Patricia Tachinardi Andrade Silva

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Orientadora: Gisele Akemi Oda
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Abstract

Studies that show discrepancy between nocturnal and diurnal activity under laboratory and field conditions, respectively, have become increasingly common in rodents and suggest that the definition of temporal niche is far more plastic than originally suspected. Recently, it has been proposed that factors that challenge the animal's energy balance play an important role in temporal niche switches. The "circadian thermo-energetics (CTE) hypothesis" suggests that daytime activity could be a response to the high energetic costs of foraging and low environmental temperatures, allowing the animal to save energy during the cooler night hours by resting and taking shelter in burrows where temperatures are higher than on the surface. In this thesis, we explored the interplay of plasticity in nocturnal/diurnal activity definition and energetic metabolism in two rodent species, tuco-tucos (*Ctenomys* aff. *knighti*) and laboratory mice (*Mus musculus*). Tuco-tucos are subterranean rodents which face peculiar energetic challenges in their habitat and were shown to be diurnal in the field and nocturnal in the laboratory. We characterized how their energy expenditure varies across day and night and described the peculiar finding of some factor inside the metabolic chamber being itself a trigger for the nocturnal to diurnal switch. Moreover, we estimated the amount of energy tuco-tucos would save by being diurnal in the field, by combining metabolic rate measurements at various ambient temperatures with records of environmental temperature in the tuco-tuco's natural habitat. We showed that, in winter, daytime activity saves a considerable amount of energy, lending support to the CTE hypothesis. However, in summer these savings are small, suggesting that other factors impact activity timing during this season. We also investigated sex-differences in both locomotor activity and body temperature patterns of laboratory mice subjected to food restriction in semi-natural conditions. The results indicate that diurnality in response to energetic challenges is sex-dependent in mice: males were more diurnal than females in all conditions and showed higher interindividual variation in the amount of daytime activity. The findings of these three studies provided valuable evidence for the discussion of the role of environmental factors, particularly energetic challenges, in the plasticity of daily rhythms.

Keywords: Circadian rhythms, Circadian thermos-energetic hypothesis, tuco-tucos, energetics, respirometry, semi-natural enclosures

Resumo

Estudos que apontam discrepâncias entre atividade noturna e diurna, respectivamente, sob condições de laboratório e de campo, estão cada vez mais comuns e sugerem que a definição de nicho temporal é muito mais plástica do que se suspeitava inicialmente. Recentemente, foi proposto que fatores que desafiam o balanço energético do animal desempenham um papel importante em mudanças de nicho temporal. A "hipótese circadiana termoenergética (CTE)" sugere que a atividade diurna pode ser uma resposta aos altos custos energéticos do forrageamento e às baixas temperaturas ambientais, permitindo que o animal economize energia durante as horas mais frias da noite, descansando e se abrigando em tocas onde as temperaturas mais quentes do que a superfície. Nesta tese, exploramos a interação entre a plasticidade da definição noturnalidade/diurnalidade e o metabolismo energético em duas espécies de roedores, o tuco-tuco (*Ctenomys* aff. *knighti*) e o camundongo (*Mus musculus*). Tuco-tucos são roedores subterrâneos que enfrentam desafios energéticos peculiares em seu habitat e verificamos que são diurnos em campo e noturnos em laboratório. Nós caracterizamos a variação de seu gasto energético ao longo do dia e da noite e descrevemos o achado peculiar de que algum fator presente no interior da câmara metabólica pode ser um gatilho para a mudança de noturnalidade para diurnalidade. Além disso, estimamos a quantidade de energia que os tuco-tucos economizariam ao serem diurnos em campo, combinando medidas de taxa metabólica em várias temperaturas ambientes com registros dessa temperatura no habitat natural do tuco-tuco. No inverno, a atividade diurna resulta em uma economia de energia, dando suporte à hipótese CTE. No entanto, no verão, essas economias são pequenas, sugerindo que outros fatores impactam o padrão de atividade durante essa estação. Também investigamos as diferenças entre sexos nos padrões de atividade e de temperatura corporal em camundongos sujeitos a restrição alimentar em condições semi-naturais. Os resultados indicam que a diurnalidade em resposta a desafios energéticos é dependente do sexo em camundongos: os machos foram mais diurnos do que as fêmeas em todas as condições e apresentaram maior variação interindividual na quantidade de atividade diurna. Os achados desses três estudos forneceram evidências valiosas para a discussão do papel dos fatores ambientais, particularmente os desafios energéticos, na plasticidade dos ritmos diários.

Palavras-chave: ritmos circadianos, hipótese circadiana termoenergética, tuco-tucos, energética, condições seminaturais.

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Chapter 1

General Introduction

1.1.Circadian rhythms

Several behaviors and physiological processes display 24-hour rhythmicity. At a first glance, one could assume that these biological rhythms are only physiological or behavioral reactions to daily environmental cycles, such as light intensity, temperature or food availability. However, this rhythmic expression persists even when the organism is exposed to conditions in which all environmental variables are held constant. In this situation, the period of the expressed rhythm (i.e., the interval of time each cycle takes to complete) is always different, although close to 24 hours, which is why this rhythm is called circadian, a Latin term meaning "around one day" (Halberg et al., 1959). Persistence under constant conditions is evidence that these rhythms are generated endogenously by circadian oscillators (Pittendrigh, 1960).

Currently, we know that circadian rhythms are present in all groups of living beings, from protists to multicellular eukaryotes (Dunlap et al., 2004). Notably, the three formal properties that define circadian rhythms are essentially the same in all species. The first one, as mentioned above, is endogenicity, evidenced by the persistence of rhythmicity under constant conditions in which the free-running rhythm expresses the endogenous period of the oscillator (called τ - "tau"), always different from 24 hours (Fig 1.1). The second property is the synchronization to environmental cycles, so that a stable phase relationship between the biological rhythm and the environmental cycle is established. Finally, the period of the circadian rhythms is temperature compensated. This means that, unlike other physiological processes whose rates or velocities vary according

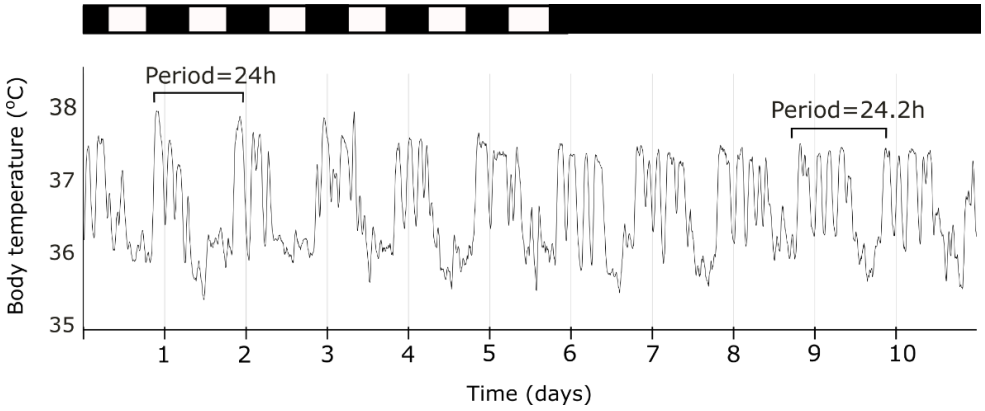

Figure 1.1. Persistence of rhythmicity under constant conditions. Body temperature records of a tuco-tuco (*Ctenomys* aff. *knighti*) kept under a 12:12 LD cycle for the first 6 days and under constant darkness from day 6 onwards. The top bar shows the duration of light (white) and dark (black). When the animal was under the LD cycle, body temperature was rhythmic with a period of 24h. Under constant darkness, the rhythm persisted, free-running with a period of 24.2h. Modified from Tachinardi et al. (2014).

to temperature (Hochachka and Somero, 2002), the τ value of the circadian oscillator is maintained stable even when there is an increase or decrease in temperature (Pittendrigh, 1954).

The basic structure the circadian system (Fig 1.2) consists of a circadian oscillator, which generates the rhythm; efferent pathways, which transmit the rhythmic signals of the oscillator to the various organs and tissues of the organism; and afferent pathways, which receive, process and transmit the temporal information from the environment to the oscillator (Moore-Ede et al., 1982).

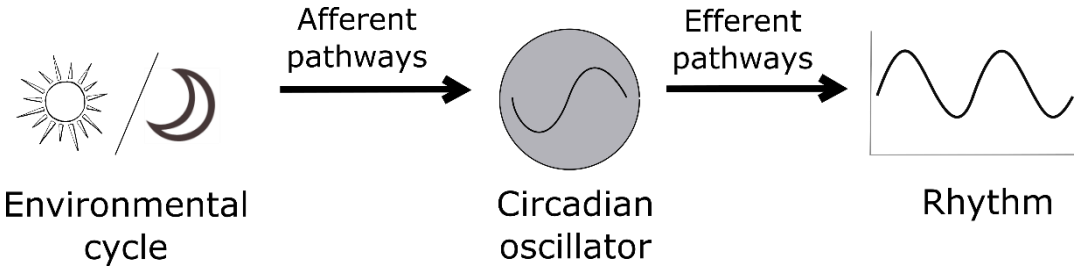

Figure 1.2. Simplified model of the circadian system. The generation of the circadian rhythmicity occurs in the circadian oscillator. The temporal information of this rhythmicity is transmitted to the organism by efferent pathways, leading to the expression of the various observed circadian rhythms. The temporal information of the environmental cycles is transmitted to the oscillator through afferent pathways.

In mammals, the central circadian oscillator is located in two small hypothalamic nuclei located above the optic chiasm, the suprachiasmatic nuclei (SCN) (Moore and Lenn, 1972). The daily light-dark (LD) is the main environmental cycle involved in the synchronization of the circadian oscillator. Light is perceived by the retina, which has direct nervous projections to the SCN, through the retinohypothalamic tract (Moore, 1983; Hattar et al. 2002; Panda et al., 2003) . The SCN have many nerve projections to other areas of the hypothalamus and the brain and chains of neurons connecting them to peripheral organs (Bartness et al., 2001; Watts, 1991). These projections can serve as efferent nerve pathways, which transmit the oscillator signals. In addition to these pathways, there is also evidence of neurosecretory efferences (Silver et al., 1996; Reppert and Weaver, 2002; Gachon et al., 2004; Mohawk et al., 2012).

1.2.Interaction between circadian rhythms and the environment: synchronization

The endogenicity of circadian rhythms allows the organism to prepare and anticipate, physiologically or behaviorally, to the cyclical and predictable environmental

variations that recur every 24 hours (Enright, 1970). For this anticipation to occur, biological processes should be allocated in specific phases of the day through the synchronization of the oscillator with the external cycles, through a mechanism called entrainment. In this process, the properties of the circadian oscillator are adjusted so that it oscillates with the same period of the environmental cycle (Aschoff, 1960). In addition to entrainment, a rhythm can also be modulated by direct stimulation or inhibition of the physiological or behavioral variable by an environmental factor. This mechanism is called masking and occurs without the properties of the circadian oscillator being altered. Often, entrainment and masking work together in the temporal integration of the organism with the environment. While entrainment results in the establishment of a stable phase relationship between the biological rhythm and the environmental cycle, thus ensuring the necessary rigidity for the biological clock function (Pittendrigh and Daan, 1976), masking allows an immediate physiological or behavioral response, for example, in case of unpredictable environmental changes. Thus, masking is an integral part of the biological timing system, as it confers plasticity to rhythmic expression and fine-tuning of synchronization (Page, 1989)

1.3.Daily rhythmicity under different conditions: laboratory *versus* field

Most of the studies on circadian rhythms that unveiled its endogenous nature and its mechanisms of synchronization to environmental cycles were performed under controlled laboratory conditions. For a long time, the patterns of daily rhythms recorded in these conditions were assumed to reflect those found in the organism's natural habitat. Many of these laboratory studies used wheel-running rhythms, the most studied in rodents due to its ease of measurement (Pittendrigh and Daan, 1976). However, the investigation of activity rhythms in rodents known to be diurnal in the field, such as the degu (*Octodon degus*) and Mongolian gerbil (*Meriones ungiculatus*), revealed that these animals become nocturnal when they have access to the wheel (Blanchong et al., 1999; Kas and Edgar, 1999; Weinert et al., 2007). These studies have raised questions not only about the meaning of wheel-running, but also about the extent to which results obtained in the laboratory can be extrapolated to what actually occurs in nature (Calisi and Bentley, 2009). Subsequently, it was noted that other species change their diurnal activity to nocturnal, regardless of the availability of wheels (Levy et al., 2007; Hut et al., 2012; Tomotani et al., 2012). Even species with rhythms very well characterized as nocturnal

in the laboratory, such as the mouse (*Mus musculus*), exhibit different patterns of activity in semi-natural conditions (Daan et al., 2011). These studies suggest that there are fundamental characteristics of the field that are not replicated in the laboratory, resulting in changes in the animal's temporal organization between these two environments.

1.4. Rhythmic plasticity as a strategy to save energy

Recently, it was proposed that the discrepancy between nocturnal and diurnal activity in lab and field might be related to differences between *ad libitum* feeding in the laboratory and the need to "work" for food in nature, with a great amount of energy spent for foraging (Hut et al., 2011; Hut et al. 2012). This proposal was based primarily on research with laboratory mice exposed to artificial conditions in which the animals had to "work" to obtain their food. In this study, nocturnal mice showed an increase in daytime activity when the workload required to obtain food was elevated (Hut et al., 2011). This increase is even more pronounced when the animals are exposed to low ambient temperatures (van der Vinne et al., 2014).

Based on these results, the "circadian thermoenergetics hypothesis" (CTE) was formulated by Hut and colleagues (2011, 2012) to explain the phase switch in locomotor activity during energetically challenging conditions. According to this hypothesis, the animals would use the light/dark cycle as a proxy (proximate factor) to ultimately reduce daily energy expenditure (ultimate factor). There are two aspects which need to be understood to explain how this could happen: the physiological mechanism of the switch in activity timing and its adaptive significance. As for the mechanism, the authors propose that a reorganization of the circadian system occurs during energetically challenging conditions, resulting in a new phase relationship between the central circadian oscillator (SCN), synchronized mainly by the light/dark cycle, and a second circadian oscillator. The expression of activity during the day would be a result of this new phase relationship between the two oscillators.

Regarding the adaptive significance, which is the main focus of this thesis, the hypothesis suggests that daytime activity could be a response to the high energetic cost of foraging, allowing the animal to rest during the cooler hours of the night. This would result in energy savings with thermoregulation, since animals at rest can adopt postures that increase their insulation and take shelter in burrows where temperatures are higher

than on the surface. In addition, they could save energy while maintaining a lower body temperature overnight.

1.5.Rhythmic plasticity in a subterranean rodent: the tuco-tuco

Evidence supporting the CTE hypothesis comes from experimental studies with strains of laboratory mice (Hut et al., 2011; Hut et al., 2012). Although the study of this species is advantageous because they are easy to be obtained and maintained and there is a great amount of knowledge accumulated about them, they have been bred in captivity for several generations, making it difficult to perform ecological correlations. The use of wild species can be extremely valuable in providing ecophysiological elements and investigating this hypothesis. In particular, the tuco-tuco (*Ctenomys* aff. *knighti*) is a particularly interesting system for this type of study due to its peculiar underground habitat, where it needs to work for its food through digging, and to the fact that it is known to present plastic daily rhythms in the field (Tomotani et al. 2012).

The member of the genus *Ctenomys* (Rodentia: Ctenomyideae) are popularly known as "tuco-tucos" or "ultutucos", onomatopoeic names in allusion to their typical vocalizations. The genus comprises more than 60 species, distributed throughout the southern cone of South America, from Peru to Tierra del Fuego, occupying a wide range of habitats, which differ in vegetation, soil type, climate and altitude (Lacey et al., 2000). Although the species of *Ctenomys* vary in body size (from 100g to over 1,000g), the external morphology is very similar among them (Luna et al., 2009). They present specialized morphological and anatomical characteristics for underground life, such as fusiform body, greater musculature of the anterior limbs and reduced ears (Stein, 2000). On the other hand, some characteristics resemble those of surface rodents, such as eye size and retinal structure (Schleich et al., 2010). There are records that show these animals perform foraging and soil removal activities on the surface (Vassallo et al., 1994, Tomotani et al., 2012). When they are in their tunnels, however, they cover their access, preventing the entry of light and predators.

The daily rhythms of the species *Ctenomys* aff. *knighti* (Fig. 1.3) have been extensively studied in both laboratory and field conditions by our research group. In the laboratory, they present circadian rhythms of locomotor activity and body temperature (T_b) (Valentinuzzi et al., 2009; Tachinardi et al., 2014). Despite the fact that these animals are not exposed to the surface environmental cycles in their natural habitat, their circadian

system synchronizes to artificial LD cycles by the same mechanisms observed for non-subterranean animals (Flôres et al., 2013, Flôres et al., 2016). Moreover, studies with freshly caught animals show that their circadian system is synchronized to natural environmental cycles in the field, even though they are exposed to light at varying times during the day (Tomotani et al., 2012; Flôres et al., 2013; Flôres et al., 2016). These animals are robustly nocturnal when synchronized to an artificial LD cycle in the laboratory (Valentinuzzi et al., 2009; Tachinardi et al., 2014). However, they display a great amount of diurnal aboveground activity in the field, showing great plasticity in their daily rhythms (Tomotani et al., 2012; Flôres et al, 2016).

Figure 1.3. Photograph of an individual of *Ctenomys* aff. *knighti* at its natural habitat.

1.6. Energetic challenges in the subterranean environment

Ctenomys are found in underground galleries consisting of a main tunnel, lateral branches and several exit holes (Antinuchi and Busch, 1992; Rosi et al., 2000). Herbivores, these animals feed mainly on aerial parts of plants and, therefore, collect their food on the surface (Busch et al., 2000). However, the detection of plants occurs in the underground through chemical tracks, and the tuco-tucos arrive at them by means of excavation (Schleich and Zenuto, 2007). Thus, they spend a great amount of energy during foraging (Luna and Antinuchi, 2006), especially in places where vegetation is sparse, as is the case of the area of occurrence of *Ctenomy*s aff. *knighti*.

In addition to energetically costly foraging, the underground environment poses other challenges to the energy budget, such as hypoxia and hypercabia. It is believed that subterranean rodents exhibit physiological adjustments to circumvent these challenges (Buffenstein, 2000). Some of these adjustments, found in *Ctenomy*s species, are the lower basal metabolic rate than predicted for surface animals of the same mass and low values

of thermal conductance (Luna et al., 2009). Despite the challenges mentioned above, underground galleries offer an energetic advantage: protection from large surface temperature variations typical of the desert environment. In light of the CTE hypothesis, tuco-tucos would save energy if they restricted their aboveground activity to the warmer time of the day, i.e., to daylight hours.

1.7.Thesis overview and objectives

The main objective of this thesis is to explore the relationship between the plasticity of daily rhythms and the energetic metabolism. We started by characterizing how energy expenditure varies across the day in tuco-tucos kept in laboratory and described the peculiar finding of the metabolic chamber being itself a trigger for the nocturnality to diurnality switch (Chapter 3). Next, we tested whether the CTE hypothesis applies to *C.* aff. *knighti* by combining metabolic rate measurements at various ambient temperatures with records of environmental temperature in the tuco-tuco's natural habitat (Chapter 4). Next, we describe further investigations of circadian plasticity in both locomotor activity and T_b of laboratory mice (*Mus musculus*) subjected to food restriction in semi-natural conditions (Chapter 5). The latter was performed during my 10-month visit in 2015 to the laboratory of Prof. Roelof Hut at the University of Groningen (the Netherlands). Finally, we combine the findings of these three studies to discuss the role of environmental factors, particularly energetic challenges, in the plasticity of daily rhythms (Chapter 6).

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Chapter 2

General Methods

This chapter presents information on the methods common to the experiments involving tuco-tucos (Chapters 3 and 4). The experimental protocols and other specific details of each experiment will be covered in the next chapters. Methods on mice (*Mus musculus*) studies will be described in Chapter 5.

2.1. Animals and Ethics statements

Initially the tuco-tucos found in the study area of this work were identified as *Ctenomys knighti* (Thomas, 1919). However, morphology of numerous collected specimens, field studies and audio-recordings indicate that our study area is occupied by a single, still unidentified, *Ctenomys* species (Amaya et al., 2016; B. Tomotani, personal communication). For these reasons, the process of species identification is still ongoing. Karyotype analyzes were carried out by the IADIZA-CCT Research Group on Biodiversity (GIB) Mendoza-CONICET and the chromosome number differs from that of the geographically close populations (Fornel, 2010). Skin and skeletons are deposited in the collections of the Patagonian National Center - CENPAT, Puerto Madryn, Chubut (specimens CNP2429 to 2432), Collection of Mammals of the Miguel Lillo Foundation, Tucumán, and IADIZA Mastozoological Collection, Mendoza. As the identity of the tuco-tucos of the study area is still under discussion, in the present work the animals will be referred to as *Ctenomys* aff. *knighti*.

Only adult individuals of *Ctenomys* aff. *knighti* were used in our studies, although their exact ages are unknown, since all were wild-caught. Both males and females were used, weighing between 140 and 220g. After the experiments were carried out, no animal was euthanized or released in the wild. These tuco-tucos remained in the laboratory and were allocated to other experiments.

All procedures performed with these animals were authorized by the Environmental Department of La Rioja (permits 028–10 and 062–08) and approved by the Ethics Committees of the Faculty of Veterinary Sciences of La Plata National University, Argentina (permit 29-2-12), the Ethics Committee of the Biosciences Institute of the University of São Paulo, Brazil (permit 164/2012) and the University of Alaska Anchorage's Institutional Animal Care and Use Committee (permit 405977-1). The techniques of capture, housing and care of the animals follow the recommendations of the American Society of Mammalogists for the use of wild animals in research (Sikes et al., 2011) and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory (NRCUS, 2011).

2.2. Area of study and trapping method

The experiments were conducted at the Regional Center for Scientific Research and Technological Transfer of La Rioja (CRILAR), located in the town of Anillaco, in the Argentine province of La Rioja (26 ° 48 'S, 66 ° 56' W, 1445 m). All animals used in this study were captured within an area of 15 km2 around the research center. This area, located in the Monte desert (Fig 2.1), presents semi-arid climate, sandy soil and vegetation composed of sparse shrubs, creeping plants and few trees (Abraham et al., 2009). In addition to occupying areas of native vegetation, the tuco-tucos are also found in grape, olive and walnut plantations, which are abundant in the region.

Figure 2.1. Photograph of a natural area of occurrence of *Ctenomys* aff. *knighti*. This area is in perimeter of Anillaco town, in La Rioja province, Argentina. It is located in the eco-region of the Monte desert.

All animals were wild-caught with live-traps, which consisted of a PVC pipe (25 cm long and 7.5 cm in diameter) containing a wire mesh at one end and a metal hatch at the other. The trap was positioned at the opening of the animal's burrow, with the door open. Inside the PVC tube there is a trigger that is activated when the tuco-tuco enters the trap, closing the door and keeping the animal inside the tube, without any injuries. After the placement of the traps, inspections were carried out at intervals of maximum 3 hours. After the capture, tuco-tucos were immediately taken to the laboratory, where they were

weighed and placed in a glass or acrylic cage. Food was offered as soon as the animals were accommodated in the cages.

2.3. Standard housing and animal husbandry

Before the experiments were performed, tuco-tucos were kept individually in acrylic cages (53x29x27cm), equipped with running wheels (23 cm in diameter, 10 cm wide, 1 cm between the bars) and lined with a layer of chopped paper. Animals were kept under an LD 12:12 cycle (12 hours of light and 12 hours of darkness). During the light phase, both the white fluorescent lamp and the red light bulbs were lit, resulting in a luminous intensity of 200-300 lux. During the darkness phase, the fluorescent lamp was turned off, but the red lamps were kept lit. Thus, the darkness condition was, in fact, characterized by the presence of low intensity red light (1-5 lux).

Food was offered *ad libitum* and the diet consisted of commercial rabbit pellets, carrots, sweet potatoes, greens, oats and sunflower seeds. Food replacement occurred on a daily basis at random times, and cleaning of cages occurred weekly. Water was not offered, a common procedure in experiments with subterranean and desert animals, which do not consume free water under natural conditions (Buffenstein, 2000; Schmidt-Nielsen, 1972). The relative humidity of the laboratory varied between 30 and 60% and the temperature was maintained at 26 ±2ºC. Data loggers (HOBO U10 / 003; Onset Computer Corporation, Bourne, MA) recorded the laboratory temperature and relative humidity every 15 minutes.

2.4. Surgical procedures

To monitor core T_b and gross motor activity, telemetric transmitters (G2 E-Mitters, Mini-Mitter, Bend, OR) were implanted intraperitoneally. Animals were anaesthetized using either ketamine/acepromazine (200 and 20 mg/Kg, respectively) or isoflurane anaesthesia (3%–5% with oxygen). Tricotomy, local disinfection and carefully prepared surgery fields reduced infection risk. The frequent post-surgical removal of suture stitches by the animals was avoided using polyglycolic acid thread (the only material that did not generate allergic itching irritation) and interrupted suture stitches (instead of continuous). The extremely thin abdominal muscular layer of this species required a small thread diameter (5-0 or 6-0). Hypothermia was avoided with thermal blankets (P010507, La-sure, São Paulo, Brazil). Immediately after surgery, tuco-tucos received a subcutaneous injection of antibiotic, enrofloxacin (Flotril® 2.5%, Schering-Plough, Rio de Janeiro,

Brazil; 10 mg/Kg), and analgesic, flunixin meglumin (Banamine® Schering-Plough, Rio de Janeiro, Brazil; 2,5 mg/Kg). After surgery, animals were allowed three to five days of recovery before returning to the animal facility where the experiments took place.

2.5. Respirometry

Central point of this thesis, the estimation of energy expenditure was carried out by measures of O_2 consumption and CO_2 production, using respirometry. We utilized an open-flow system using excurrent flow measurement (Fig. 2.2). The basic principle of this system is to pull air though a chamber holding the animal and calculate the difference of gas content between the air entering the chamber and the air exiting it. Due to the animal's respiration, the air inside the chamber is depleted of O_2 and enriched with CO_2 (Lighton, 2008; Tøien, 2013). Chapter 3 details the equations used for this gas analysis.

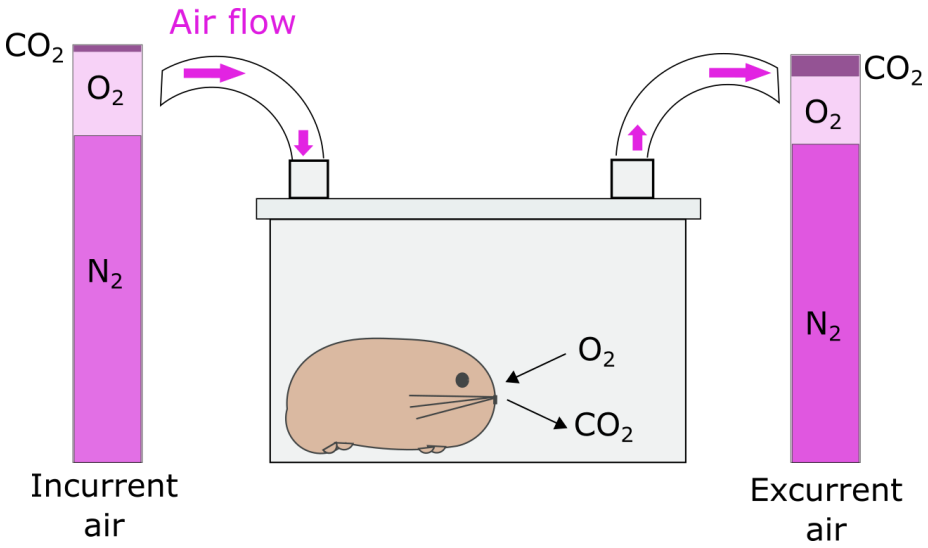

Figure 2.2. Simplified scheme of an open-flow, pull mode, respirometry system, indicating changes in gas composition of the air. Air is pulled through the chamber holding the animal, where its gas composition is changed due to the animal's respiration. Purple bars depict gas composition of incurrent and excurrent air. The N_2 fraction also comprises other inert gases not altered by the animal's respiration. O_2 is depleted and CO_2 is increased in the excurrent air. Changes in gas composition result in air volume change and need to be taken into account in the O_2 consumption calculations. (Modified from Tøien, 2013)

The use of this technique was unprecedented in our laboratory. For this reason, it was necessary to set up a new system that could meet our needs. This process was carried out in collaboration with two researchers, Prof. Loren Buck (Northern Arizona University) and Dr. Øivind Tøien (University of Alaska Fairbanks), who kindly granted us and adapted the software of his authorship used in these measures. Two systems were

set up, one in 2013 (Fig. 2.3), using equipment borrowed by Prof. Buck, and another since 2014 (Fig. 2.4), already with the new equipment acquired by our research group. In 2013, we used a FoxBox (Sable Systems, Las Vegas, NV) and Molecular Sieve 3Å (8–12 mesh, Sigma-Aldrich, Saint Louis, MO) as a desiccant with O_2 measurement only. This system required that we manually changed the air flow from the animal chamber to reference ambient air to calibrate the O_2 analyzer every hour, continuously for several days. The system we have been using since 2014 consists of the Field Metabolic System (Sable Systems, Las Vegas, NV) and a Nafion Dryer (Tøien, 2013) to remove moisture from the air. The greatest advantage of the new setup was the ability to automatically perform the hourly calibrations, through a computer-controlled solenoid valve. Since $\dot{V}O_2$ data collected in the two systems did not significantly differ (two-tailed t-test, $p>0.05$), we merged data from both years for further analysis.

2.6. Visual analysis of daily rhythms

To visualize rhythmic patterns, we constructed actograms with the ElTemps software (Díez-Noguera, Universitat de Barcelona, 1999). This form of graphical representation is traditionally used for records of locomotor activity, but can also be used to visualize other biological variables. The concept behind an actogram is simple. It is as if a large plot of the time series were cut every 24 hours and each of these stretches were stacked, so that each line of the graph corresponded to one day (Moore-Ede et al., 1982). For better visualization, the actograms are constructed in duplicate, with two identical graphs presented next to each other, and the graph on the right is positioned one row upwards, so that 48-hour records are displayed on a single line (Fig. 2.5). To facilitate visualization of the data, the plot shows only the values of T_b or locomotor activity that are between minimum and maximum limits established for each animal.

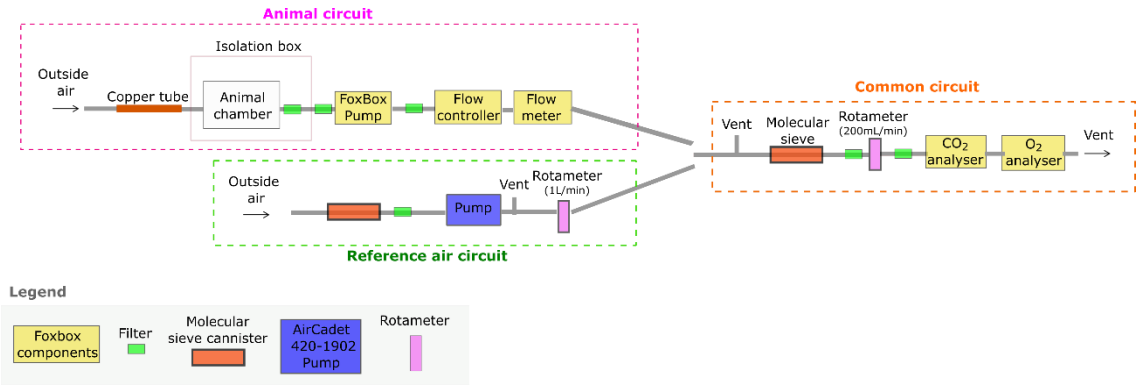

Figure 2.3. Scheme of respirometry setup used in 2013. Depicts an open-flow system using excurrent flow measurement (pull mode). When measurements are being taken, the animal circuit is connected to the common circuit containing the gas analyzers. Air withdrawn from outside the building is passed through a copper tube for its temperature to equilibrate with the air temperature in the animal room. Afterwards it is pulled through the animal chamber by a pump with a flow controller and mass flow meter (part of the FoxBox, Sable Systems, NV). A subsample of this flow has its water vapor removed by passing through a molecular sieve canister and is then passed through the CO_2 and O_2 analyzers (also part of the FoxBox). To calibrate the O_2 analyzer, the reference air circuit is manually connected to the common circuit every hour, passing outside air through the gas analyzers.

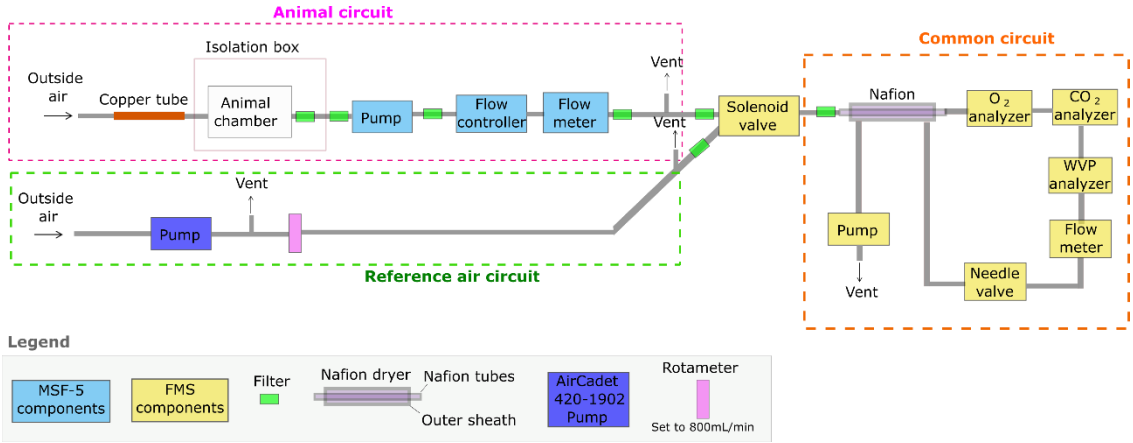

Figure 2.4. Scheme of respirometry setup used since 2014. Depicts an open-flow system using excurrent flow measurement (pull mode). When measurements are being taken, the animal circuit is connected to the common circuit containing the gas analyzers. Air withdrawn from outside the building is passed through a copper tube for its temperature to equilibrate with the air temperature in the animal room. Afterwards it is pulled through the animal chamber by a pump with a flow controller and mass flow meter (Mass Flow System-5, Sable Systems, NV). A subsample of this flow has its water vapor removed by passing through Nafion tubes, which bind to water molecules and is then pulled through the O_2, CO_2 and water vapor (WVP) analyzers by a pump (all components of the Field Metabolic System, Sable Systems, NV). Before exiting the system, the dry subsampled air passed through the outer sheath of the Nafion dryer, removing the water molecules outside the Nafion tubes due to WVP pressure and thus creating a countercurrent gas exchanger (detailed in Tøien, 2013). To calibrate the O_2 analyzer, the reference air circuit is connected to the common circuit every hour by the computer controlled solenoid valve, passing outside air through the gas analyzers.

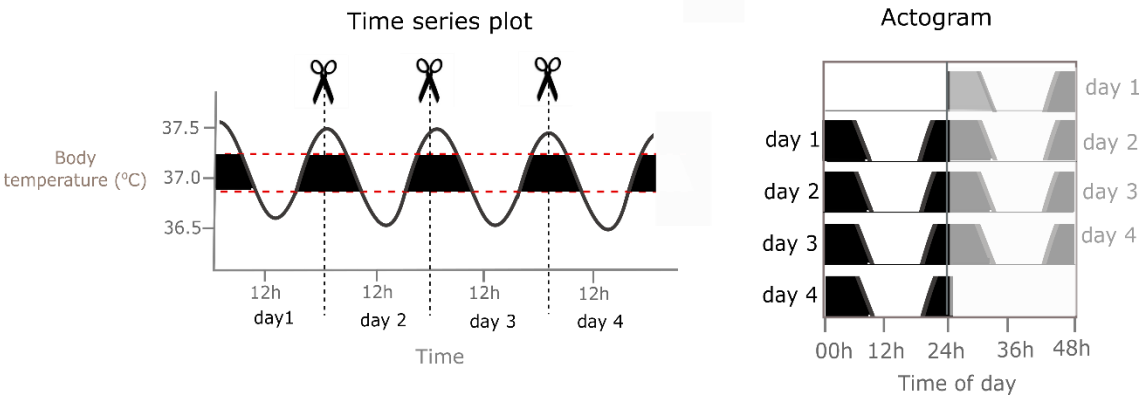

Figure 2.5. Construction of an actogram from hypothetical T_b data. In the left plot the raw 4 days temporal series of T_b data are presented as a function of time. The red horizontal lines represent the maximum and minimum limits of the T_b values that will be indicated in the actogram (black areas). The vertical dotted lines mark the beginning of each day, where the time series graph will be "cut". These excerpts corresponding to 24 hours of data are then stacked, sequentially, forming an actogram (left), shown here in duplicate. Locomotor activity maps are constructed in the same way (see text).

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Chapter 3

Nocturnal to Diurnal Switches with Spontaneous Suppression of Wheel-Running Behavior in a Subterranean Rodent

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3.1. Abstract

Several rodent species that are diurnal in the field become nocturnal in the lab. It has been suggested that the use of running-wheels in the lab might contribute to this timing switch. This proposition is based on studies that indicate feed-back of vigorous wheel-running on the period and phase of circadian clocks that time daily activity rhythms. Tuco-tucos (*Ctenomys* aff. *knighti*) are subterranean rodents that are diurnal in the field but are robustly nocturnal in laboratory, with or without access to running wheels. We assessed their energy metabolism by continuously and simultaneously monitoring rates of oxygen consumption, body temperature, general motor and wheel running activity for several days in the presence and absence of wheels. Surprisingly, some individuals spontaneously suppressed running-wheel activity and switched to diurnality in the respirometry chamber, whereas the remaining animals continued to be nocturnal even after wheel removal. This is the first report of timing switches that occur with spontaneous wheel-running suppression and which are not replicated by removal of the wheel.

3.2. Introduction

The tuco-tuco (*Ctenomys* aff. *knighti*), a South American subterranean rodent, is among the several mammals described as having discrepant activity timing between field and laboratory conditions (Labyak et al., 1997; Blanchong and Smale, 2000; Levy et al., 2007; Weinert et al., 2007; Daan et al., 2011; Hut et al., 2012; Barak and Kronfeld-Schor, 2013). Whereas they are active during the day in semi-natural field enclosures, all individuals are nocturnal under laboratory conditions, with or without access to running-wheels (Valentinuzzi et al., 2009; Tomotani et al., 2012; Tachinardi et al., 2014) suggesting that a fundamental feature of their natural environment is not reproduced in the laboratory. Both ecological and physiological studies indicate the critical role of daily energy balance in constraining the timing of activity, which is primarily determined by the circadian clock (Daan, 1981; Kenagy and Vleck 1982; Halle, 2000; Ruf and Heldmaier, 2000; Kronfeld-Schor et al., 2001; Hut et al., 2011; Hut et al, 2012; van der Vinne et al., 2014). Thus, differences in energy demand between field and laboratory conditions could be the fundamental feature ultimately leading to inversion in the timing of daily activity (Hut et al., 2011; Hut et al., 2012; van der Vinne et al., 2014).

Subterranean rodents are excellent subjects to explore this proposition because their foraging activity in the field involves intense underground excavation, an intense energy

demanding activity (Buffenstein, 2000; Lacey et al., 2000; Luna et al., 2002; Luna and Antinuchi, 2007; Luna et al., 2009; Persinotti et al., 2009). Moreover, the periodic day-time emergence of tuco-tucos to the surface (Tomotani et al., 2012; Flôres et al., 2013) might indicate economy in thermoregulation costs, allowing allocation of the rest phase inside burrows during the coldest hours of the desert night (Burda et al., 2007; Hut et al., 2012).

Few studies have addressed continuous, 24h rhythms of metabolism in subterranean rodents (Kenagy and Vleck, 1982; Riccio and Goldman, 2000). We set out to assess the daily patterns of metabolic rate and its interrelationships with body temperature (T_b) and activity in wild-caught tuco-tucos. We hypothesized that tuco-tucos would show higher metabolic rates, levels of activity and T_b during darkness under laboratory light/dark cycles. Furthermore, we predicted that availability of a running wheel in the respirometry chamber would modulate amplitude but not timing of metabolic rate, as shown before for T_b (Tachinardi et al., 2014). To accomplish our investigation, we monitored individuals continuously for oxygen consumption ($\dot{V}O_2$), T_b, general motor activity and wheel-running over several consecutive days. Measurement of $\dot{V}O_2$ occurred inside a respirometry chamber which, to our surprise, revealed a new triggering factor for activity phase inversion and a novel association between wheel-running and switches in timing of activity. Cause and effect between activity timing and wheel-running appear now in a reformulated and challenging perspective.

3.3. Material and Methods

Ethics statement

All procedures followed the guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes et al., 2011) and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory (NRCUS, 2011). All experiments were performed in Anillaco and were authorized by the Environmental Department of La Rioja (permits 028-10 and 062-08) and approved by the Ethics Committees of the Biosciences Institute of the University of São Paulo, Brazil (permit 164/2012), the University of Alaska Anchorage's Institutional Animal Care and Use Committee (405977-1) and of the Faculty of Veterinary Sciences of La Plata National University, Argentina (permit 29-2-12).

Animals

Tuco-tucos were trapped within a 3 km radius of the town of Anillaco (28° 48´ S; 66° 56´ W; 1350 m) in the ecoregion of the Monte Desert, Argentina. Nine adult individuals (140-220 g) were used, of which five were females and four were males. Because these animals are solitary, they were housed individually in plastic cages (53×29×27 cm) equipped with running wheels (23 cm diameter, 10 cm wide, 1 cm between bars). Food (grass, native plants, carrot, sweet potato, rabbit pellets, oat, sunflower seeds) was provided *ad libitum* and replaced daily at various times. Water was not offered because subterranean rodents do not drink free water (Buffenstein, 2000).

Cages were placed inside light-tight boxes equipped with one incandescent red light bulb providing continuous dim red light (1–5 lux) to facilitate animal care, and one fluorescent bulb of 200–250 lux at cage lid level connected to a timing device. Unless specified otherwise, tuco-tucos were kept under an LD cycle with 12 hours of "darkness" (1 - 5 lux) followed by 12 hours of light (LD 12:12), with lights on at 07:00 AM (local time, GMT -3).

Relative humidity ranged from 30 - 60% and room temperature was maintained at 25±2°C, which is within the thermoneutral zone of other *Ctenomys* species (Busch, 1989; Tachinardi, unpublished) Data loggers (HOBO U10/003, Onset Computer Corporation, Bourne, MA) recorded room temperature and relative humidity every 15 minutes.

Monitoring of wheel-running, general activity and body temperature

Tuco-tucos were surgically implanted with temperature sensitive transponders (G2 E-Mitters, Mini-Mitter, Bend, OR) to allow for continuous monitoring of core T_b and gross motor activity. Animals were anaesthetized using either ketamine/acepromazine (200 and 20 mg/Kg, respectively) or isoflurane anaesthesia (3%–5% with oxygen). Transponders were inserted into the peritoneal cavity through 1.5-2 cm vertical midline incision (1 cm bellow the rib cage) and sutured with poliglicolic acid thread (for more surgical details, see Tachinardi 2014). All surgeries were completed at least eight weeks prior to initiation of experiments.

Each cage was placed above a receiver (ER 4000, Mini-Mitter, Bend, OR) and data were collected and processed using the software VitalView (Mini-Mitter, Bend, OR); averages of T_b and activity were recorded each five minutes. Wheel-running was recorded as total revolutions in each 5-min interval by the ArChron Data Acquisition System (Simonetta System, Universidad Nacional de Quilmes, Buenos Aires, Argentina).

Respirometry

Rates of O_2 consumption were measured by open-flow respirometry during February and March of 2013 and 2014. In 2013, we used a FoxBox (Sable Systems, Las Vegas, NV) and Molecular Sieve 3Å (8-12 mesh, Sigma-Aldrich, Saint Louis, MO) as a desiccant with O_2 measurement only. In 2014 we used the Field Metabolic System (Sable Systems, Las Vegas, NV) and a Nafion Dryer to remove moisture from the air (Tøien, 2013) Since $\dot{V}O_2$ data collected in the two years did not significantly differ (two-tailed t-test, $p>0.05$), we merged data from both years for further analysis.

During the experiments, animals were individually kept inside a respirometry chamber (volume= 40L). It is important to note that the respirometry chamber is the home cage with the following modification: the wire lid of the home cage is replaced with a sealed clear acrylic lid with fittings for in-flow and out-flow of air for the respirometry measures. Outside air was pulled through the metabolic chamber at 450-650 mL/min, depending on the size of the animal. Before entering the chamber, outside air was passed through copper tubing (2m length) to facilitate equilibration of incurrent air temperature with air temperature of the animal room. Flow was generated by a vacuum pump and measured by a mass flow meter (part of the FoxBox System or the Mass Flow System-5, Sable Systems, Las Vegas, NV).

Excurrent air was drawn through Molecular Sieve 3Å or the Nafion dryer to remove moisture prior to measurements of gas concentrations. A subsample was passed through oxygen and carbon dioxide analyzers. The O_2 analyzer was calibrated with ambient air every hour. Averages of flow rate and O_2% were logged onto a computer each minute and corrected for baseline drift by linear interpolation using modified version of LabGraph (Tøien, 2013).

Mass specific rate of oxygen consumption (mL g^{-1} h^{-1}) was calculated using the following equations (Withers, 1977; Tøien, 2013):

$$\dot{V}O_2 = (\dot{V}_E * (FIO_2 – FEO_2)/(1 – FIO_2*(1\text{-}RQ)))/BM$$

$\dot{V}_E$ = airflow exiting chamber (mL/min), FIO_2 = fraction of O_2 entering chamber, FEO_2 = fraction of O_2 exiting chamber, RQ= respiratory quotient (assumed to be 0.85, BM = body mass (Kg).

Integrity of the respirometry system was tested before the 2014 trials using alcohol burns (Tøien, 2013).

Sufficient food for at least three days was placed inside the chamber at the beginning of the experiment. For trials lasting more than three days, additional food was

supplied during the experiment by quickly opening and re-sealing the chamber. Chamber temperature was 25±1°C, recorded every 15 minutes by a data logger (HOBO U10/003, Onset Computer Corporation, Bourne, MA). Animals were weighed before and after each trial.

Experiments

We performed continuous 5-9 day long respirometry trials for each animal, previously entrained by CE12:12, using two protocols. In the first (N=4), respirometry trials were initiated without animal access to a running wheel and wheels were added on day three inside the chamber. In the second protocol (N=5), trials started with a running wheel inside the chamber but removed on the third day. Activity and T_b were monitored continuously for at least 3 days before, during, and for 3 days after the respirometry trials.

Data analysis

Animal activity and T_b were firstly depicted in double-plotted actograms using the software El Temps (Díez-Noguera, Universitat de Barcelona, Spain, 1999). Actograms allowed visual estimation of phase and rhythmic pattern.

To quantify phase changes in different conditions, we used a modified version of the diurnality index (D) proposed by Hoogenboom et al. (1984) (Daan et al., 2011; van der Vinne et al., 2014):

$$D = \sum [(T_L - M) - (T_D - M)]_i / \sum [(T_L - M) + (T_D - M)]_i$$

where T_{Li} and T_{Di} correspond respectively to each T_b measure during the light and dark phase (only values above the mean were considered) and M corresponds to the mean T_b during light and dark. This index is symmetric around 0 and runs from -1 (no high T_b during the day) to +1 (high T_b only during the day). We used T_b to calculate the D-Index because it was a variable recorded throughout all conditions.

$\dot{V}O_2$ data are presented as means±SEM. We tested for the significance (α=0.05) of differences in variables under different conditions using one-way ANOVAs (for multiple group comparisons) or two-tailed Student's t-test (when only two conditions were compared). To test for significant associations among D-Indices and measured variables, we ran Pearson's product-moment correlation tests. All analyses were performed with R version 2.11.1 (R Development Core Team, 2010).

3.4. Results

Before the start of the respirometry trials, all animals displayed a nocturnal pattern with high T_b, general activity and wheel-running concentrated in the dark phase. When animals were placed into the respirometry chamber, some animals showed a radical and immediate change in their timing of peak $\dot{V}O_2$, T_b and general activity. While some (N=3) remained clearly nocturnal (D-Indices<-0.4; Fig 3.1a), the majority (N= 6) changed their rhythmic pattern (Fig 3.1b) and became either robustly diurnal (D-Indices>0; N=3) or did not show clear nocturnality or diurnality (D-Indices between -0.1 and 0.1; N=3).

D-indices ranged from -0.98 to -0.39 when animals were outside the respirometry chamber. Inside the respirometry chamber, D-indices ranged from -0.23 to +0.29 in the absence of the running wheel and from -0.61 to +0.83 when the wheel was available (Fig. 3.2). One individual showed a particularly dramatic change in the D-Index, switching from -0.83 outside the chamber to +0.83 inside the chamber in the presence of the wheel. Differences in D-Indices among the three conditions were statistically significant ($p<0.001$).

$\dot{V}O_2$ followed the same rhythmic patterns as general activity and T_b (Fig. 3.1). In addition to the daily variation, $\dot{V}O_2$ periodically peaked for episodes of more than one hour corresponding to bouts of high general activity and T_b. Mean $\dot{V}O_2$ of tuco-tucos was 1.305± 0.073 mL g^{-1} h^{-1}. Mean $\dot{V}O_2$ of females (1.235± 0.060 mL g^{-1} h^{-1}, N=5) and males (1.384± 0.151 mL g^{-1} h^{-1}, N=4) did not significantly differ ($p>0.05$). In Table S3.1, we present the mean values of $\dot{V}O_2$ and T_b for each individual, during days with and without access to running wheels.

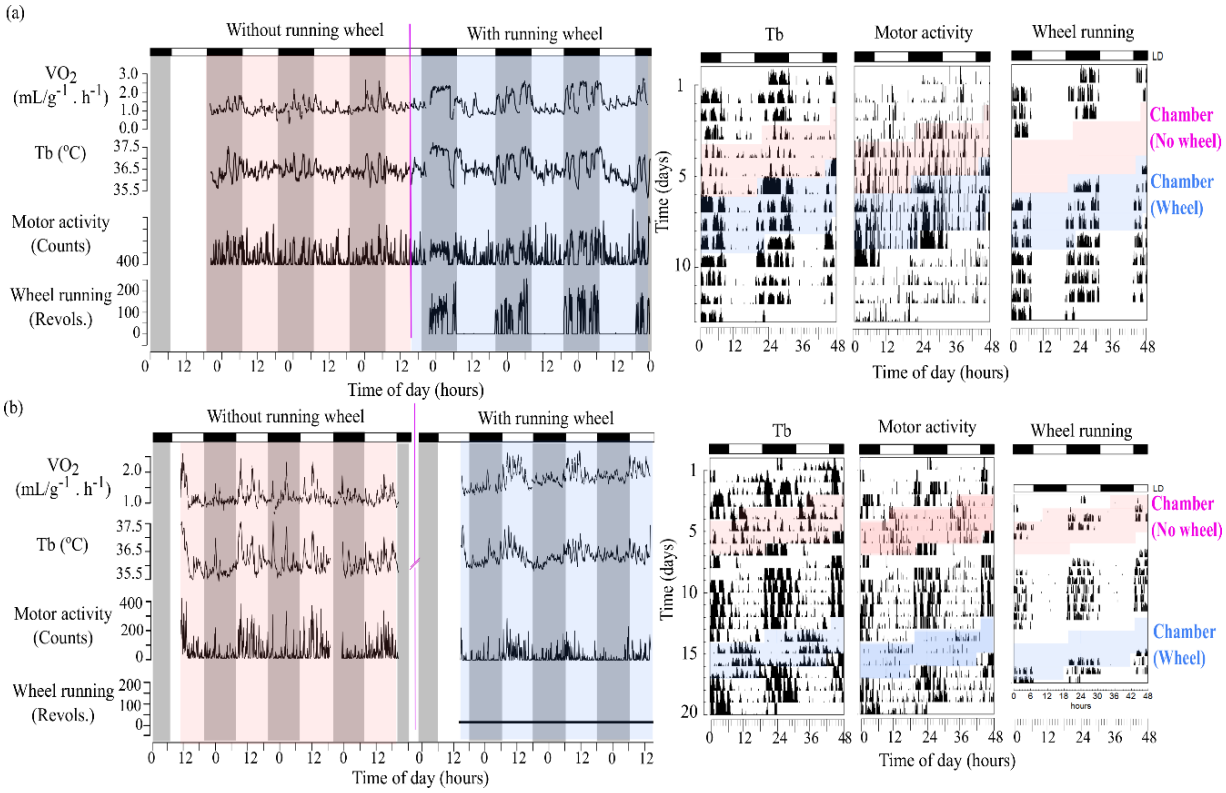

Figure 3.1: **Simultaneous measurements of daily rhythms in oxygen consumption ($\dot{V}O_2$), body temperature (T_b), gross motor and wheel-running activity of tuco-tucos.** Left: temporal series collected when the animal was inside the respirometry chamber, with and without a running-wheel. Shaded areas indicate dark phases and white areas light phases. Right: actograms along across experimental conditions. Pink and blue backgrounds indicate data from animals inside the respirometry chamber, with and without access to wheels, respectively. (a) Representative individual that did not switch phase inside the respirometry chamber. Pink line in the left figure indicates introduction of the wheel to the chamber. (b) Representative individual that switched from nocturnal to diurnal inside the respirometry chamber. There was a 7 day interval outside the respirometry chamber before the wheel introduction due to technical problems. Pink broken line in the right figure separates days with and without wheels. General conditions: LD12:12 (L=200-250 lux), 25±2°C and food *ad libitum*.

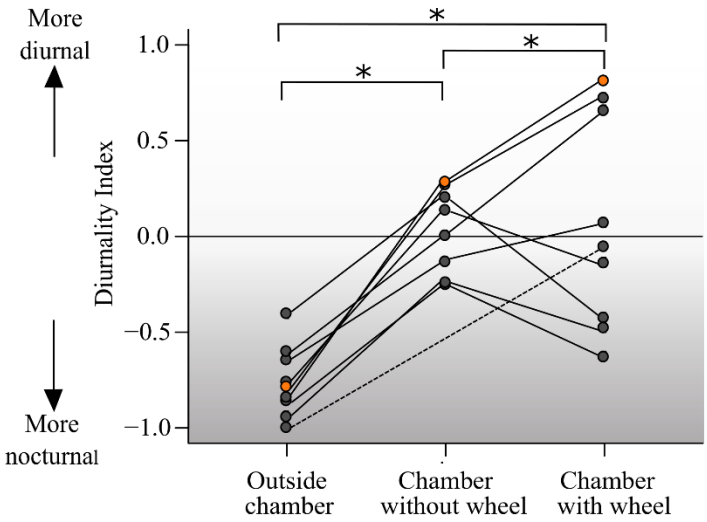

Figure 3.2: **Variation of diurnality indices across the stages of the experiment.** D-index for individual tuco-tucos (N=9) along days outside and inside the respirometry chamber both with and without running-wheels. D-Index for each individual in the different conditions are connected by a line. The points in orange indicate the values for animal #143 which showed the highest discrepancy in D-Indices across conditions. Dashed line connects the values for animal #146, which was not submitted to the "chamber without wheel" condition.

Total amount of wheel-running revolutions per day was significantly reduced when animals were housed in the respirometry chamber ($p<0.001$). Whereas all individuals completed >5000 wheel revolutions/day outside the respirometry chamber, only one displayed such intense running while inside the chamber (Fig. 3.3a). Daily amount of wheel-running correlated negatively with D-index ($r = -0.73$, $p<0.001$) with the most strongly nocturnal animals displaying the greatest amount of wheel-running (Fig. 3.3a). Lower wheel-running and associated phase inversion occurred both in the animals exposed to the wheel immediately upon being placed in the respirometry chamber and in those animals that were provided a wheel after three days in respirometry chamber. Despite the drastic decrease in wheel-running activity, mean daily general activity, T_b and $\dot{V}O_2$ did not differ significantly among conditions ($p>0.05$) and neither correlated with D-Indexes ($p>0.05$) (Fig. 3.3b, 3c and 3.3d, Table S3.1).

3.5. Discussion

Despite showing day-time activity under field conditions, tuco-tucos consistently display nocturnal patterns when housed in the laboratory irrespective of access to running-wheels (Valentinuzzi et al., 2009; Tomotani et al., 2012; Tachinardi et al., 2014). In the present study, we report the first displays of diurnality in the lab, which occurred exclusively during our respirometry experiment (Fig.3.1). Some individuals in the new

environment of the sealed respirometry chamber completely suppressed running-wheel activity and switched to diurnality as revealed by T_b, $\dot{V}O_2$ and general activity rhythms; while others remained nocturnal as usual in the laboratory and continued to run on the wheel (Fig. 3.3).

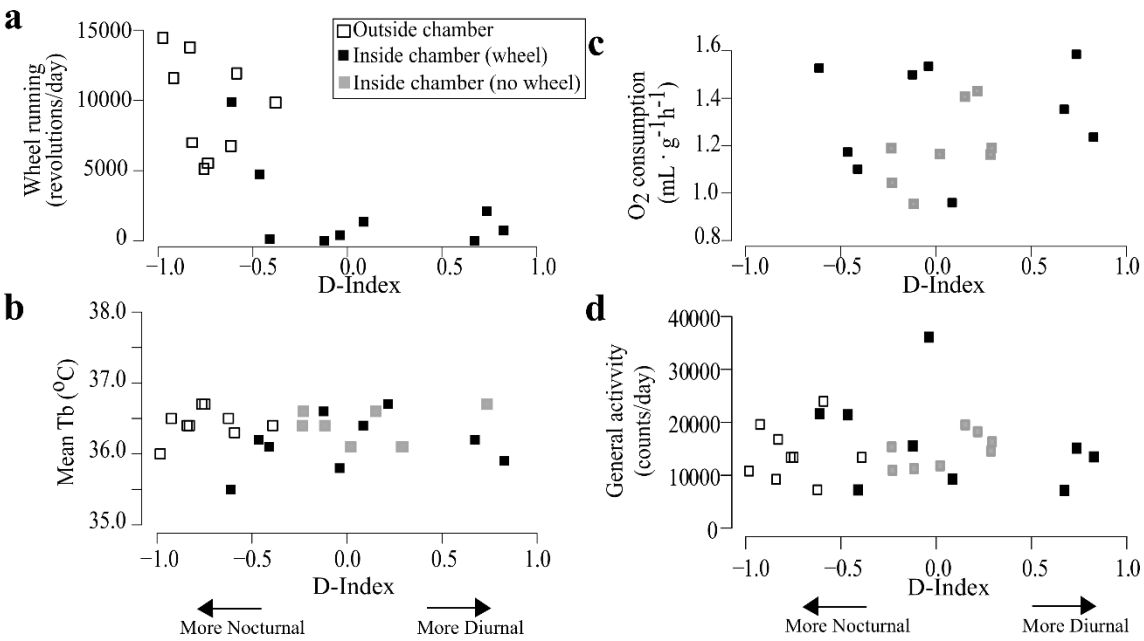

Figure 3.3: **Wheel-running, mean T_b and mean Oxygen consumption of tuco-tucos in relation to diurnality indexes.** Measurements for each individual (N=9), across the stages of the experiment including days outside (white squares) and inside (black square) the respirometry chamber both with and without (grey squares) running-wheels. (a) Mean daily wheel-running levels are associated to nocturnality. (b) Mean body temperatures during each stage. There is no clear correlation with D-Indices. (c) Mean $\dot{V}O_2$ during each stage. There is no clear correlation with D-Indices. (d) Mean amount of general activity per day during each stage. There is no clear correlation with D-Indices.

The search for the critical factors which trigger the nocturnality/diurnality switch observed in other species often converge upon the issue of the meaning of the running-wheel activity in the laboratory (Mather, 1981; Sherwin, 1998; Novak et al., 2012; Meyer et al., 2014). In some rodent species, all individuals are diurnal in the field whereas in the laboratory some become nocturnal while others remain diurnal. Interestingly, when offered unrestricted access to running wheels, the diurnal individuals become nocturnal (grass rats, *Arvicanthis niloticus* (Blanchong, 1999); degus, *Octogon degus* (Kas and Edgar, 1999); and mongolian gerbils, *Meriones ungiculatus* (Weinert et al., 2007). This phenomenon has been associated with a line of research devoted to investigating the "effect of" vigorous wheel-running on the period and phase of circadian activity rhythms

(Yamada et al., 1988; Kas and Edgar, 1999; Reebs and Mrosovsky, 1989; van Reeth and Turek, 1989; Edgar et al., 1991; Mrosovsky, 1995; Redlin, 2001). Phase shifts in the free-running suprachiasmatic nuclei (SCN) due to "pulses" of intense running wheel activity are reported but are of very small magnitude (Reebs and Mrosovsky, 1989; Reebs and Mrosovsky, 1995). Downstream from the SCN, however, wheel-running activity could act directly on the activity/inactivity signaling between the SCN and locomotor centers, as proposed by Kas and Edgard (1999). Their proposal was based on investigations of degus, a species that is known to switch phase from nocturnal to diurnal activity when provided access to a running wheel while in DD yet without any change to the basic free-running rhythm period.

The spontaneous suppression of wheel-running activity was displayed by all individuals that switched to diurnality (i.e., animals which showed D-Index>0.5) when exposed to the new environment of the respirometry chamber (Fig.3.3a). This phenomenon occurred in both of our trials in two consecutive years. It is noteworthy that general motor activity was maintained and switched to a diurnal pattern in all individuals that stopped running on the wheel (Fig. 3.1)

Our finding of a phase inversion (nocturnal to diurnal) in tuco-tucos when housed within a respirometry chamber illustrates a novel association between running-wheels and timing of activity not observed in any of the previous work on degus, grass rats and Mongolian gerbils. In common with the above species, the greatest levels of activity are always associated with nocturnality (Fig. 3.4). Although phase inversion inside the respirometry chamber occurs concomitantly with suppression of wheel-running behavior, it is not "a response" to removal of the wheel (Fig. 3.3) because when the wheel is removed from the respirometry chamber the nocturnal individuals do not switch to diurnality. Robust nocturnal patterns have been previously observed in 100% of 18 animals with wheels (Valentinuzzi et al., 2009; Tomotani et al., 2012) and of 5 without wheels (Tomotani et al., 2012). Additionally, wheel removal without switch in activity phase in tuco-tucos has been reported before in 100% of 6 animals, during experiments performed in other contexts (Tachinardi et al., 2014). These results suggest that the previously reported switches in activity timing upon introduction or removal of a running wheel may not necessarily be caused by feedback from wheel-running itself, when these two processes occur simultaneously.

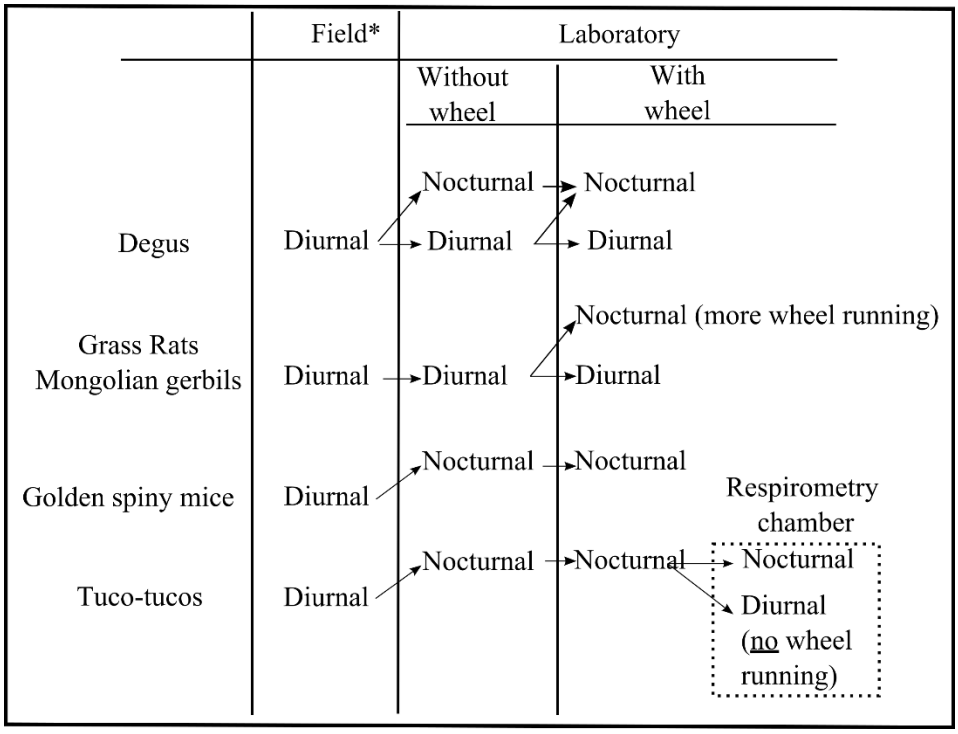

Figure 3.4: **Schematic view of different phase switch patterns associated to the presence of running wheels**. Based on: Labiak et al. (1997), Kas and Edgar (1999), Fulk (1976) and Hagenauer and Lee (2008) for *Octogon degus*; Blanchong and Smale (2000) and Blanchong et al. (1999) for *Arvicanthis niloticus*; Levy et al. (2007) and Cohen et al., 2009 for *Acomys russatus*; Weinert et al. (2007) for *Meriones ungiculatus*; Tomotani et al., 2012 and Flôres et al., 2013 for *Ctenomys* aff. *knighti*. *For each species, field data were collected using different methods and do not necessarily reflect activity patterns of whole populations

Diurnal/nocturnal switches in the laboratory occur so rapidly that it has been argued that this flexibility might represent an adaptive mechanism to sudden changes in the species' natural environment (Blanchong et al., 1999; Kas and Edgar, 1999). The switches in activity timing in tuco-tucos were triggered by the novelty of a respirometry chamber (Fig. 3.4) where tuco-tucos face mild alteration of gas composition of the ambient air (< 1% decrease in O_2 and <0.5% increase in CO_2). A survey of the literature of the effects of O_2 and CO_2 content of air on circadian patterns reveal mostly changes in amplitude, with rhythmic depression as a consequence of hypoxia or hypercapnia in rats (Mortola and Seifert, 2000). However, minute phase changes have been observed in free-running golden hamsters exposed to pulses of hypoxic air (Jarsky and Stephenson, 2000). These studies involved more extreme hypoxia/hypercapnia than faced by our tuco-tucos in the respirometry chamber and they were conducted with non-subterranean animals. It is conceivable that tuco-tucos, which live in sealed underground tunnels, are able to detect even small changes in gas composition and/or humidity. Perceived changes in the gas composition of the environment could serve as a triggering mechanism to incite an

alertness response needed for predator avoidance or tunnel maintenance and, possibly, lead to changes in the temporal pattern of activity, as suggested by our results in the sealed chamber.

Several interesting insights have emerged from our simultaneous measurements of the interconnected $\dot{V}O_2$, T_b, general motor and wheel-running rhythms. Our results clearly demonstrate that switches in timing of activity phase can occur concomitantly with spontaneous suppression of wheel-running. Apparently, in tuco-tucos cause and effect of activity timing and wheel-running have been shuffled, reappearing now in a reformulated and perhaps illuminating perspective.

3.6. Acknowledgements

The authors thank Danilo Flôres for valuable discussions and help during experimental measurements, Melanie Richter and Barbara Joos for help with the respirometry setup, Jose Paliza for technical assistance and Vinícius Dokkedal for assisting with data collection.

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3.8. Supplementary material

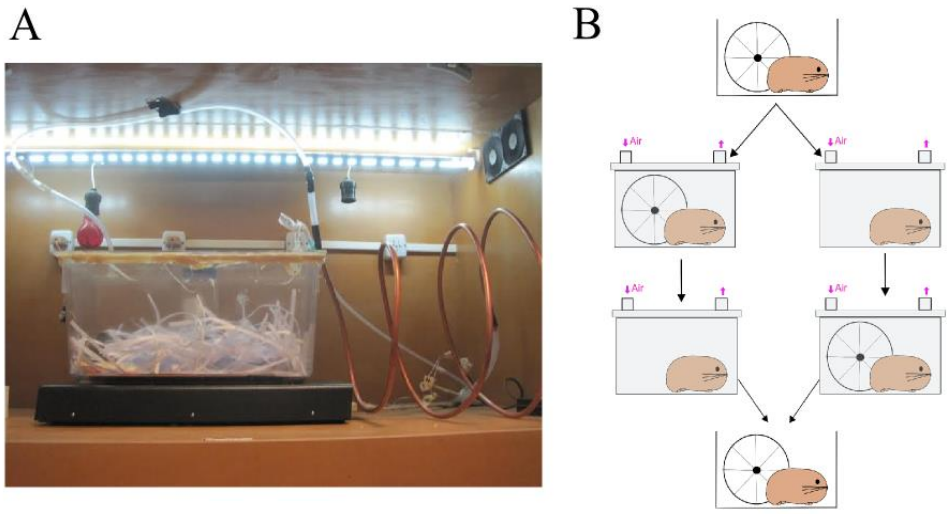

Figure S3.1. Respirometry chamber and schematic illustration of the experimental protocol. A. Photography of the respirometry chamber without the running wheel. The chamber consists in a standard home cage with an acrylic lid with fittings to allow the airflow. The chamber was kept in a light-tight cabinet, which was the same used in the non-respirometry steps of the experiments. B. Scheme of the experimental protocol. At first, the animal was kept in its home cage with access to a running-wheel. Then, it was placed in the respirometry chamber. One group was put in a chamber with running-wheel and the other in a chamber without a wheel. The group that started with the wheel would then have it removed, while the other would have the wheel added to the chamber. After the respirometry trials, measurements would continue in a standard home cage

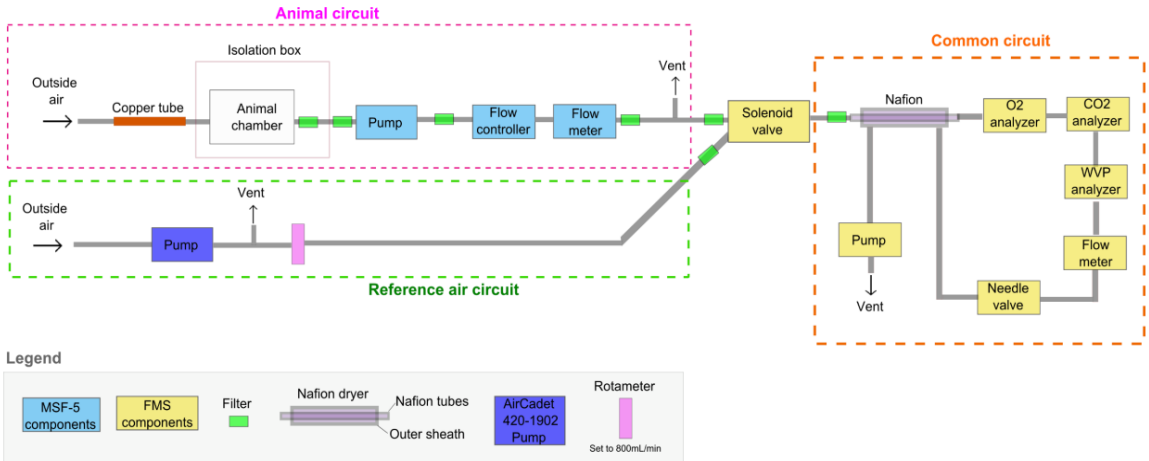

Figure S3.2. Scheme of the respirometry system.

Table S3.1. Summary of the variables measured under different conditions, for each individual. [1]In mL · $g^{-1}h^{-1}$,represented as mean±SD . [2]In °C. [3]Mean total daily revolutions.

			Outside chamber			Chamber with wheel						Chamber without wheel				
									Mean O2 consumption[1]					Mean O2 consumption[1]		
Animal	Sex	Mass (g)	Mean Tb[2]	D-Index	Wheel Rev.[3]	Mean Tb[2]	D-Index	Wheel Revol.[3]	Daily	Light	Dark	Mean Tb[2]	D-Index	Daily	Light	Dark
99	F	140	36.3	-0.592	12064	36.2	0.673	0	1.33 (±0.20)	1.41 (±0.23)	1.24 (±0.11)	36.1	0.02	1.15 (±0.28)	1.17 (±0.25)	1.12 (±0.21)
98	M	201	36.4	-0.841	13916	35.5	-0.613	9885	1.50 (±0.55)	1.22 (±0.36)	1.78 (±0.56)	36.4	-0.234	1.17 (±0.28)	1.14 (±0.24)	1.21 (±0.31)
46	F	207	36.4	-0.391	9980	36.1	-0.411	144	1.09 (±0.14)	1.10 (±0.15)	1.07 (±0.13)	36.1	0.287	1.15 (±0.25)	1.20 (±0.25)	1.09 (±0.24)
69	M	180	36.7	-0.748	5640	36.6	-0.123	0	1.47 (±0.33)	1.47 (±0.33)	1.47 (±0.33)	36.6	0.152	1.38 (±0.40)	1.43 (±0.45)	1.33 (±0.45)
123	F	156	36.7	-0.765	5265	36.7	0.737	2109	1.56 (±0.41)	1.80 (±0.37)	1.31 (±0.26)	36.7	0.217	1.40 (±0.36)	1.55 (±0.39)	1.26 (±0.26)
146	M	174	36	-0.983	14601	35.8	-0.039	408	1.51 (±0.17)	1.50(±0.17)	1.51 (±0.35)	--	--	--	--	--
141	F	152	36.5	-0.925	11724	36.2	-0.464	4745	1.16 (±0.34)	1.07 (±0.16)	1.23 (±0.43)	36.6	-0.23	1.03 (±0.23)	1.17 (±0.25)	1.07 (±0.26)
137	M	220	36.5	-0.624	6897	36.4	0.085	1358	0.94 (±0.20)	1.00 (±0.18)	0.89 (±0.21)	36.4	-0.117	0.94 (±0.17)	0.90 (±0.15)	0.98 (±0.18)
115	F	160	36.4	-0.83	7084	35.9	0.827	735	1.21 (±0.34)	1.42 (±0.35)	1.01 (±0.14)	36.1	0.293	1.17 (±0.32)	1.28 (±0.30)	1.06 (±0.30)

Chapter 4

The Interplay of Energy Balance and Daily Timing of Activity in a Subterranean Rodent: A Laboratory and Field Approach

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4.1 Abstract

The tuco-tuco (*Ctenomys* aff. *knighti*) is among the rodent species known to be nocturnal under standard laboratory conditions and diurnal in natural conditions. The circadian thermo-energetics (CTE) hypothesis postulates that switches in activity timing are a response to energetic challenges; daytime activity reduces thermoregulatory costs by consolidating activity to the warmest part of the day. Studying wild animals under both captive and natural conditions can increase understanding of how temporal activity patterns are shaped by the environment and could serve as a test of the CTE hypothesis. We estimated the effects of activity timing on energy expenditure for the tuco-tuco by combining laboratory measurements of metabolic rate with environmental temperature records in both winter and summer. We showed that, in winter, there would be considerable energy savings if activity is allocated at least partially during daylight, lending support to the CTE hypothesis. In summer, the impact of activity timing on energy expenditure is small, suggesting that during this season, other factors, such as predation risk, water balance and social interaction may have more important roles than energetics in the determination of activity time.

4.2. **Introduction**

Daily rhythms of physiology and behavior are driven by outputs of endogenous circadian clocks, which are synchronized to environmental cycles. Recent studies, combining investigations of animals under laboratory and natural conditions, are providing new insights into the adaptive significance of daily rhythmicity and its plasticity (Kronfeld-Schor et al. 2013). The immediate and dramatic switch from diurnality when in the field to nocturnality in the laboratory displayed by some rodent species is a striking example of this plasticity (Levy et al. 2007; Blanchong et al., 2009; Daan et al. 2011; Hut et al. 2012; Tomotani et al. 2012). Survival and fitness of free-living animals requires integration of a far more complex suite of biotic and abiotic factors than is found in the laboratory and that can serve to shape the expression of daily rhythms (Hut et al. 2012). These factors include environmental conditions known to impact energy balance, particularly food availability and ambient temperature (T_a). A recently formulated circadian thermo-energetics (CTE) hypothesis (van der Vinne et al 2014; van der Vinne et al. 2015) posits switching from nocturnal to diurnal activity is a response to energetic challenges; diurnality reduces thermoregulatory costs by consolidating activity and higher body temperature (T_b) to the warmest part of the day and rest and lower T_b to the coldest hours of the night.

Most evidence supporting the CTE hypothesis comes from studies of laboratory mice, which clearly respond to energetic challenges of low T_a and working for food by increasing daytime activity (Hut et al. 2011; van der Vinne et al. 2014). Utilizing wild animals under both captive and natural conditions can increase understanding of how temporal activity patterns are influenced by the environment and could serve as a test of the CTE hypothesis. The tuco-tuco (*Ctenomys* aff. *knighti*) is a subterranean rodent that is known to peak in activity and T_b during the day under natural conditions whereas under

standard laboratory conditions it is strongly nocturnal (Tomotani et al. 2012; Tachinardi et al. 2014).

Energetic challenges are much greater in the field than in the lab. In field conditions, this herbivorous rodent relies upon intense digging through hardened soils in a semi-arid habitat where vegetation is sparse (Luna et al., 2002; 2009). Moreover, daily changes in T_a can exceed 15ºC and might present thermoregulatory challenges at certain times of day and year. To investigate whether consolidation of activity during the day could provide energy savings in this species, we measured metabolic rate (MR) across a range of T_a's relevant to natural conditions (i.e., Scholander curve; Scholander et al. 1950). Additionally, we recorded air (T_{air}), operative (T_e) and soil (T_{soil}) temperatures across one year. Using these field data in conjunction with estimates of MR from laboratory held animals, we estimated the impact of aboveground activity timing on minimum daily energy expenditure (MDEE), taking into consideration T_e, T_s and daily durations animals were active above or below ground. This study adds a subterranean rodent perspective to the recent body of studies modelling energetics in light of the plasticity of activity timing (Levy et al. 2012; Van der Vinne et al. 2014; van der Vinne et al. 2015; Levy et al. 2016)

4.3. Methods

Animals, measurement of core body temperature (T_b) and general considerations

Four male and four female *Ctenomys aff. knighti* (150–212 g) were trapped within a 3 km radius of the town of Anillaco (28° 48´ S; 66° 56´ W; 1350 m) in the Monte Desert, Argentina. Animals were initially housed individually in plastic cages under LD12:12, 23 ± 2 °C and provided *ad libitum* sweet potato, carrot and commercial pellets, for 8 to 12 months. Animals were implanted with temperature sensitive transponders (G2 E-Mitters,

accuracy of ±0.1°C, Mini-Mitter, Bend, OR) for continuous measurement of T_b (details in Tachinardi et al. 2014). Data were recorded every five minutes and analyzed using VitalView software (Mini-Mitter, Bend, OR).

Throughout the manuscript time is expressed as UTC-3 (the time zone of the study area). When averages are mentioned, mean values are reported with standard deviation.

Metabolic measurements

We measured rates of O_2 consumption ($\dot{V}O_2$) and CO_2 ($\dot{V}CO_2$) production at rest using open flow respirometry (Sable Systems, Las Vegas, NV, detailed in Tachinardi et al. 2015) to estimate MR across a range of T_a's thus obtaining a Scholander curve (Scholander et al. 1950). Briefly, individual animals were placed in a 7.4 L plastic respirometry cage within an environmental chamber at a fixed T_a. Animals were allowed to acclimate for two hours after which $\dot{V}O_2$ and $\dot{V}CO_2$ were recorded each minute simultaneous with telemetric measures of T_b. Measurements were made at T_a's approximating 8, 16, 20, 24, 28 and 32°C as recorded within the respirometry chamber with a temperature logger (HOBO U10/003, accuracy of ±0.53°C, Onset Computer Corporation, Bourne, MA). $\dot{V}O_2$ was calculated using LabGraph (Tøien, 2013), and described by Withers' (1977) equation 3b with the approximation of $\dot{V}CO_2 = \dot{V}_E*(FICO_2 - FECO_2)$, where $\dot{V}_E$ = airflow exiting chamber (mL/min), $FICO_2$ = fraction of CO_2 entering chamber, $FECO_2$ = fraction of CO_2 exiting chamber. $\dot{V}O_2$ and the respiratory quotient ($RQ=\dot{V}CO_2/\dot{V}O_2$) were used to calculate energy expenditure in Watts (Withers 1977). For each individual, measurements of MR at each T_a were conducted at the same hour of the day, during the light hours, corresponding to the resting phase of tuco-tucos in standard laboratory conditions.

The average MR of the last hour of measurement at each T_a was used for data analysis. We performed linear regression analysis by means of least-squares for several T_a ranges, all comprising measurements between the lowest T_a measured and a given tested break-point T_a (T_a's between 20 and 30^oC were tested, at 1^oC steps). The best-fit model was used for the slope of the Scholander curve below thermoneutrality and the break-point T_a for that model was considered the lower critical temperature (LCT). We tested the correlation between T_b and T_a using Pearson's correlation test to assess if animals exhibited hypothermia, torpor or hyperthermia at the various T_a's. All analysis were performed with R version 3.3.2 (R Development Core Team. 2013).

Daily variation of environmental temperature in the tuco-tuco habitat:

All environmental temperatures were measured in a location where tuco-tucos naturally occur and previous field experiments took place (Tomotani et al. 2012) using data loggers (HOBO UA-002-08). T_{air} was measured at 1m aboveground, inside a radiation shield (RS3, Onset Computer Corporation, Bourne, MA). For T_{soil}, the temperature loggers were buried and placed in the soil at depths tuco-tucos of this region are known to occupy (20, 40 and 60 cm; personal observations). Loggers were not placed inside the burrow system itself by design to avoid damage from the animals and the potential of acquisition of spurious results should the animal rest on or near the logger. Although not placed directly into the burrow chamber, temperature data obtained should correspond to burrow temperature, since the airspaces below ground are sealed and quite small, in thermal equilibrium with the soil.

We used a taxidermic mount to obtain operative temperature (T_e), which is a result of the combined effects of conduction, convection and radiation on an inert body of the same size, shape and color as our study animal (Bakken 1980; Chappell and Bartholomew

1981; Long et al. 2005). The taxidermic mount consisted of a temperature logger surrounded by copper and fitted inside a tuco-tuco pelt and placed adjacent to the experimental outdoor enclosures.

T_{air} and T_{soil} were measured at hourly intervals for 12 consecutive months (from January 1 through December 31, 2016). T_e was measured at 20-minute intervals from February 16 through June 6, 2016 and at 30-minute intervals from July 3 through November 9, 2016.

Estimation of MDEE for nocturnal and diurnal strategies:

We modeled MDEE in both summer and winter for aboveground activity allocated at different times of the day. To estimate the temperature to which our model animals would be exposed (<T>), we calculated hourly averages of T_e and T_{soil} at 60cm below ground from February 17th to March 17th (Summer) and from August 15th until September 13th (Winter) and the average T_{soil} at 60cm below ground, the presumed depth of animals rest (Fig. S4.1). We assumed the duration above ground was the average of the duration observed for individuals released in semi-natural enclosures during each season (Winter: 186±71 min/day, n=8, data from Flôres et al. 2016; Summer: 82±36 min/day, n=8, data from Jannetti et al. 2016). For simplicity, we assumed activity would occur in a single continuous block of time. To estimate the impact of aboveground activity time on energy expenditure, we estimated MDEE 24 times, each having the aboveground activity centered at one hour of the day. Using the equation obtained for the slope of our tuco-tuco Scholander curve (DEE= 9.09955- 0.25063 * <T>), the average T_e or T_{soil} and the hypothetical information of whether the animal was underground or aboveground at that given time, we calculated the amount of energy the animal would spend each hour of the day and then summed the 24 hour values to obtain MDEE (minimum daily energy

expenditure excluding energetic costs of activity). To estimate the impact of timing of aboveground activity, we estimated MDEE 24 times, each having the aboveground activity centered at one hour of the day, except for the hours of the day in which T_e exceeded the 34°C, above which tuco-tucos cannot maintain constant euthermic T_b and become hyperthermic (Tachinardi 2012).

4.4. Results and Discussion

We estimated the potential effects of activity time on energy expenditure in a subterranean rodent by combining laboratory measurements of MR and field environmental temperature in both winter and summer. The lower critical temperature (LCT) of the tuco-tuco was 23°C, below which MR increased linearly with decreasing T_a (r^2=0.78, $p<0.05$, Fig. 4.1, Table S4.1), suggesting that the thermoneutral zone (TNZ) for these animals ranges from approximately 23 to 33°C. MR did not increase with decreasing T_a across this range and the T_b of tuco-tucos increases at T_a's above 34°C (Tachinardi 2012). T_b did not correlate with T_a across the range measured range of 4°C to 32°C (Pearson's r = 0.36, $p>0.01$) and averaged 35.87±0.36°C. The TNZ and LCT for *Ctenomys* aff. *knighti* are similar to those described for another *Ctenomys* species (TNZ for *C. talarum*= 25-30°C; Busch 1989; Baldo et al. 2015).

T_{air} and T_e exhibited wider daily and seasonal variation than T_{soil} at any depth (Fig. 4.2). Across the year, minimum daily T_e and T_{air} were lower than the LCT of tuco-tucos. Tuco-tucos spend more time outside the burrows during the coldest months, when T_a is always below their LCT. This might be due to increased foraging needs; winter is the dry season in the Monte desert and food availability is lower, with higher energy requirements due to low temperature. Alternatively, tuco-tucos may spend less time above ground in summer to avoid daytime high temperatures and incident solar radiation, since in summer T_e is often above the TNZ.

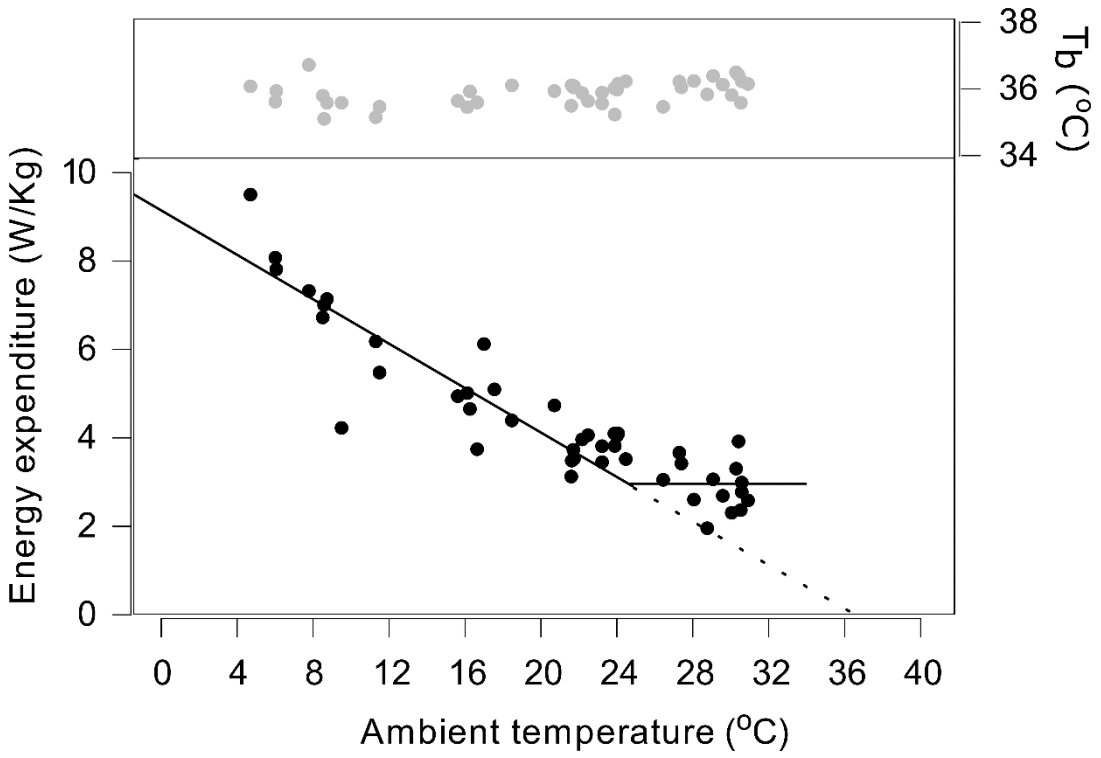

Figure 4.1. Metabolic rate and T_b as a function of ambient temperature. Each point represents the MR (black) or T_b (grey) measured for one individual at a given T_a. The horizontal line is traced along the TNZ (MR= 3.3 W/Kg). The inclined line represents the linear regression model calculated for T_a below 23ºC. The dashed line extrapolates the linear model to MR=0 and T_a=T_b (Average T_b of individuals in the 5 days prior to the metabolic measurements=36.3 ±0.4ºC). Tuco-tucos maintained a stable T_b throughout all the T_a's used during the MR measurements.

The results of our model indicate that the effect of activity timing varied between seasons (Fig. 4.3). In summer, there is almost no difference in MDEE related to the timing of activity, the time of activity with the highest MDEE (295.6 kJ/Kg, 10:00) was only 2.68% higher than the time with lowest MDEE (287.7 kJ/Kg, 21:00). In winter, the differences in MDEE are much larger, with a difference of 11.48% between highest (463.2 kJ/Kg, activity at 09:00) and lowest (410 kJ/Kg, 20:00). In summer, T_e exceed 34ºC from 13:00 until 20:00, which we considered non-permissive for aboveground activity. In both summer and winter, the time of aboveground activity with the highest MDEE is early morning. In winter, MDEE reached a low constant in the afternoon and early evening (14:00 – 20:00). Because activity phase in winter averages 186 minutes,

the onset of activity when the center is 20:00 is at least one hour before sunset. This indicates that aboveground activity during daylight hours can be energetically beneficial for tuco-tucos. Besides the seasonal differences of temperature, the duration of the activity phase has quite an important impact on the magnitude of the differences in MDEE between seasons (Fig. S4.2).

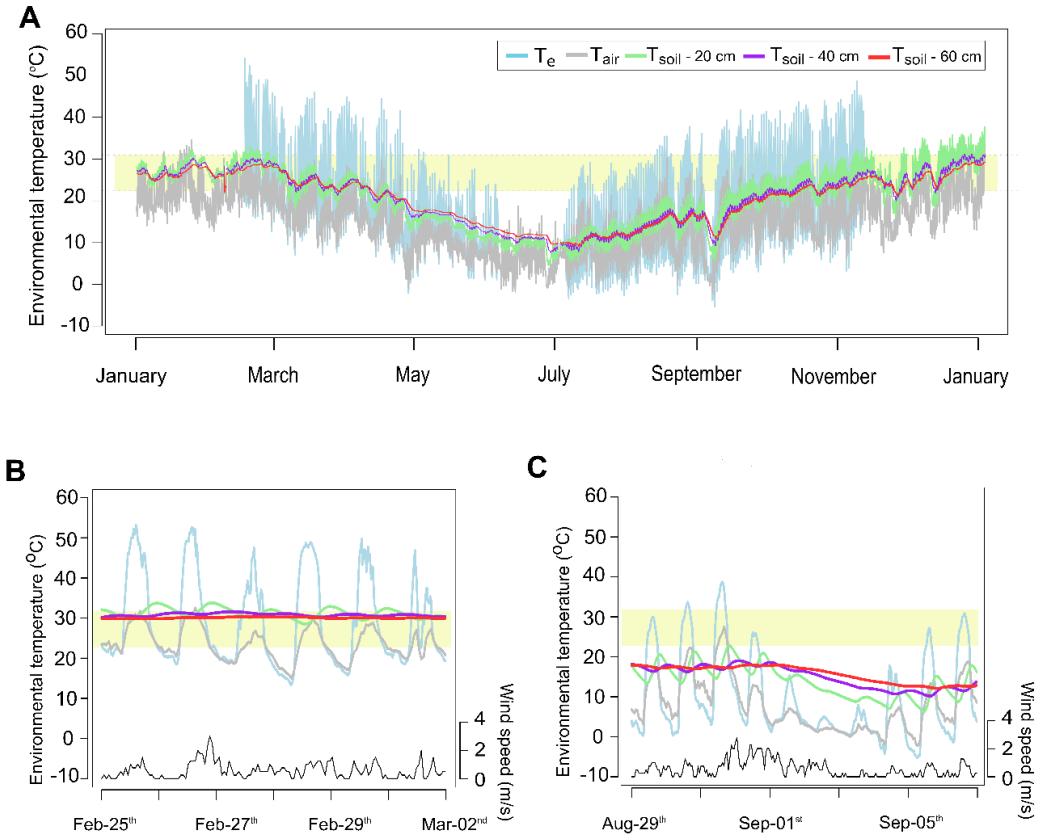

Figure 4.2. Environmental temperatures at the tuco-tuco's natural habitat. Blue line indicates T_e measured using a taxidermic mount, gray line indicates T_{air} 1m aboveground (measured inside a radiation shield), green line indicates T_{soil} at 20 cm deep, purple line indicates T_{soil} at 40 cm deep and red line indicates T_{soil} at 60 cm deep**.** The black line in B and C depicts the wind speed. Shaded yellow area indicates the TNZ of the tuco-tuco. Te was only measured from February 16th 2016 and from July 03rd until November 09th 2016. **A)** Temperatures measured from January 2016 until December 2016. **B)** Close-up of temperatures during six summer days (February 27-March 2). **C)** Close-up of temperatures during ten winter days (August 29 – September 7). From May until September, T_{air} and T_{soil} remained mostly below the TNZ of the tuco-tuco. T_e was above the TNZ during daylight hours in most measured days. Most of the time, underground T_{soil} was higher than T_{air} and the daily variation in T_{soil} was minimal compared to T_{air} and T_e.

We acknowledge that our model has limitations. MR was measured during the rest phase of the animals, but used for both activity and rest phases in our MDEE estimations. Taking into consideration that MR is higher during the active phase than during the rest phase (up to 50% in non-primate mammals, Aschoff 1982) taking this issue into account would further increase the impact of activity timing on MDEE (Fig S4.3). Although T_e integrates the effects of conduction, convection and radiation, it assumes the animal is metabolically inert and does not take into account wind induced changes in resistance to heat flow (Bakken 1980, Chappell and Bartholomew 1981). Adding those variables to the model would also increase the differences in MDEE according to timing of activity (Fig. S4.4). Finally, we estimate MDEE in field settings while using MR measured in laboratory conditions, which is known to change many aspects of physiology (Calisi and Bentley 2009). However limited, this approach allows fair estimations and has been widely used as a first step towards estimating energy budgets in the field (e.g., Kenagy and Hoyt 1989, Kenagy et al., 2002). Ideally, this approach should be followed and validated by other independent estimates of energy expenditure in the field such as doubly labeled water (e.g., Weather et al., 1984. Buttemer et al., 1989, Goldstein et al 1988, Kronfeld-Schor et al., 2001), heart rate (e.g., Portugal et al. 2016) or accelerometry (e.g., Williams et al. 2016a,b). Furthermore, while the absolute MDEE values are probably not precise, our goal was to assess the relative differences in energy expenditure for different times of activity.

While we did not take into account seasonal changes in both animal and nest insulation, we think that changes in these parameters would have minor impact on MDEE. Seasonal variations in nest insulation was not observed in excavated burrows (personal observations) and model calculations based on data from marmots (Webb and Schnabel 1983) and arctic ground squirrels (Buck and Barnes 1999) suggest that it is unlikely that

variation in conductivity of nests of subterranean rodents plays a significant role in energy expenditure. Although seasonal changes in fur density and length were observed in other tuco-tuco species (Cutrera and Antinuchi 2004), they are quite small compared to species from temperate and arctic climates (Scholander et al. 1950; Underwood and Reynolds 1980). Also, it has been noted that for small mammals, seasonal pelage variations have only modest effects on mass-specific metabolism (Steudel et al. 1994), whereas they can have substantial effects on large mammals.

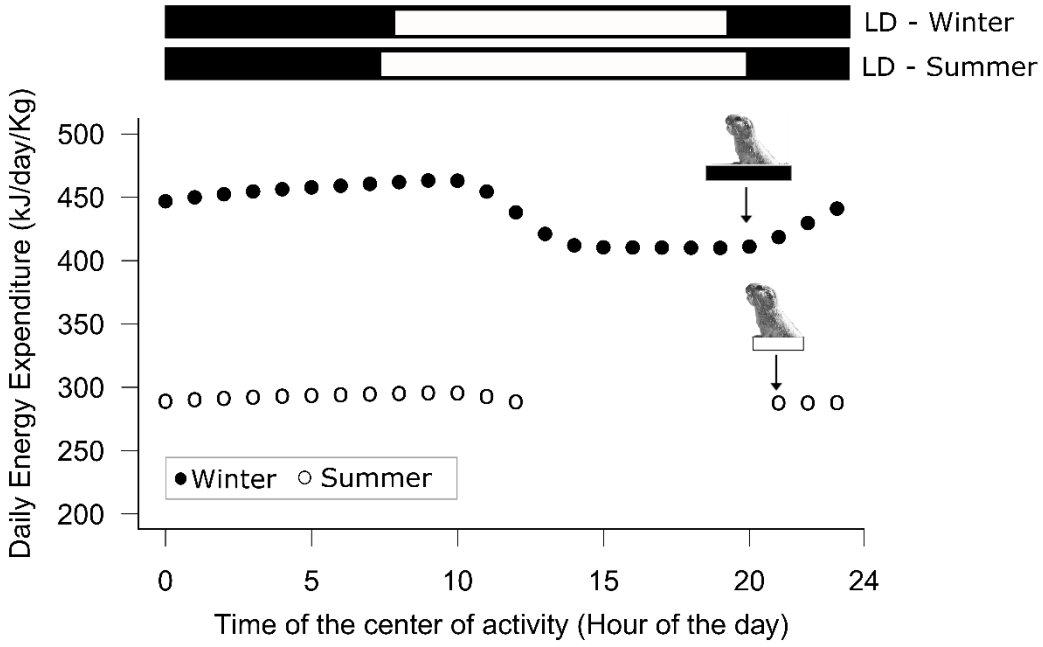

Figure 4.3. Estimated MDEE for different times of activity. Points are the MDEE values estimated by our model when aboveground activity is centered around a given time, winter (full circles) and summer (open circles). Small bars above the points indicate the duration of the aboveground activity phase centered at the time of the lowest MDEE value (Winter activity phase length: 186 minutes, lowest MDEE at 19:00; summer activity length: 82 minutes, lowest MDEE at 21:00). Bars above the plot indicate the average light (white) and dark (black) phases for the days used in the model (Summer: sunrise at 07:20 and sunset at 19:58; winter: sunrise at 07:40, sunset at 19:14).

Collectively, our results suggest that, in winter, there would be considerable energy savings if activity was allocated at least partially during daylight, lending support to the CTE hypothesis, similar to the results reported for house mice (van der Vinne et al.

2015). In summer, however, the impact of activity timing on energy expenditure is small, suggesting that in this season, some combination of other factors, such as predation risk (Tyler 2016), water balance (Levy et al. 2016) and social interaction (Kronfeld-Schor and Dayan 2003) may play more important roles than energetics in the timing of activity. Interestingly, our model indicates that duration of aboveground activity time can greatly increase the impact of daytime activity on MDEE, which was also reported for mice (Van der Vinne et al. 2015). Since during winter, food is scarce and ambient temperatures are low (especially at night), tuco-tucos spend more time foraging above ground (Tomotani et al. 2012). Consolidation of aboveground activity to the daytime decreases thermoregulatory costs and increases the animal's ability to cope with these energetic challenges. This would be especially evident in years with low precipitation and suggests that projected changes in rainfall in South America for the next decades (Boulanger et al. 2007; Labraga and Villalba 2009) may ultimately impact activity patterns of tuco-tucos. Finally, energetic benefits of daytime aboveground activity might be more significant for tuco-tucos inhabiting higher latitudes and altitudes (van der Vinne et al. 2015).

4.5. Acknowledgments

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Electronic Supplemental Information

Table S4.1. Average values for oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$) and respiratory quotient (RQ), for four T_a ranges.

	04-10ºC	**04-10ºC**	**20-24ºC**	**24-32ºC**
VO_2	1.32±0.27	0.93±0.15	0.69±0.08	0.56±0.12
VCO_2	0.92±0.21	0.66±0.08	0.52±0.05	0.43±0.09
RQ	0.69±0.04	0.73±0.10	0.77±0.05	0.77±0.09

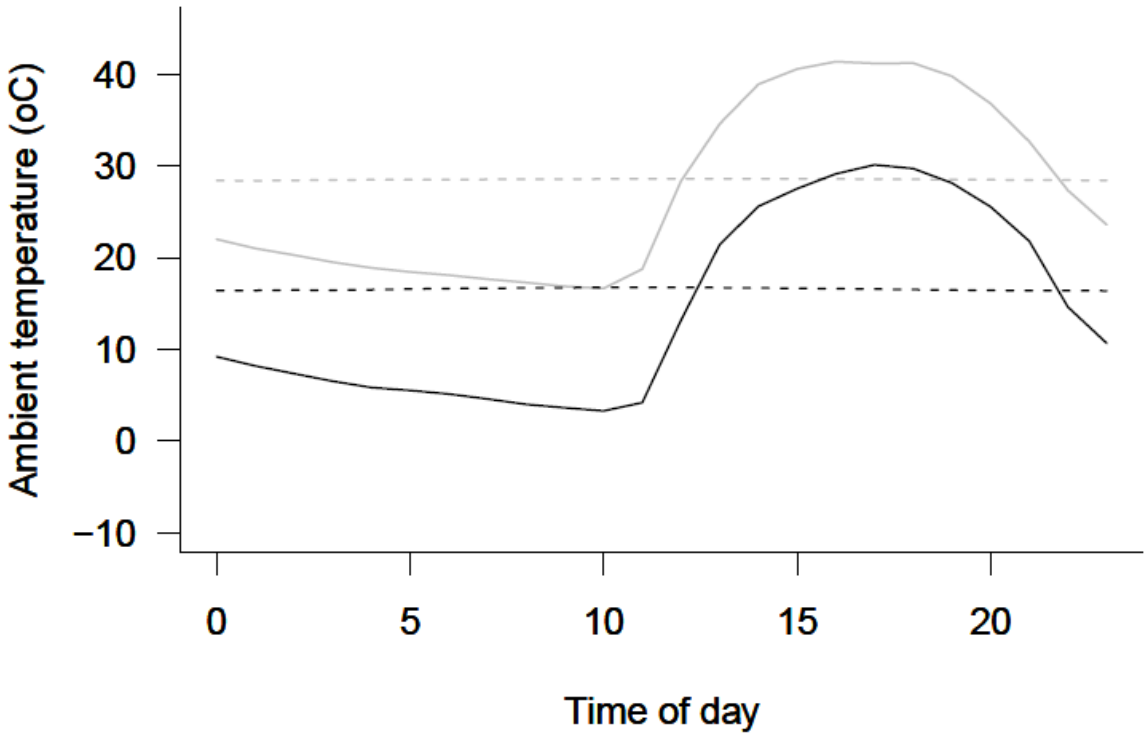

Figure S4.1. Hourly averages temperatures used in the model to estimate MDEE. Grey line indicates summer temperature; black line indicates winter temperature. Dashed lines indicate T_{soil} at 60 cm deep and solid line correspond to T_e.

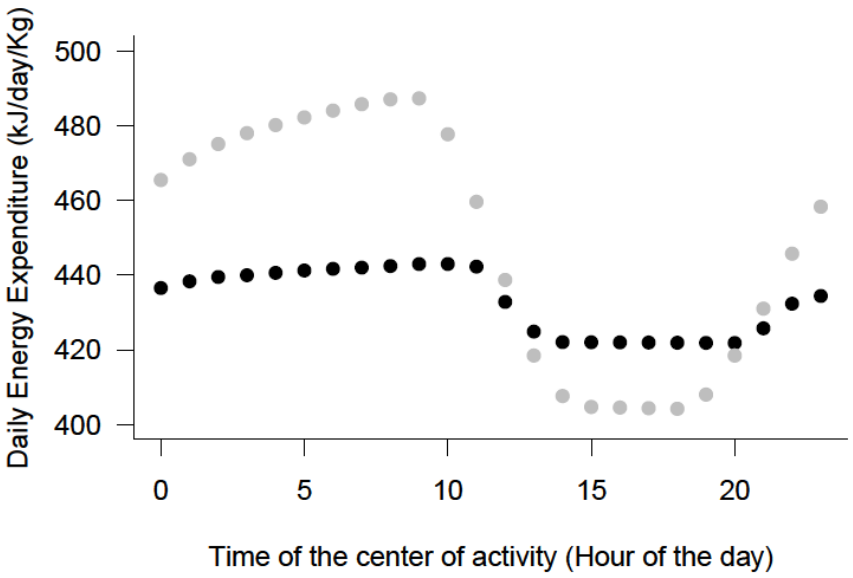

Figure S4.2. MDEE estimation in winter for two activity phase lengths. The durations tested are 60 (black) and 240 (gray) minutes (both based on actual extreme values reported for aboveground activity during the winter, in Flôres, 2016). For 60-minutes of activity, the difference between highest and lowest MDEE is 4.78%. For 240-minute phase, this difference is 17.07%.

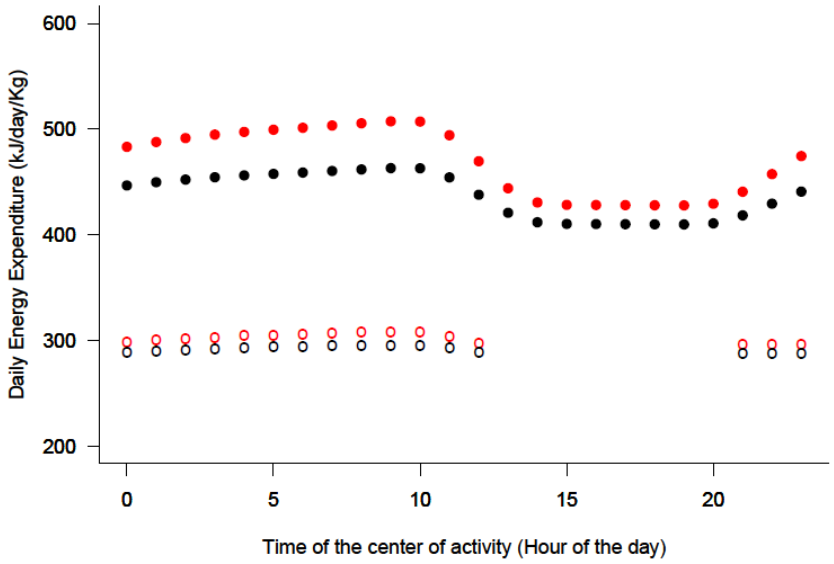

Figure S4.3. MDEE estimation using higher MR in the active phase. Black points are the values for MDEE obtained using the model described in the text, with no variation in MR according to activity or rest phase. Red points are estimates of MDEE assuming that during the activity phase MR is 50% higher, as it is predicted for non-primate mammals (Aschoff 1982). Open circles: summer. Full circles: winter.

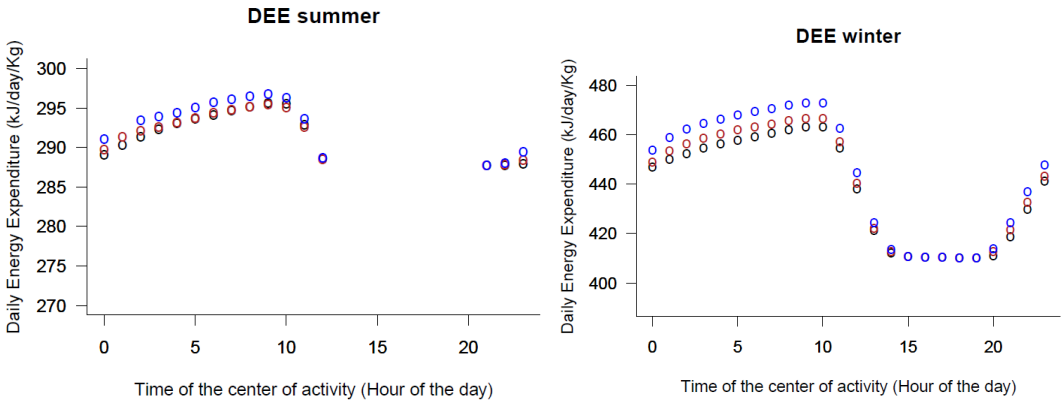

Figure S4.4. Estimated MDEE using standard operative temperatures (T_{es}). Because wind induced changes in resistance are not known for tuco-tucos, T_{es} was calculated based on published resistance values of two rodents from distinct environments: the arctic ground squirrel (*Urocitellus parryii*, Chappel 1981) and the antelope ground squirrel (*Ammospermophilus leucurus*, Chappel and Bartholomew, 1981). Blue: MDEE using T_{es} based on arctic ground squirrel resistance. Red: MDEE using T_{es} based on antelope ground squirrel resistance. Black: MDEE using T_e. Open circles: summer. Full circles: winter.

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Chapter 5

Sex Differences in Plasticity of Daily Rhythms of Mice Under Energetic Challenges

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5.1. Abstract

Studies that show discrepancy between daily activity patterns between laboratory and field have become increasingly common. Recently, it has been proposed that food availability could be one of the fundamental differences between field and laboratory that could alter the temporal daily pattern of activity. The "circadian thermo-energetics hypothesis" suggests that daytime activity could be a response to the high energetic cost of foraging, allowing the animal to rest during the cooler evening hours in burrows where temperatures are higher than on the surface, resulting in energy savings with thermoregulation. Taking into account that strategies to cope with energetic constraint vary between males and females and that social interactions and reproduction also play a role in shaping daily activity patterns, we hypothesize that plasticity in timing of activity may vary between the sexes. In this study, we assessed activity patterns of male and female populations of mice kept in a semi-natural enclosure, under several food conditions. The results support the CTE hypothesis, since the animals responded to increased energetic challenges by allocating activity during daytime. Males were more diurnal than females in all conditions and showed higher interindividual variation in the amount of daytime activity. Furthermore, body temperature (T_b) of males and females kept in a mixed-sex breeding population was measured, allowing the investigation of sex-differences in T_b patterns in reproductively active individuals and the investigation of occurrence of daily torpor in both sexes under semi-natural conditions. Overall, males displayed more robust daily T_b patterns males and longer torpor bouts than in females. The differences in activity patterns of females in the breeding population and females in the one-sex populations indicate that reproductive status may play an important role in activity timing.

5.2. Introduction

There are several sex differences in the circadian system regarding its morphology, physiology and sensitivity to gonadal hormones (Bailey and Silver, 2014; Turek and Gwinner, 1988). However, most of the studies investigating daily activity rhythms show that non-gonadectomized rodents display little or no sex differences in their free-running period (Kuljis et al., 2013; Krizo and Mintz, 2015) and only small differences regarding time of activity onset, related to the estrous cycle with females showing more variability (Albers et al., 1981; Kuljis et al., 2013; Morin et al., 1977; Takahashi and Menaker, 1980; Wolnik and Turek, 1988). Noteworthy, these studies were

conducted under standard laboratory conditions. Under natural conditions, sex differences in activity rhythms might be more pronounced due to the trade-off involving survival, reproductive success and energy balance.

Some studies indicate that the strategies to cope with energetic challenges may differ between the sexes, especially if reproductive costs are considered (Gittleman and Thompson, 1988). In a "work for food" protocol, in which house mice (*Mus domesticus*) need to run on the wheel to obtain their food, high workloads result in females increasing their total activity time and shutting down reproductive development, whereas males do not show such pronounced increase in total activity and maintain their sexual functions (Perrigo and Bronson, 1995).

This same "work for food" protocol has been implemented in more recent studies and revealing interesting phenomena regarding the timing of activity under energetic challenges (Schubert et al., 2010; Hut et al., 2011; van der Vinne at al., 2014).Notably, laboratory mice (*Mus musculus*) under this protocol become more diurnal with increasing workloads (Hut et al., 2011). This results gave rise to the Circadian Thermo-Energetics (CTE) hypothesis, which postulates that daytime activity could contribute to energy savings by allowing the animal to rest during the cooler hours of the night, when the animals can shelter themselves and adopt postures that increase their insulation. Ongoing laboratory work show that this response might vary between the sexes, since males are prone to become diurnal faster than females (SJ Riede, V van der Vinne, RA Hut., unpublished).

Daytime activity could also save energy by allowing the animal to lower body temperatures (T_b) overnight. Indeed, mice not only allocate the lower values of their circadian T_b rhythm during the night, but also display daily torpor when exposed to high workloads in the "work for food" protocol (Schubert et al., 2010; Hut et al., 2011). Torpor is characterized by the regulated lowering of metabolic rates, which is accompanied by lowering T_b (Heller & Hammel 1972). Unlike hibernation, daily torpor can be a short-term response to environmental challenges and low foraging success, allowing the animal to balance their energy budget in such conditions (Levy et al., 2013). In the laboratory, torpor is observed in mice exposed to low temperatures (Hudson and Scott, 1979; Tomlinson et al., 2007) and food restriction (Hudson and Scott, 1979). Records of house mice entering torpor in the field are scarce. Morton (1978) observed torpid mice during daytime in nests shared with the marsupial *Sminthopsis crassicaudata*, but there are no records during the night.

Although depressing T_b and metabolic rate may enhance fitness at energetic challenging conditions, it also decreases performance in many physiological and behavioral processes (Kronfeld-Schor and Dayan, 2013). Because of these disadvantages, the theory of adaptive thermoregulation proposes that torpor should only be displayed when the costs of homeothermy outweighs its benefits (Angilleta et al., 2010). These costs and benefits might be different between males and females, especially during reproduction. This hypothesis can be supported by the fact that the use of torpor is sex-dependent in many mammals, such as bats (Audet and Fenton, 1988; Grinivitch et al. 1994; Cryan and Wolf, 2003), ternrecs (Poppit et al., 1994), lemurs (Schmidt, 2001) and the pouched mouse (Lovegrove and Raman, 1998) Therefore, it is possible that the propensity to enter torpor is also sex-specific in mice, further increasing the differences in plastic strategies to cope with energetic challenges.

In addition to differences in the energy balance, activity patterns of males and females may vary due to inter- and intraspecific interactions. When predation risk was simulated under semi-natural conditions, in which individuals allocate part of the activity during day-time, female mice turned completely nocturnal, while males still showed some diurnal activity even under predation risk (Van der Vinne, 2015). Moreover, intraspecific social interactions, which differ between males and females, especially regarding social hierarchy, can play an important role in activity timing (Bovet, 1972; Calhoun, 1975; Farr and Andrews, 1975; Blanchard et al., 1995). For instance, a study reported disruptions not only in locomotor activity, but also in heart rate and Tb rhythms caused by social stress. These disruptions were more severe in individuals that were subjected to stress associated with defeat and subordination (Meerlo et al., 2002).

In this study, sex differences in the plasticity of daily activity and T_b patterns were investigated in populations of mice kept in semi-natural enclosures. First, we measured activity of male mice kept in male-only populations under various food conditions and compared to the activity patterns of female mice which underwent the same conditions (van der Vinne, 2015). To stay in line with earlier observations, it was expected that male mice display more day-time activity than females. Furthermore, T_b of males and females kept in a mixed-sex population was measured, allowing the investigation of sex-differences in both activity and T_b patterns in reproductively active individuals and the investigation of torpor under semi-natural conditions

5.3. Material and Methods

Animals

CBA / CaJ mice (*Mus musculus*) bred at the animal facility of the University of Groningen were used in this project. All animals, which were at least 10 weeks old, had subcutaneous microchips (Passive Integrated Transponders, PIT-tag, 11.5 x 2.2 mm; Trovan ID100) injected bellow the skin at the back, between the shoulder blades. Microchip injection was performed under light isoflurane anesthesia. Procedures were authorized by the ethics committee of the University of Groningen (DEC 5454).

Semi-natural enclosures

The experiments were conducted in four outdoor enclosures (7 x 9 m, Fig 5.1), located at the University of Groningen (53º 14' N, 6º 32' E). They were filled with sand and covered by a net, which provided protection from predators but allowed rain to get through. Each enclosure (Fig 5.2) contained a nest box (100 x 65 x 55 cm) filled with hay. A custom-made feeding system (Fig 5.3) provided controlled delivery of food pellets (rodent chow diet AM II, 10 mm, 17.3 kJ/g). It consisted of a conveyor belt which rotated 2.6 mm every 15 minutes, filling a PVC tube with food evenly across the day. The tube end to which the animals had access was covered by a wire-mesh grid, allowing the animals to feed but preventing them from removing the pellets for hoarding. A tunnel-shaped cover made of overturned gutters (diameter: 10 cm) connected most of the 6m between the feeder and the nest box. Water was available *ad libitum* in drinking towers designed for chicken (Welkoop, Netherlands). Each enclosure had three antennas able to read and record data of the implanted PIT-tags (Acumen system), two in the middle of the cover and the other around the feeder, allowing monitoring of each individual.

Data of female-only populations were collected by Van der Vinne (2015) during autumn of 2014. The same experimental procedures were then repeated for males beginning February 2015. Adult male CBA/CaJ mice were released in 3 outdoor enclosures (population 1: n=28, population 2: n=29, population 3: n=26). They were allowed to acclimate for one week before the start of the experimental protocol, under *ad libitum* food conditions.

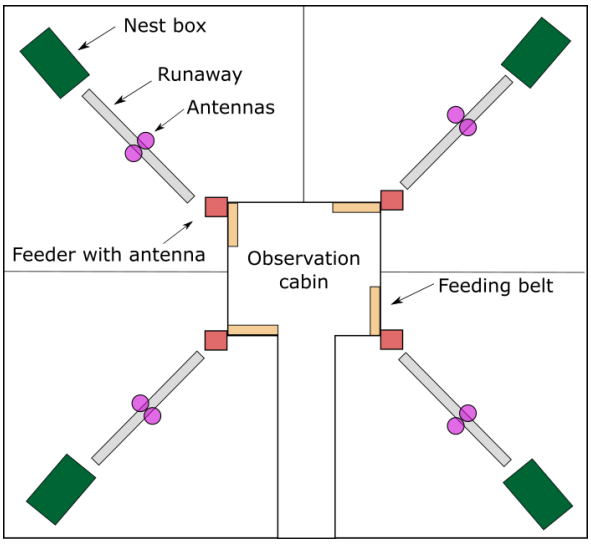

Figure 5.1. Schematic representation of the outdoor facility, with 4 enclosures. The green rectangles represent the nest boxes, purple circles represent cover antennas, red squares represent the feeders with antenna, and the orange rectangles inside the observation cabin represent the conveyor belts used to deliver food evenly across the day.

Figure 5.2. Photograph of one enclosure. It shows a nest box filled with hay, the runway cover leading to the feeder and the cover antennas.

Figure 5.3. Photographs of the custom-built feeding system. A) Conveyor feeding belt filled with pellets. B) Feeder tube surrounded by an antenna. C) Mice feeding at the end of the tube covered by a wire-mesh grid.

Assessment of activity patterns in male populations

The experimental protocol consisted in manipulations, either of food amount or of population size. In the well-fed condition (WF), 4.5g food/mouse/day were delivered. During simulated food scarcity (SFS) the amount of food was 2.5g food/mouse/day**.** In the half population condition (HP), the total amount of food from the previous condition was kept constant, but half of the mice were removed from the enclosure. Therefore, the amount of food per mice would be double the amount in the previous, SFS conditions. Mice taken out of the enclosure had similar body weights than the ones left (Table 5.1). The experiment was concluded with an overfed condition (OF), with 8g food/mouse/day, a condition in which there were always pellets left in the feeding tube.

Each condition lasted two weeks and was rotated over the different enclosures to decrease the influence of specific weather events. Mice from every population endured SFS at least three times, HP at least one time and WF conditions at least three times (Table 5.2). At the end of each condition, mice were manually caught and weighted. The catching event lasted 2.5 hours during which as many mice as possible were caught. Number of mice caught and weighed at the end of each manipulation can be found in Table 5.3, average capture rate was 59%.

Table 5.1. Average body weights for animals separated from the population and those remaining in the population during all half population manipulations. The first manipulation in population 2 is classified as HP1 and subsequent HP manipulations are numbered HP2, HP3 and HP4. Of note is that body weights are highly similar between remaining and removed populations and that remaining populations are roughly the same size during all HP manipulations. All data are averages with SEM.

	Separated from population		**Remaining population**	
	Body mass (g.)	**Animals removed**	**Body weight (g.)**	**Animals remaining**
HP1	25.1 (+- 1.0)	11	26.3(+-0.7)	10
HP2	29.9(+-1.5)	11	29.5(+-1.4)	11
HP3	28.9(+-0.9)	12	28.0(+-0.6)	11
HP4	29.1(+- 0.3)	11	28.3(+-1.1)	11

Table 5.2. The experimental protocol per population. All mice received 4.5g /mouse per day of food for the first two weeks (WF) and were then, followed by two weeks of 2.5g/mouse per day (SFS). Afterwards mice were either well fed (WF), underwent simulated food scarcity (SFS) or half the population was taken away (HP). In the end., all populations were overfed (OF), receiving 8g/mouse per day.

Manipulation #	Population 1	Population 2	Population 3
1	WF	WF	WF
2	SFS	SFS	SFS
3	WF	HP	SFS
4	SFS	SFS	WF
5	HP	WF	SFS
6	SFS	SFS	HP
7	HP	WF	SFS
8	OF	OF	OF

Table 5.3. Number of animals that were captured and weighed per capture round. Capture yields range from 23.1% in population 2 during the first capture round to 91.3% in population 3 during the 6th capture round. The average capture rate was 59%.

Capture round	Population 1		Population 2		Population 3	
1	13/24	(54.2%)	6/26	(23.1%)	13/26	(50%)
2	11/23	(47.8%)	9/26	(34.6%)	10/26	(38.5%)
3	14/22	(63.6%)	14/21	(66. 7%)	15/26	(57.7%)
4	10/22	(45.5%)	2/8	(25.0%)	10/25	(40%)
5	15/22	(68.2%)	11/17	(64.7%)	13/24	(54.2%)
6	7/12	(58.3%)	10/15	(66.7%)	21/23	(91.3%)
7	17/22	(77.3%)	12/15	(80%)	10/12	(83.3%)
8	9/10	(90%)	11/15	(73.3%)	17/23	(73.9%)

Body temperature measurements in a mixed-sex breeding population

T_b of eight females and eight males were measured in a mixed-sex population, which was naturally growing due to breeding. The amount of food provided to this population remained fixed, which meant that the amount of food per mouse would decrease with the increase in population size.

Intraperitoneal temperature dataloggers (Anipill, Bodycap, France) were used to measure and record T_b. Implant surgeries were performed in animals anesthetized with isoflurane (3%-5% oxygen). The logger was inserted into the peritoneal cavity through a 1.5-2 cm vertical midline incision (5 mm bellow the rig cage), which was sutured with

polyglycolic acid thread and tissue adhesive (Vetbond, 3M, St. Paul, MN). Throughout the surgical procedure, mice were kept on a thermal blanket to prevent hypothermia.

After the implant surgeries, the animals were allowed to recover for one week before being released into the enclosures. During this recovery period, the animals were kept in individual cages in an outdoor facility, where they were sheltered from rain and direct radiation, but were exposed to environmental air temperature changes. They were then released, in the beginning of July 2015, in an enclosure which already had other male and female mice. Recordings took place until the battery of the temperature loggers ran out, which occurred in mid-October. The data-logger used could also transmit data (taken at 5-min intervals) to a monitor placed near the nest box and data was downloaded every week. Activity was also recorded by PIT-tag readings, as described in the previous section.

Data analysis:

Locomotor activity and T_b data were plotted as actograms using the ElTemps (Diez-Noguera, Universitat de Barcelona, 1999) software for visual analysis of rhythmic parameters. Statistics was performed using SPSS statistical software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) and R scripts (R Development Core Team, 2010).

Daytime activity percentage was calculated using the following equation over the final 7 days of a food condition (HP, WF, SFS or OF).

$$\boldsymbol{D}\% = \frac{\frac{\sum \boldsymbol{\alpha^d}}{\boldsymbol{t^l}}}{\frac{\sum \boldsymbol{\alpha^d}}{\boldsymbol{t^l}} + \frac{\sum \boldsymbol{\alpha^n}}{\boldsymbol{ts^n}}}$$

This equation corrects for the changes of daylength throughout the year. Variables $\boldsymbol{\alpha^d}$ and $\boldsymbol{\alpha^n}$ are total activity counts during the day and night respectively and $\boldsymbol{t^l}$ and $\boldsymbol{t^n}$ are the average hours of light and darkness respectively. Since day and night length differences in the 7-day period used to calculate daytime activity percentage were negligible (2-3 minutes per day), average values of day and night length were used. This daytime activity percentage (**D%)** ranges from 0% to 100%, where 0% and 100% mean no activity or all activity during the day, respectively.

Survival was estimated using PIT tags recordings; an animal was considered to be dead when it was no longer registered by the antennas for more than three days. A custom

R script was used to determine the final day an animal was scanned.

Differences in body mass were assessed using a general linear model with food condition (HP, SFS or WF) as a fixed variable and animal ID as a random variable in SPSS. Posthoc Tukey's HSD tests were performed to further analyze the data.

Daytime activity was analyzed by fitting a mixed linear model by restricted maximum likelihood in R using the lme4 package. For fitting, food condition (SFS, HP or WF) was considered as a fixed factor and PIT-tag ID number and population as random effects. Interaction effects were not included. Post-hoc analysis was performed in the lsmeans package in R using a Tukey's HSD test. Male and female data were compared by performing one way ANOVAs in SPSS.

Data from this experiment were compared to earlier data from experiments in female CBA/CaJ mice which went through the same protocol in autumn 2014 (van der Vinne, 2015).

5.4. Results

Sex differences of activity patterns in response to food conditions

Survival varied greatly among the 3 male populations (Fig. 5.4A) and between males and females (Fig. 5.4; van der Vinne, 2015). Population 2 showed the highest death rate with only 59.3% surviving until the end of the experiment (Figure 5.4A). Death rates in female mice (van der Vinne, 2015) were much lower with only 16.7% dying in the population with the highest death rate (Figure 5.4B). In female populations 1 and 2 (van der Vinne, 2015), only 1 animal died per population, leading to an overall survival rate of 96.2% in these populations. In both male and female populations, deaths occurred primarily during the first half of the experiment.

All manipulations led to changes in body mass (Fig. 5.5). SFS resulted in a decrease in body mass, whereas in manipulations in which food availability was increased (WF, HP and OF) there was an increase in body mass. During the first 19 days of the protocol, the average body weight of all 3 populations dropped, even though they were in the WF condition (Fig. 5.5).

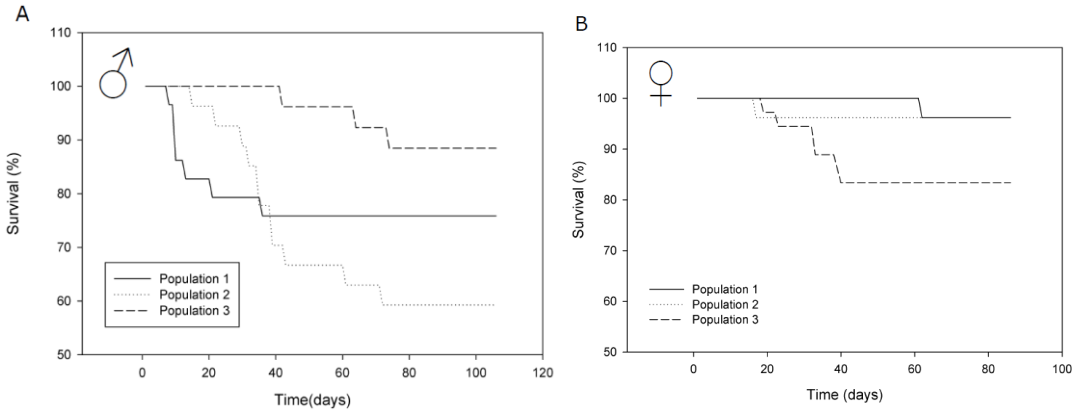

Figure 5.4. Survival in male and female mice populations. Survival was lower in male mice during spring 2015 than for female mice in autumn 2014. **A)** Survival in male mice during spring 2015**:** survival was lower in population 1 and 2, respectively 75% and 59%; 88% survived in population 3. A decrease in survival in population 1 can be observed from day 10 until day 26 (17.2% mortality). **B**) Survival in female mice during autumn 2014 (van der Vinne 2015). Survival rates were lowest in population 3 (83.3%). In population1 and 2 most animals survived (96.2% in both population 1 and 2). (Figure produced by Jildert Akkerman).

Overall day-time activity was increased during SFS condition and decreased during WF, HP and OF (Fig. 5.6 and 5.7). This is similar to the results obtained in the female experiments (van der Vinne, 2015). In male mice, the HP condition had a stronger effect on the decreased daytime activity than WF did ($p<0.0001$, Fig. 5.7). whereas the response to these two conditions was not statistically significant in females (Fig. 5.7B). In all manipulations, daytime activity was higher for males than females (van der Vinne, 2015; Fig. 5.7B).

Body mass and daytime activity percentage correlated negatively in all food conditions, except WF (Fig. S5.1). However, they were only statistically significant in the HP condition ($p=0.018$). In females, negative correlation between body mass and day-time activity at the end of the experiment (in which mice were under SFS condition) was highly significant ($p=0.0015$, van der Vinne, 2015). The higher amount of day-time activity displayed by male mice might explain a less pronounced correlation than the one observed in females.

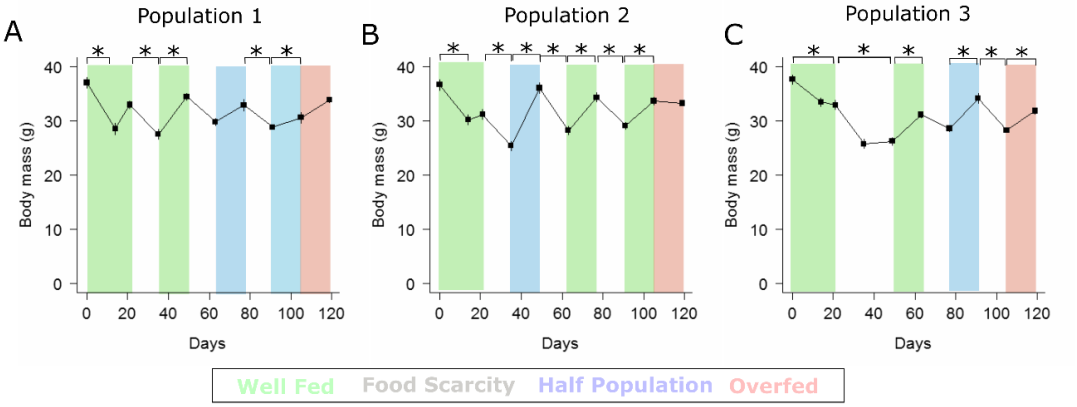

Figure 5.5. Average body mass of male CBA/CaJ mice in a semi-natural enclosure under four food conditions. Green shadings represent duration of WF, blue of HP and red of OF conditions. When no colored shadings are shown, animals underwent SFS. Asterisks indicate a significant ($p<0.05$) difference between the averages, which are plotted with SEM. **A)** Average body mass of population 1. **B)** Average body mass of population 2**. C)** Average body mass of mice in population 3. Body mass was dependent on manipulation (HP, WF or SFS) in all populations ($p<0.0001$). All populations lost weight under the first 20 days of WF conditions ($p<0.0001$ in all populations). In both population 1 and 2, the feeding tube got cluttered for approximately 2.5 days in the second week, which led to decreased food availability. Body masses were significantly lower in population 1 compared to population 3 ($p=0.001$). Population 2 also had lower body masses compared to population 3, albeit non-significant ($p=0.087$). After one week of recovery (day 13 until day 20) the differences in average body mass among populations were no longer significant ($p>0.9999$ and $p=0.297$ respectively). During overfed food conditions body mass raised in population 1 and 3 but not in population 2.

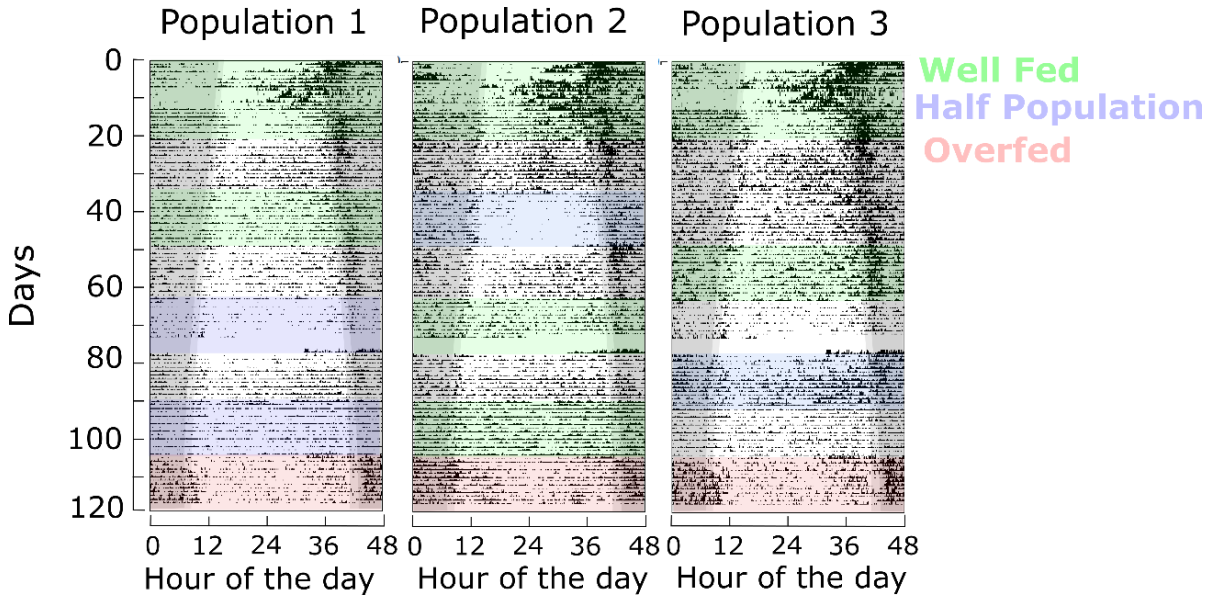

Figure 5.6. Actograms for populations 1, 2 and 3 of male mice during spring 2015. All data were normalized per mouse per day. Green shadings indicate WF conditions, while blue indicate HP and red indicate OF. The areas without shading are SFS condition. Dark grey shadings indicate the time between sunset and sunrise while light grey shading indicates the civil twilight. Activity was measured by PIT tag readings at the feeder antenna and both central PIT-antennas) Day 1 of this plot is 21st of February 2015.

Males displayed a higher degree of individual variation in the amount of the daytime activity than females. Compared to the females, they showed higher distribution of percentage of daytime activity (Fig. 5.8). The average day activity percentage in each condition was also higher in males under all conditions. Under SFS, average day-time activity was 48.39% for males and 31.14% for females. Under WF conditions, it was 42.7% for males and 22.79% for females. Finally, under HP it was 30.57% for males and 22.98% for females. Figure 5.9 shows six individual actograms (2 per population) as an example of the individual variation. Of note, during the end of the experiment, when photoperiods were very long, almost all individuals remained relatively day-active (figure 9). This can be observed both in individual mice, as well as in overall population activity (Fig. 5.6). Whereas most animals showed their activity peak close to sunset or during dusk, animal#79 showed a predominant peak of activity in the early morning, just after dawn (Fig. 5.9F). This peak was most pronounced during high food conditions (HP and WF). Some individuals seem to "avoid" each other at certain times of the day. For instance, animal #34 (Fig. 5.9C) displayed much of its daytime activity during the first part of the day,, whereas animal #45 (Fig. 5.9D) displayed most of its activity during the second part of the day.

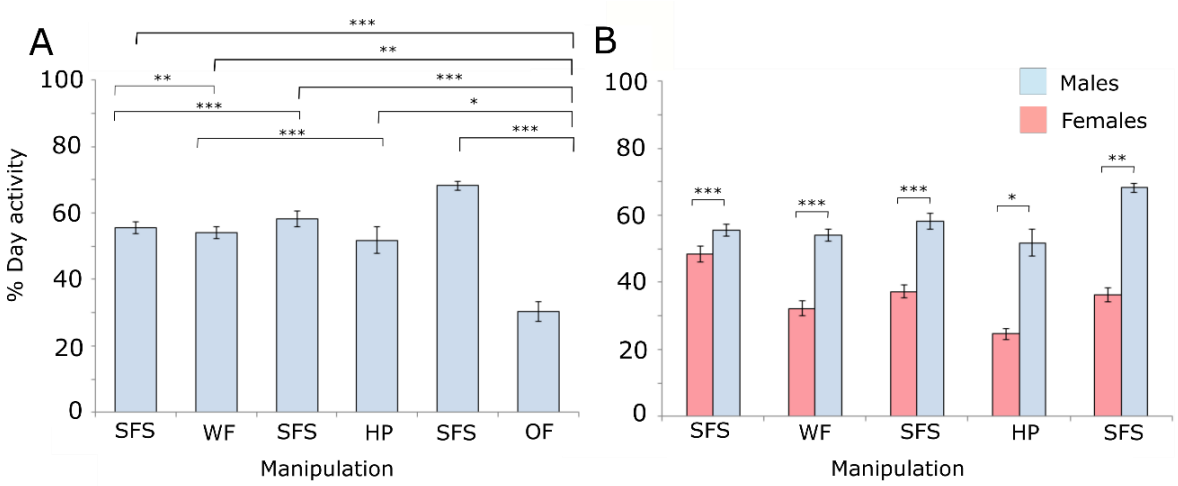

Figure 5.7. Daytime activity during the last 7 days of each food condition for both male and female mice.. **A)** Daytime activity of male mice. Males increased their daytime activity during conditions of decreased food availability. During the HP condition daytime activity was significantly lower than during the WF conditions. During the final OF food conditions the daytime activity % reduced even more, with significantly lower daytime activity than during SFS ($p<0,0001$) and significantly lower daytime activity than during WF ($p=0.0013$) and HP ($p=0.014$). **B)** Daytime activity for both males and females. Males showed significantly more daytime activity in every food condition. Under all conditions males were significantly more dayactive than females (Significance levels: ***$p<0.0001$, **$0.01<p>0,0001$, *$0.01>p<0.05$, averages are plotted with SEM).

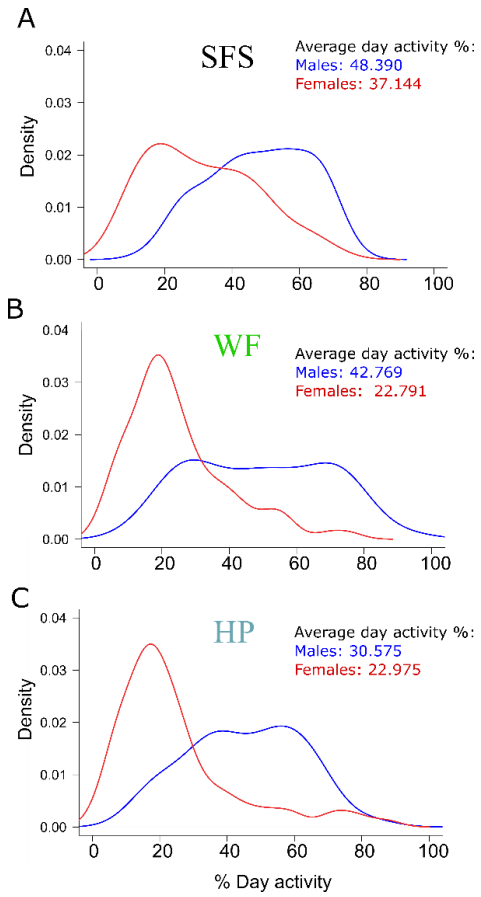

Figure 5.8. Kernel density plots comparing male and female diurnal activity in three conditions. Plots show relative densities, calculated by a kernel function, of diurnal activity percentages. Females show higher densities for low day-activity percentages, while males show more diurnal activity. males showing a much wider distribution of day-activity percentages than females, indicating higher individual variation in males than in females. **A)** Density plots of daytime activity during SFS. **B)** Density plots of daytime activity during WF conditions. **C)** Density plots for daytime activity during HP condition.

Body temperature measurements in a mixed-sex breeding population

Two females and two males died within the first two weeks of the experiments and were not used in the data analysis. In the first month of the experiment, the T_b recording system had several malfunctioning issues, resulting in data loss. For this reason, only data from the end of August onwards were analyzed. T_b patterns across the experiment are shown in Figure 5.10. Males clearly displayed a more robust diurnal rhythm than females (Fig. 5.10 and Fig S5.2). All animals had episodes in which T_b fell below 32ºC (Blue markings in Fig. 5.10). In females, these episodes were usually short (lasting less than two hours). However, males showed longer and more frequent torpor bouts (Fig. 5.10). Most torpor bouts started late-night and ended early morning.

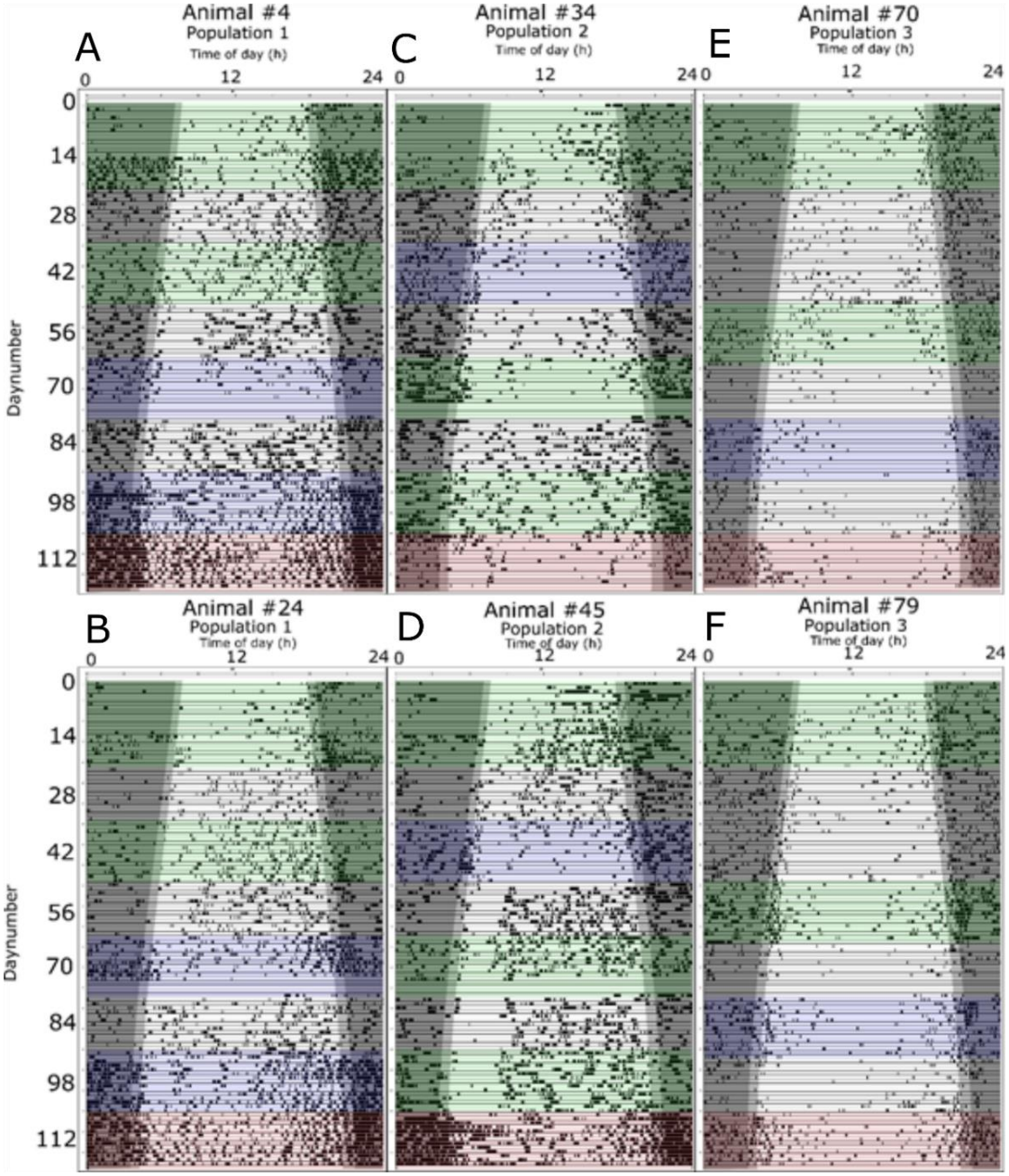

Figure 5.9. Actograms for activity of single individuals in male-only populations. Two animals per population ae depicted. Example profiles were chosen among those that survived the whole experiment and those being in remaining population during HP conditions. Green shadings indicate WF conditions, while blue indicate HP and red indicate OF. The areas without shading are SFS condition. Dark grey shadings indicate the time between sunset and sunrise while light grey shading indicates the civil twilight. Individual differences in the phase of daytime activity were present.. Figure produced by Jildert Akkerman.

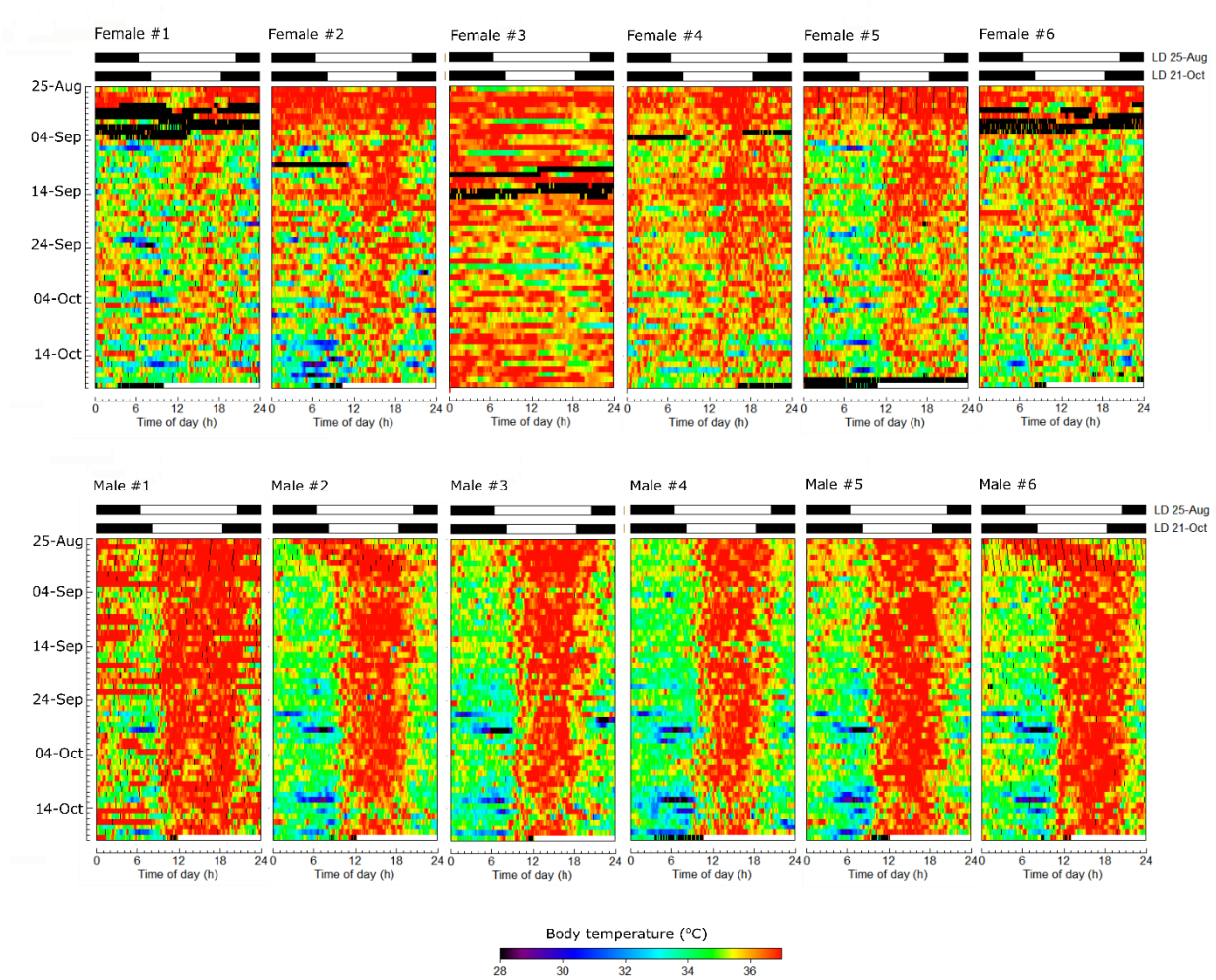

Figure 5.10. **Body temperature patterns in mice under semi-natural conditions.** Actograms of six females (top row) and six males (bottom row) kept in the mixed-population enclosure, from August 25th until October 21st. T_b is color coded according to the gradient legend at the bottom of the figure. Black bars indicate data loss. Bars above the plots indicate the light (white)-dark (black) cycle at the first and last day of the measurements. Higher T_b occur mostly during the day. Males show a more robust daily rhythm than females. Torpor bouts are more common towards the end of the experiment.

As the days got colder and shorter (Fig. S5.3), the frequency of torpor bouts increased and the mean T_b decreased. Average daily mean T_b was significantly different comparing data measured at the end of summer and the mid-autumn in both males and females ($p<0.05$; Fig. 5.11). Amplitude, however, did not change significantly ($p>0.05$; Fig. 5.11). Despite the sex differences in Tb patterns seen in figure 5.10, mean T_b ann T_b amplitude were not significantly different between males and females.

Daily activity patterns coincided with T_b patterns, with most activity happening during times when T_b was higher. When T_b was very low, such as during the torpor bouts, animals were inactive (Figures, S5.4, S5.5 and S5.6).

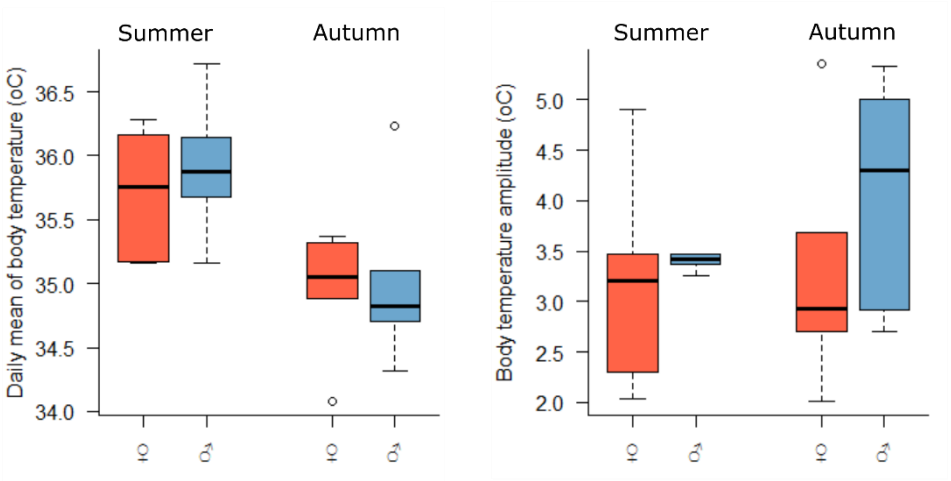

Figure 5.11. Boxplots of daily mean (left) and amplitude (right) of Tb in males (blue) and females (red) during summer and autumn.

5.5. Discussion

Under semi-natural conditions, mice show sex-differences in most variables examined in this study, such as survival, daily activity and T_b patterns. The lower survival rate in males (Fig. 5.4) was also previously reported by Daan et al. (2011) under similar semi-natural conditions. DeFries and McGlearn (1970) reported that one of the main causes of death in males is the fighting behavior between dominant and subordinate individuals. None of the corpses that were found intact had any signs that the death had been a result of direct aggression. However, death might still have been associated to male-male competition, with some mice not getting enough food and shelter. Another possibility is that males may be more sensitive to the changes in environmental conditions between the lab and the enclosures.

The CTE hypothesis predicts that diurnal activity would increase in response to energetic challenges, allowing the animal to rest during the colder times of the day (Hut et al., 2012). Body mass measurements (Fig. 5.5) indicate that the manipulations performed in the first experiment affected the energetic state of the animals. Average body mass decreased during reduced food availability (SFS), suggesting that mice were challenged energetically in this condition, while HP, WF and OF conditions led to significant recovery in average body mass. Changes in activity patterns in all manipulations were very similar in direction between males and females, with scarcer food conditions leading individuals of both sexes to display more of their activity during the day, as expected by the CTE hypothesis (Fig. 5.6 and 5.7)., Males were more diurnal

than females in all conditions (Fig. 5.7B) and showed higher interindividual variation in the amount of daytime activity (Fig. 5.8). These results suggest that even though both sexes tend to shift their activity to the day during energetic challenges, other factors, which are sex-specific, also play a role in temporal niche selection.

Of particular interest is the high degree of interindividual variation in males. Since all mice came from the same background (CBA/CaJ), they are assumed to be genetically similar. However, individuals responded differently to the manipulations, with some increasing greatly the amount of daytime activity during the day and others remaining mostly nocturnal. The fact that some individuals "avoid" to overlap activity (e.g. animals #34 and #45, Figure 5.9) suggest that mice in these populations may have "temporal territories". In a study with long-tailed field mice (*Apodemus sylvaticus*), Bouvet (1972) also reports avoidance of simultaneous activity by some individuals, noting that in most of these episodes the individuals with the lowest rank avoided the ones with the highest rank.

Considering that body size is positively correlated to ranks in social hierarchy (DeFries and McGlearn, 1970), a possible hypothesis could be that males that are more active during the day can maintain a body size as large as possible, which in turn would increase fitness by increasing the chances of socially dominant position in the hierarchy. This hypothesis assumes that, for males, the reproductive benefits of social dominance outweigh the negative effects of being active during the day, when predation risk is higher (Gerkema et al., 2013). It is supported by the fact that when increased predation risk was simulated by removing the runways between the feeder and nest box, females became almost completely nocturnal, while males still displayed some activity during the day (van der Vinne, 2015), suggesting that males are indeed more prone to take risks than females.

Sex-differences in the second experiment, in which T_b was measured in a mixed-sex population, were quite intriguing. Daily T_b patterns were much more robust in males than in females (Figure 5.10 and S5.2). This contrasts with laboratory studies which show no to very slight differences in daily T_b patterns between the sexes (Mortola, 2017). Moreover, activity patterns observed in females in this experiment were notably less marked than the ones obtained in the experiment with female-only enclosures (van der Vinne, 2015). This is probably due to the fact that in the mixed-sex enclosure the animals were reproductively active, and the individuals might have been pregnant or lactating. It has been shown in laboratory studies that activity patterns during different reproductive

stages could alter activity patterns, especially when the animal faces energetic challenges (Perrigo, 1987). During lactation, when energy expenditure can be three times higher than during no reproductive state (Speakman and McQueenie, 1996), activity of house mice is more scattered throughout the day, with several bouts of activity occurring during the light phase of the day (Perrigo, 1987).

Males displayed longer torpor bouts than females. Studies with bats reported similar findings (Audet and Fenton, 1988; Grinevitch et al., 1995). It is argued that in females the energy-saving benefits of torpor might not outweigh the consequences of low T_b to gestation and lactation, such as slow fetal development, delayed parturition and slow growth of pups (Racey and Swift, 1981; Grinevitch et al., 1995). Data from other species support the hypothesis that it is advantageous to maintain higher Tb during pregnancy and lactation. Hedgehog tenrecs (*Echinops telfairi*), which are usually heterothermic, become homeothermic during pregnancy and lactation, maintaining their T_b higher than environmental temperature. In males, to which, as mentioned above, a large body size might increase reproductive success, the energetic benefits of torpor probably outweigh its possible negative consequences in spermatogenesis (Grinevitch et al., 1995).

Overall, the results described in this chapter support the CTE hypothesis, since the animals responded to increased energetic challenges by allocating activity during daytime and resting and displaying lower T_b during the night, when activity would be energetically costlier due to lower ambient temperatures. However, interindividual variation and sex-specific differences in daily activity and T_b patterns suggest that, under semi-natural conditions, there are other behavioral and physiological factors which also play a role in the plasticity of activity timing. To further investigate the hypothesis raised here, assessment of social rank and reproductive state would be of great importance.

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5.7. Supplementary material

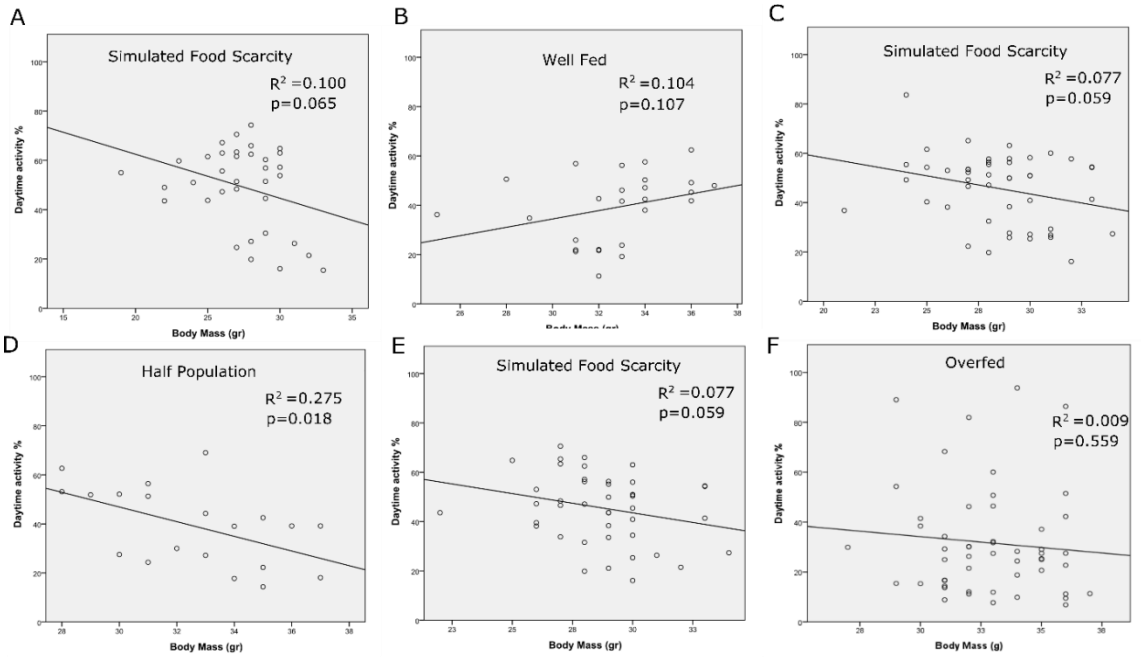

Figure S5.1. Correlations between body weights and the average amount of daytime activity 7 days prior to the end of every food condition in male mice. Body masses were assessed during the final day of every manipulation. Activity measurements during the capture day were excluded from the correlations. A negative correlation between daytime activity and body mass was observable in during every food condition except during WF conditions. **A)** Daytime activity was not significantly correlated with body mass during the final 7 days of simulated food scarcity preceding Well Fed conditions (p=0.065, n=35, r=-0.316). **B)** The correlation between daytime activity and body weight during the final 7 days of WF conditions. There was no significant correlation (p=0.107, n=26, r=0.323) this correlation appears to be slightly positive. **C)** Correlation between daytime activity and body mass in final 7 days of the SFS condition preceding the Half Population condition. The correlation was not significant (p=0.059, n=47, r=-0.277) and negative. **D)** The correlation between daytime activity of the remaining mice during the final 7 days of half population condition. The negative correlation between daytime activity and body mass was positive during the half Population. The correlation during the half population condition is the only significant correlation observed (p=0.018, n=20, r=-0.525). This is in line with the stronger response to Half Population conditions compared to Well Fed conditions observed in males. **E)** Correlation of daytime activity and body mass. Just as the other correlations during food scarcity, this correlation is not significant (p=0.093, n=43, r=-0.259) and negative. **F)** During Overfed (OF) food conditions the correlation between body mass and daytime activity is even less pronounced, with an insignificant correlation between body mass and Daytime activity % (p=0.559, n=52, r=-0.093). (Figure produced by Jildert Akkerman)

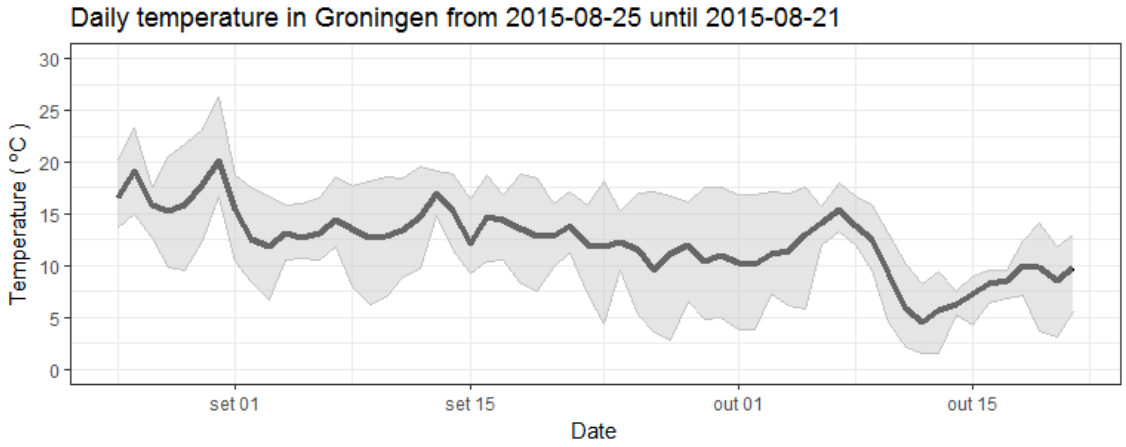

Figure S5.2. Environmental temperature in Groningen from August 25st 2015 until October 21st 2015. The black line represents average daily temperature. The upper limit of the gray are represents the maximum temperature of that day, while the lower limit represents the minimum temperatures. Data from the Eelde meterological station, obtained from the Royal Netherlands Meteorological Institute (KNMI) website (http://www.knmi.nl/nederland-nu/klimatologie/daggegevens, accessed 18th February 2017)

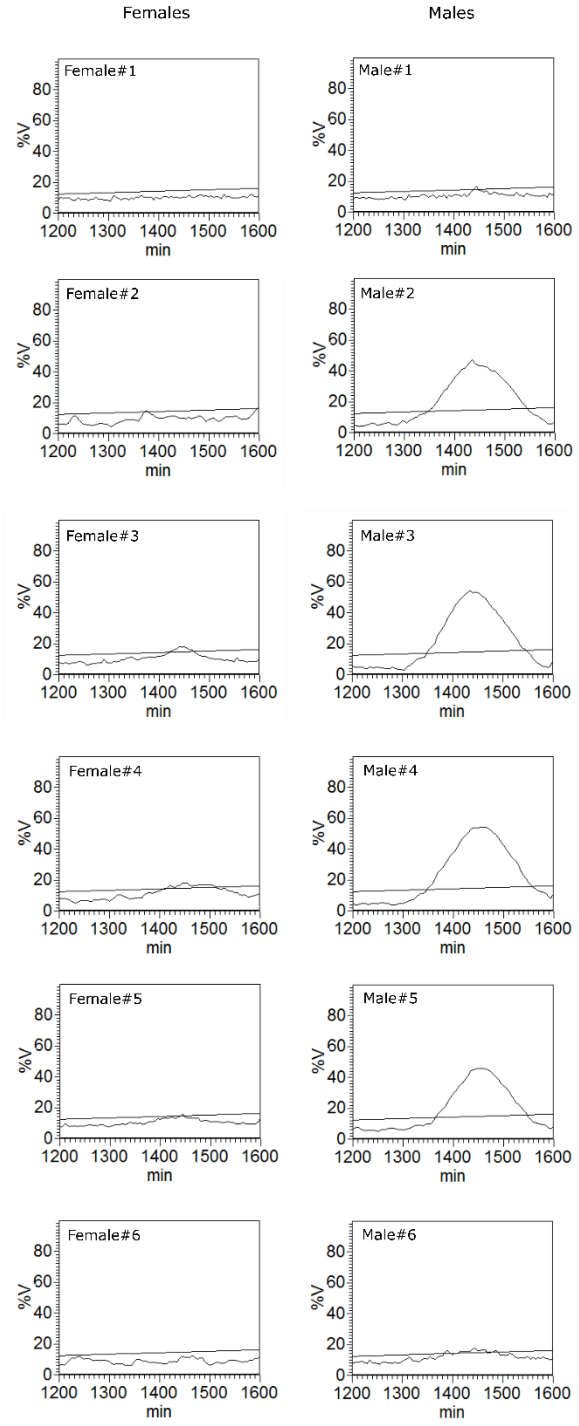

Figure S5.3. Period analysis of the rhythms from Fig. 5.10. Sokolove-Buschell periodogram analysis was conducted over 10 days of data for each individual (from September 14th until September 24th). Each graph depicts the probability (%V) of a given tested period (x-axis), which are significan when above the significance line. When there is a robust rhythm, the analysis shows a peak at the most probable period. No females (left) show significant periods, while most males (right) show significant periods around 24 hours.

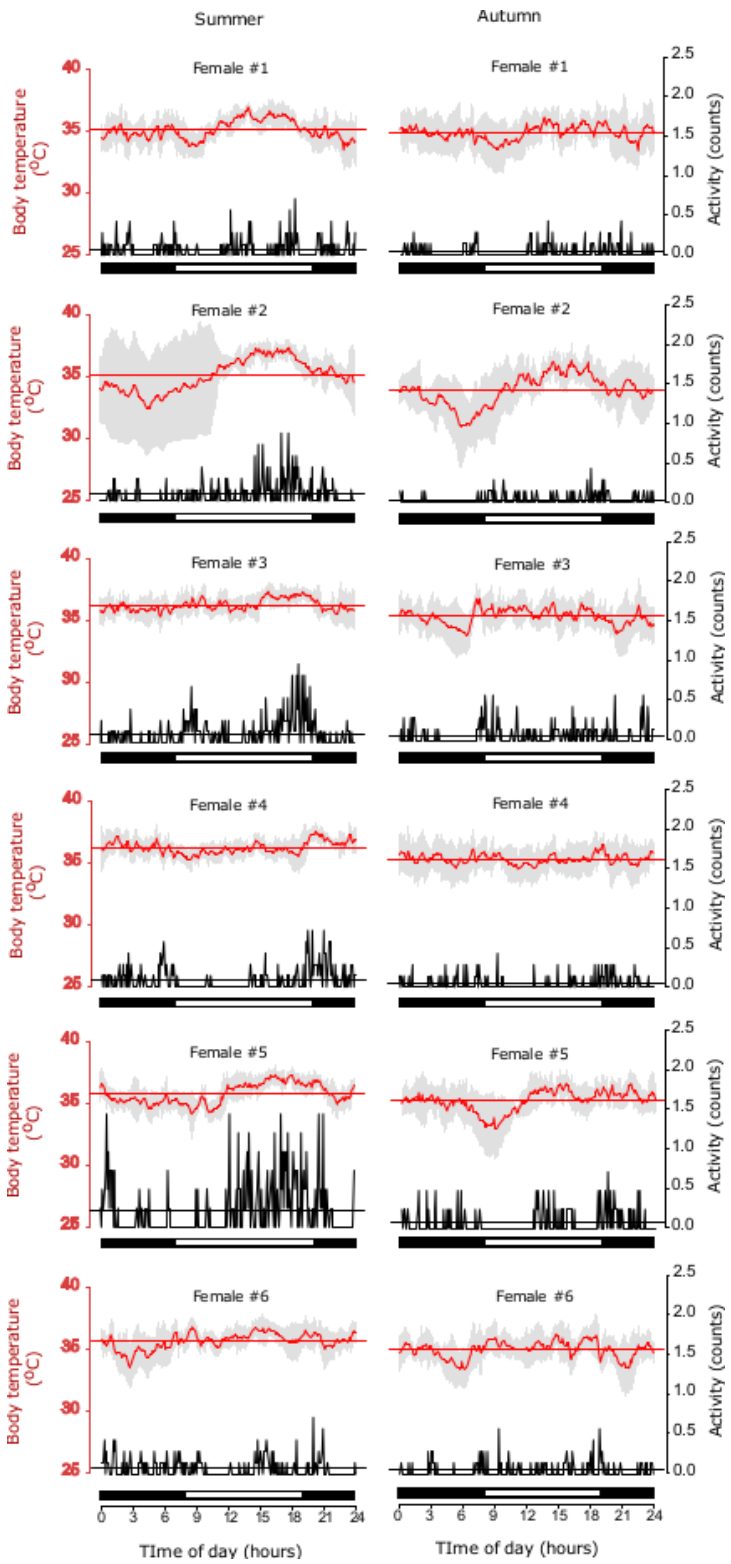

Figure S5.4. Temporal relationship between Tb and motor activity rhythms in female mice. Daily profiles of Tb (red) and gross motor activity (black) rhythms of six females under semi-natural conditions, during summer (left) and autumn (right). Each point represents the average of 7-day measures for the corresponding time of the day. Horizontal lines indicate the mean of the total values obtained for each variable and vertical gray line represent the standard deviation for Tb for each time over the 7 days. The bar bellow the x-axis indicated the light (white) and dark (black) phases, determined by sunset and sunrise.

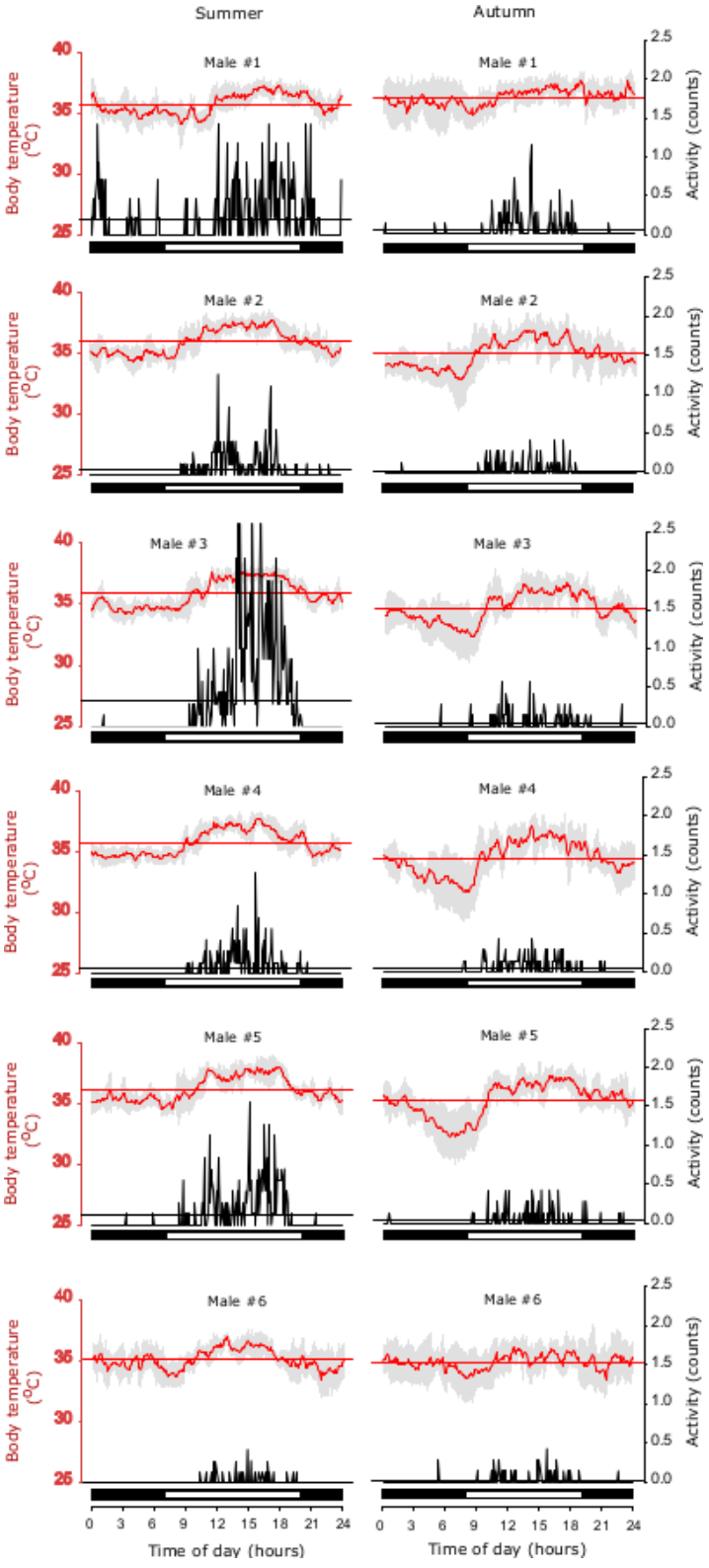

Figure S5.5. Temporal relationship between Tb and motor activity rhythms in male mice. Daily profiles of Tb (red) and gross motor activity (black) rhythms of six males under semi-natural conditions, during summer (left) and autumn (right). Each point represents the average of 7-day measures for the corresponding time of the day. Horizontal lines indicate the mean of the total values obtained for each variable and vertical gray line represent the standard deviation for Tb for each time over the 7 days. The bar bellow the x-axis indicated the light (white) and dark (black) phases, determined by sunset and sunrise.

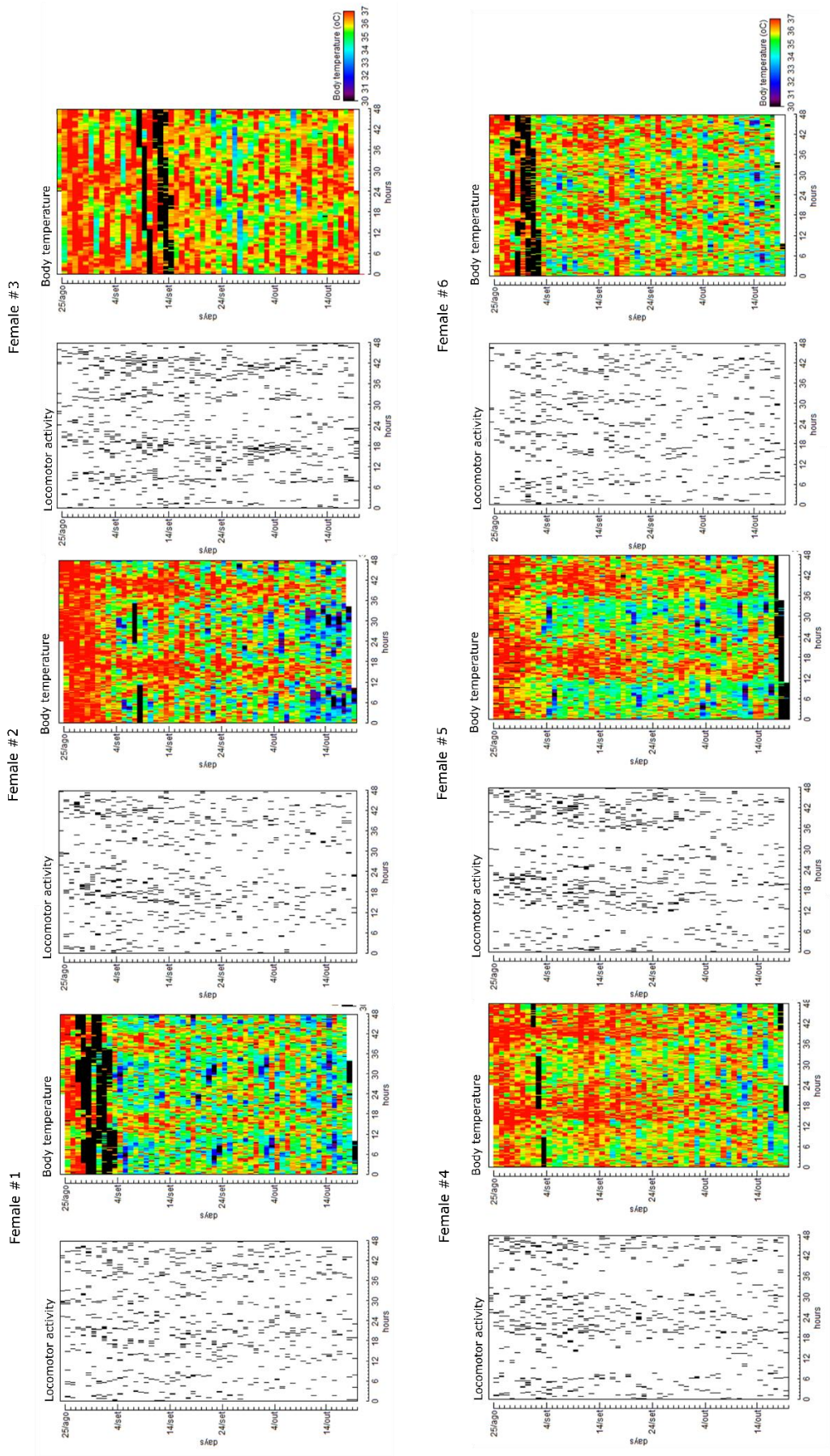

Figure S5.6. Double-plotted actograms of Tb and activity in female mice kept in the mixed-population enclosure, from August 25th until October 21st. T_b is color coded according to the gradient legend at the bottom of the figure. Black bars indicate data loss.

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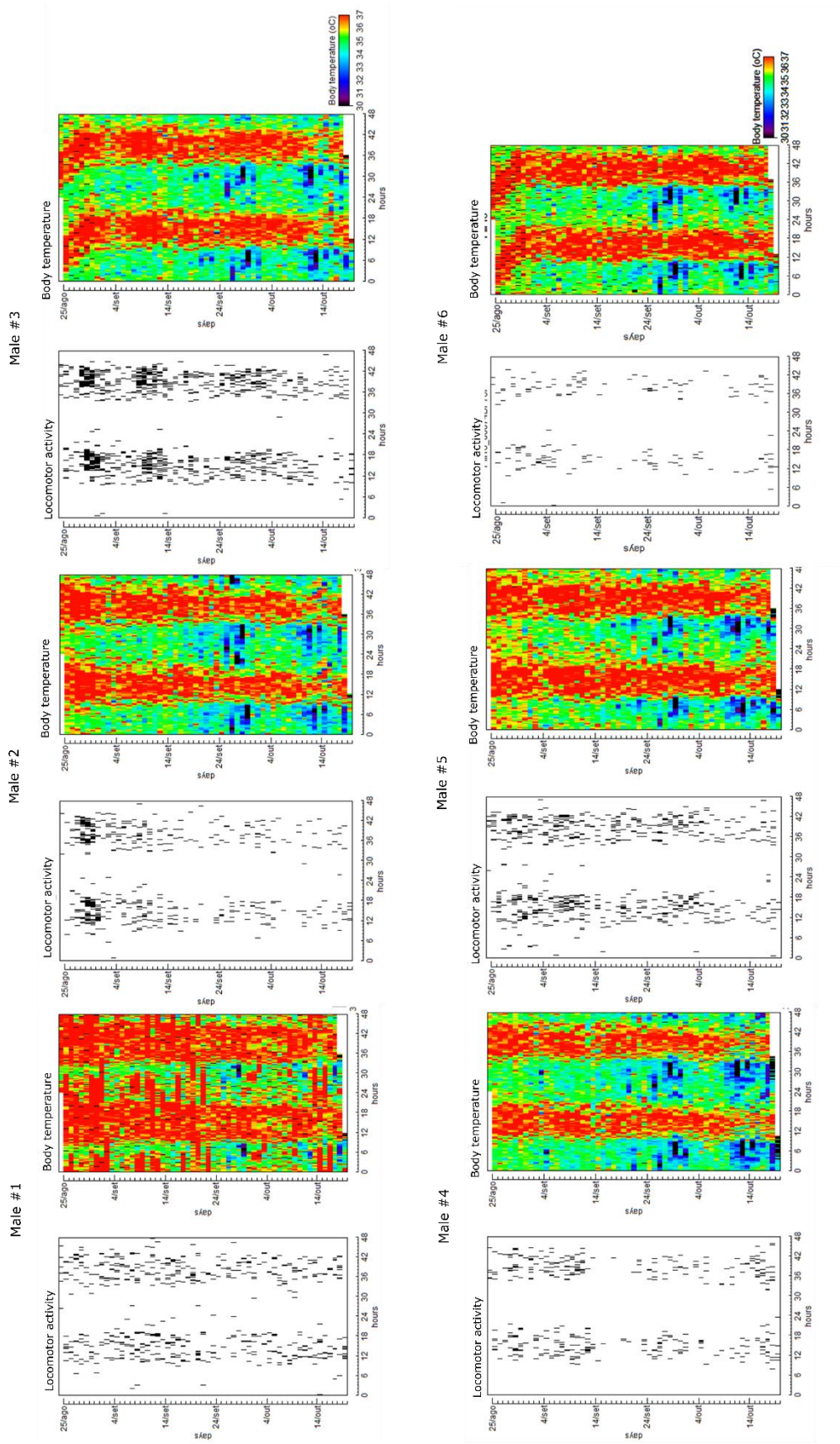

Figure S5.7. Double-plotted actograms of Tb and activity in male mice kept in the mixed-population enclosure, from August 25th until October 21st. T_b is color coded according to the gradient legend at the bottom of the figure. Black bars indicate data loss

Chapter 6

General Discussion

6.1. Temporal niche plasticity

The time of the day at which an animal allocates its activity, which can be defined as temporal niche, may be decisive for its survival and reproductive success (e.g. Daan, 1981; Kronfeld-Schor and Dayan, 2003; Hut et al., 2012). Allocation of activity during the daytime or nighttime defines diurnality and nocturnality, respectively. Characterization of a species as diurnal or nocturnal can be straightforward under controlled, narrow, laboratory conditions. This standard characterization, together with anatomical and morphological specializations, such as eye size and rod-cone ratio of the retina (Hut et al., 2012), can lead to the conclusion that temporal niche is an inherent and fixed characteristic of the species. However, activity patterns are much more plastic in nature (Hut et al., 2012; Gerkema et al., 2013), which was empirically shown in both tuco-tucos and mice (Daan et al., 2011; Tomotani et al., 2012; van der Vinne, 2015; Chapter 5).

Studies combining field and laboratory approaches are of great importance towards our understanding of how biotic and abiotic variables in the animal's habitat shape activity patterns. In the present thesis, we focused on the impact of environmental factors affecting the animal's energy budget (particularly food availability and temperature) on the temporal niche it occupies, and how the activity patterns themselves might impact the animal's energetic state.

6.2. Energy balance as a factor impacting temporal niche

One of the best elaborated hypothesis for temporal niche switches is the one which relates these switches to the energy balance of the animal. The "circadian thermo-energetic (CTE) hypothesis" (Hut et al., 2011, 2012) associates the phase switch in locomotor activity with energetically challenging conditions. According to this hypothesis, daytime activity would allow the animal to rest during the cooler hours of the night, counteracting the high energetic costs of foraging. Nighttime rest would result in energy savings with thermoregulation, since animals at rest can adopt postures that increase their insulation and take shelter in burrows where temperatures are higher than on the surface. Moreover, animals could save energy while maintaining a lower T_b and entering torpor overnight

In chapter 5, we showed that mice under semi-natural conditions respond to a decrease in food availability by increasing the amount of daytime activity and displaying

torpor, in line with previous studies in the laboratory (Hut et al., 2011). Daily torpor is an efficient strategy to reduce energy turnover in small mammals (Geiser, 2004). However, it comes at the cost of decreased social interactions and reproductive rates, as well as possible reduced nutrient assimilation due to the low T_b (Racey and Swift, 1981; Carey, 1989; Grinevitch et al., 1995; Ruf and Heldmaier, 2000). Switching from nocturnal to daytime activity can be an alternative to save energy without such costs, by allowing the animal to rest during the coldest hours of the day. Nevertheless, when daily energy expenditure surpasses maximum possible energy intake, mice need to additionally enter daily torpor to maintain their energy balance (Ruf and Heldmaier, 2000). For females, the costs of daily torpor on their offspring might prevent them to enter it unless strictly necessary (Racey and Swift, 1981; Grinevitch et al., 1995).

In tuco-tucos from Anillaco, daily torpor was never observed when T_b was recorded in the field, even during winter (personal observations) and is possibly not used at all , since they are much larger than mice, and energy savings by torpor significantly decreases with increasing body size (Heldmaier et al., 2004). For this reason, tuco-tucos should adopt other strategies to cope with energy constraints. In Chapter 4, we estimated that allocating aboveground activity to daytime instead of nighttime in tuco-tucos may be a strategy to save small amounts of energy. Another adjustment may be a decrease in mean T_b, which would allow a decrease in overall thermoregulatory heat production. Indeed, we observed that some individuals reduce their mean Tb when transferred to a semi-natural enclosure (Fig. 6.1). Other possible strategies may involve modification of the digestive tract and in the gut microbiome to increase nutrient uptake, since tuco-tucos are strictly herbivorous and eat foods that are not rich in energy (Martino et al., 2007). All these strategies can be combined when the animal's is energetically challenged. As proposed by Ruf and Heldmaier (2000), "activity, food intake, and energy expenditure during locomotion, rest, and torpor, represent linked variables of energy balance, that continuously feed-back on each other".

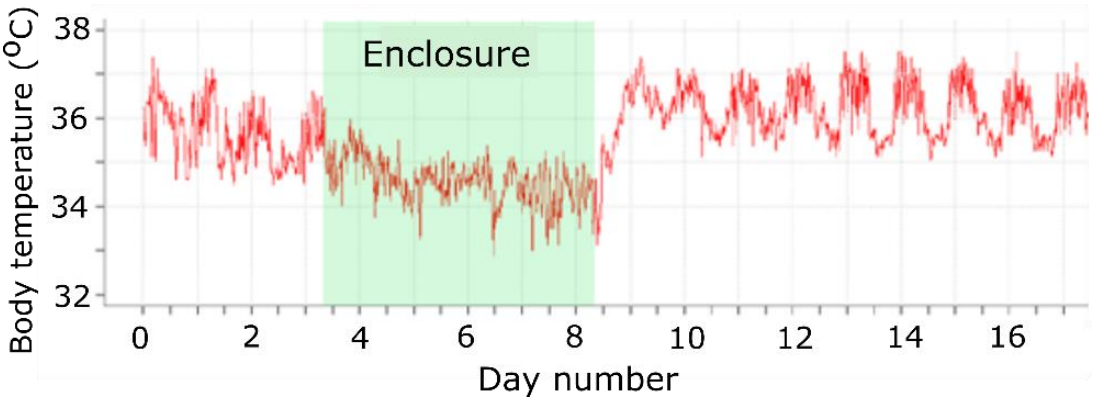

Figure 6.1. Tuco-tuco's T_b in the laboratory and in the field. Measurements of T_b in a tuco-tuco kept in the laboratory (white background) and in the semi-natural enclosure (green background).

6.3. Other factors impacting temporal niche

Mice in semi-natural enclosures show a great variability in activity patterns, especially males, even when all individuals have the same genetic background and are kept under the same environmental conditions (Chapter 5). This implies that other social and environmental factors might play important roles in shaping the timing of activity patterns. In Chapter 5, we discussed the possible role of social rank in the temporal niche. It is important to remember that competition and social rank themselves can impact the energy balance, since competition most probably causes variation of food intake among individuals.

Other social interactions may also change the temporal niche of mice (Castillo-Ruiz et al., 2012). The fact that females spread activity throughout the day when in a breeding population suggests that parental care can alter activity patterns of the mother. Previous studies in the same semi-natural enclosures suggest that perceived predation risk also plays an important role in the shape of activity patterns (van der Vinne, 2015). When the runway cover between the feeder and the nest box is removed daytime activity is greatly reduced, especially in females.

In tuco-tucos, we showed that diurnality reduces energy expenditure (Chapter 4). However, the small magnitude of these savings suggests that factors other than energetics may be important in the temporal niche tuco-tucos occupy in nature. Earlier works by our group have suggested some weather variables, such as wind and rain, may be factors that modulate activity, as well as the presence of predators (Tomotani et al., 2012). We know from personal observations and reports of people living the area that among the predators

of tuco-tucos there are either animals considered diurnal (e.g. raptors and snakes) or nocturnal (e.g. cats, foxes and owls). Therefore, the variations in population density and activity patterns of each of these predators may modulate the tuco-tucos´ timing of activity. Moreover, despite being solitary in their burrows, tuco-tucos interact with conspecifics constantly through vocalizations, making intraspecific interactions another item on the list of possible temporal modulators of activity patterns.

This multitude of environmental factors may explain the great variability of daily T_b patterns in the field recently recorded by our research group. Although the first, 2012 field T_b recordings showed strong diurnal patterns, later measurements revealed a wide range of temporal T_b patterns in the field, such as nocturnal, crepuscular and arrhythmic. It is worth noting that rainfall in our study area increased significantly along the later years, thus increasing the amount of food availability. We hypothesize that in 2012 energetic challenges were so intense that even the smallest amount of energy saving through daytime activity might have been needed. However, when food availability increased, predation risk and territory defense may have been more relevant in the determination of activity time.

6.4. Mechanisms underlying temporal niche switches

Many studies exploring plasticity of temporal niches have investigated the possible anatomical and physiological mechanisms that underlie the switch in activity timing. While some focused on differences of the circadian system between species considered diurnal and nocturnal (Smale et al., 2003; Kalsbeek et al., 2008; Hagenauer and Lee, 2008; Cohen et al., 2010), this section will focus on studies exploring mechanisms behind the switches within the same individual, either due to energy balance factors described in section 6.2 (e.g. Hut et al., 2011; van der Vinne et al., 2014; van der Vinne, 2015) or to social and environmental factors listed in section 6.3 (e.g. Kas and Edgar, 1999; Fernandéz-Duque et al., 2010, Chiesa et al., 2010; Cohen et al., 2010).

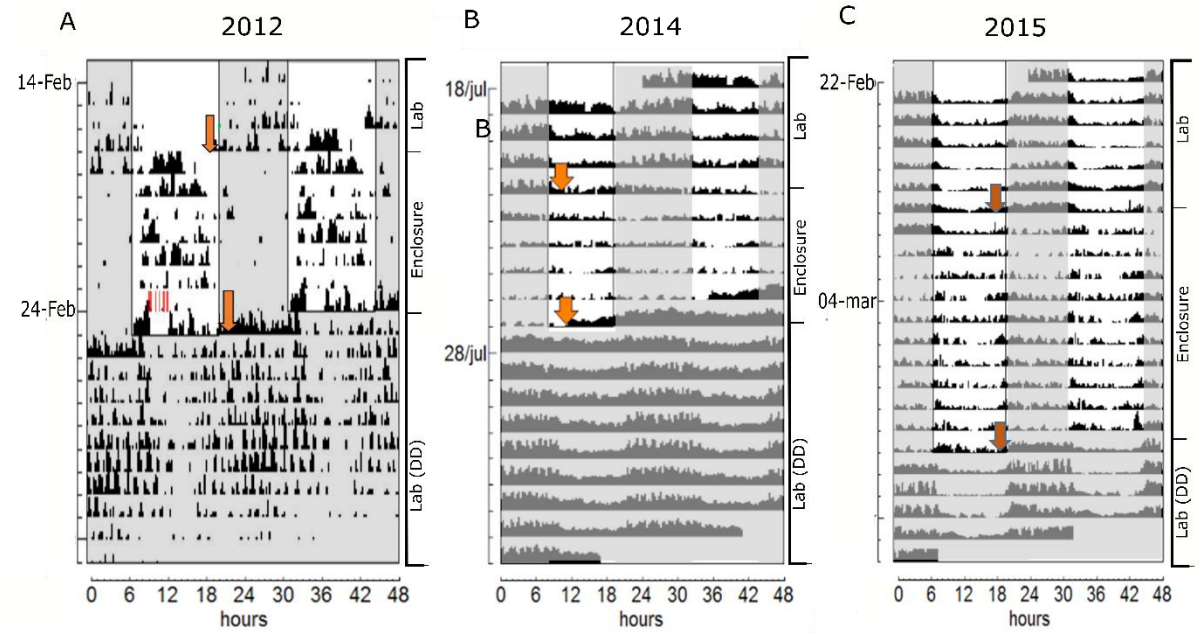

Figure 6.2. Double-plotted actograms of T_b of three tuco-tucos released in an experimental enclosure. Each line of the diagram represents two days and the black markings indicate the times when the T_b exceeded the mean. In the first days, the animals were kept in the laboratory under open-window condition and then released (first arrow) into the enclosure built in its natural habitat for 5-10 days. The animals were then recaptured (second arrow) and returned to the laboratory under constant darkness. The gray shaded areas indicate the dark phase in the field and in the laboratory. A) Measurement from 2012, the individual shows a diurnal T_b pattern in the field. B) Measurement from 2014, the individual shows an arrhythmic pattern in the field. C) Measurement from 2015, the individual shows a nocturnal pattern in the field

In Chapter 1, we described the basic structure of the circadian system (Fig. 1.2), consisting of a central circadian oscillator (the SCN in mammals), which receives information from the environment from afferent pathways and deliver rhythmic signals to the rest of the organism through efferent pathways, generating output rhythms (Moore-Ede et al., 1984). The use of locomotor activity rhythms as a proxy of period and phase of the oscillator itself has provided valuable discoveries about how this circadian system works, even prior to the anatomical identification of the central circadian oscillator (e.g. Pittendrigh and Daan, 1976).

More recently, it has been shown that in mice under laboratory conditions, the timing of SCN circadian electrical activity is indeed strongly related to the onset and offset of behavioral, locomotor activity (Houben et al., 2009). One might then assume that the switch from nocturnality and diurnality occurs due to a change in the phase of general rhythmicity within the SCN. However, studies contrasting laboratory and field

conditions (Halle and Stenseth, 2000), along with the gained knowledge on the anatomical basis of the circadian system (Smale et al., 2003) have indicated, with increasing evidence, that the temporal niche determination in nature may occur downstream from the circadian oscillator and that this determination presents far more plasticity than originally suspected.

The literature proposes at least three mechanisms through which temporal niche switches may occur (Fig. 6.3; Chiesa et al., 2010; Hut et al., 2012; van der Vinne, 2015):

1) *A phase switch occurs in the circadian oscillator* (Fig. 6.3-1) – As mentioned above, it is unlikely that this occurs in the cases presented in this thesis, as the SCN seems to be fairly insensitive to metabolic changes, ambient temperature and some stressful social interactions (van der Vinne et al., 2014; Buhr at al., 2010; Meerlo et al., 2002). Direct assessment of SCN gene expression in mice that become diurnal under the work for food protocol shows that it does not change its phase compared to when the animals display nocturnal activity rhythms (van der Vinne et al., 2014). We do not have direct assessment of the SCN in tuco-tucos. However, indirect assessments suggest that it does not change its phase when the animal becomes diurnal in the field (Tomotani et al., 2012). Similar results were also reported in golden spiny mice (*Acomys russatus*) transferred from the field to the laboratory (Levy et al., 2007), reinforcing the hypothesis that intra-individual temporal niche switches in most, if not all, rodents are not dependent on the phase of the SCN.
2) *The change in activity phase is determined downstream from the oscillator* (Fig. 6.3-2)– There are several possibilities for a downstream switch. One is that under certain conditions, such as when the animal is energetically challenged (as in section 6.2), the time signals sent by the SCN to the rest of the organism have opposite effects when compared to "standard" conditions (Fig 6.3-2A). For instance, a given level of electrical activity in the SCN can stimulate activity in one energetic condition and inhibit it in the other (Kas and Edgard, 1999). Another possibility is that there is another non-SCN oscillator involved in the determination of activity timing (Fig. 6.3-2B). This second oscillator would be coupled to the SCN and their phase relationship would change under different energetic conditions (van der Vinne, 2015).
3) *Activity in the field is a result of masking* (Fig. 6.3-3)- There is a possibility that daytime activities of nocturnal animals observed in the field are not clock-

controlled but due to masking (Marques and Waterhouse, 1994). In this case, an environmental factor in the field would directly stimulate activity during the day or presumably inhibit it during the night, without affecting the phase of the SCN and overriding its signals.

These mechanisms are not exclusive of each other. Indeed, we believe that the activity patterns observed both in tuco-tucos in the field (Tomotani et al., 2012) and in mice under semi-natural conditions (van der Vinne, 2015; Chapter 5) may be a combination of a switch downstream from the oscillator and masking. Laboratory experiments using the work for food protocol strongly suggest that energetic challenges cause downstream switches in activity timing (van der Vinne et al., 2014; van der Vinne, 2015). However, these experiments were done under controlled laboratory conditions in individually housed mice. In the field, there is an interplay of environmental and social factors that may stimulate or inhibit activity directly. We suggest that when food is scarce and there is a need to make adjustments to maintain the energy balance, the circadian system signals stimulate daytime activity through a downstream switch. Meanwhile, other factors, such as predation risk, social interactions and weather conditions fine tune the timing of activity through masking, resulting in a far more complex activity pattern than the one observed in the laboratory. This plasticity would allow the animal to endure not only predictable environmental challenges, but also unpredictable ones.

6.5. Triggering factors for temporal niche switches

The mechanistic explanation of the CTE hypothesis suggests that metabolic signals might trigger the downstream switch of activity timing and this seems to be the case in the laboratory "work for food" experiments in mice (Hut et al., 2011; van der Vinne, 2015). However, the diurnal/nocturnal switches can occur so rapidly in some conditions, such as in the case of animals transferred from the field to the lab (Fig 6.3) that one can argue whether this plasticity is acutely triggered by sudden changes in the

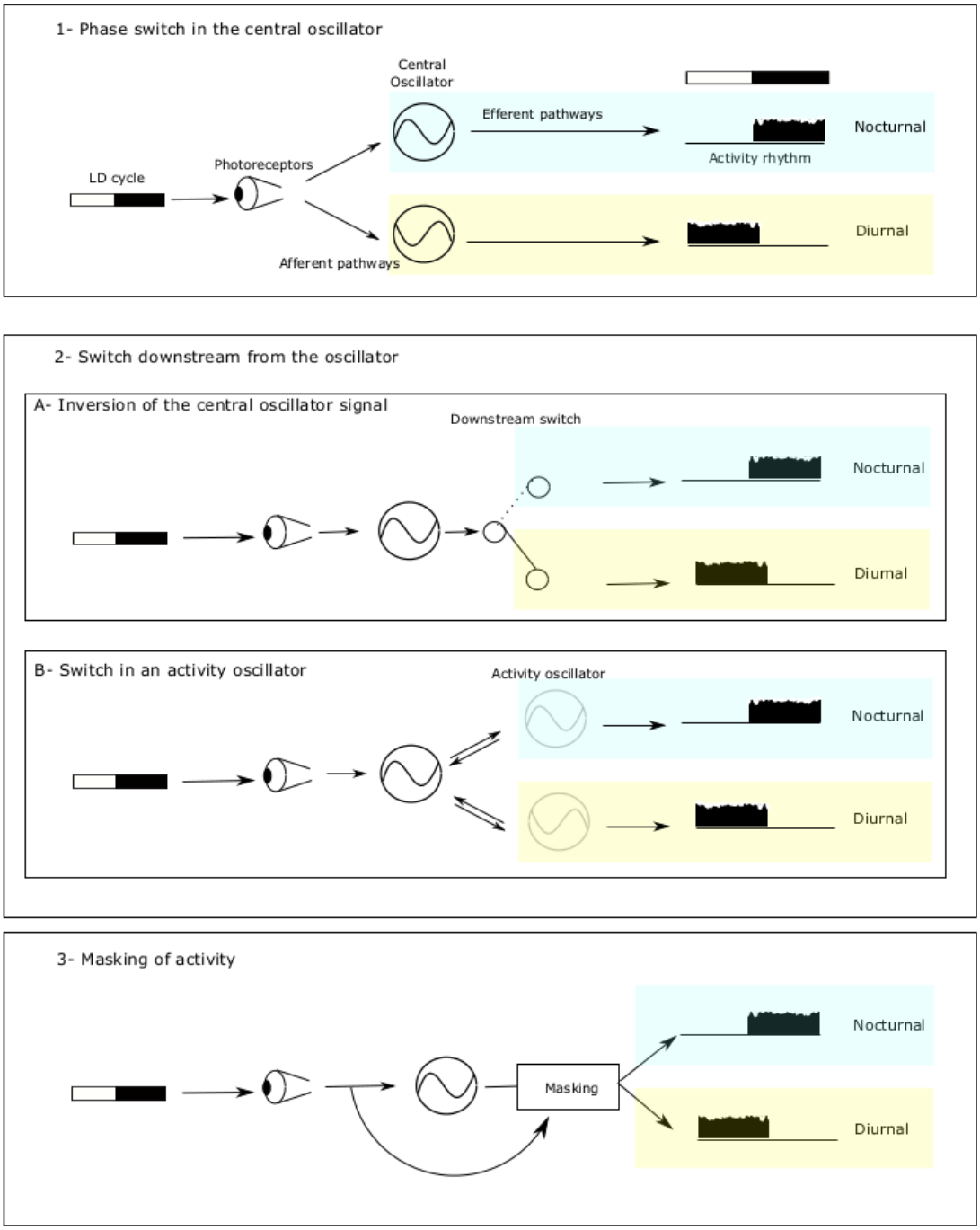

Figure 6.3. Scheme of the possible mechanisms underlying temporal niche switches. See text for details. Modified from Chiesa et al. (2010).

animal's environment. It is virtually impossible to identify a single environmental factor responsible for triggering temporal niche switches by only studying transfers from field to laboratory conditions. Thus, laboratory studies which allow manipulation of isolated environmental variables are valuable. Sudden switches in activity timing were reported, for instance, when a running-wheel was added to or removed from the home cage of degus (*Octodon degus*), Mongolian gerbils (*Meriones unguiculatus*) and unstriped nile rats (*Arvicanthis niloticus*). (Kas and Edgar, 1999; Smale et al., 2003; Weinert et al., 2007)

No changes in activity timing were detected due to the presence of running wheels in tuco-tucos (Tomotani et al., 2012; Tachinardi et al., 2014). Nevertheless, we did observe nocturnal to diurnal switches when the animals were introduced into a respirometry chamber (Chapter 3) where they face mild alteration of gas composition of the ambient air (< 1% decrease in O_2 and <0.5% increase in CO_2). Although these gas alterations are minor for non-subterranean animals, it is conceivable that tuco-tucos, which live in sealed underground tunnels, are able to detect even small changes in gas composition and/or humidity.

To test whether gas changes in the atmosphere are responsible for the temporal switches, we performed preliminary pilot tests using two individuals that had switched to diurnality inside the respirometry chamber (Fig. 6.3). Because we could not directly change the gas content of the chamber atmosphere, we increased the air flow going through the chamber and slightly opened the lid, what would drive O_2 and CO_2 to levels close to the ones in the outside atmosphere. To avoid any direct masking effects of light on the observed rhythms, the light regimen was changed from LD 12:12 to constant darkness (DD). The diurnal pattern of T_b rhythm persisted in DD, with the highest T_b and general activity occurring during the time lights were previously on. On the fourth day under DD, the lid was open and airflow was also increased for at least 8 days. As a result, both individuals displayed a shift of T_b, general activity and running-wheel activity back to the subjective night. One animal was allowed a running wheel throughout the entire experiment whereas the other animal had its wheel removed five days after the increase in airflow rate. Removal of the wheel did not affect its phase of activity (Fig 6.3a). We believe these preliminary results are potentially very interesting. Although we are still not able to discriminate if the cause of the switch back to nocturnality upon lid opening was the O_2/CO_2 content and/or humidity, we do consider we have an indication that a factor inside the chamber is causing the temporal switch.

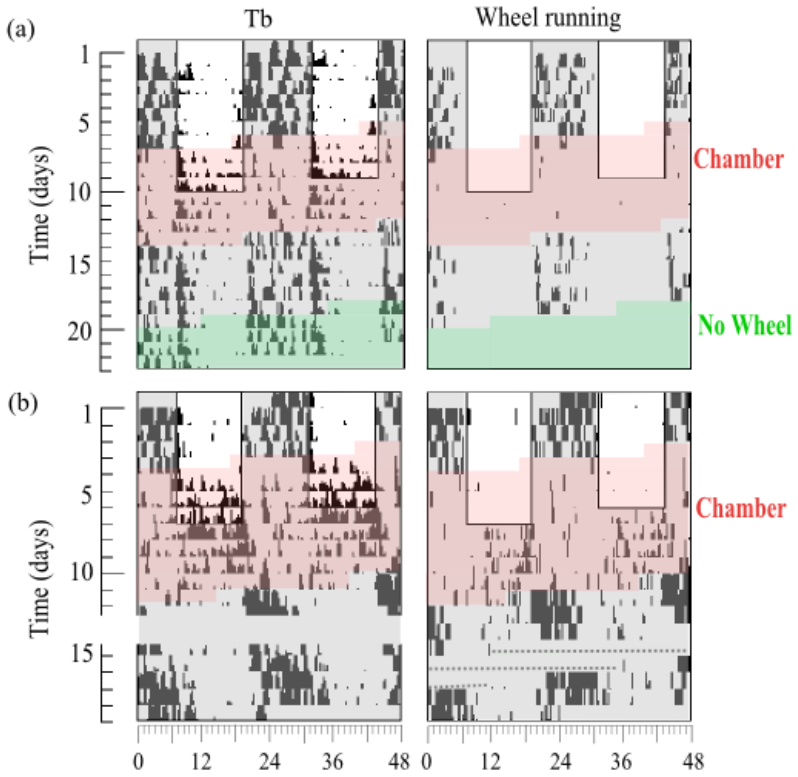

Figure 6.3. Actograms highlighting phase of Tb and wheel-running in two individuals (panels a and b) that switched from nocturnal to diurnal inside the respirometry chamber. Shaded areas indicate darkness whereas light phases are indicated by white. The switch in phase, indicated by Tb and gross motor activity, occurred on days 7-13 (panel a) and 4-12 (panel b). When animals were in the respirometry chamber (red background) wheel-running was suppressed in both animals. Under DD conditions, (days 10-13 (panel a) and 8-12 (panel b)) wheel-running resumed during the subjective day in one animal (panel b) but remained suppressed in the other animal (panel a). Upon opening of the lid and increase in flow rate (days 13 (panel a) and 12 (panel b)) the phase of Tb, motor and wheel-running activity switched back to subjective night. Wheel-running reappeared in one animal (panel b) under this condition. On day 19 the wheel was removed from one of the animals (panel a, green background). This caused no change in the Tb rhythm. Grey dotted line indicates missing data due to technical problems.

6.6. Final remarks and perspectives

Understanding why and how activity is allocated at certain times of the day in nature demands extensive investigations involving both field and laboratory studies. These investigations would benefit from using both laboratory animal models, which are well characterized regarding their anatomy, physiology and genetics, and wild animals, which can add valuable ecological context to the study. Many questions await to be answered in future studies on the plasticity of temporal niche, such as what are the exact mechanisms behind it, what environmental factors trigger temporal switches and how can this plasticity enhance the animal's fitness.

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