodal pattern emerged. This coincided with the first time the cubs left the den (at 33 ± 0.3 days old, mean $\pm$ SE). Activity increased at both dawn and dusk with peaks at 6:00h ($58 \pm 2.4\%$) and 19:00h ($73 \pm 2.9\%$). In the evening, the activity peak was shorter and higher than at dawn, when activity lasted longer but never surpassed 60%. From the third to the seventh month of life, the bimodal activity pattern was fully established. The maximum activity peak at dawn and at dusk exceeded 80% ($83 \pm 2.2\%$ between 4:00h and 5:00h and $85 \pm 1.5\%$ between 18:00h and 19:00h, respectively) and activity at midday was reduced (below 22% from 12:00h to 14:00h).

Males and females lynxes exhibited a similar circadian rhythm pattern (fig. 3a), with a Δ_4 overlap coefficient of 0.96 ± 0.006 . The MWW test showed no significant differences in the distribution of activity between sexes throughout the day ($W = 1.93$, $p = 0.381$). On the other hand, there were significant differences in the activity circadian pattern depending on the rearing method (MWW test; $W = 43.73$, $p < 0.01$) and the breeding center (MWW test; $W = 10.92$, $p < 0.01$), although both Δ_4 overlap coefficients were high (0.88 ± 0.016 ; 0.90 ± 0.015 , respectively). At night, hand-reared cubs exhibited less activity than those reared by mothers. At dawn, the latter developed their maximum activity at 4:00h, while hand-reared cubs had their maximum at 6:00h. During midday, the activity of mother-reared cubs decreased quickly and stayed below the activity of hand-reared cubs until the dusk peak of activity, in which the groups displayed a similar level of activity (fig. 3b). The activity of the cubs born in LO was less than that of EA cubs for much of the day (from 9:00h to 24:00h; fig. 3c). Only the activity pattern of mother-reared cubs was compared with the circadian rhythm of non-adult wild lynxes (Fig. 3d), because hand-reared cubs were not intended for reintroduction. Wild animals were more active at night than captive animals (around 60% between 21:00h and 1:00h). After that, the activity of the wild lynxes decreased, until it increased again just before the dawn. This peak reached 53% of activity at 6:00h, being much lower than the maximum observed in captive animals. During midday activity remained low in both groups (about 20%). During the dusk, captive animals began to increase their activity earlier than wild animals and they reached a higher maximum value. However, the peak of activity was also observable in wild animals. The maximum was reached at 20:00h with 75% of activity. The Δ_4 overlap coefficient was 0.85 ± 0.016 , but the difference was significant (MWW test, $W = 30.09$, $p < 0.01$).

4. Discussion

4.1 Daily activity

Daily activity of captive-born Iberian lynx cubs varied during their development. Changes in Iberian lynx activity occurred in a similar manner to that observed in the domestic cat

(Hoppenbrouwers and Sterman, 1975), although cats develop slightly faster. This variation was closely related to the sensory and locomotor development of cubs and with their ability to interact with their environment. During the first month of life, while the cubs remained in the den, they were active 30% of the day. During this time, the sensory and locomotor development of lynx cubs was low (Yerga et al., 2014), so their ability to respond to external stimuli was diminished. During this first month of life, nursing was the only activity that cubs displayed, as they remained asleep the rest of the day.

A second period in the ontogeny of the activity of the lynx, between the six and the eight weeks of age, can be described. The beginning of this period coincided with the first explorations outside the den. At this age the cubs had reached a weight of 1kg and were able to walk, and the senses of sight and hearing were fully developed (Yerga et al., 2014). Cubs began to exhibit new behaviors, like playing and exploring. The time that they spent engaging in these new behaviors led to an increase in activity from 30% to 50%. A third period in the ontogeny of the activity of the Iberian lynx cubs began at 9 weeks of age. Cubs honed their motor skills, spent more time playing and exploring the environment, lactation ended and they learned to hunt. The activity of the cubs then remained around 50% for a number of weeks. The slight decrease of activity observed between weeks 21 and 25, which correspond to the warmest period of the summer, was probably due to an increase in temperature that typically leads to a decrease in the activity of lynxes (Beltrán and Delibes, 1994; McNab, 1970).

Even though hand-reared cubs were raised in a very different environment during the first month of life and they were subsequently housed alone, their level of activity was similar to that of cubs reared with their mother and siblings. We did not study whether there are specific differences during development or in the type of behaviors they performed by the cubs when they are active, but from the second month of life, we would expect hand-reared and mother-reared cubs to show a different ethogram, as the former cannot perform some behaviors (e.g. social interactions). It would be necessary to study more thoroughly the behavior of both groups to assess how hand-reared cubs modify their time budget.

The effect of sex on daily activity has been studied in adult mammals, but not in cubs. It seems that differences in daily activity between sexes are related to variations throughout the year that depend largely on differences in behavior between breeding and non-breeding seasons (Schmidt et al., 2009). Therefore, it is expected that this effect does not occur in animals that have not reached sexual maturity. In the Iberian lynx, adult males are slightly more active than females, both in captivity (Penabad et al., 2012) and in the wild (Beltrán, 1988), but the differences are not significant. The animals in our study were juveniles, in which sex-specific behaviors are still not observed, thus consistent with the absence of differences in daily activity. The differences in daily activity between the breeding centers are probably related to the climate of each site. Cubs reared in LO spent less time active than those born in EA as the average daily and maximum temperatures are higher in LO during summer (García, 2011). Oftentimes, captive felids are generally less active than their wild conspecifics, which is probably due to the fact that the lack of stimuli in the enclosures does not allow them to carry out all of the typical behaviors of the species (Weller and Bennett, 2001). However, the activity of captive Iberian lynxes, once it was stabilized after 9 weeks, was similar to that observed in the free-ranging Iberian lynxes. Anyway, a further study would be necessary to know if the type of behavior they perform when they are active are similar in both groups.

4.2 Circadian rhythm

The development phases of the circadian pattern in the captive-born Iberian lynx cubs coincided with the development of the amount of daily activity. The lack of a defined pattern of activity in newborns has been widely observed in other altricial species (Ibuka, 1984). The appearance of overt rhythms depends on the developmental stage (Vargas & Anderson, 1998; Weinert, 2005). Iberian lynx cubs had not developed their sensory and locomotor systems and the main *zeitgebers*, namely light and temperature, did not have any influence inside the dens. In addition, mothers nursed the cubs regularly for 24 hours a day, so that the activity of the cubs was almost constant throughout the day. Once the cubs were out of the den, they were subjected

to the environmental factors that regulate the circadian cycle and they gradually established the characteristic bimodal pattern of the species.

The overlap of circadian rhythms obtained in pairwise comparisons by sex, rearing method and breeding center were very high and the bimodal pattern of circadian activity was maintained in all groups. However, some significant differences were observed. The peak of maximum activity at dawn occurred two hours later in hand-reared cubs than in mother-reared ones. This may be due to the fact that the hand-reared cubs had imprinted on humans. During the morning, keepers performed the tasks of cleaning and maintenance in the area where the enclosures were located. Their presence was a positive stimulus for these cubs, so hand-reared cubs spent more time active trying to interact with the keepers. On the other hand, mother-raised cubs had little contact with keepers during development, so humans were a negative or at least neutral stimulus, and during the morning these cubs were resting. As occurred with daily activity, the effect of the breeding center is likely to be related to the higher temperatures recorded at LO (García, 2011), which resulted in the lower activity, from midday until night, of cubs born at this center.

The comparison of activity patterns between wild and captive lynxes has some limitations. The way the data was recorded for the two groups is markedly different, the records were made in different years with different weather conditions, and also the wild lynxes had to adapt their activity to a changing and less predictable environment, especially concerning the availability of food. Wild lynxes were more active at night, as are rabbits (Monterroso et al., 2013), their preferred prey. Despite all this, there was a bimodal pattern in wild and captive lynxes, both exhibiting peaks of activity at dawn and at dusk. This is consistent with numerous studies which have shown that the circadian rhythm is regulated by an endogenous component (Aschoff, 1960; Minors and Waterhouse, 1984), apart from environmental factors.

5. Conclusions

Many studies have found that captive animals often develop a modified circadian cycle and exhibit less activity than those who live in the wild (Berger, 2011; Weller and Bennett, 2001).

However, our results showed that, once the lynx cubs had completed their development, the time they remained active and the daily distribution of this activity over a 24 hour period was similar to that observed in free-living adults. This is probably related to the type of husbandry and the size and level of enrichment of the enclosures. The husbandry of the animals was minimized to reduce the influence of human presence on their behavior. The enclosures far exceeded the minimum size recommended by the Association of Zoos and Aquariums (AZA), which is 8 m² for small felids between 10 and 20 kg (Mellen, 1997). Furthermore, it has been shown that the degree of enrichment is closely related to the prospect of developing a wide variety of natural behaviors and therefore increases the activity and welfare of animals in captivity (Bashaw et al., 2003; Young, 2003). The ILCBP enclosures included a wide variety of vegetation, vertical structures, visual barriers and burrows where prey could hide, and as a result hunting was more difficult and the lynxes had to perform various behaviors, such as stalking. These actions appear to be essential to ensure that the captive-born Iberian lynxes at the ILCBP are suitable, from the point of view of circadian activity rhythms, to contribute to the reintroduction projects of the species in the wild.

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Model	df	logLik	AICc	Δ AICc	W
A + BrC	6	2968.80	-5925.59	0,00	0.96
A + BrC + ReM	7	2966.28	-5918.54	7.05	0.03
A + BrC +S	7	2965.38	-5916.73	8.86	0.01
Null model	4	2911.10	-5814.20	111.39	0.00

Table 1. Comparison of the different models for describing the ontogeny of the daily activity of Iberian lynx cubs. A is the age, BrC is the breeding centre, ReM is the rearing method and S is the sex. Degrees of freedom (df), log-likelihood (logLik), AICc (corrected Akaike's Information Criterion), Δ AICc (difference between the AICc of a model and the lowest value of AICc for the set of models) and Akaike weight (W) for each model is provided. The table includes those models in which Δ AICc<10 and the null model.

Figure 1. Daily activity (proportion of daily records where the lynxes were active, mean $\pm$ SE) of the Iberian lynx cubs born in captivity between birth and seven months old.

Figure 2. Circadian activity pattern of Iberian lynx cubs born in captivity during the first seven months of life. Activity is presented as the proportion of records cubs were active for each hourly interval.

Figure 3. Pairwise comparison of circadian activity rhythms. Activity is presented as the ratio of time that the animals were active: a) males (solid line) vs females (dashed line), b) hand-reared (solid line) vs. mother-reared (dashed line), c) EA (solid line) vs LO (dashed line), d) wild (solid line) vs captive (dashed line).