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Resumo geral

Ritmos diários em insetos são gerados por um sistema circadiano localizado no *protocerebrum* e nos lobos ópticos do sistema nervoso central. O sistema circadiano é composto por osciladores acoplados às vias de aferência e eferência. O oscilador gera ritmos através de mecanismos moleculares, integrantes de alças de retroalimentação. Nas vias de aferência estão envolvidos componentes que participam da transdução mediada da luz. Diversos neuropeptídeos fazem parte das vias de eferência.

Forrageiras da abelha sem ferrão *Melipona quadrifasciata* exibem um ritmo diário de atividade. Forrageiras foram utilizadas neste trabalho para identificar componentes circadianos, através de três diferentes abordagens: I) análise da expressão gênica, II) identificação de estruturas no sistema nervoso central, III) estudo comparado de neuropeptídeos possivelmente relacionados com o sistema circadiano, utilizando como espécie referência *Apis mellifera*.

I) Fragmentos de prováveis genes do relógio foram clonados. Somente o gene *period* (*per*) mostrou expressão rítmica, o pico ocorreu 1h após o início do escuro. *cryptochrome (cry)*, *Clock* e *cycle* não apresentaram diferença estatística na expressão rítmica.

II) Anticorpos contra PER, CRY (proteína da via de aferência) e "pigment dispersing hormone" (PDH, neuropeptídeo da via de eferência) marcaram diversas áreas no cérebro e nos lobos ópticos. PER e CRY foram localizados nos lobos ópticos, em fibras da região protocerebral, com um padrão rítmico. PDH foi observado em corpos celulares no *protocerebrum* lateral, em projeções no cérebro e em algumas fibras nos lobos ópticos.

III) Neuropeptídeos, provavelmente relacionados com o sistema circadiano, foram detectados em *A. mellifera* e *M. quadrifasciata*. Alguns deles: "tachykinins-related peptides", alatostatinas, e "FMRF-related peptides" são rítmicos, com padrões espécie-específicos.

O sistema circadiano de *M. quadrifasciata* mostrou particularidades nos prováveis componentes do relógio quando comparados com *A. mellifera* e outros insetos. A expressão,

localização, distribuição e dinâmicas temporais apontam para características específicas da organização do sistema circadiano.

General abstract

Daily rhythms of insects are generated by a circadian system localized in the *protocerebrum* and in the optic lobes of the central nervous system. The circadian system is composed by coupled oscillators connected to input and output pathways. The oscillator generates rhythms by molecular processes, linked in feedback loops. In the input pathways the components are involved in light mediated-transduction. In the output, several neuropeptides are involved.

Foragers of the stingless bee *Melipona quadrifasciata* exhibit a daily activity rhythm. Foragers have been used here to identify circadian components, through three different approaches: I) analysis of gene expression; II) identification of structures in the central nervous system; III) comparative study of neuropeptides possibly related with the circadian system, using *Apis mellifera* as the reference species.

I) Fragments of putative clock genes were cloned. Only *period (per) gene* showed rhythmic expression, peaked at 1h after lights off. Non statistically significant rhythms were detected in *cryptochrome (cry), Clock* and *cycle* genes expression.

II) Antibodies against PER, CRY (an input pathway protein) and pigment dispersing hormone (an output pathway neuropeptide) evinced several areas in the brain and in the optic lobes. PER and CRY were localized in the optic lobes and in fibers in the protocerebral region, in a rhythmic pattern. PDH was observed in cell bodies in the lateral *protocerebrum*, in projections in the brain and in some fibers in the optic lobes.

III) Neuropeptides probably related to the circadian system, were found in *A. mellifera* and *M. quadrifasciata*. Some of them: tachykinin-related peptide, allatostatin, and FMRF-related peptide were rhythmic and present in specie-specific patterns.

The circadian system of *M. quadrifasciata* showed particularities in the putative clock components when compared with *A. mellifera* and other insects. The expression, localization, distribution and temporal dynamics of the circadian system point out a novel, specific feature.

General Introduction

Stingless bees

The tribe Meliponini comprises the stingless bees distributed in tropical and southern subtropical areas of the world (Michener, 2007). The oldest known bee fossil is a stingless bee – *Cretotrigona prisca* – an amber from the late Cretaceous with over 65 million years, discovered in Kinkora, New Jersey (Camargo & Pedro, 1992; Engel, 2000).

Meliponini can be recognized by the reduction of the forewing venation; the presence of a jugal lobe in the hind wing; the presence of one or two rows of long setae in the hind tibia, a structure called *penicillum*; simple claws; and reduction or absence of the sting (Wille, 1983).

Camargo and Pedro (2013) recognize 33 genera and 417 species-groups in the Neotropical region. Like the honeybees (Tribe Apini), the stingless bees have a complex social organization. Their colonies are eusocial which implies: reproductive division of labor with caste differentiation, overlap of at least two generations, and cooperative care for the young (Wilson, 2000). Eusocial bees establish large perennial colonies with elaborated nest architecture (Sakagami, 1982).

In the stingless bee colony is possible to recognize the simultaneous presence of different members, with specific roles. Despite cases of facultative polygyny in *Melipona bicolor* and transients episodes of polygyny in other bee species (Carvalho-Zilse & Kerr, 2004; Alves *et al.*, 2010), in Meliponini colony there is only one queen (Sakagami *et al.*, 1965), males and workers. Like in other Hymenoptera, sex determination is genetic and related to haplodiploidy: diploid females (of fertilized eggs) and haploid drones (of unfertilized eggs). The production of males is a high investment for the colony, being restricted to the reproductive phase (Velthuis *et al.*, 2005).

The production of the workers and queens (caste determination) involves different mechanisms among the species of stingless bees. In the genus *Trigona*, a quantitative difference in

larval food determines castes (larvae of future queens have access to high amounts of food). In *Melipona* genus, Kerr (1946, 1950) proposed a genetic predisposition to determine the castes. The author suggested a two *loci* model, each with two alleles each, where double heterozygote females would determine fertile females, queens; and homozygote for any of them would develop into a worker. This genetic mechanism would explain the larger proportion of produced queens in *Melipona* colonies (up to 20-25%). Also the participation in the process of trophic factors is not excluded, but the mechanisms are still un-resolved (Hartfelder *et al.*, 2006). None of these mechanisms are observed in the honey bees, in which a nutritional switch in the fourth to the fifth larval instar from worker jelly to royal jelly determines the queen's development (Hartfelder *et al.*, 2006).

Among the stingless bee workers, Kerr and Santos-Neto (1956) observed a temporal dynamic in the activities performed by the individuals according to age-correlated patterns, similar to the dynamics seen in honey bees (Robinson, 1992). The division of labor is not a rigid process, but rather reflects tendencies toward certain activities at certain ages (Wille, 1983). The sequence of tasks carried out along a worker's life generally has four stages: 1) the youngest bees (nurses) perform incubation and repairs in the brood chamber; 2) a bit later, they are in charge of the construction and provisioning of brood cells, cleaning of the nest and feeding young workers and the queen; 3) bees of intermediate age are responsible for cleaning the nest, repairs of structural elements, reception of nectar and guard duty at the entrance of the nest; 4) the oldest bees are the foragers, that collect pollen, nectar, resin and other materials important to the colony maintenance (Wille, 1983; Cepeda, 2006). All the activities are integrated and related to the spatial elements of the colony.

The structural elements of the nest architecture are arranged in a unique conformation for each species. Usually, the nest is built inside cavities in the ground, or in the hollow of trees, either trunks or branches (Michener, 2007). The primary material used to construct nests is the *cerumen*: a mix of wax (secreted by the abdominal glands of the youngest workers), with large amounts of resin (collected in the field). In some groups, the bees use the *cerumen* with vegetal matter or mud

to form the *batumen* (Nogueira-Neto, 1997). Both, *cerumen* and *batumen* are used to build the structural elements of the nest.

In the nest, clear divisions are observed: the entrance tube, the food pots area and the brood cells region. Connecting the nest to the external environment is the entrance tube, recognizable by the members of a colony for its unique, curved shape and external ornamentations (Nogueira-Neto, 1997; Roubik, 2006). The interior wall of the nest is lined with *batumen* (Wille 1983), which has an important role in the thermoregulation process. The *batumen* (fig. 1) coating and the bends of the entrance tube prevent the entrance of light in the nest that is always completely dark. The alimentary components, pollen and honey, are stored in food pots (fig. 1) made of *cerumen* in the periphery of the nest; their number, shape and size vary in accordance with the species and season (Wille & Michener, 1973 *apud* Wille, 1983).

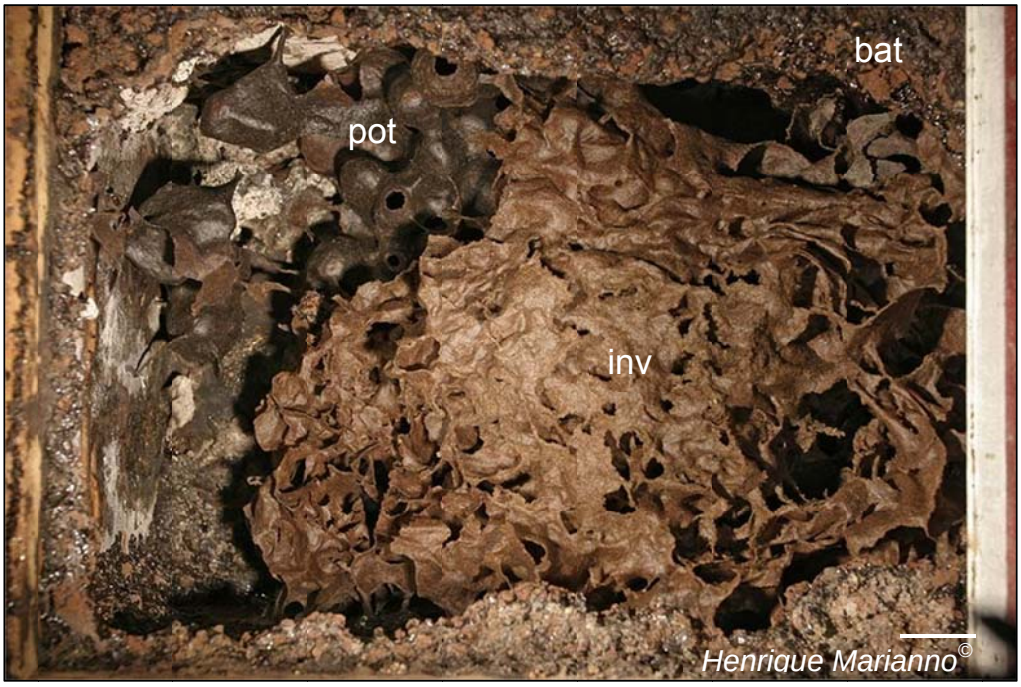

Figure 1 – Architecture of the inner nest of a stingless bee, *Melipona quadrifasciata*. The interior wall is covered by *batumen* (bat), in the periphery are the food pots (pot) and in the central portion of the nest are the brood cells, which are covered by the *involucrum* (inv). Bar scale = 5cm.

The *cerumen* is also used to build brood cells. As brood cells are used only once, after the emergence of the adult, the material is reallocated in other structures, like food pots and pillars (Sakagami, 1982). Differently from the honeycombs of *A. mellifera* in which food pots and brood cells have all the same hexagonal format, brood cells (fig. 2b) and food pots (fig. 2a) of Meliponini exhibit distinct shapes (Nogueira-Neto, 1997). The disposition of brood cells and food pots is species specific. They are assembled together in clusters, in spiral plates, or in horizontal combs and all structure is supported and interconnected by pillars, depending on the species (Sakagami, 1982; Roubik, 2006). The brood cells are localized in the central part of the nest and in some species, they are wrapped in several layers of *cerumen* (fig 1) (Wille, 1983). The wrapping provides a tighter temperature control for the brood than for the rest of the colony.

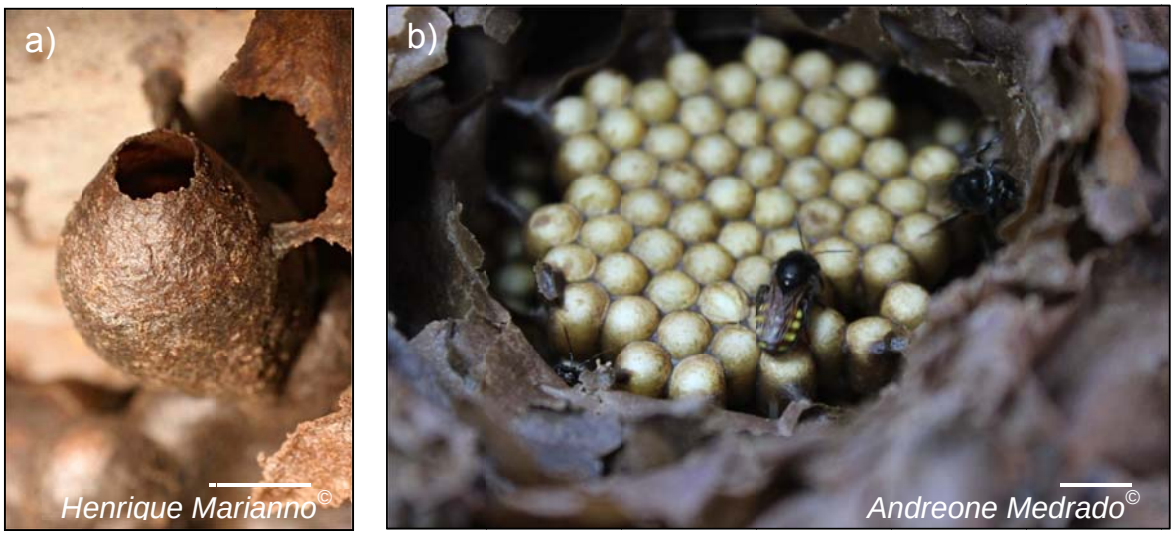

Figure 2 – Structural elements of a stingless colony (*Melipona quadrifasciata*). a) Image of an isolated food pot, in detail. Bar scale = 2.5cm. b) the *involucrum* (inv) was removed and the brood cells can be observed (bro). Bar scale = 1cm.

The spatial information conferred by the architecture of the nest is intimately related to temporal dynamics that can be detected in several processes and behaviors. One of the most evident is the division of labor, which spatially follows a centrifugal sequence (Wilson, 1985; Bourke & Franks, 1995). Younger bees perform their activities in the central region of the nest, in or around

brood cells; as the aging process continues, they go progressively toward the periphery of the nest, until they become foragers and fly out for resources in the external environment. This progression is followed by a transition of micro-climates: from completely darkness and low temperature variation in the central area, to direct contact with the light/dark cycle and the pronounced temperature variation of the external environment. Before they become foragers, the workers leave the nest sporadically when they transport wastes to the outside. In these moments, short flights around the colony entrance are observed and they occur always during the light phase (Kerr & Santos-Neto, 1956). As for the foragers, their activity in the field takes place also during the day, characterizing diurnal species.

Temporal dynamics and rhythmic components in the stingless bee colony

The organization of a stingless bee colony depends on the architectural structure and behavioral activities that must occur in rather precise time intervals.

Reproduction, and all the behaviors and processes linked to it, is one of the examples in which the temporal adjustment is essential. Adjustments start much before the nuptial flight. Virgin queens emerged from the brood cells are immediately killed by the workers (Kerr & Krause, 1950), unless a new queen is required by the colony. Precisely in this circumstance, males are produced (Velthuis *et al.*, 2005), an essential condition for the synchronization of the sexes. Males emerge before and the fecundation of the queen occurs in the air, during the flight. In *Melipona quadrifasciata*, the queen mates with only one drone, that dies immediately after the copulation (Kerr *et al.*, 1962; Peters *et al.*, 1999).

Oviposition is a step pf a process known as POP – Provisioning and Oviposition Process – which entails a sequence of steps: the construction of brood cells by the workers, the oviposition by the queen and the cells closing by the workers. Despite its resemblance to the oviposition process of solitary bees, it is characteristic of the Meliponini (Sakagami, 1982). The POP completion

depends on the adjustment of the time intervals of the workers and the queen. The consequence is the rhythmic occurrence of oviposition (Oda *et al.,* 2007). The period of this rhythm is specific for each species and varies widely, as examples: *Melipona quadrifasciata* the period is 3-4 hours (Teixeira, 2006) and *Frieseomelitta duoderleini*, 24 hours (Fernandes, 2004).

Another clear rhythmic pattern is present as a daily rhythm, detected in the activity of foragers. This rhythm has already been studied in two stingless bee species. The rhythmic expression remained under constant environmental conditions, evincing the existence of an endogenous, circadian rhythm in *Scaptotrigona* aff. *depilis* (Bellusci & Marques, 2001) and in *Frieseomelitta varia* (Fernandes, 2004). In *F. varia*, the forager circadian activity rhythm was fully characterized. The rhythm was entrained both by light/dark and by warm/cold environmental cycles, and its period showed temperature compensation, being 23h under 28^{o}C and 23.5h under 38 ^{o}C (Fernandes, 2004).

In *A. mellifera* also, the expression of circadian rhythms is related to the division of labor. Nurse bees perform brood care “around the clock” what led Moore *et al.* (1998) to the conclusion that circadian rhythms were not present in the nurses, while the oldest bees, the foragers, show an evident circadian activity rhythm (Moore & Rankin, 1993). A more recent study corrected the former interpretation and described strong behavioral and molecular circadian rhythms in young *A. mellifera* nurses kept outside the hive (Shemesh *et al.,* 2010). In stingless bees, rhythms of oxygen consumption were detected in very young nurses (24h-old) of *Melipona quadrifasciata* kept in constant environmental conditions (Teixeira *et al.*, 2011; Camargo, 2012). Foragers submitted to the same treatment, equally showed a circadian respiratory rhythm but ten times more robust than the nurses’ (Teixeira *et al.*, 2011).

In addition to the rhythms described above, in the stingless bee colonies, high frequency (ultradian) rhythms are also observed in physiological and behavioral processes (Teixeira, 2006). In the opposite range, seasonal rhythms have been detected. Changes in the internal architecture of the colony were observed along the year. These changes were mainly in the proportions of the

regions, being the nest area larger during the summer. The area of the food pots increases significantly during the fall, becoming smaller by the winter end. These changes are accompanied by the cessation of activity on the brood cells and oviposition by the queen during part of the autumn and winter (Cosignani, 2006).

So far, the studies on stingless bees' circadian rhythms contemplated behavior and physiology. The registration of behavioral expressions has enabled the detection of rhythmic components in individuals and in colonies (Bellusci & Marques, 2001; Fernandes, 2004). Physiological procedures allowed the refinement of the knowledge on the mechanisms involved in the rhythmic expression studied in isolated individuals (Teixeira *et al.*, 2011; Camargo, 2012). The present work was designed to detect and localize rhythmic components of the circadian system analytically. Putative elements that compose the circadian system of other insects were used as references in the research of the oscillation generating elements present in the central nervous system of the stingless bee. Different tools have been used to tackle the problem from diverse angles. A comparative approach, using the honey bee clock components, provided the bases for speculations on the similarities and differences of circadian system features of the two bee species.

The stingless bee species chosen was *Melipona quadrifasciata* (popular name: mandaçaia). It is one of the most studied stingless bees. It can be found along the coastal area of Brazil, from Paraiba until Rio Grande do Sul state (Moure & Kerr, 1950). There are two sub-species: *Melipona quadrifasciata quadrifasciata* (distributed in warmer regions) and *Melipona quadrifasciata anthidioides* (found in sub-tropical areas, southern Brazil). Considering that results on daily and circadian rhythms of *Melipona quadrifasciata anthidioides* are already available (Teixeira *et al.*, 2011; Camargo, 2012) as well as a histological map of its central nervous system (Yamashita, 2009), this was the species chosen for this work and only foragers have been used.

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Concluding Remarks

The elaborate temporal features observed in individuals and in the colony of *Melipona quadrifasciata* must be, at least in part, controlled by the circadian system. In this work, to try and access its components three different approaches have been adopted: 1. the study of gene expression; 2. the description and identification of structures in the central nervous system where clock proteins were detected; and 3. the comparative study of neuropeptides possibly related with the circadian system, using *Apis mellifera* as the reference species. The results represent the first evidences on the organization of a stingless bee temporal system.

The most significant results are listed below. They reflect the diverse tools employed in the project as they show different aspects of the several components studied.

1) Attempts to clone and purify fragments of putative clock genes were successful with the genes: *period*, *cryptochrome*, *Clock* and *cycle*, but not with: *pdf*, *corazonin*, *vrille*, *pdp1* and *timeless*.
2) The expression profile of the fragments showed that only *period* was rhythmic. It peaked in the beginning of the scotophase. Despite the expression of other genes also showed peaks, they were not statistically different: *cryptochrome* expression was higher in the dark phase, one hour after lights-off; *Clock* largest expression was in the middle of the photophase. Gene *cycle* did not show a rhythmic expression.
3) The topology of the optic lobes and the brain of *M. quadrifasciata* is similar to that of *A. mellifera*. Notwithstanding the similarities, some particularities were observed, as the spatial location of the median *calyces* of *corpora pedunculata* and the *lobula* shape in the optic lobes.
4) Neurosecretory material was detected in the *pars intercerebralis*, in the *nervi corporis cardiaci I* and in the recurrent nerve.

5) Immunohistochemical preparations using antibodies against different clock proteins showed the neuropeptide *Pigment Dispersing Hormone* (PDH) not co-localized with *period* protein (PER) and neither with *crypthochrome* (CRY) protein. Although PDH and PER, in some areas occurred in the same group of neurons, they were not in the same region of the cell; when one was in the cell body, the other was in the axon and vice-versa.
6) PDH was present in the cell bodies of a group of neurons localized in the lateral *protocerebrum*; in neuron projections along the brain midline; and in several fibers next to *corpora pedunculata*, under the *calyces* and around the α lobe. In the optic lobe, it was observed in some processes of the *lamina* and *medulla.* PDH was also seen in the *deutocerebrum*.
7) PER was present in several areas of the *protocerebrum*, in fibers around the α lobe and the calyces of *corpora pedunculata*; in fibers descending from the *ocelli* and others proceeding from the *lobula*. In the *deutocerebrum*, marked fibers were present around the *glomeruli* of the antennal lobe.
8) CRY immunolabeling was seen in the protocerebral area in fibers that constitutes the β lobe and around the α lobe of *corpora pedunculata.*
9) PER and CRY were localized in columnar-shaped cells, probably glial cells, in the optic lobes. These cells were tightly assembled together, composing continuous structures between *lamina* and *medulla* and between *medulla* and *lobula.* Both proteins showed a rhythmic pattern, being more abundant during the scotophase. CRY, unlike PER, was concentrated in the basal region of the columnar cells. No co-localization assays for the two proteins have been performed.
10) PER and CRY showed a rhythmic oscillation in their amount throughout the day. The number of marked fibers in the *protocerebrum* and cell bodies in the optic lobe was larger during the scotophase.

11) Mass spectrometry assays were done using *M. quadrifasciata* and *A. mellifera* brains. These assays allowed the identification of some neuropeptides, possible components of the circadian systems of both species.

12) Tachykinin related peptides, allatostatin A and FMRFamide-like were possibly found in the profiles of honeybees and stingless bees. However specific differences emerged in the daily oscillations showed by the peptides ion masses. The tachykinin related peptide (977m/z) in *M. quadrifasciata* peaked one hour after lights off (ZT13) and the nadir was at ZT9. For the same peptide in *A. mellifera*, the acrophase was at ZT9 and the lowest amount at ZT21. Another tachykinin related peptide (993m/z) displayed a similar rhythmic profile in both species, the acrophase was at ZT9. Allatostatin A (1021m/z) peaked at ZT13 and the nadir was at ZT9 in *M. quadrifasciata*, and in *A. mellifera*, the acrophase was at ZT9 and the nadir was at ZT21 (like tachykinin related peptide 977m/z). The FMRFamide-like was only detected in *M. quadrifasciata* (1175m/z) at ZT13 only.

The many results already published on the *A. mellifera* circadian system facilitated the realization of this work, because the *M. quadrifasciata* genome is not yet available. In addition to operational facilities, conserved features of the clock components made identifications easier. However, particularities observed in the expression, localization, distribution and temporal dynamics of putative components of the circadian system point out a novel feature that can determine the specific rhythmic patterns only seen in *M. quadrifasciata*. If differences in the genes controlling behavioral expressions were expected because of the differences between the two species, a certain similarity of their circadian clocks was foreseen because both are eusocial, diurnal species. Nevertheless, the results obtained did not confirm the conjectures. The particularities observed in *M. quadrifasciata*'s putative clock components seem to indicate that the social organization of the nest plays a role more important to the determination of the temporal organization of the species than the general, diurnal activity of the colony.