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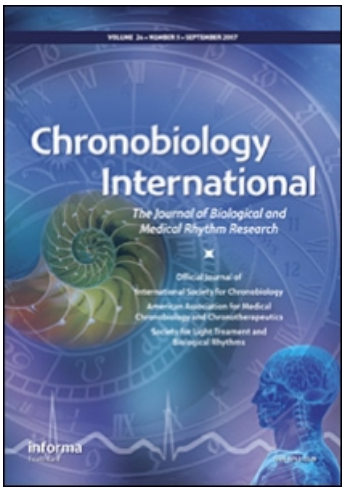

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Circadian Pattern of Wheel-Running Activity of a South American Subterranean Rodent (Ctenomys cf knightii)

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CIRCADIAN PATTERN OF WHEEL-RUNNING ACTIVITY OF A SOUTH AMERICAN SUBTERRANEAN RODENT (*CTENOMYS CF KNIGHTII*)

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Circadian rhythms are regarded as essentially ubiquitous features of animal behavior and are thought to confer important adaptive advantages. However, although circadian systems of rodents have been among the most extensively studied, most comparative biology is restricted to a few related species. In this study, the circadian organization of locomotor activity was studied in the subterranean, solitary north Argentinean rodent, *Ctenomys knightii*. The genus, *Ctenomys*, commonly known as Tuco-tucos, comprises more than 50 known species over a range that extends from 12°S latitude into Patagonia, and includes at least one social species. The genus, therefore, is ideal for comparative and ecological studies of circadian rhythms. *Ctenomys knightii* is the first of these to be studied for its circadian behavior. All animals were wild caught but adapted quickly to laboratory conditions, with clear and precise activity-rest rhythms in a light-dark (LD) cycle and strongly nocturnal wheel running behavior. In constant dark (DD), the rhythm expression persisted with free-running periods always longer than 24 h. Upon reinstatement of the LD cycle, rhythms resynchronized rapidly with large phase advances in 7/8 animals. In constant light (LL), six animals had free-running periods shorter than in DD, and 4/8 showed evidence of "splitting." We conclude that under laboratory conditions, in wheel-running cages, this species shows a clear nocturnal rhythmic organization controlled by an endogenous circadian oscillator that is

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Address correspondence to Verónica Sandra Valentinuzzi, Centro de Investigación Científica y de Transferencia Tecnológica—CRILAR Entre Rios y Mendoza s/n, (5301) Anillaco, La Rioja, Argentina. Fax: 54 3827 494231; E-mail: vvalentinuzzi@crilar-conicet.com.ar, veroval@hotmail.com

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entrained to 24 h LD cycles, predominantly by light-induced advances, and shows the same interindividual variable responses to constant light as reported in other non-subterranean species. These data are the first step toward understanding the chronobiology of the largest genus of subterranean rodents. (Author correspondence: vvalentinuzzi@crilar-conicet.com.ar)

Keywords Circadian rhythm, Tuco-tuco, *Ctenomys knightii*, Subterranean rodent, Rhythm splitting

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INTRODUCTION

Circadian rhythms are essentially ubiquitous in nature, having been demonstrated in almost all organisms that have been examined. Rhythmicity is produced endogenously by a circadian pacemaker that is synchronized to environmental cycles. Circadian pacemakers have free-running periods (τ) that are close to 24 h and are entrained by natural zeitgebers, mainly the 24 h light/dark (LD) cycle of the Earth (Aschoff, 1960). In mammals, the central pacemaker is the suprachiasmatic nucleus, located in the hypothalamus (Moore, 1983). Circadian rhythms are thought to have evolved mainly because they provide organisms with the ability to "anticipate" and thus to prepare physiologically for the relatively predictable environmental challenges associated with the progression of the day-night cycle (Enright, 1970). Additionally, circadian systems provide a source of internal temporal organization that might have adaptive value, even in the absence of regular environmental cycles (Pittendrigh, 1961).

The underground environment presents one such situation where day-night environmental differences and light exposure are minimal. There have been few detailed investigations of circadian rhythms in fossorial organisms. Most studies have been done in the different species of rodents commonly grouped as mole rats from Africa and Asia (Ben-Schlomo et al., 1995; Goldman et al., 1997; Lovegrove & Muir, 1996; Lovegrove & Papenfus, 1995; Nevo et al., 1982; Ooisthuizen et al., 2003; Rado et al., 1993; Riccio & Goldman, 2000; Tobler et al., 1998). These rodents show numerous physiological, morphological, and behavioral adaptations for their underground life. The circadian organization of these species reflects the temporal adaptation to the subterranean environment, and the activity-rest temporal patterns are the emergent rhythm of this organization. Variable activity patterns have been shown, both inter- and intra-specific, thus indicating that polymorphisms in rhythmic expression seems to be common in subterranean species. Possible sources of this variability might be related to the degree of daily exposure to light and also the degree of sociability of the species.

The only South American burrowing rodent studied chronobiologically in the laboratory has been *Spalacopus cyanus*, which has an endogenous circadian rhythm entrained by the LD cycle, with activity

concentrated during the dark phase (Begall et al., 2002). The most numerous of all subterranean rodents in terms of species is the genus *Ctenomys*. The genus comprises more than 50 species that occupy much of South America, south of 12°S latitude (Cook & Lessa, 1998; Morgan & Verzi, 2006; Pearson, 1984; Reig, 1970). Chronobiological studies in this genus have not yet been performed. Recent ecological studies analyzing one of its species, *Ctenomys talarum*, during a 24 h recording interval suggested arrhythmicity as the general tendency of activity temporal patterns. In captivity and in field studies, only 25% of the individuals analyzed presented activity concentrated in a definite phase of the LD cycles, some being diurnal, others nocturnal, and other crepuscular (Cutrera et al., 2006; Luna & Antinuchi, 2003; Luna et al., 2000).

Among the known species that comprise the genus *Ctenomys*, all but one are solitary (Chan et al., 2006). Compared with other subterranean species, this genus exhibits more activity above the surface, venturing away from their tunnels during short bouts to gather vegetation (Vassalo et al., 1994). However, once inside their burrows, they immediately cover the used entrance, avoiding predators and light, and remain underground for unknown periods of time, presumably for as long as food sources are available. The selective pressure, therefore, on the circadian system to drive anticipation of light and dark may be reduced in some species and not in others, depending on the need for surface activity. Given the number of species and the range and variability of habitat, the tuco-tuco is a genus for which circadian systems are subject to numerous selective pressures, and the strategies for remaining synchronized with the outside world may be varied. The present study was aimed at characterizing the temporal activity/rest patterns of one solitary species, *Ctenomys cf knightii*, under laboratory conditions, using classical chronobiological protocols to assess endogenous circadian rhythms and entrainment to artificial LD cycles.

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MATERIALS AND METHODS

Ctenomys cf knightii is one of the three species of tuco-tuco believed to inhabit the province of La Rioja in Argentina (Barquez et al., 2006; Braun & Díez, 1999). Eight tuco-tucos were easily captured within a restricted 20,000 m^2 area (except one, Archivaldo, captured in Aminga, 3 km south) between June and October of 2007, during the daytime hours, with traps that maintain the animals alive and uninjured, in Anillaco, Castro Barros Department, province of La Rioja, Argentina (26° 48′ S; 66° 56′ W; 1445 mts). Six were males and two were females (Martina and Cutú) with mean body weight 158.4 ± 14.3 g and length 15.6 ± 0.5, plus 8.4 ± 0.2 cm tail length. Animals were adults but ages were unknown because all were wild-caught. Upon arrival at our

laboratory, the animals were housed individually in acrylic cages (53 × 29 × 27 cm) with computer monitored running wheels (23 cm diameter, 10 cm wide, 1 cm between bars) and filled with a 5 cm layer of chopped desk paper. Temperature in the animal facility was maintained constant at 22 ± 1°C. Rat pellets were available ad libitum. Supplementation (carrots, sweet potatoes, salad greens, and oat meal) was offered ad libitum each day at random times. *Ctenomys sp* does not drink and obtains all required water from its food. Cage cleaning occurred every four to five days, at random times. CRILAR received authorization from the Environmental Department of La Rioja (Secretaria de Ambiente, Ministerio de Producción y Desarrollo Local), registration no. 062-08, and the methods and procedures were consistent with the ethical standards established by the journal (Portaluppi et al., 2008).

Animals were exposed to four lighting regimes:

1. LD 12:12 h with lights on at 07:00 h, for 21 days.
2. DD (constant darkness). Technically, we used constant dim light to facilitate animal care; animals were kept in 3 to 5 lux, which corresponds to the light intensity of a full moon night, for 38 days.
3. Reestablishment of the previous LD cycle, maintained for 29 days.
4. LL (constant light) with lights on at 150 to 250 lux for 87 days. Two animals (Winnipeg and Clementino) were left longer in LL (134 days) because their activity patterns did not seem stable at the time.

Light during the light phase of the LD cycles and during the constant light phase was provided by a 36W, 1.2 m-long fluorescent bulb (Cool Day Light, OSRAM L) controlled by a mechanical timer (TBCin-China) when necessary. Light intensity varied from 150 to 250 lux at the cage lid level of the eight cages. During the constant dim light condition, dim light was provided by two incandescent red lamps (Philips 40/25W) connected to a dimmer (TECLASTAR Milano, 200W). Light intensity was measured with a digital Luximeter Model TM-201 (Tenmars Electronics CO., Ltd, Taiwan).

Wheel-running activity was continuously recorded with the ArChron Data Acquisition System (Simonetta System, Universidad Nacional de Quilmes, Buenos Aires). Each turn of the wheel activated a microswitch that was registered as one pulse of activity. Graphical output (actograms) and rhythm analysis were conducted using the *El Temps* software (A. Díez-Noguera, Universitat de Barcelona, 1999). Analysis of the actograms was done by visual inspection assisted by the "point-and-read" tools of *El Temps*. Determination of the daily activity onset was required to calculate the phase angle of entrainment and endogenous period in constant conditions, as well as to establish the rate of re-entrainment. We fitted a line through the onsets, and another was placed at the time of lights off, and

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the difference between both was calculated. The phase angle of entrainment to the LD cycle was defined as the number of minutes that activity onset preceded (positive phase relationship) or followed (negative phase relationship) the onset of darkness. The period of the activity rhythm was estimated using the original chi-square periodogram analysis described by Sokolove and Bushell (1978) with a global risk level of $p < 0.05$. This method provides information about the significant periods as well as the percentage of variance (PV) explained by the rhythm. The PV is an indicator of the prominance (amplitude) of the rhythm. Determination of daily offsets of wheel-running activity was required in two animals in which onsets were not adequate for periodogram analysis. Rate of re-entrainment following reinstatement of the LD cycle was determined. Entrainment was considered complete when the phase relationship between activity onset and the beginning of the dark phase became stable. The number of days between reinstatement of the cycle and stable phase relationship was quantified. Results are reported as means $\pm$ standard errors ($\bar{x} \pm SE$).

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RESULTS

Figure 1 shows the complete actograms of the eight animals studied. During the first experimental phase of synchronization, the patterns of all individuals were similar. A clear 24 h rhythm of activity-inactivity was present in which wheel-running was always concentrated in the dark phase of the external cycle. Only minor inter-individual differences were seen in the characteristics of the active phase or in the details of entrainment. In some animals (Aputik, Archivaldo, and Winnipeg), the inactive phase was totally clear with essentially zero activity. Others (Gizhii, Clementino, and Martina) showed some scattered bouts of activity during the light phase. It can be stated that the greater number of bouts observed during an animal's inactive phase, the less consolidated activity was during the active phase. Entrainment appeared stable, with the stationary front of activity onsets following a 24 h rhythm. In general, activity onsets occurred at the time of lights-off, with the exception of Gizhii and Martina; these animals showed a positive phase relationship starting activity 30 and 40 min before lights off, respectively. The other important exception in this parameter is Clementino; this animal showed a negative phase relationship, starting activity 130 min after lights-off. As is common in most studied rodents, offsets tend to be more variable; they occurred after (Gizhii, Archivaldo, Cutú, Zac, and Martina) or before (Aputik, Clementino, and Winnipeg) lights off. That is, the activity phase was in general 12 h or more, with the exception of these three animals that showed a shorter consolidated active phase.

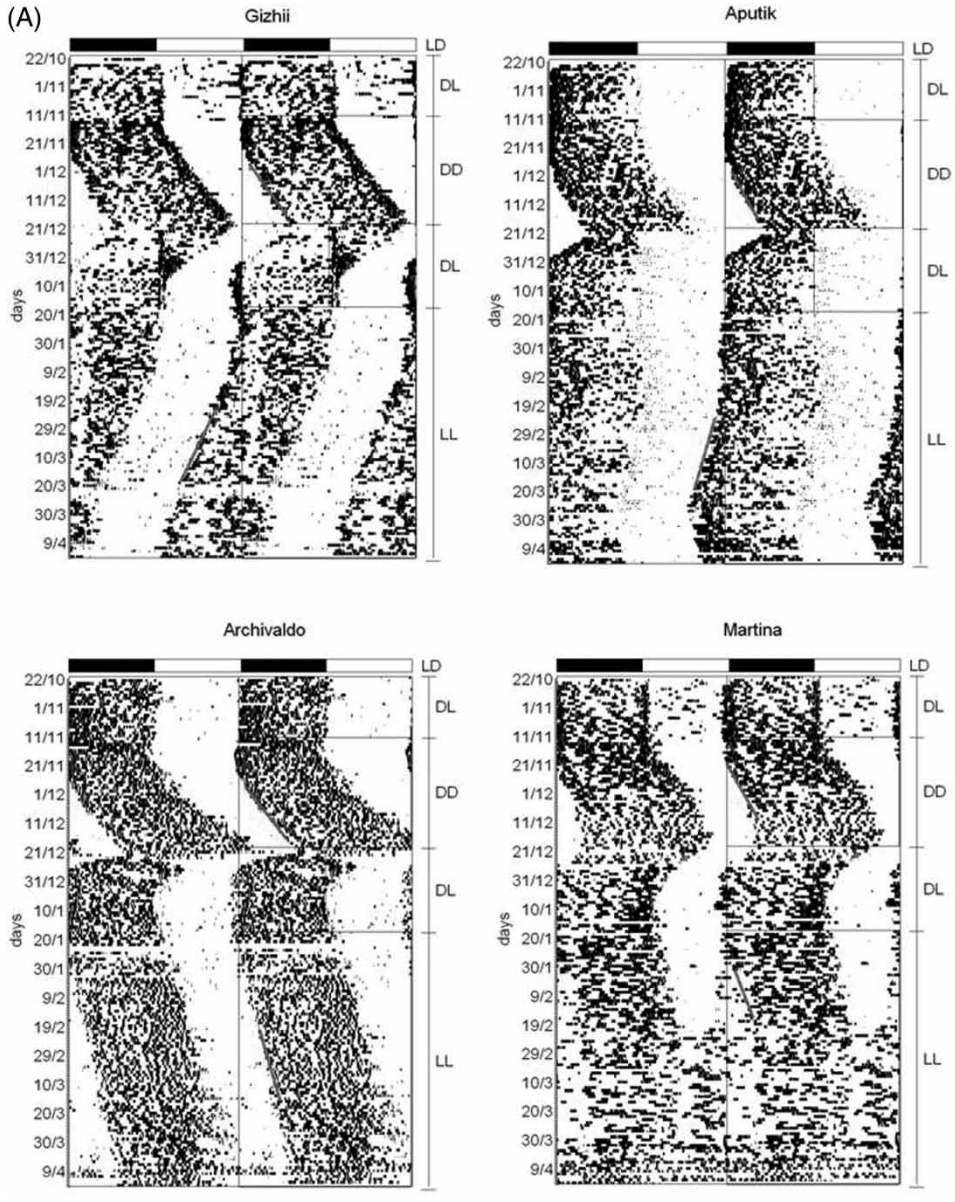

FIGURE 1 Complete double plotted actograms of the eight animals. Time is plotted across the horizontal axis (48 h per line), and successive days are plotted beneath one another. The bar on the top indicates the light-dark cycle during phases I and III. The vertical line on the left indicates the duration of each experimental phase. Lines through onsets indicate the cycles used to calculate period. (A) Three animals that did not split in LL and one (Martina) that became arrhythmic in LL. (B) Splitters: two animals in LL that showed abrupt splitting (Cutu and Zac) and two (Winnipeg and Clementino) that showed slowly evolving splitting, but that later on showed refusion of both components.

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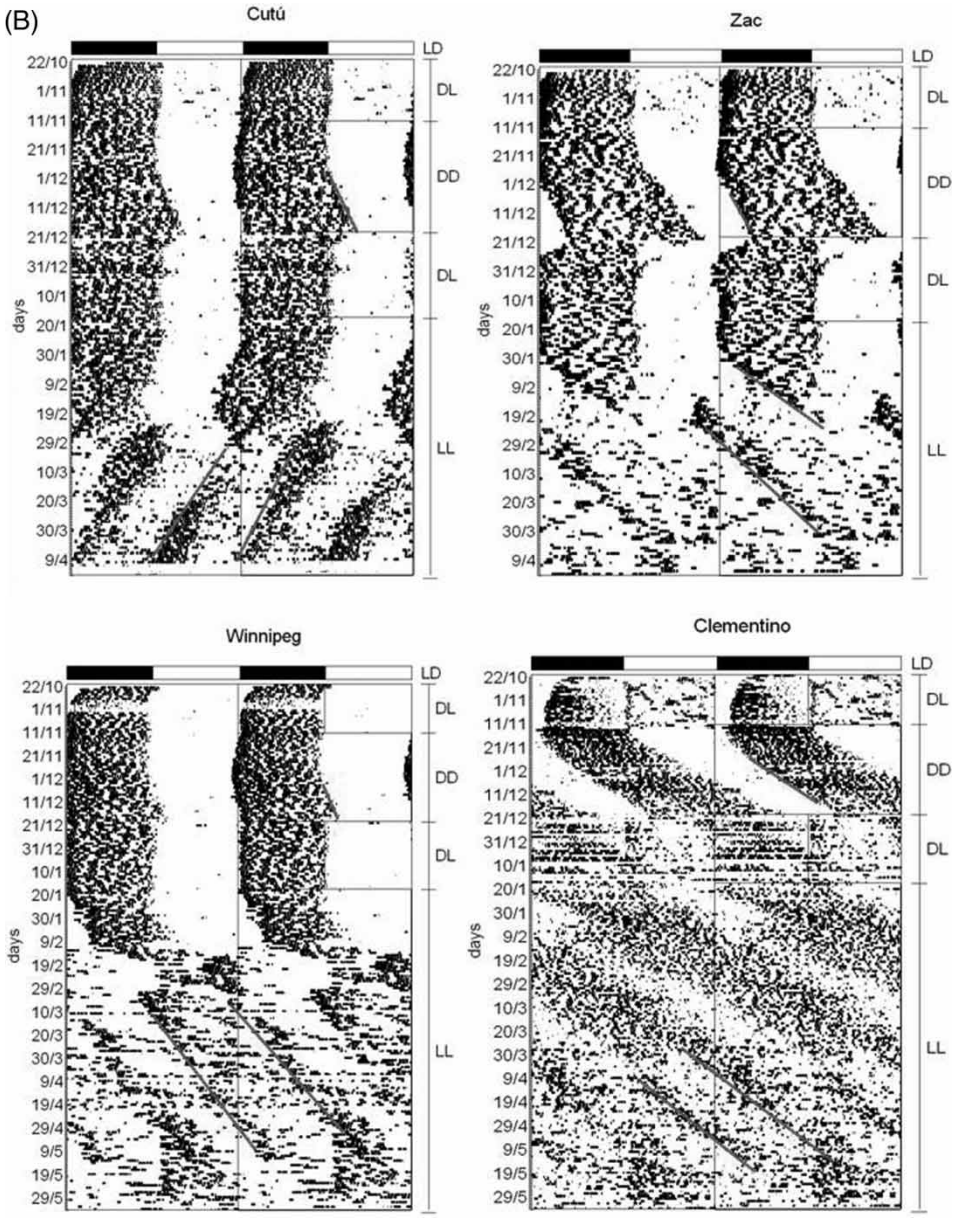

FIGURE 1 Continued

In constant darkness (DD), each animal continued to alternate a long span of wheel-running bouts with a quiescent period on a daily basis. The 24 h period (τ) of the previous entrainment schedule (24.01 ± 0.00) remained under this constant condition for many days before the final expression of the endogenous period. This interval demonstrating

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"aftereffects" of entrainment ranged from 6 (Clementino) to 24 days (Winnipeg). The mean value of aftereffects was 13.38 ± 1.94 days.

The free-running period was calculated for each animal for intervals that varied between 19 to 24 cycles after excluding the aftereffects (see Table 1). The period was always longer than 24 h, with τ varying from 24.09 to 24.48 (24.25 ± 0.04) h. The active phase (α) was characterized by an abrupt start of wheel turning, and subsequent bouts of rapid wheel running with daily patterns that varied both within and among individuals. In most cases, the onset of α was used as a reference measure for period calculations. In two cases (Cutú and Winnipeg), offsets were used because onsets coincided with the previous lights-off time while offsets seemed to free-run. In DD, the active and inactive phases became much clearer; the bouts of activity present during the light phase of the previous LD cycle totally disappeared in DD, and activity became concentrated solely during the well-defined active phase. The active phase tended to expand as time in DD increased.

During the subsequent LD cycle, the tuco-tucos re-entrained quickly to the lights-off transition. The pattern of adjustment depended on the relationship of activity onset to the imposed light schedule. When the newly initiated light overlapped the start of activity, entrainment occurred by daily delay phase-shifts until activity onset occurred shortly after lights-off; this was the case of Clementino. Cutú and Winnipeg barely had any change because activity onsets had not deviated significantly from the lights-off phase of the previous LD cycle. The other animals re-entrained with phase advances that varied between 4 (Zac) to 12 days (Gizhii), the mean value being 5.50 ± 1.49 days.

The phase relationship between activity onset and lights-off was different under the second LD schedule. Animals showed, in general, a more positive phase relationship with the exception of Aputik and Winnipeg, which

TABLE 1 Circadian periods (τ) expressed in hours for each animal during DD and LL

		LL	LL
Animal	DD	First or single component	Second component
Gizhii	24.25 (23)	23.83 (32)	–
Aputik	24.22 (22)	23.90 (31)	–
Archivaldo	24.37 (20)	24.10 (31)	–
Martina ♀	24.16 (23)	24.09 (20)	–
Cutú ♀	24.18 (23)	23.72 (42)	23.75 (42)
Zac	24.21 (19)	24.46 (35)	24.52 (26)
Winnipeg	24.09 (13)	24.26 (61)	24.27 (64)
Clementino	24.48 (24)	24.43 (34)	24.42 (42)

In parenthesis is the number of cycles that were used in the periodogram analysis. The two females are identified with♀. A paired t-test between DD and LL revealed no significant difference.

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assumed the same phase as in the previous LD cycle, and Clementino, which showed a large change in its phase relationship from a positive value of 100 to 0 min. However, onset determination was difficult in this animal during this particular phase due to great variability in activity onset.

When released into LL, an aftereffect was observed (as in the case of DD), in which seven animals continued to show a period close to 24 h from 1 day (Winnipeg) to 29 days (Aputik) after release (9.13 ± 3.25 days).

Various temporal patterns were observed under the prolonged maintenance of animals in LL. Gizhii, Aputik, and Archivaldo remained with one rhythmic bout of activity (see Figure1A and Table 1). Archivaldo and Clementino showed a longer than 24 h period, while Gizhii and Aputik presented a shorter than 24 h period under LL. Further, Aputik turned to a lengthening of period after 67 days in this lighting regime. One animal, Martina, became arrhythmic after 34 days in LL. On the other hand, four animals showed a splitting of their activity pattern (see Figure 1B). In other words, the main nocturnal bout of activity was divided (split) in two bouts (components), 12 hours apart. Within a splitted animal, both components had basically the same period (see Table 1). One, Cutú, had a τ shorter than 24 h in both components, while the other three splitters had a τ longer than 24 h in both components. While Zac and Cutu showed abrupt splitting onset, Winnipeg (after 40 days in LL) and Clementino (after 70 days in LL) showed slowly evolving splitting. Both Winnipeg and Clementino rejoined both components after several cycles (90 and 50 days, respectively).

DISCUSSION

Most chronobiology studies on subterranean animals have come from strictly subterranean African and Asian-Middle East rodents (mole rats from the families Bathyergidae and Spalacidae, respectively). Rhythms were characterized by a highly variable pattern of activity: inter- and intra-specific variability is common in subterranean species, with some individuals being rhythmic and others arrhythmic. Additionally, among the rhythmic animals, some are diurnal, others nocturnal, and others crepuscular. This remarkable rhythmic variability is thought to be related to the degree of daily exposure to light in these subterranean species (Ben-Schlomo et al., 1995; Goldman et al., 1997; Lovegrove & Muir, 1996; Nevo et al., 1982; Ooisthuizen et al., 2003; Rado et al., 1993).

All wild caught individuals studied here exhibited similar, clear, and precise rhythms of wheel-running activity. The rhythms readily entrained to a LD cycle with activity concentrated during the dark phase of the external LD cycle in all individuals. The rhythms were also expressed under constant dark (DD), with periods longer than 24 h in all individuals, demonstrating the endogenous nature of the wheel-running activity timing.

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Synchronization by the light-dark cycle illustrated many new behavioral details of the *Ctenomys*'s circadian day. Daytime was characterized by no running in the wheel, and activity onset tended to occur abruptly and almost always a few minutes after lights-off. Vertical alignments of the activity onsets reflect stable synchronization of an animal in full view of the photoregimen to an abrupt on-off light signal. We can say without a doubt that, at least in the laboratory conditions of this study, *Ctenomys cf knightii* is a nocturnal species that is clearly sensitive to the synchronizing effect of a LD cycle.

That this light synchronization was not a direct reaction to the external stimulus (Aschoff, 1960) but entrainment of the endogenous oscillator was definitely shown by the "aftereffects" of entrainment and the "transient" cycles that preceded the resynchronization to the previous LD cycle (Pittendrigh & Daan, 1976a). Interestingly, re-entrainment from DD occurred rapidly. One animal, Archivaldo, advanced about 10 h in 5 days. While we have not yet determined the phase-response curves to light, it is apparent that this species, under the experimental lighting conditions (long periods of DD), is capable of rapid re-entrainment, and predominantly in the advancing direction. Given the nocturnal and largely subterranean behavior, this may prove to be a critical adaptation for retaining synchrony with natural diurnal cycles, as well as other members of the species. Data from wheel-running has the advantage of, generally, revealing very clear and precise rhythms of activity that facilitate visualization of the rhythms and their analysis. However, we acknowledge that we should be cautious with the conclusions drawn from these types of data. First, other types of activities are ignored. Second, the presence of a wheel tends to induce activity, much more than expected in the absence of a wheel, which can exert a significant feedback effect on the circadian system (Edgar et al., 1991; Pratt & Goldman, 1986; Reebs & Mrosovsky, 1989) as well as affect upper and lower limits of entrainment to LD cycles (Chiesa et al., 2007). Third, some species even change their temporal allocation according to the presence or absence of a wheel, being nocturnal or diurnal accordingly (Blanchong et al., 1999). These are issues that merit further investigation in *Ctenomys*.

Although constant conditions are required for the expression of the endogenous, free-running rhythmicity of organisms, constant light has physiologically different effects compared to constant darkness (Daan, 1977), especially in a nocturnal animal, and they were expected to be even greater in a subterranean species. In this context, it is remarkable that a 200 lux constant condition turned out to be permissive for the expression of rhythmicity (Enright, 1981) in *Ctenomys cf knightii*, and it was in this condition only that they showed variable inter-individual rhythmic expressions. This is the first report of the same variable rhythmic patterns presented by hamsters (*Mesocricetus auratus*) in the classical work of Pittendrigh & Daan

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(1976b), under the same constant light condition, occurring in the same temporal scale (around 40 days).

Splitting of activity into two bouts in antiphase under constant light (LL) has been shown in a variety of organisms, ranging from insects (Wiedenmann, 1983) to primates (Hoffman, 1971), and as reported for two subterranean rodents under DD (Lovegrove & Papenfus, 1995; Tobler et al., 1998). However, variability in rhythmic patterns under LL had been systematically observed and analyzed in hamsters only (Earnest & Turek, 1982; Pittendrigh & Daan, 1976b). The main patterns consist of abrupt splitting (observed in Cutu and Zac), slowly evolving splitting (observed in Winnipeg and Clementino), splitting followed by refusion (Clementino and Winnipeg), no splitting (observed in Gizhii, Aputik, and Archivaldo), and arrhythmicity (observed in Martina). The splitting phenomenon reflects the existence of at least two functional oscillators composing the central circadian pacemaker of rodents, which expressed themselves under the dissociated states caused by the constant light (LL) condition. These two oscillators might be located in the left and right suprachiasmatic nucleus (De la Iglesia et al., 2002). The occurrence of a set of exactly the same variable splitting patterns in two different species supports the proposition of a common mechanism underlying all these variable patterns within a set, including the ones that do not split (Oda & Friesen, 2002).

Considering the non-strictly subterranean characteristic of *Ctenomys* and consequently greater exposure to the natural day-night cycle, all the rhythmic characteristics shown above, indicating *Ctenomys cf knightii*s closer similarity to non-subterranean species, seem reasonable. In this respect, some eye characteristics, including size, are also clearly distinct from strictly subterranean species, such as the blind-mole rats. These species have reduced eyes and only below the skin (Cooper et al., 1993). Although, a direct correlation between eye size and photosensitivity cannot be absolutely stated (Stein, 2000), it is noteworthy that the *Ctenomys* species present bigger (around 6 mm) than average eyes (4 ± 2 mm) compared to other fossorial rodents, when measures were taken from 23 species of Patagonia (Pearson, 1984).

Other than the degree of daily exposure to light, however, there is an interesting proposition that the temporal organization of subterranean species might also be related to the degree of their sociability. As mentioned before, the genus *Ctenomys* has more than 50 species inhabiting totally different soils and climates, from the Puna desert at 4000 m to the Atlantic coastal sand dunes. Because of this, and in spite of the tendency to think in similarities of the subterranean niche, the variability observed in the rhythmic expression in subterranean African rodents may also apply to this South American genus due to historical as well as ecological constraints. Remarkably, of the more the 50 species, only one is social.

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The rare and threatened Patagonian tuco-tuco (*C. sociabilis*) has evolved a complex social structure that is unique among all the closely related tuco-tuco species (Lacey et al., 1997, 1998). How is the circadian system of this species? Will the social characteristic relate to some special temporal organization? The Bathyergidae family of African subterranean mole rats presents a continuum of sociality, ranging from strictly solitary, social, to eusocial. Oosthuizen et al. (2003) studied the daily patterns of activity of these three species in the laboratory under different illumination conditions and suggested that more sociality implied less sensitivity to the external LD entraining agent. Furthermore, inter-individual variability in rhythmic patterns in an eusocial colony might be related to the division of labor between individual animals. This has been suggested to be case for naked-mole rats, in which it appears that only the disperser morph individuals tend to be rhythmic (Riccio & Goldman, 2000), and in insect colonies (Bloch et al., 2001; Oda et al., 2007; Yerushalmi et al., 2006). This raises many questions as to the rhythmic expression of the more than 50 species of closely related *Ctenomys*, all of which inhabit very different soil, weather, and vegetation conditions. The data presented here on the temporal organization of one of the solitary species of tuco-tuco open the way to further studies on this chronobiologically unknown South American genus.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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