Mila Maria Pamplona Barbosa

Ritmos diários de forrageamento e corte de folhas em uma colônia de *Atta sexdens*

Daily rhythms of foraging and leaf-cutting on an *Atta sexdens* colony

São Paulo

2022

Mila Maria Pamplona Barbosa

Ritmos diários de forrageamento e corte de folhas em uma colônia de *Atta sexdens*

Daily rhythms of foraging and leaf-cutting on an *Atta sexdens* colony

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências, na Área de Fisiologia Geral.

Orientador(a): André Frazão Helene
Coorientador(a): Gisele Akemi Oda

São Paulo

2022

Ficha Catalográfica

Barbosa, Mila Maria Pamplona
Ritmos diários de forrageamento e corte de folhas em uma colônia de *Atta sexdens*
86 pgs.

Dissertação (Mestrado) - Instituto de Biociências da Universidade de São Paulo. Departamento de Fisiologia.

Palavras-chave: 1. Ritmos biológicos 2. Formigas cortadeiras 3. Software de Video-tracking.
Key-words: 1. Biological rhythms 2. Leafcutter ants 3. Video-tracking software.
Universidade de São Paulo. Instituto de Biociências. Programa de Pós-graduação em Ciências (Fisiologia Geral).

Comissão Julgadora:

Prof(a). Dr(a). _____

_____

Prof(a). Dr(a). _____

_____

Prof(a). Dr(a). _____

_____

Orientador(a) _____

_____

Coorientador(a)_____

_____

Dedicatória

Aos meus pais, Janinha e Carlinhos, pelo apoio incondicional na busca pela realização dos meus sonhos.

Epígrafe

Stonehenge

One of my goals in these closing sections has been to illustrate - and enjoy! - what I take to be the real adventure in the scientific enterprise, which is, among other things, a search for pattern and meaning in what one observes. It is the necessarily conjectural nature of that search, at least at the outset, which entails hazard and makes the enterprise adventurous, not only for the observer himself, but for his observations and ideas. “Escape from light” is just such an adventure, which one enjoys for its own sake, and possibly will prove useful, perhaps even correct.

Stonehenge

Um de meus objetivos nestas seções finais tem sido ilustrar - e aproveitar! - o que considero ser a verdadeira aventura no empreendimento científico, que é, entre outras coisas, uma busca por padrão e significado no que se observa. É a natureza necessariamente conjectural dessa busca, pelo menos no início, que acarreta perigos e torna o empreendimento aventureiro, não apenas para o próprio observador, mas para suas observações e idéias. "Fugir da luz" é exatamente uma tal aventura, que se desfruta por si mesma, e que possivelmente se revelará útil, talvez até correta.

Colin S. Pittendrigh,
TEMPORAL ORGANIZATION: Reflections of a Darwinian Clock-Watcher
(tradução nossa)

Agradecimentos

Primeiramente, gostaria de agradecer aos meus orientadores Frazão e Gi, por todos esses anos de mentoria durante o mestrado e a graduação. Vocês são grandes inspirações para mim e não tenho palavras para descrever o quanto o apoio de vocês foi acima da média diante de todos os meus desafios durante esses anos.

Em segundo lugar, gostaria de agradecer aos meus pais, Jane e Carlos, a quem essa dissertação é dedicada. Sem o apoio incondicional deles, a graduação e a pós-graduação (com meses sem bolsa) na USP jamais seriam uma possibilidade. Vocês nunca hesitaram em me apoiar no caminho que eu escolhi e eu sou imensamente grata por esse privilégio que sei que é a exceção no nosso país. Agradeço também a CAPES pela bolsa de mestrado.

Agradeço também ao Marcelo, pessoa sem a qual esse projeto jamais seria possível. Obrigada por me “adotar” como IC e me ensinar tantas coisas que nunca pensei que saberia, querido. Aqui também deixo meus agradecimentos a todos os membros do LabCog, especialmente Julia, MaFer e Ana Clara, que me ajudaram com as anotações de formigas, e também Pedro, Paty, Yani, Karla, Clevinho, Dani, Fer e Salsa, pela amizade, companheirismo, ensinamentos, sofrimentos, churrascos e bolos compartilhados. Incluo também aqui Bia, Giovane, Jefferson, Milene, “cronoamigos” com quem partilhei tanto nos últimos anos e também os amigos do nosso “lab irmão da neuro”, Elisa, Mateus, Luan, Bruna e Isa.

Não poderia deixar de mencionar também os demais professores do departamento de fisiologia, que sempre estiveram de portas abertas para que eu tirasse dúvidas, ou discutisse temas relacionados à universidade pública, desde a graduação. Cito aqui especialmente Gilberto, Silvinha, Fernando, Renata, Zé Gui, Maíra, Michael e Ana, com quem tive muito contato por diferentes projetos e disciplinas. Agradeço também aos membros com quem partilhei meus meses como representante discente da CCP, pois eles me ensinaram muito sobre como acontece a construção de uma universidade pública e acessível para todos.

Também gostaria de estender meus agradecimentos a alguns professores de outros departamentos. Alê, Camila e Dri por me introduzirem a uma nova paixão, a estatística e me darem a oportunidade de vivenciar uma monitoria que foi muito marcante para mim. Agradeço também ao Paulo Inácio e à Rê, por me aceitarem em

uma disciplina como ouvinte que me da caixinha e me ajudou a traçar futuros passos.

Ao longo dos meus anos de mestrado e graduação, participei de muitas atividades e por isso, gostaria de agradecer a todos os professores que partilharam comigo tantas comissões ao longo desses anos e especialmente, a todas as mulheres extraordinárias com quem convivi através do IB Mulheres. Foram muitas as batalhas e sei que juntas - ainda que distantes - estaremos sempre lutando por uma universidade mais justa e igualitária, com a qual sonhamos. Estendo aqui meus agradecimentos à COST, à Atlética, ao CA e à LAAUSP, entidades que me permitiram o privilégio de viver as minhas mais felizes e loucas experiências na universidade, ao lado de pessoas extremamente especiais. Deixo um abraço especial àqueles com quem compartilhei todas essas experiências, elas com certeza tornaram meu mestrado possível e me ajudaram a definir meus sonhos.

Por último e não menos importante, não poderia deixar de lembrar todos aqueles que foram tão presentes nesses anos de pandemia e de desgoverno, pois a pós-graduação pareceu menos impossível graças a eles. Agradeço à minha terapeuta, Gabi, cujo trabalho dedicado possibilitou a finalização desse mestrado ao me ajudar a enfrentar a depressão nos últimos meses. Deixo um agradecimento especial para os meus glebinhos (que são muitos e só por isso não vou listar nomes, mas vocês sabem quem são), amigos com quem partilhei a maior parte do tempo do meu mestrado e que espero continuar partilhando toda a minha vida. Agradeço ainda aos “Piranhas”, aos costinhos e a todas as pessoas especiais trazidas à minha vida pelo Hand Barango, Utsal e Fôlei. Incluo aqui também, toda a minha família querida, a Barbosada, e destaco especialmente meus irmãos, Manu, Maria, vovó Jadir, Helena, Jade e meus padrinhos, tio Wa e tia Giu, que foram as pessoas com quem mais partilhei essa trajetória de mestrado. Amo muito todos vocês e obrigada por tudo!

Índice

Resumo Geral

Ritmos biológicos têm um papel importante nos aspectos fisiológicos e comportamentais dos organismos. A compreensão do ritmo diário de insetos sociais nos permite realizar análises desde o indivíduo até o nível da colônia. O estudo dos ritmos diários das formigas, especialmente formigas cortadeiras, tem sido um desafio devido ao tamanho das colônias e ao polimorfismo. O presente trabalho teve como objetivo entender como as colônias de formigas cortadeiras se comportam coletivamente considerando o corte de folhas e o forrageamento, desenvolvendo uma ferramenta de vídeo-tracking para monitorar o fluxo de formigas. Foram utilizadas câmeras para registrar o forrageamento (vídeo) e como a área foliar diminuiu (fotografias). Uma colônia de *Atta sexdens* foi exposta a um ciclo LD 12:12 (500 lux durante a fase de claridade e luzes vermelhas acesas durante todo o experimento; temperatura = 23ºC, umidade = 60%). O reabastecimento de folhas (*Acalypha sp.*) aconteceu uma vez por dia usando um protocolo aleatório. Registramos a atividade da colônia durante 30 dias, realizando 2 deslocamentos de fase de 6 horas (atraso e avanço). Observamos que embora a colônia esteja ativa durante as fases de claro e escuro, sua atividade é maior durante a fase de escuro, tanto em relação ao forrageamento quanto ao corte de folhas. O software de rastreamento de vídeo desenvolvido neste projeto nos permitiu contar 143904 formigas após analisar 2133 vídeos. Também analisamos a relação entre o número de forrageadoras e as taxas de corte de folha sob condições sincronizadas e dessincronizadas. Concluímos que esta relação pode ser um parâmetro interessante para discutir estratégias de eficiência a nível individual e coletivo após distúrbios na ordem temporal interna da colônia. Também acreditamos que usando os protocolos e software desenvolvidos neste projeto muitos outros aspectos da organização temporal em formigas cortadeiras podem ser identificados, revelando uma compreensão mais profunda de como a ressincronização ocorre após rupturas da ordem temporal interna.

Abstract

Biological rhythms play an essential role in organisms' physiological and behavioral aspects. Understanding daily rhythmicity on social insects allows us to analyze the individual to the colony level. Studying ants' daily rhythms, especially leaf-cutter ants, has been challenging due to the colony sizes and polymorphism. The present work aimed to understand how colonies of leaf-cutter ants rhythmically behaved collectively considering their leaf-cutting and foraging activity, developing a machine learning video tracking tool to analyze the ant's flow. Cameras were used to register the foraging activity (video) and how the leaf area decreased (photographs). An *Atta sexdens* colony was exposed to an LD cycle 12:12 (500 lux during the light phase and red lights on during all the experiments; temperature = 23ºC, humidity = 60%). Leaves replenishments (*Acalypha sp*.) happened using a random protocol once a day. We recorded the colony activity during 30 days, performing two 6 hours phase shifts (delay and advance). We have observed that even though the colony is active during both light and dark phases, the colony activity is higher during the dark phase, regarding both the foraging and the leaf-cutting activities. The video-tracking software developed in this project allowed us to count 143904 ants after analyzing 2133 videos. We have also analyzed the relationship between the number of ants foraging and the leaf-cutting taxes under synchronized and desynchronized conditions. We concluded that this relationship might be a sensitive parameter for discussing efficiency strategies at the individual and collective levels after disturbances on the colony's internal temporal order. We also believe that using the protocols and software developed in this project, many other aspects of temporal organization in leaf-cutter ants may be identified, building a deeper understanding of how resynchronization occurs after disruptions in the internal temporal order.

Chapter 1: General Introduction

BIOLOGICAL RHYTHMS

Throughout evolution, organisms have developed the ability to anticipate cyclic environmental changes in physiology and behavior (Pittendrigh, 1993). The development of endogenous rhythmicity generated by biological oscillators and their ability to synchronize with environmental cycles are significant aspects of this adaptive feature, which increases organisms' fitness (Jabbur *et al*., 2021). In association with the regularity of environmental events, which occurs in the most diverse time scales, several biological rhythms also occur with short duration up to annual scales. In this context, circadian rhythms stand out (with periods near 24h), subject to synchronization by 24h environmental cycles such as light-dark, which synchronize them to daily, 24h rhythms (Aschoff, 1979; Pittendrigh, 1993 and Bloch *et al*., 2013). Such rhythms are observed in all groups of living things, from single-celled to multicellular organisms (Dunlap, 2004). Considering even a longer time scale, it is worth noting the aspects involved with seasonality. Several polar animals, such as Arctic squirrels and birds, exhibit behaviors related to annual synchronization, such as hibernation and migratory behaviors, respectively (Bloch *et al*., 2013). Some rhythms are synchronized by the tidal cycle, lunar cycles, and several others (Refinetti *et al*., 2007; Wagner *et al*., 2007). Given all this, we can verify the extent of these mechanisms of temporal regulation in nature so that the biological temporal organization occurs through different forms, ranging from cellular mechanisms to behavioral ones (Bloch *et al*., 2013).

The organism's daily rhythmicity rhythm observed may result from several synchronizers. Among the most common daily synchronizers, the light-dark (LD) cycle, feeding cycles, and temperature cycles are worth mentioning. There is also, for social animals, the presence of a daily social cycle as a significant synchronizer, which is observed in social insects (Bloch, 2013; Lone and Sharma, 2011), bats

(Marimuthu, 1981; Marimuthu, 1983), and even humans (Roenneberg et al., 2019) - Figure 1.

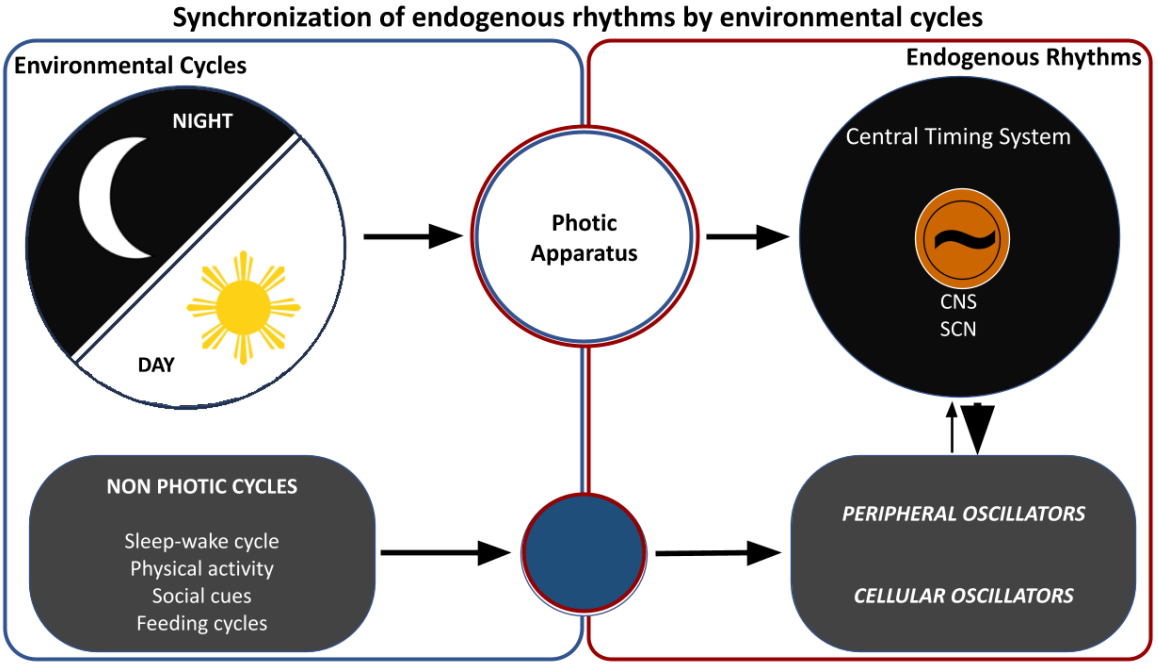


Figure 1) Schematic representation of the synchronization principles of endogenous rhythms by environmental cycles. Environmental cycles are sensed by afferent systems, which transmit timing information to central oscillators that transmit this information to the other peripheral oscillators in the organism, leading to synchronization (adapted from Buttgereit *et al*., 2015).

The observed daily rhythm can be synchronized through two mechanisms: entrainment and masking. These two have great adaptive value since they provide the temporal organization system with precision and flexibility, respectively (Pittendrigh and Daan, 1976; Page, 1989). Entrainment occurs when the oscillator that generates the observed rhythm starts to exhibit the same period as the environmental cycle (which we call *Zeitgeber*). On the other hand, masking is an acute modulation of the amplitude of the rhythm, either by stimulation or by inhibition, without changing the properties (period and phase) of the oscillator that generates the rhythm. Thus, the activity of an organism can have effects of more than one synchronizer and has both anticipatory and direct response aspects to environmental phenomena (Pittendrigh and Daan, 1976; Page, 1989; Tachinardi, 2012). Another

relevant piece of information is that after perturbations on its internal temporal order, such as those due to phase shifts of the LD cycle, an organism takes several days to resynchronize with the new environmental cyclic conditions. This temporary oscillatory state between two synchronized and steady states is called “transients” and reflects the dynamics of the oscillators during the resynchronization processes (Pittendrigh, 1960).

Biological rhythms on social insects

Social insects are groups of insects that organize themselves in colonies, such as termites, bees, wasps, and ants. In these groups, characterized by eusociality (Wilson, 1971), we find a division of reproductive labor so that a caste is capable of reproducing and another infertile. Those systems also show a complex division of labor corresponding to the other tasks of the colony. Thus, the infertile class (workers) is divided into sub-castes with specific functions within the colony, such as foraging, care of the offspring, defense of the colony, garbage removal, and maintenance of the fungus (Wilson, 1980).

A colony of social insects can have hundreds to millions of individuals. It is worth noting that individual survival depends on belonging to the colony, as the colony’s survival relies on the organized functioning of the individuals. Because there are individual and collective aspects, these systems can be considered complex (Boccara, 2003). In multicellular organisms, not all cells are exposed to light-dark cycle cues; however, a group of cells sensitive to photic information can be synchronized and transmit this information to the whole organism, which can then present a fully synchronized rhythm (Figure 1). Social insect colonies often present groups of individuals exposed to photic environmental cues and can inform them to the rest of the colony that lives in constant conditions (Figure 2). That social interaction within the colony also allows its synchronization, similar to what we observe in many multicellular organisms. Social insect colonies allow us to investigate daily synchronization due to environmental cycles (light/dark) and the internal synchronization due to social interactions (Southwick and Moritz, 1987;

Moritz and Sakofski, 1991). In addition, the worker class of a leafcutter ant colony has a high level of genetic similarity, around 75% (Hamilton, 1964). All these features reinforce the idea of ant colonies as “superorganisms,” making them, therefore, excellent objects for studying the processes involved in the emergence of biological rhythms in colonies and possibly even in other organisms. However, the knowledge built about this topic is mainly resulting only from chronobiological studies conducted in bees (Frisch and Koeniger, 1994; Moore, 2001, Bloch *et al*., 2001; Toma *et al*., 2000; Bloch and Robinson, 2001), and few studies have been conducted with ant colonies, and there are still many questions to be explored about their synchronization mechanisms (Figure 2).

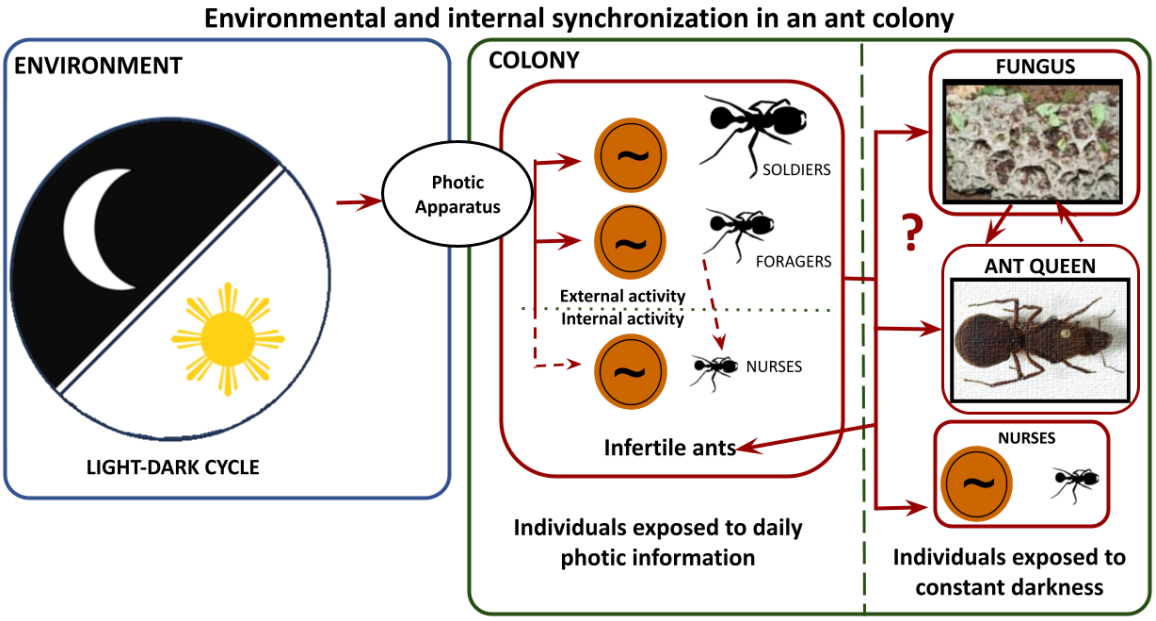


Figure 2) Possible relationships involved in the light-dark cycle synchronization of a leafcutter ant colony. Individual rhythms emerge into rhythms of the subcastes, and these emerge as the colony rhythm. Furthermore, the role of elements such as the fungus and queen in synchronizing the rest of the colony is unknown.

.

Although leafcutter ant societies, such as *Atta*, offer an excellent opportunity to extend comparative studies (McCluskey 1958; 1965; Roces and Nunez 1996), two main aspects make the task of measuring collective behavior potentially with the support of video recordings difficult. The first is a large number of individuals since *Atta* colonies can have up to 8 million individuals. The second is the variety of sizes among ants, since several species show polymorphism (Figure 3), with individuals ranging in size from millimeters to a few centimeters.

Given these challenges, the rare studies of rhythmicity in ants have been carried out exclusively in isolated individuals (Sharma *et al*., 2004) or small groups (McCluskey 1965, 1967), often deprived of the collective context offered by the colony. Such an approach, although practical, ignores one of the fundamental aspects of colony regulation: the existence of the collective and individual organization, inseparable in regulatory processes.

Recent work has explored ant rhythmicity at the molecular level, from clock genes present in individual circadian oscillators located in the brain (Ingram *et al*., 2009, Kay *et al*., 2018). Some studies look at rhythmicity in nurses, showing that the presence/absence of eggs modulates their activity (Fuchikawa *et al.*, 2017). This aspect is fascinating since this sub-caste (and the queen) is not directly exposed to light-dark effects as their activities are restricted to the nest, where there is no light input in a natural environment. In this sense, despite the significant advances made in the study of the spatial organization of ant societies (Clark, 2006), less is known about the temporal organization of their activity and its distinct individual and collective aspects, especially important in these eusocial organisms.

An interesting additional factor in these collective systems is the presence of a symbiont fungus present in some ant groups. The *Neoattini* ants (belonging to the tribe *Attini*), with the genera *Atta* (“saúvas”) and *Acromyrmex* (“quenquéns”), are known as leafcutters and have a high distribution in Neotropical areas. They are characterized by growing a fungus garden, cutting leaves, foraging on a large scale, and colonies that can number in the millions. Particularly, species of the genus *Atta* also exhibit a gradual polymorphism among their subcastes (Weber, 1972 and Wilson, 1980). Thus, larger ants are generally associated with colony defense tasks

and are called soldiers. On the other hand, smaller ants tend to be responsible for tasks internal to the nest, such as bringing water to the colony. Foraging is usually related to the intermediary ants (Wilson, 1980) (Figure 3). It is also worth noting the dynamics of this division of labor among the subcastes, which is not deterministic, and the function of an individual may vary according to the colony's requirements.

Our laboratory also observed that larger and intermediate ants tend to suffer more significant influence from daily environmental cycles (Toledo, 2014) since they perform tasks outside the nest, being more exposed to environmental variations (Bloch *et al.*, 2013). Recent work from our laboratory also shows that leafcutter foragers organize themselves in work shifts, with two distinct states of the colony organization considering the light and darkness phases (Constantino *et al*., 2021).

https://upload.wikimedia.org/wikipedia/commons/thumb/7/7d/Atta.cephalotes.gamut.selection.jpg/220px-Atta.cephalotes.gamut.selection.jpg

Figure 3) Polymorphism in *Atta*. The figure shows the gradual variation in size between the castes. The two most prominent individuals comprise the reproductive caste, representing a queen and a virgin queen. The others correspond to the distinct sub-castes within the workers.

As mentioned, one of the main challenges in dealing with the individuals of a vast colony with a great morphometric variety are methodological aspects. In order to observe the ants in these conditions, computational techniques need to be created, especially considering the number of ants that had to be evaluated (15.000). As discussed later, a method based on machine learning techniques to evaluate large numbers of ants was developed.

A LOOK INTO PRODUCTIVITY, ENERGETIC EFFICIENCY, AND BEHAVIOR

Social insects' literature comprehends a significant number of uses of the terms "productivity" and "efficiency." Sometimes they are used almost as equivalents, but through a more profound look across the decades, we can observe some changes in the guiding ideas that drove the discussion of productivity or efficiency in social insects. In this sense, discussing "productivity" within a social context is not trivial since it can take into account individual aspects (productivity per capita) as well as global aspects of the colony (colony productivity). The productivity of a social insect colony should be thought of within an evolutionary context (Montagna *et al*., 2010 and Naug and Wenzel, 2006). Keeping in mind the idea of inclusive fitness (or kin selection) within social insects (Hamilton, 1964), the ability to obtain food for the colony should be a key component in determining its productivity (Naug and Wenzel, 2006). Given this, foraging becomes an essential process, as larger foraging areas should imply a greater number of food sources available to the colony (Mailleux *et al*., 2003, Montagna *et al*., 2010 and Naug and Wenzel, 2006). The number of individuals recruited to forage is directly related to the colony's foraging area, so the greater the number of foragers, the greater the total area foraged by the colony (Mailleux *et al*., 2003) Naug, and Wenzel, 2006).

In this way, we have an increased probability of success in finding new food sources while also increasing the number of ants that cannot find new food sources (Mailleux *et al*., 2003 and Naug, and Wenzel, 2006). Thus, we get two essential concepts for thinking about aspects of colony productivity. Firstly, we have an idea of

“Colony Productivity,” which can be inferred through parameters such as the total food obtained by the colony, the total number of individuals recruited for foraging activity, in addition to aspects related to colony size, and consequently reproductive parameters associated with the queen, with their particularities within social insect species (Michener, 1964, Mattila *et al*., 2012, Montagna *et al*., 2010 and Naug and Wenzel, 2006).

Secondly, we have the concept of “Productivity per capita,” which aims to understand how individual components act within the overall colony context (Modlmeier, 2013). Productivity per capita can be assessed through the efficiency of individual transportation or even the time an individual uses to perform the task (Naug and Wenzel, 2006). The big question associated with these two concepts, brought about by Michener’s paradox, consists in the fact that increasing the number of foragers is able to increase the overall productivity of the colony, even if the per capita productivity suffers a reduction (Michener, 1964 and Naug and Wenzel, 2006).

Similar to what we observe with the term “productivity,” “efficiency” can also refer to many different aspects of social insects’ literature. Efficiency can refer to reproductive efficiency, considering how many individuals can be generated per reproductive female and comparing colony sizes (Connell, 1961; Michener, 1964). Meanwhile, more recent studies focus the “efficiency” discussion in social insects on aspects related to task efficiency (Dussutour *et al*., 2007; Bernadou *et al*., 2016; Bouchebti *et al*., 2015; Yerushalmi *et al*., 2006; de Toledo *et al*., 2016). While the first relied on the idea that colonies with higher numbers of individuals were more efficient, task efficiency refers to workers’ individual performance, similar to what we observe in the idea of productivity per capita. For example, if a forager cuts and carries more leaves than others, it could be considered more efficient, assuming an isolated parameter (leaves cut per ant). However, it is possible to add more variables considering task performance: ant sizes, speed, distance to the food source, type of source, and time are some of the aspects that can increase the complexity of discussing foraging efficiency. All of those are deeply related to the energetic aspects involving foraging activity. Despite the intrinsic difficulty of defining efficiency, considering that behavior change over time can alter the strategy for obtaining resources in a way that can be observed and described (based on measurable

inputs and outputs), it is possible to assume that efficiency has changed being increased or decreased.

Although this idea considering social insects contains several particular features, as discussed above, the main discussion about energetic efficiency is similar to many other species. An organism homeostatic balanced spends less energy to maintain all its activities than their energetic supplies available and, thanks to this optimal relationship, increases its adaptiveness and its fitness (Stewart, 2007). Nevertheless, we are aware that this optimal relationship is constantly being threatened as natural environments offer a variety of challenges to the organism's survival. Thus, all these aspects also increase the complexity of discussing energetic efficiency since physiological mechanisms and distinct behaviors might offer different advantages in different contexts.

Behavioral plasticity

It is possible to argue that organisms' behavior can be resulted from different strategies to predict and respond to environmental stimuli. In this sense, the ability to anticipate cyclic environmental changes relates to an endogenous component and rhythmic behaviors, while responding to specific cues can be related to an exogenous component, which can involve an acute or even an anticipatory response based on previous experiences, mediated by the cognitive system.

A great yet curious example of these ideas is the octopus *Abdopus aculeatus*. Its habitat involves rhythmic challenges, as it faces diurnal changes of tides. Predators are more present during the high tidal level. During the low tide, they search for food sources (primarily crustaceans) in the intertidal reef, which usually requires several types of locomotion, as these marine animals move from pool to pool walking on land (Huffard, 2005, 2006, 2007). The behavioral set of these animals is vast, as they can quickly adapt from aquatic to aerial environments, besides the constant state of alertness to promote escape from predators. Considering all these challenges, they chose different locomotion strategies that vary from aerobic to anaerobic and have different energetic costs, according to their necessities (Huffard, 2005). Escaping from a predator or attacking a prey usually

involves locomotion strategies that require much energy; however, success in these tasks is mandatory for the survival of these octopuses (Huffard, 2005).

Considering this example, we observe the high level of importance to combine rhythmic anticipatory behaviors (endogenous component) and fine-tuning behaviors resulting from the cognitive system (exogenous component). The optimal combination of these physiological components is crucial to an organism's survival, increases its fitness and adaptiveness, and is deeply related to the dynamic variations of behaviors and their specific energetic requirements. The present work aims to discuss the combination of these physiological components and the dynamic strategies involved in the efficiency of a leafcutter ant colony when facing temporal disruption on its internal temporal order and random feeding events.

MACHINE LEARNING TECHNIQUES APPLIED TO BEHAVIORAL RESEARCH

Machine learning is defined as the study of algorithms that allow computers to perform tasks automatically and improve their performance thanks to previous data. Machine learning uses neural networks and is the basis of artificial intelligence (Naqa and Murphy, 2015; Zhang, 2017). The modern and globalized world uses machine learning techniques and artificial intelligence (AI) to perform several activities, from social media on our cellphones to defining public policies based on big data. Whether we understand it or are aware of this presence, it is everywhere.

Machine learning uses input data to extract features that can "learn" and perform a task based on this learning. Machine learning can be divided into two main groups: shallow and deep learning (Figure 4). The main difference between shallow and deep learning refers to feature extraction. While deep learning involves a more complex architecture and an automatic selection of features, in shallow learning, humans select the features that will be used for training the models. In this sense, deep learning offers an important tool to solve and deal with many biological questions since variability is an intrinsic component of biological data. Selecting the

best features for model training may be challenging when researchers do not have enough knowledge about their system of study.

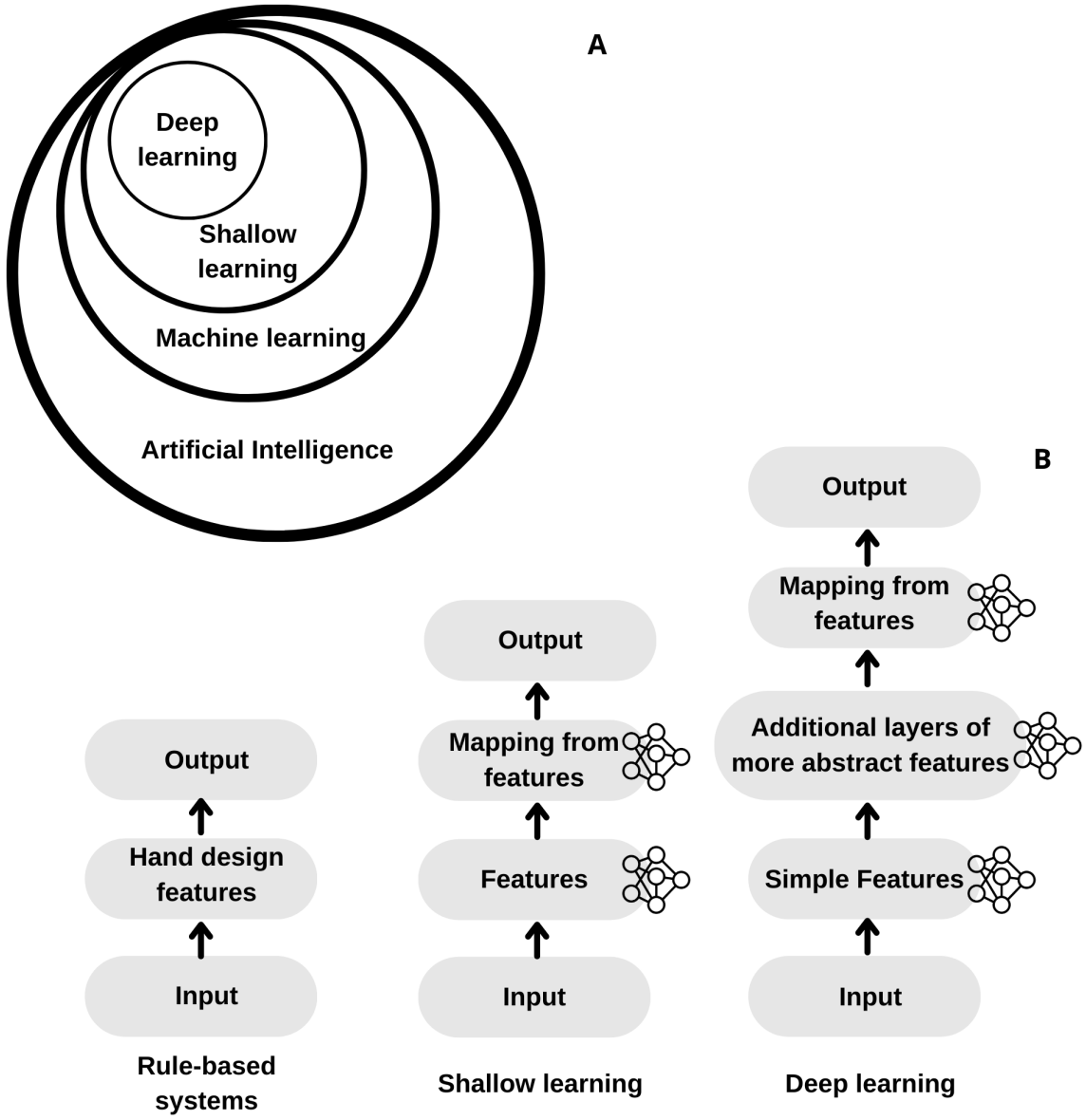


Figure 4) Schematic representation showing machine learning structure. Venn diagram of the relationship between different components of Artificial Intelligence. Flow chart of different AI parts used in machine learning. Small network symbols indicate components that can learn from data (adapted from Goodfellow et al., 2016).

OBJECTIVES

1. Developing a video-tracking software able to analyze the foraging behavior of a leaf-cutter ant colony inside the laboratory.
2. Applying this video-tracking tool to a synchronization experiment using a leaf-cutter ant colony. We also aim to monitor the daily leaf consumption and leaf-cutting rhythm in the same experiment.

Objective 1 is treated briefly below (for more detailed information, please check the Tutorial present in the Annexes section). Objective 2 is the central theme discussed in Chapter 2 of this manuscript.

DEVELOPMENT OF AN OBJECT DETECTION AND COUNTING TOOL FOR ANALYZING ANT'S FORAGING ACTIVITY

Our group has been developing tools for analyzing ant foraging trails through video-tracking, allowing size discrimination, and monitoring a population of about 10-15 thousand individuals. This software uses machine learning and deep learning techniques to automate the counting of high flows of ants on foraging trails, being an essential tool for studies with this type of system, enabling deeper investigation of temporal aspects at the colony level (Toledo, 2014 and 2018). For developing our final video-tracking tool, many steps were involved in the machine learning process (Figure 5).

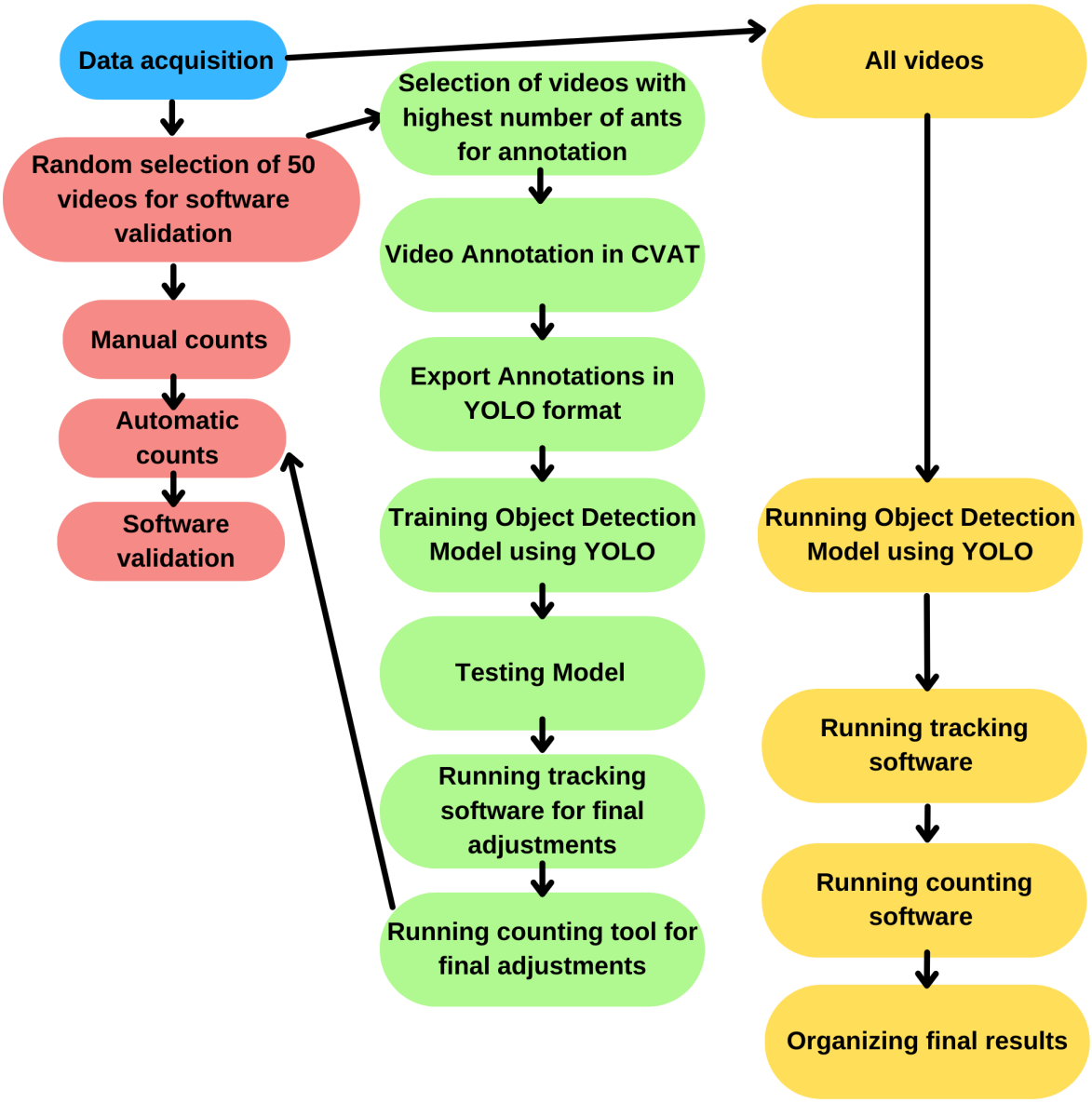
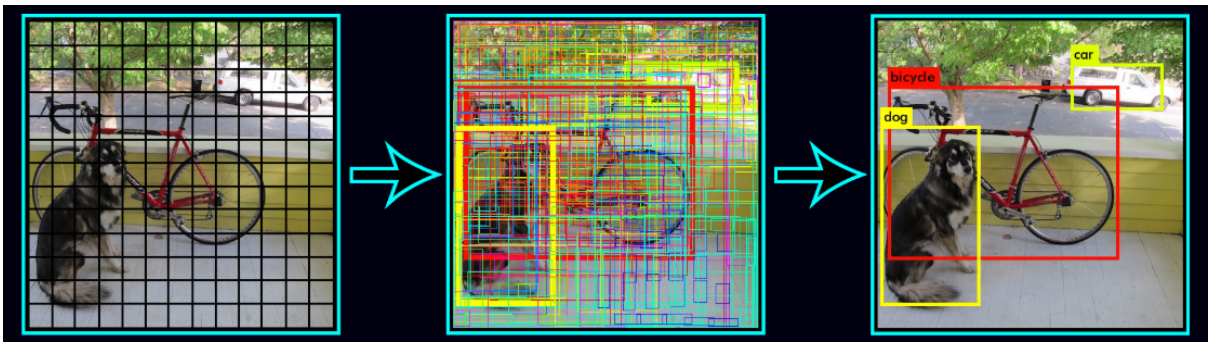


Figure 5) Flowchart of all the steps involved in our video-tracking software development. Blue box involves data acquisition (experiments). Red boxes show all steps involved in the software validation stage. Green and yellow boxes show the deep learning train model stage and run our model results for all our data.

The main challenges regarding using this type of tool involve object detection. In this case, it is necessary to make the computer recognize the object (ant) and follow it throughout the video, accounting for it in the flow counts. However, the foraging trail is a complex environment that involves different elements, albeit in the laboratory context. We have, for example, ants of various sizes and functions, such

as those seeking or carrying leaves, as well as those removing obstacles ("waste") from the trail itself. In addition, the flow of ants can be very high at certain times, especially when the colony is feeding and during the night. Thus, this software needs to be trained to recognize the ants in different frame contexts in the video image. It is precisely in developing this kind of tool that part of the present work took place, making use of powerful object detection techniques such as YOLOv2 (You Only Look Once - versão 2, Redmon *et al.*, 2016) and YOLOv5 (You Only Look Once - version 5, Zhu *et al*., 2021). When detecting objects in a video, this technique observes each frame once and calculates the probability that each object found in the frame belongs to a previously trained class (Figure 6). This way allows the analysis of a large amount of video data, being an important tool used, for example, for monitoring public security cameras.



https://towardsdatascience.com/implementing-yolo-on-a-custom-dataset-20101473ce53

Figure 6: Frame-by-frame object detection mechanism realized by YOLO.

During this project, we developed two versions of this software, and they required several complex steps which are detailed and organized in our supplementary material. The first version used YOLOv2 and counted ants in our pilot experiment with *Acromyrmex* ants (Figure 7). However, in our main experiment using *Atta* ants, the number of ants in the trail became extraordinarily high, reaching hundreds of ants per 2 minutes video. Since our first version was powerful enough to detect and count until 50 ants per video, and the software error increased the higher the number of ants per video, we decided to develop a second version of the

software using one of the most powerful and recent object detection techniques ever developed, which was YOLOv5 (Zhu *et al*., 2021; Fang *et al.,* 2021).

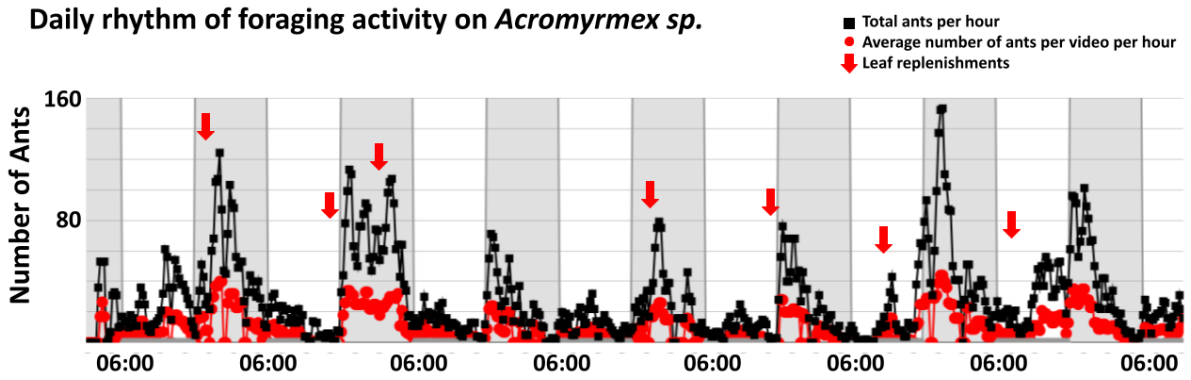


Figure 7: Daily rhythm of foraging. Recordings of 8 days of activity of a colony of Acromyrmex sp. exposed to a 12:12 LD cycle (lights on at 06:00h). Humidity was kept at 60% and room temperature at 23ºC. Leaf replenishments occurred randomly once a day.

YOLOv5 provided us with great results, as we were able to count over 143.000 ants during the main experiment. Nevertheless, it is essential to acknowledge that developing a second version of the software in such a shorter time (we developed version 1 during a year and version 2 in 4 months) and obtaining these results was only possible due to a recently open-source project for data annotation, called Computer Vision Annotation Tool (CVAT - Intel corporation). CVAT enables video annotation using a tracking mode function and online annotation that can be accessed from different devices simultaneously. Our software was developed using data from our video experiments. A precise object annotation in each image plays a vital role in software performance. Poor data annotation makes machine learning models decrease their accuracy, and it may be computationally hard to boost their performance with optimization tools.

We emphasize the importance of data selection for the annotation step, as it is important for biologists to select data that represents our object of study satisfactorily. In our first version of the software, we faced two issues. First, we transformed all our data in binary and ran algorithms that extracted 10.000 frames (the minimum required to train an object detection model) according to the probability of containing objects that could be identified as ants. Our pilot experiment did not

contain a high number of ants per video (only 16% of the videos contained more than 20 ants), but also many video frames did not contain any objects (27% of the videos had 0 ants). Thus, a significant number of frames extracted by the algorithm had no ants. That was a second issue because a lot of the images used to train the YOLOv2 model could not provide a higher pool of ant annotations, making the model accuracy smaller. When we developed the second version of the software using YOLOv5 and CVAT, we selected a pool of videos with a high number of ants from both light and dark phases. Since we knew how computationally powerful YOLOv5 was, we trusted it would solve videos with smaller numbers of ants and trained our model on the worst-case scenario: hundreds of ants per video. We chose to share this information here because we believe that many ethological researchers using video-tracking software may not understand the importance of clear image data (with high contrast between image background and objects and no unnecessary objects in the video), as machines are not able to solve issues that are easily detected by the expert human eye. Also, we believe sharing this experience may help programmers who collaborate with biologists to develop tools to solve challenging experimental questions such as ours. In our experience, YOLOv2 allowed us to count 5052 *Acromyrmex* ants in our pilot experiment (1152 minutes of video), while YOLOv5 allowed us to count 143.904 *Atta* ants in our final experiment (4320 minutes of video recordings).

REFERENCES

Aschoff, J. (1979). **Circadian rhythms: influences of internal and external factors on the period measured in constant conditions.** Zeitschrift fur Tierpsychology. 49:225–**249.**

Boccara, N. (2003). **Modeling Complex Systems (Graduate Texts in Contemporary Physics).** http://www.amazon.com/exec/obidos/redirect?tag=citeulike07-20&path=ASIN/0387404627

Bouchebti, S., Ferrere, S., Vittori, K., Latil, G., Dussutour, A., and Fourcassié, V. (2015). **Contact rate modulates foraging efficiency in leaf-cutting ants.** Nature Publishing Group, 1–5. https://doi.org/10.1038/srep18650

Bernadou, A., Felden, A., Moreau, M., Moretto, P., and Fourcassié, V. (2016). **Ergonomics of load transport in the seed harvesting ant Messor barbarus : morphology influences transportation method and efficiency.** 2920–2927. https://doi.org/10.1242/jeb.141556

Bloch, G., Robinson, G.E., (2001). **Chronobiology: Reversal of honeybee behavioural rhythms.** Nature. 410, 1048.

Bloch, G., Toma, D.P., Robinson, G.E. (2001) **Behavioural rhythmicity, age, division of labor, and period expression in the honeybee brain.** Journal of Biological Rhythms 16:444–456.

Bloch, G., Barnes, B. M., Gerkema, M. P., & Helm, B. (2013). **Animal activity around the clock with no overt circadian rhythms: Patterns, mechanisms and adaptive value.** Proceedings of the Royal Society B: Biological Sciences, 280(1765).

Bloch, G., Herzog, E. D., Levine, J. D., & Schwartz, W. J. (2013). **Socially synchronized circadian oscillators.** Proceedings of the Royal Society B: Biological Sciences, 280(1765). https://doi.org/10.1098/rspb.2013.0035

Connell, J. H. (1961). **The Influence of Interspecific Competition and Other Factors on the Distribution of the Barnacle *Chthamalus Stellatus*.** Ecology, 42(4), 710–723. https://doi.org/10.2307/1933500

Clark, E. (2006). **Dynamic matching of forager size to resources in the continuously polymorphic leaf - cutter and, *Atta colombica.*** Animal Cognition 12: 21-30.

Dunlap, J.C., Loros, J.J., DeCoursey, P.J. (2004). **Chronobiology: Biological Timekeeping.** Sinauer, Massachusetts, USA.

Dussutour, A., Beshers, S., Deneubourg, J., and Fourcassié, V. (2007).**Crowding increases foraging efficiency in the leaf-cutting ant Atta colombica.** Insectes Sociaux 54, 158–165. https://doi.org/10.1007/s00040-007-0926-9

Fang, Y., Guo, X., Chen, K., Zhou, Z., & Ye, Q. (2021). **Accurate and automated detection of surface knots on sawn timbers using YOLO-V5 model.** *BioResources*, *16*(3), 5390–5406. https://doi.org/10.15376/biores.16.3.5390-5406

Frisch, B., Koeniger, N. (1994). **Social synchronization of the activity rhythms of honey bees within a colony.** Behavioral Ecology and Sociobiology. 35:91–98.

Fuchikawa, T., Fujioka, H., Shimada, M., Tsuji, K., Okada, Y., and Abe, M. S. (2017). **Ant circadian activity associated with brood care type.** Biology Letters, 13(2), 20160743. https://doi.org/10.1098/rsbl.2016.0743

Goodfellow, I. J., Bengio, Y., Courville, A. (2016). **Deep learning.** MIT Press. www.deeplearningbook.org

Hamilton, W. 1964. **The genetical evolution of social behaviour.** I. J Theor Biol 7:1–6.

Huffard, C. (2005). **The Behavioral Ecology and Locomotion of *Abdopus aculeatus* (d'Orbigny, 1834).** December 2005. https://doi.org/10.13140/RG.2.1.3489.8960

Huffard, C. L. (2006). **Locomotion by *Abdopus aculeatus* (Cephalopoda: Octopodidae): Walking the line between primary and secondary defenses.** Journal of Experimental Biology, 209(19), 3697–3707. https://doi.org/10.1242/jeb.02435

Huffard, C. L. (2007). **Ethogram of *Abdopus aculeatus* (d'Orbigny, 1834) (Cephalopoda: Octopodidae): Can behavioural characters inform octopodid taxomony and systematics?** Journal of Molluscan Studies, 73(2), 185–193. https://doi.org/10.1093/mollus/eym015

Ingram, K.K., Krummey, S., LeRoux, M. (2009) **Expression patterns of a circadian clock gene are associated with age-related polyethism in harvester ants, *Pogonomyrmex occidentalis*.** BMC Ecology 9:7.

Jabbur, M.L., Zhao, C., Johnson, C. H. (2021) Insights into the Evolution of Circadian Clocks Gleaned from Bacteria. **Circadian Rhythms in Bacteria and Microbiomes.** https://doi.org/10.1007/978-3-030-72158-9

Kay, J., Menegazzi, P., Mildner, S., Roces, F., and Helfrich-Förster, C. (2018). **The Circadian Clock of the Ant *Camponotus floridanus* Is Localized in Dorsal and Lateral Neurons of the Brain.** Journal of Biological Rhythms, 33(3), 255–271.

Lone, S. R., and Sharma, V. K. (2011). **Timekeeping through social contacts: Social synchronization of circadian locomotor activity rhythm in the carpenter**

ant *Camponotus paria*. Chronobiology International, 28(10), 862–872. https://doi.org/10.3109/07420528.2011.622676

Marimuthu, G., Rajan, S., and Chandrashekaran, M. K. (1981). **Social entrainment of the circadian rhythm in the flight activity of the microchiropteran bat *Hipposideros speoris.*** Behavioral Ecology and Sociobiology, 8(2), 147–150. https://doi.org/10.1007/BF00300827

Marimuthu, G., and Chandrashekaran, M. K. (1983). **Continuous light inside a cave abolishes the social synchronization of the circadian rhythm in a bat.** Behavioral Ecology and Sociobiology, 12(4), 321–323. https://doi.org/10.1007/BF00302900

McCluskey, E.S. (1958) **Daily rhythms in Male Harvester and argentine ants.** Science 128: 536-537.

McCluskey, E.S. (1965) **Circadian rhythms in male ants of five diverse species.** Science 150: 1037-1039.

McCluskey, E.S. (1967) **Circadian rhythms in female ants, and loss after mating flight.** Comparative. Biochemistry and Physiology 23:665-677.

Michener, C.D. (1964) **Reproductive efficiency in relation to colony size in hymenopterous societies**. Insectes Soc 4:317–342

Moore, D., Siegfried, D., Wilson, R., and Rankin, M. A. (1989). **The Influence of Time of Day on the Foraging Behavior of the Honeybee, *Apis mellifera*.** Journal of Biological Rhythms, 4(3), 305–325.

Moore, D. (2001). **Honey bee circadian clocks: behavioral control from individual workers to whole colony rhythms.** Journal Insect Physiology 47:843–857.

Moritz, R.F.A., Sakofski, F., 1991. **The role of the queen in circadian rhythms of honeybees (*Apis mellifera* L.).** Behavioral Ecology and Sociobiology. 29, 361–365.

Naqa, I. El, & Murphy, M. J. (2015). **Machine Learning in Radiation Oncology.** Machine Learning in Radiation Oncology, 3–11. https://doi.org/10.1007/978-3-319-18305-3

Naug, D., and Wenzel, J. (2006). **Constraints on foraging success due to resource ecology limit colony productivity in social insects.** Behavioral Ecology and Sociobiology, 60(1), 62–68. https://doi.org/10.1007/s00265-005-0141-5

Page, T.L. (1989) **Masking in invertebrates.** Chronobiology International 6 (1):3-11
Pittendrigh CS, Daan S (1976a) **A functional analysis of circadian pacemakers in nocturnal rodents. IV.** Entrainment: pacemaker as clock. Journal of Comparative Physiology A 106:291 -331

Pittendrigh, C.S. (1960). **Circadian rhythms and the circadian organization of living systems.** Cold Spring Harbor Symposia on Quantitative Biology, *25*, 159–184. https://doi.org/10.1101/SQB.1960.025.01.015

Pittendrigh, C.S. and Daan, S. (1976b) **A functional analysis of circadian pacemakers in nocturnal rodents: V.** Pacemaker structure: A clock for all seasons. Journal of Comparative Physiology A 106 (3):333-355.

Pittendrigh, C.S. (1993). **Temporal organization: reflections of a Darwinian clock-watcher.** Annual Review of Physiology. 55:17–54.

Refinetti, R., Lissen, G.C., Halberg, F. (2007). **Procedures for numerical analysis of circadian rhythms.** Biological Rhythm Research. 38(4): 275–325

Redmon, J., Divvala, S., Girshick, R., and Farhadi, A. (2016). **You only look once: Unified, real-time object detection.** Proceedings of the IEEE Computer Society Conference on Computer Vision and Pattern Recognition, 2016–December, 779–788.

Roces, F., Nunez, J.A. (1996) **A circadian rhythm of thermal preference in the ant *Camponotus mus*: masking and entrainment by temperature cycles.** Physiological Entomology. 21:138-142

Roenneberg, T., Pilz, L. K., Zerbini, G., & Winnebeck, E. C. (2019). **Chronotype and Social Jetlag - A (self-) critical review.** Preprints, 2019050092, 1–19. https://doi.org/10.20944/preprints201905.0092.v1

Schindelin, J., Arganda-Carreras, I., Frise, E. (2012). **Fiji: an open-source platform for biological-image analysis.** Nature Methods 9(7):676-682.

Sharma, V.K., Lone, S.R., Goel, A., Chandrashekaran, M.K. (2004a) **Possible evidence for shift work schedules in the media workers of the ant species *Camponotus compressus*.** Chronobiology International 21:297–308.

Sharma, V.K., Lone, S.R., Goel, A., Chandrashekaran, M.K. (2004b) **Circadian consequences of social organization in the ant species *Camponotus compressus*.** Naturwissenschaften 91:386–390.

Southwick, E.E., Moritz, R.F.A.. (1987). **Social synchronization of circadian rhythms of metabolism in honeybees (*Apis mellifera*).** Physiological Entomology 12:209–221.

Stewart, J. M. (2007). **Functional metabolism: Regulation and adaptation. In Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology (Vol. 148, Issue 1).** https://doi.org/10.1016/j.cbpb.2007.07.001

Stokkan, K.-A. (2001). **Entrainment of the Circadian Clock in the Liver by Feeding.** Science, 291(5503), 490–493. https://doi.org/10.1126/science.291.5503.490

Tachinardi, P. (2012). **Ritmo circadiano de temperatura corporal no tuco-tuco (Ctenomys aff. knighti), um roedor subterrâneo sul-americano.**. [https://doi.org/10.11606/D.41.2012.tde-11102012-111208].São Paulo: Instituto de Biociências, Universidade de São Paulo. Dissertação de Mestrado em Fisiologia Geral.

Toledo, M. A. (2014). **Aspectos Temporais da organização coletiva do forrageamento em formigas saúvas (*Atta sexdens rubropilosa*).** São Paulo: Instituto de Biociências, Universidade de São Paulo. Dissertação de Mestrado em Fisiologia Geral.

Toledo, M. A., Ribeiro, P. L., Carrossoni, P. S. F., Tomotani, J. V., Hoffman, A. N., Klebaner, D., Helene, A. F. (2016). **Two castes sizes of leafcutter ants in task partitioning in foraging activity.** Ciencia Rural, 46(11), 1902–1908. https://doi.org/10.1590/0103-8478cr20151491

Toledo, M.A.F. (2018). **Orientação espacial e comportamento coletivo em formigas saúvas.** [doi:10.11606/T.41.2018.tde-20092018-110950]. São Paulo: Instituto de Biociências, Universidade de São Paulo. Tese de Doutorado em Fisiologia Geral.

Toma, D.P., Bloch, G., Moore, D., Robinson, G.E. (2000). **Changes in period mRNA levels in the brain and division of labor in honeybee colonies.** Proceedings of the National Academy of Sciences of the United States of America 97:6914–6919.

Wagner, H. J., Kemp, K., Mattheus, U., and Priede, I. G. (2007). **Rhythms at the bottom of the deep sea: Cyclic current flow changes and melatonin patterns in two species of demersal fish.** Deep-Sea Research Part I: Oceanographic Research Papers, 54(11), 1944–1956. https://doi.org/10.1016/j.dsr.2007.08.005

Weber, N.A. (1972). **The Fungus-Culturing Behavior of Ants.** American Zoologist, v. 12, n. 3, p. 577-587.

Wilson, E.O. (1971). **The insects societies.** [S.l]: Cambridge, Massachusetts, USA, Harvard University Press [Distributed by Oxford University Press].

Wilson, E.O. (1980). **Caste and Division of Labor in Leaf-Cutter Ants (Hymenoptera: *Formicidae: Atta*) II. Ther Ergonomic Optimization of Leaf Cutting.** Behavioral Ecology and Sociobiology, v. 7, n. 2, p. 143-156.

Yerushalmi, S., Bodenhaimer, S., and Bloch, G. (2006). **Developmentally determined attenuation in circadian rhythms links chronobiology to social organization in bees.** Journal of Experimental Biology, 209(6), 1044–1051. https://doi.org/10.1242/jeb.02125

Zhang, X.-D. (2017). **Chapter 6 Machine Learning.** In *A Matrix Algebra Approach to Artificial Intelligence* (Vol. 45, Issue 13). https://books.google.ca/books?id=EoYBngEACAAJ&dq=mitchell+machine+learning+1997&hl=en&sa=X&ved=0ahUKEwiomdqfj8TkAhWGslkKHRCbAtoQ6AEIKjAA

Zhu, X., Lyu, S., Wang, X., and Zhao, Q. (2021). ***TPH-YOLOv5: Improved YOLOv5 Based on Transformer Prediction Head for Object Detection on Drone-captured Scenarios*.** 2778–2788. https://doi.org/10.1109/iccvw54120.2021.00312

CHAPTER 2 - Unpublished Manuscript

Effects of disturbances on the internal temporal order of a leafcutter ant colony: daily rhythms of foraging and leaf-cutting activities

ABSTRACT

Biological rhythms are an essential component of organisms' physiological and behavioral aspects. On social insects, biological rhythms can be studied considering the individual and the collective level. Studying ants' daily rhythms, especially on leafcutter ants, has been challenging due to the colony sizes and polymorphism. The present work aimed to understand how colonies of leafcutter ants rhythmically behaved collectively considering their leaf-cutting and foraging activity, developing a machine learning video tracking tool to analyze the ant's flow. We also aimed to understand how disturbances on the internal temporal order of the colony affected foraging, leaf-cutting activities, and daily leaf intake. We recorded the colony activity during 30 days, performing two 6 hours phase shifts (delay and advance). Although the colony is active throughout the day, the colony activity is higher during the dark phase. Our video tracking software allowed us to count 143904 ants (4320 minutes of recording). Feeding events (leaf replenishments) show an acute effect on both leaf-cutting and foraging rhythms. Daily leaf intake was higher after the phase shifts. We have also analyzed the relationship between the number of ants foraging and the leaf-cutting taxes under synchronized and desynchronized conditions. This relationship might be a sensible parameter to discuss efficiency strategies at the individual and collective levels after disturbances on the colony's internal temporal order. Many questions remain unanswered about how synchronization arises from the individual to the colony level; however, we believe that the techniques developed in our work could be used to further studies regarding biological rhythms on ant colonies.

INTRODUCTION

Colonies of social insects are interesting for circadian rhythm studies due to the opportunity of investigating the roles played by external

environmental cycles and internal social interactions (Southwick and Moritz, 1987; Moritz and Sakofski, 1991) in their daily synchronization. Circadian rhythmicity can be identified at multiple levels, from individual behavior and physiology (North, 1987; Moore 2001), through caste-related group activities (Crailsheim *et al*., 1996; Bloch *et al.*, 2001; Moore *et al.*, 1989; Oda *et al*., 2007) and up to the emergent rhythm of the whole colony (Moritz and Sakofski, 1991; Frisch and Koeniger, 1994). Despite the variety of eusocial insect species, such as ants, wasps, termites, and bees, most investigations have been developed in social bee colonies (Toma *et al*., 2000; Bloch and Robinson, 2001).

Ant societies offer an excellent counterpart for comparative studies (McCluskey, 1958; 1965; North, 1993; Roces and Nunez, 1996; Sharma *et al*., 2004), but the much larger number and smaller size of individuals make it difficult to follow individual activities within a colony throughout the day continuously. Rhythmicity in ants has in most cases been measured either in individuals (North 1987; 1993; Sharma et al., 2004a;2004b) or small groups (McCluskey 1965, 1967) isolated from the colony context. Recent works have explored ant rhythmicity at the molecular level, focusing on clock genes in individual circadian oscillators located in the brain, in *Camponotus floridanus* (Kay *et al*., 2018; Das and de Bekker, 2021) and *Pogonomyrmex* ants (Ingram *et al.*, 2009 and 2016). Regarding the temporal organization within ant castes, some studies show that the presence/absence of eggs can modify the rhythmicity of *Diacamma* nurses (Fuchikawa *et al*., 2017), and *Atta sexdens* foragers seem to organize themselves in work-shifts (Constantino *et al*., 2021).

To investigate rhythmicity at the colony level, we developed an automated video-tracking software based on machine learning techniques that enabled monitoring the ant trail activity in laboratory conditions. We used this system to track rhythmicity within a forager trail displayed by a colony of South American ant *Atta sexdens.* The colony context was thus maintained in the laboratory under artificial light/dark cycles and constant temperature. As a first step, we characterized the daily activity rhythm of the forager group along

a trail that emerged between the colony and a food source. We simultaneously monitored the daily rhythm of leaf-cutting activity of the same colony. Forager ants responded to leaf replenishments with increases in acute activity, and we verified that these acute responses varied throughout day and night. Then we proceeded to analyze the hypothesis that a temporal shift of the light/dark cycle would perturb the internal temporal order of the whole colony by verifying its effects on the rhythms under synchronization and during transient states (temporary oscillatory states between two steady states, usually the first days after phase shifts) after perturbation. Our results indicate the potential of this procedure for future studies aiming at uncovering the fine networks of internal synchronization within the colony.

METHODS AND MATERIALS

Colony, food intake, and room conditions

An *Atta sexdens* colony (10.000-15.000 individuals) older than 3 years, raised in the bioterium of the Institute of Biosciences USP (São Paulo, Brazil), was used in this work. The colony was maintained in the center box of a maze with three arms respectively connected to a food, a waste, and an empty box (Figure 1A) under an LD 12:12 cycle (lights on at 06:00 hours; L= 500 lux) and controlled temperature (23°C) and humidity (60%) maintained by two humidifiers and one air conditioner (monitored with the use of a thermigometer). Red lights were on during the whole experiment.

The colony was fed with *Acalypha sp*. leaves once a day (approximately 750 cm^2), according to a semi-random protocol that distributed feeding times along light and darkness hours, as shown in Figure 1B. Each replacement schedule results from a time selection conducted in two stages and occurs both during the acclimatization and the experiment days. In the first stage, we randomly select whether feeding would take place during the morning (06:00-11:00 h), afternoon (12:00-17:00 h), evening (18:00-23:00 h),

or early morning (00:00-05:00 h). In the second stage, we randomly select which of the 6 hours of each interval the replenishment may occur. The leaves were arranged side by side inside the food box without overlapping (Figure 2A). Before each feeding event, the remaining leaves from the last replenishment were removed. During the experiment, the colony never completely consumed the whole amount of the *Acalypha sp.* leaves available. The fungus size was steady during the whole procedure.

Experimental Protocol

The colony was maintained in the maze for 15 days under the initial LD cycle for acclimatization before recording. The colony activity was recorded under the initial LD cycle for 10 days (Stage I - lights on at 06:00h). The LD cycle 6h phase delay was applied in Stage II by a 6h lengthening of the light phase on day 11 (lights on at 12:00h). This new stage was recorded for 10 days. Finally, the LD cycle 6h phase advance was applied in Stage III by shortening the light phase of the LD cycle on day 21 (Figure 1C).

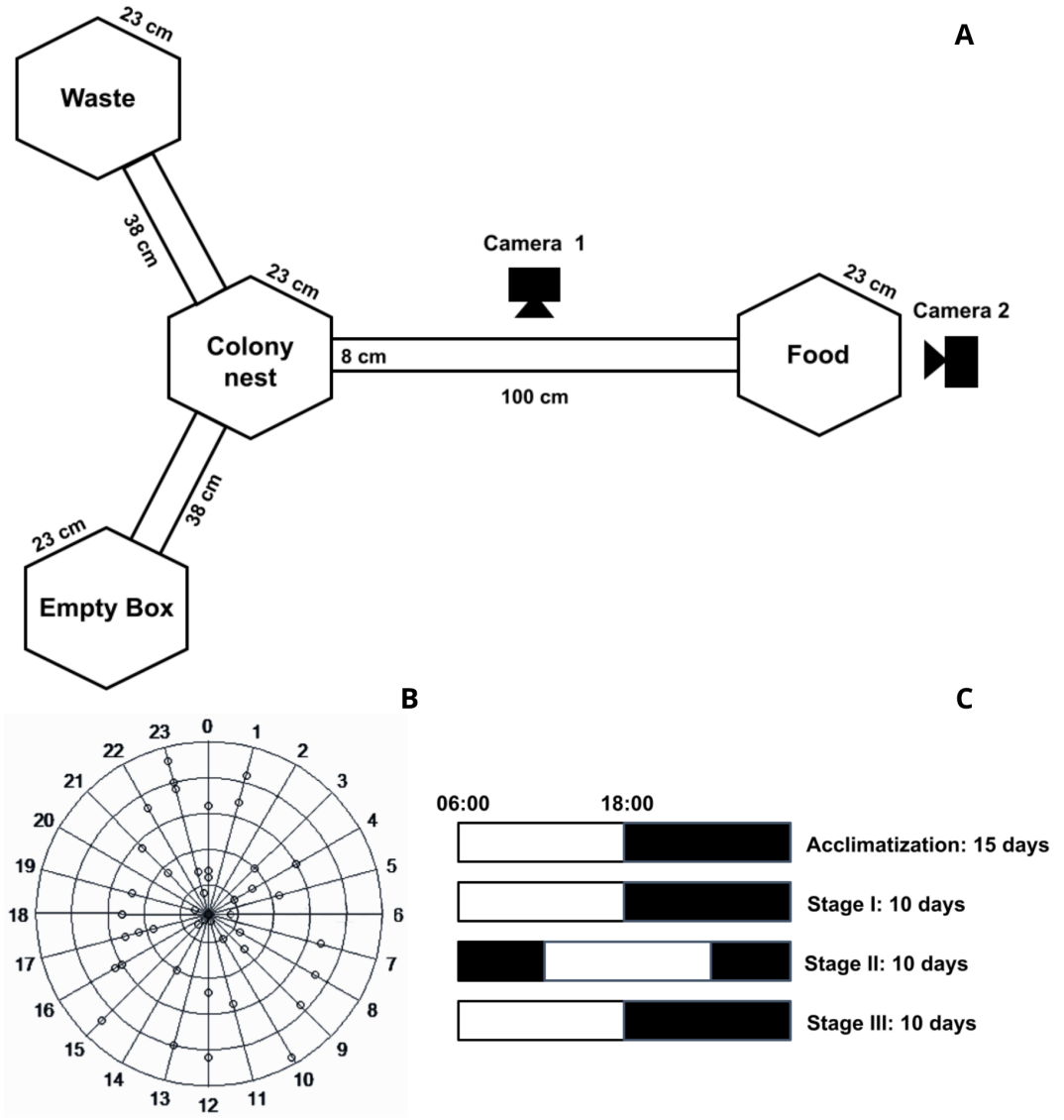


Figure 1: Experimental setup. A) Schematic arrangement of the experimental maze setup with three trails leaving the central colony box. Camera 1 records videos of the foraging ant flow, while Camera 2, positioned above the food box, takes pictures of the leaf area. **B)** Leaf offering events distributed over 24h. **C)** Schematic representation of the Experimental Protocol. Light-dark cycle conditions throughout the experiment are shown. White and black bars represent, respectively, light and dark hours. Acclimatization and Stage I had the same LD cycle conditions (Lights on at 06:00h). Stage II is initiated by a 6h phase delay of the LD cycle on day 11, and Stage III begins with a phase advance of the LD cycle on day 21.

Daily Rhythm of Activity

We monitored two activity rhythms: leaf-cutting and foraging activity. All data were obtained by images recorded using two Logitech C920 HD Pro webcams. Camera 1 was located in the middle of the trail connecting the nest to the food source, and Camera 2 was positioned above the food box (Figure 1A). All videos and pictures were taken using the software iSpy (copyright iSpyConnect.com).

Leaf-cutting

A picture of the leaves was automatically taken every 20 minutes for 30 days. Using FIJI (ImageJ2 distribution) (Schindelin *et al.* 2012), we measured the leaf area on each photograph and calculated the leaf-cutting taxes for each hour (Figure 2A).

Two measures were made from the data. We considered the leaf-cutting tax as the difference between the leaf area in a photo and the remaining leaf area after 60 minutes every 20 minutes (resulting in 72 tax values for each day). We considered the hourly difference between the leaf area and the remaining leaf area for the leaf intake measures after 60 minutes (24 points for each day). Daily leaf intake is calculated by the total sum of these 24 area values.

Foraging activity

Foraging activity was recorded based on a 2 minutes video within a sequence of 20 minutes intervals (72 videos per day). We developed a video tracking software using machine learning techniques (Figure 2B). For the software validation, we randomly selected 50 videos from 2160 recorded. We manually counted the number of ants in these 50 videos and further compared these values with those obtained by the automatic counts generated by the software (Figure 2C). Developing a video tracking and counting tool based on machine learning techniques requires data annotation,

which involves identifying the object of interest as a class in an image dataset extracted from our video data. Video annotations were realized using the tracking mode function in the open-source CVAT (Computer Vision Annotation Tool - Intel Corporation). We selected the 4 videos (2 from the dark phase and 2 from the light phase) with the highest number of ants from the 50 videos mentioned above. We used the minimum bounding box method for every video frame annotated to identify each ant present on each image, which conferred an identification number for every ant (both bounding boxes and identification numbers were further used to train the object detection model and the video tracking tool). Video annotations were exported in the YOLO format and subsequently used to train an object detection model using YOLOv5 (You Only Look Once version 5) (Zhu *et al*., 2021). Software codes, hardware requirements, and all steps performed in the counting procedure are described in the Supplementary Material.

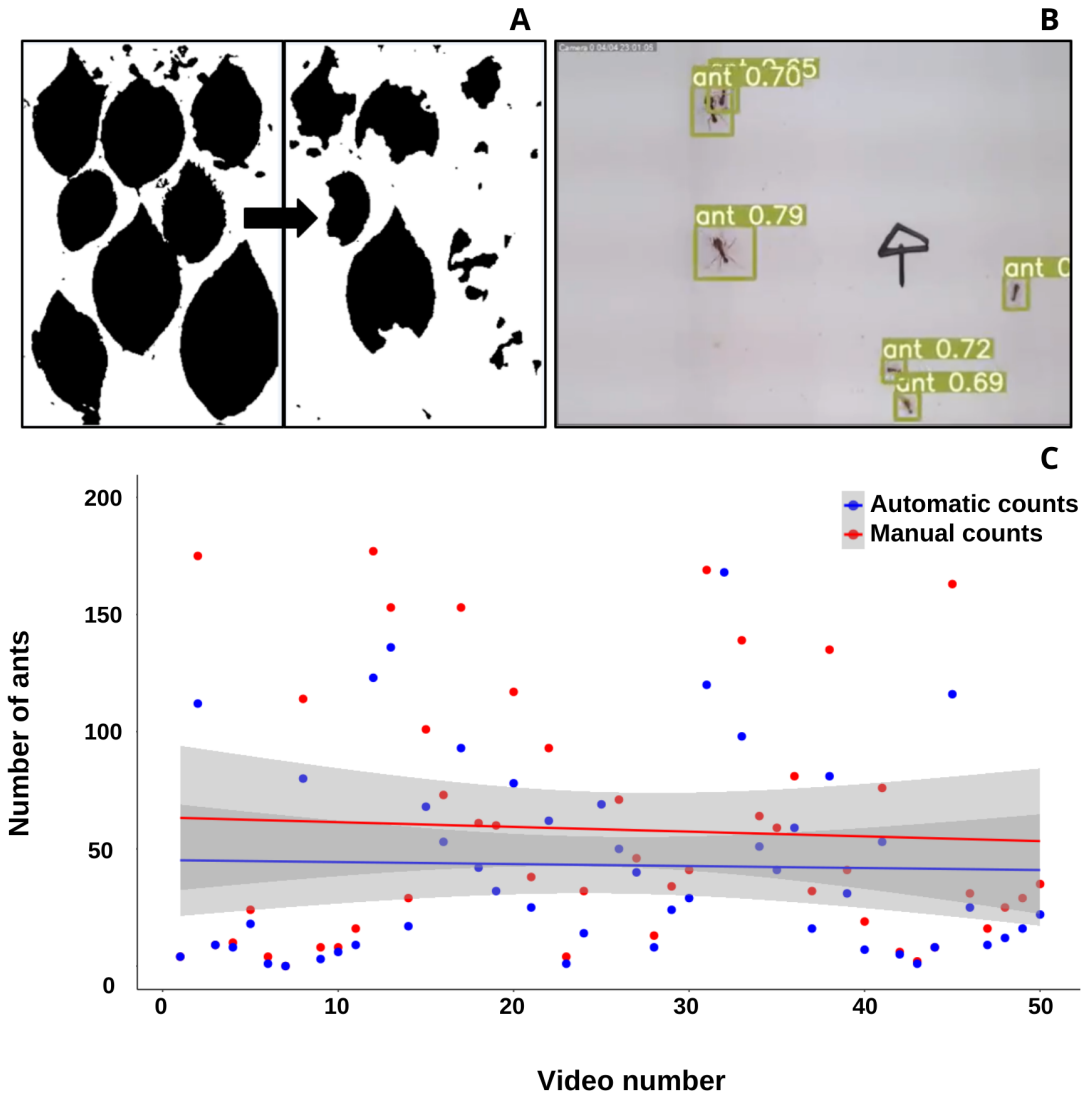


Figure 2) Software analysis of Daily Rhythms. A) Representative sequence of two pictures showing how leaf area decreased through time (analyzed in FIJI). **B)** Frame extracted from a light phase video exhibiting how our video tracking software detected ants using YOLOv5. Each box identifies an ant, and each number shows the probability (varying from 0 to 1) of that object belonging to the class previously trained as "ant." **C)** Software validation with the number of ants in 50 videos with 2 min duration. For each video, manual counts are presented in red and automatic counts in blue. Linear regressions show the relationship between the values of each category.

Data analysis

We have recorded for 30 days both daily rhythms of foraging and leaf-cutting activities. Lomb-Scargle periodogram analyses were performed to verify if data from Stage I were rhythmic. However, those rhythms are masked by the acute responses of ants to leaf replenishment. Therefore, we chose to analyze the temporal variation of the response to leaf replenishments observed in both foraging and leaf-cutting activities. Since leaf replenishment events occurred randomly once a day, we observed the response effects by monitoring each activity during the 120 minutes before the leaf offering and the 120 minutes after it, considering Stage I (synchronized) and events within the days of Stages II and III. The first 5 days of these stages, which presumably display more pronounced transients, were analyzed separately to verify how the LD shift affected the two activity responses to leaf replenishments. We calculated the Area Under the Curve (AUC) considering the 120 minutes interval after the leaf offering for the foraging activity and the 100 minutes interval for the Leaf-cutting taxes (we exclude the first data point after leaf replenishments as leaf-cutting tax is negative in this particular moment, due to the increase of leaf area available for cutting). In order to identify the differences between stages and local time, we fitted a general linear mixed model (GLMM) for the AUC measures of foraging activity (using a Poisson distribution). The number of ants was a function of the categorical variables: Stage (in this particular case, synchronized days from Stage I, the last 5 days of Stage II, and the last 4 days of Stage III; and also the first 5 days from Stage II and the last 6 from Stage III), hour (divided in 00:00h, 03:00h, 06:00h, 09:00h, 12:00h, 15:00h, 18:00h, and 21:00h) and the interaction between these two factors. Days were included in the model as random effects affecting the intercept, as they contain different random intervals between leaf replenishment events. As the AUC measures of Leaf-cutting taxes were normally distributed, we performed a linear regression model with the same categorical variables (Stage and hour) and the interaction between them. Minimum adequate models were selected using parsimony criteria and analysis of variance for model comparison.

We examined daily leaf intake considering each Stage of the experiment, including daily changes after the phase shifts and differences between light and dark phases. An analysis of variance (ANOVA) was performed to check if the differences between stages were statistically clear.

We performed linear regression analyses to estimate the relationship between leaf-cutting taxes and the number of ants foraging during both light and dark phases. We have also calculated the leaf-cutting taxes and the number of ants ratio, which was analyzed considering light and dark phases by performing an analysis of variance. All graphs and analyses were performed in R version 4.1.2. Areas Under Curves were obtained through the function ‘trapz’ from the ‘pracma’ package. We used the function ‘glmer’ from the package ‘lme4’ for GLMM analyses and the function ‘lm’ from the package ‘R stats’ to create the linear regression models. The periodogram analyses were built using the function ‘lsp’ from the package ‘lomb .’ Graphs were built using ‘ggplot2’.

RESULTS

Daily rhythms of leaf-cutting and foraging activities

We have successfully measured the colony activity for 30 days, based on both the leaf-cutting and foraging activity (Figure3). The Lomb-Scargle periodogram analyses confirmed rhythmicity for both leaf-cutting ($p<0.01$) and foraging activity ($p<0.01$). Our video-tracking system allowed us to count a total of 143.904 ants crossing the trail, even though our software validation process shows that our software tends to underestimate the total number of ants at each video (Figure 2C). The colony activity was typically nocturnal.

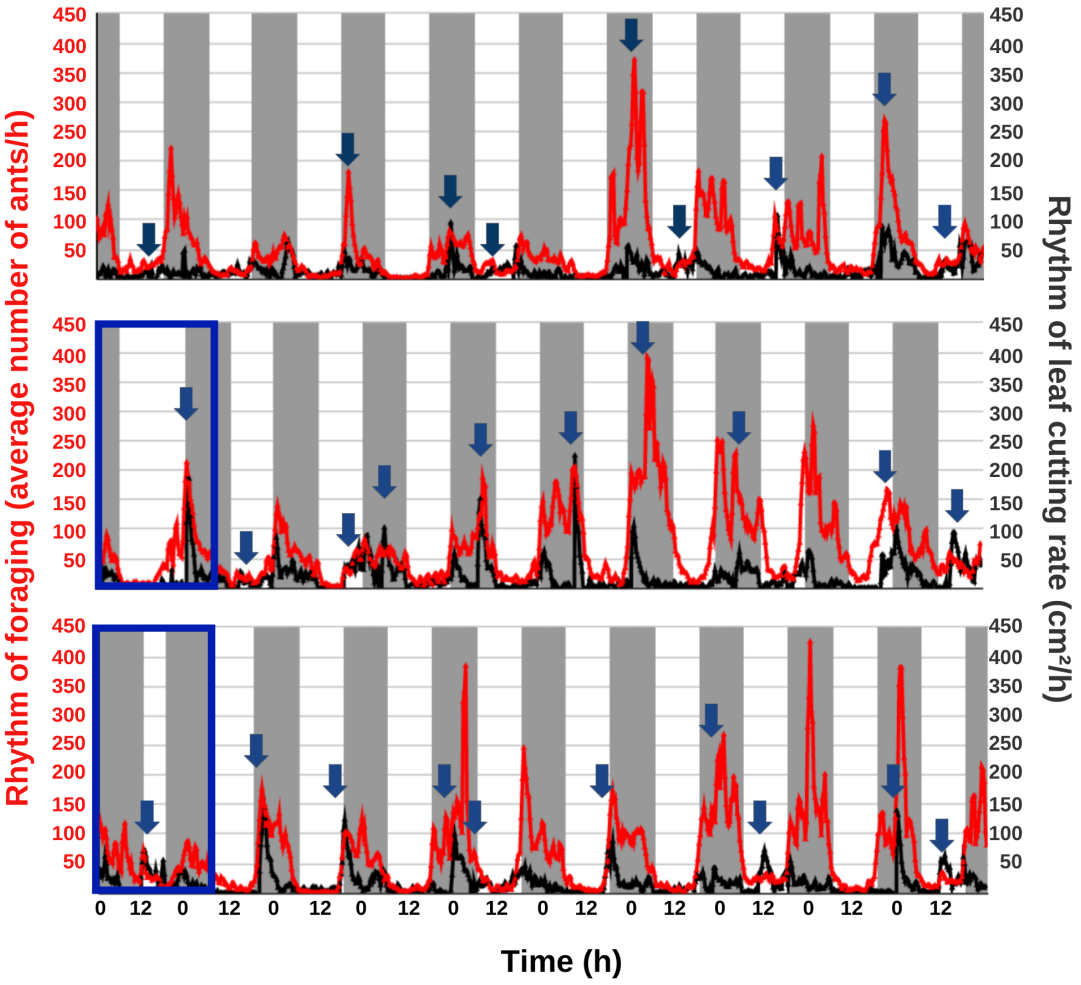


Figure 3: Daily rhythms of foraging and leaf-cutting activities. Time series of foraging (red line) and leaf-cutting rate (black line) across the three stages of the experiment. Gray areas represent the dark phase, while white areas correspond to the light phase of the LD cycle. Blue arrows indicate the hours when leaf replacement occurred, generating masking effects. The first ten days of the experiment (Stage I) had the light phase lasting between 06:00h and 18:00h. Blue boxes indicate the 6h phase delay of the LD cycle (on the 11th day, initiating Stage II) and the 6h phase advance of the LD cycle (on the 21st day, starting Stage III).

Analysis of acute responses to leaf replenishments

Although we randomly fed the colony to avoid synchronization by leaf replenishment time, both leaf-cutting and foraging daily rhythms displayed acute increases as responses to leaf offering. As these exogenously

generated amplitude increases deeply influenced our rhythms, we chose to focus our rhythm analysis on these responses themselves, verifying whether they were higher or lower across the 24h. To this end, we first quantified the acute responses to leaf replenishments in each experiment stage, separately for synchronized and assumed to be “transient” days. Transients were estimated by visual analysis and comparison with Stage I, which contains only synchronized days (Figure 4). Because the time interval between leaf offerings was not regular, we further analyzed if any observed response variations could be alternatively influenced by longer intervals between feeding events (Table I). There was no clear association among longer intervals between feeding events and higher response effects to leaf offering on both leaf-cutting and foraging activities.

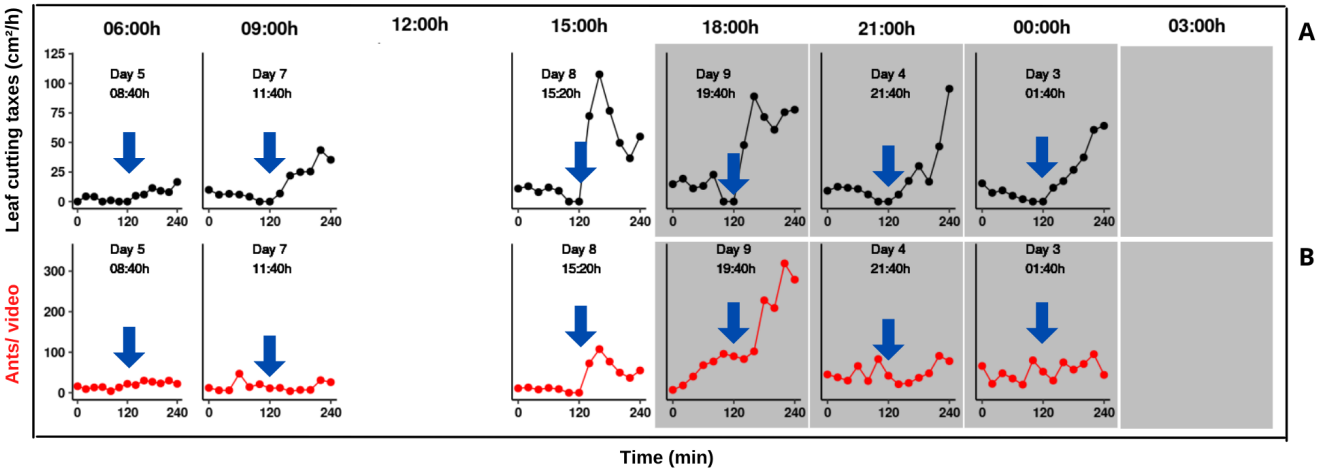


Figure 4: Acute effects of leaf replenishment on foraging and leaf-cutting activities across the day during Stage I. Acute responses at different times of the day are represented by the 120 minutes before and 120 minutes after each leaf offering. In each graph, leaf replenishment events occurred at minute 120 (black arrows). Day numbers represent when leaf replenishment occurred and are organized according to the local time subdivided into eight 3h intervals for better visualization. When no feeding events occurred in that interval during that stage, we represented it as blank. Only one is represented when more than one feeding event occurred in the same interval (even when they referred to different hours). The exact leaf replenishment time is indicated in all graphs. **A)** Acute effect on leaf-cutting taxes

due to leaf replenishment events across the day. **B)** Acute effect on the foraging activity caused by leaf replenishment events across the day.

Day	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Interval Between Feeding (h)	44	11	38.3	12.6	27.6	28.3	15.6	12.3	38	28.3	10.3	26	26.3	15.3
Day	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	
Interval Between Feeding (h)	28.3	39.3	18.6	20.3	32	21.6	29.6	6.6	34.6	29.6	12.3	37.3	11.3	

Table I: Time interval between feeding events. We calculated each interval by the difference between the time when the new leaf offering occurred on that day and the time when the last event occurred (preceding day).

We calculated the Area Under the Curve for each day after the leaf offering and used this to quantify the response effect to each leaf replenishment event. If the response to leaf replenishment events were uniform across the day, we would expect that there would not be significant differences between the calculated AUCs. We visually inspected the AUCs calculated for both leaf-cutting taxes and the foraging activity, and as we found distinct response effects alongside daily hours for each stage, we proceeded to inspect these differences statistically. Shapiro-tests were calculated for AUCs measures obtained by leaf-cutting taxes ($p=0.10$) and the number of ants per video ($p<0.05$, Poisson distribution).

Foraging response

Our minimum adequate model for the foraging response's AUC measures included the interaction between stages and hour (^{2} =46.536, df=11, $p<0.001$). Foraging response to leaf offering was clearly higher in the last days of Stages II ($z=2.819$, $p<0.01$) and III ($z=3.097$, $p<0.01$) when compared to Stage I. Foraging response during the first 5 days of Stage II, when transients are expected to be more pronounced, was also clearly higher

(z=2.153, p<0.05) than during Stage I. However, the first 6 days of Stage III showed a slightly positive increase in the forage response, and this difference was not statistically significant. Considering the effects of the variable hour, when compared to the 00:00h response from Stage I, there was a clear negative difference for the light phase hours such as 06:00h (z=-2.707, p<0.01), 09:00h (z=-4.101, p<0.001) and 12:00h (z=-3.879, p<0.001), showing that foraging response was smaller in this phase. An increase of foraging response was observed for hours 15:00h, 18:00h, and 21:00h; however, the only statistically significant response was around 18:00h (z=3.258, p<0.001) regarding the response observed for 00:00h.

Table II shows the results for the interaction between stages and hours. We found an increase in the foraging response in the hour 06:00h of the first 5 days from Stage II and 09:00h in the first 6 days from Stage III. This increase in the foraging response was also found in the last 5 days of Stage II. Nevertheless, a decrease in the foraging response was observed in the hour 18:00h of the first 5 days from Stage II and in the hour 15:00 for the first 6 days of Stage III. The only clear decrease of foraging response for the last days (assumed to be synchronized) was observed in the hour 18:00h from Stage II.

HOUR	STAGE II		STAGE III
	First 5 days	Last 5 days	First 6 days
06:00	z=1.787 p=0.07		
09:00		z=3.075 p<0.01	z=2.015 p<0.05
15:00			z=-2.530 p<0.05
18:00	z=-6.245 p<0.001	z=-3.090 p<0.01	

Table II: GLMM results for the interaction between Hours and Stages. Z-values and p-values are shown according to each interaction. We have only shown values that are statistically or marginally significant.

Leaf-cutting response

Our minimum adequate linear regression model for the AUCs obtained by the leaf-cutting taxes response to feeding events contained only the factor stages as a predictor variable. Although we found increases and decreases in the leaf-cutting response between hours in relation to 00:00h, those were not clearly significant. Comparing the model with both stages and hours and the one with only stages, the more complex model did not offer a better prediction for our data ($F=0.71$, $p=0.66$). When compared with Stage I ($t=3.283$, $p<0.01$), we see that all stages (assumed transients and synchronized days) show an increase in the leaf-cutting response, although this increase is only marginally significant for the first five days from Stage II ($t=1.894$, $p=0.07$). Comparing only the differences between hours in the leaf-cutting responses, we see that 00:00h shows the highest values ($t=2.205$, $p<0.05$).

Daily leaf Intake

As we observe in Figure 5A, daily leaf intake varied between Stages I, II, and III. Stages II and III showed a higher daily intake when compared to Stage I ($F=3.9738$, $p=0.05$). There were no clear differences between Stages II and III ($F=0.307$, $p=0.58$). Regarding the differences between light and dark phases, leaf intake was higher during the dark phase ($F=6.585$, $p<0.05$).

We have also measured the proportional leaf intake during light and dark phases for each day, as shown in Figure 5B. In the synchronized state (Stage 1), leaf intake was higher during the dark phase. After the 6h lengthening of the light phase of the LD cycle on day 11, we observe that most of the proportional daily leaf intake occurs in the dark phase on the first days, and this pattern seems to be reversed after day 5. On day 21, after the 6h shortening of the light phase, we observe higher values during the dark phase in the first days, reverting in the last 5 days. The pattern present in Stage I did not seem to reappear in Stages II and III.

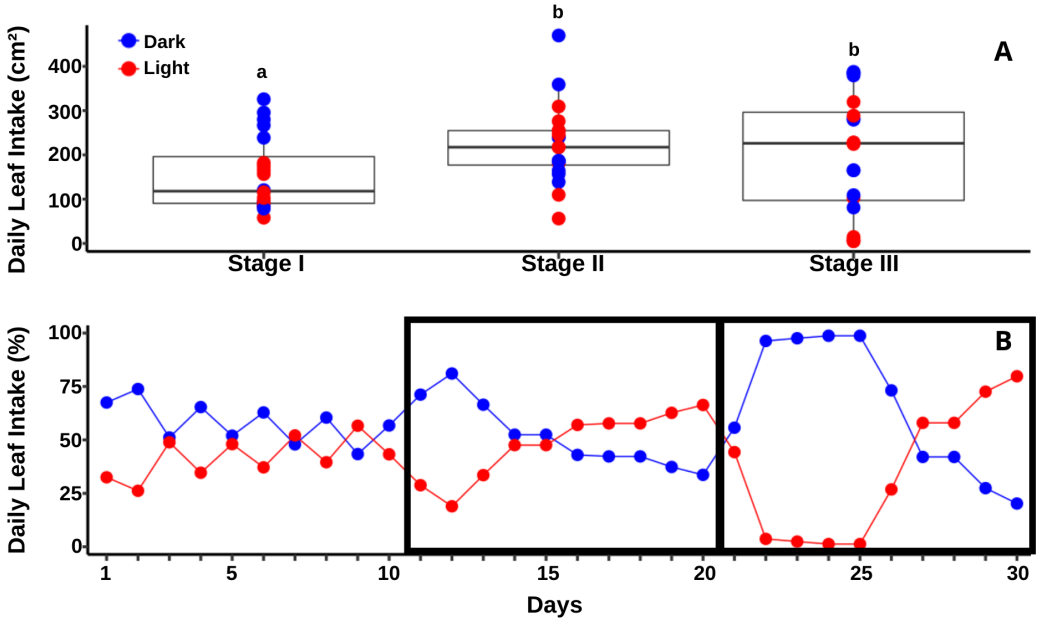


Figure 5: Temporal profile of daily leaf intake. Red points indicate values consumed in the light phase, while blue points refer to dark phase values. **A)** Boxplots showing daily leaf intake during Stages I, II, and III. **B)** Daily variation of leaf intake during light and darkness phases over the 30 days of the experiment. Boxes indicate the stages on which phase shifts of the LD cycle occurred.

Relationship between leaf-cutting taxes and the number of ants per video

We analyzed the relationship between leaf-cutting taxes and the number of ants foraging, considering both light and dark phases (Figure 6A). We observed that in Stage I, a higher number of ants were measured crossing the trail in the dark phase. However, these higher ant numbers were not related to higher leaf-cutting taxes. On the other hand, in the light phase, an increase in the ant flow appears to be proportionally related to an increase in leaf-cutting taxes.

After the first phase shift, we observed that during days 11 to 15, the higher ant frequencies found in the dark phase of Stage I disappeared. We also observed changes in the light phase pattern with milder increases in the

relationship between higher ant counts and higher leaf-cutting taxes, which seemed similar to the one found during the dark phase. As of the 6th day of Stage II (days 16 to 20), we recover the pattern described for Stage I in both light and dark phases.

After the 6h shortening of the light phase performed on Stage III (transients are assumed from days 21 to 26), the most noticeable changes occurred in the light phase, which appeared to involve a decrease in the number of ants counted. Nonetheless, this small number of ants was associated with high leaf-cutting taxes. During days 27 to 30 (presumably synchronized), we did not observe notable changes when compared to days 21 to 26 considering the light phase; however, the proportional increase in leaf-cutting taxes associated with the number of ants foraging in the dark phase appeared to reduce its slope.

Daily differences between the ratio between leaf-cutting taxes and the number of ants are shown in Figure 6B. Regarding the light and dark phases, this ratio is higher for the light phase during the whole experiment ($F=143.99$, $p<0.001$), and no clear differences were observed between the three stages ($F=0.6229$, $p=0.43$).

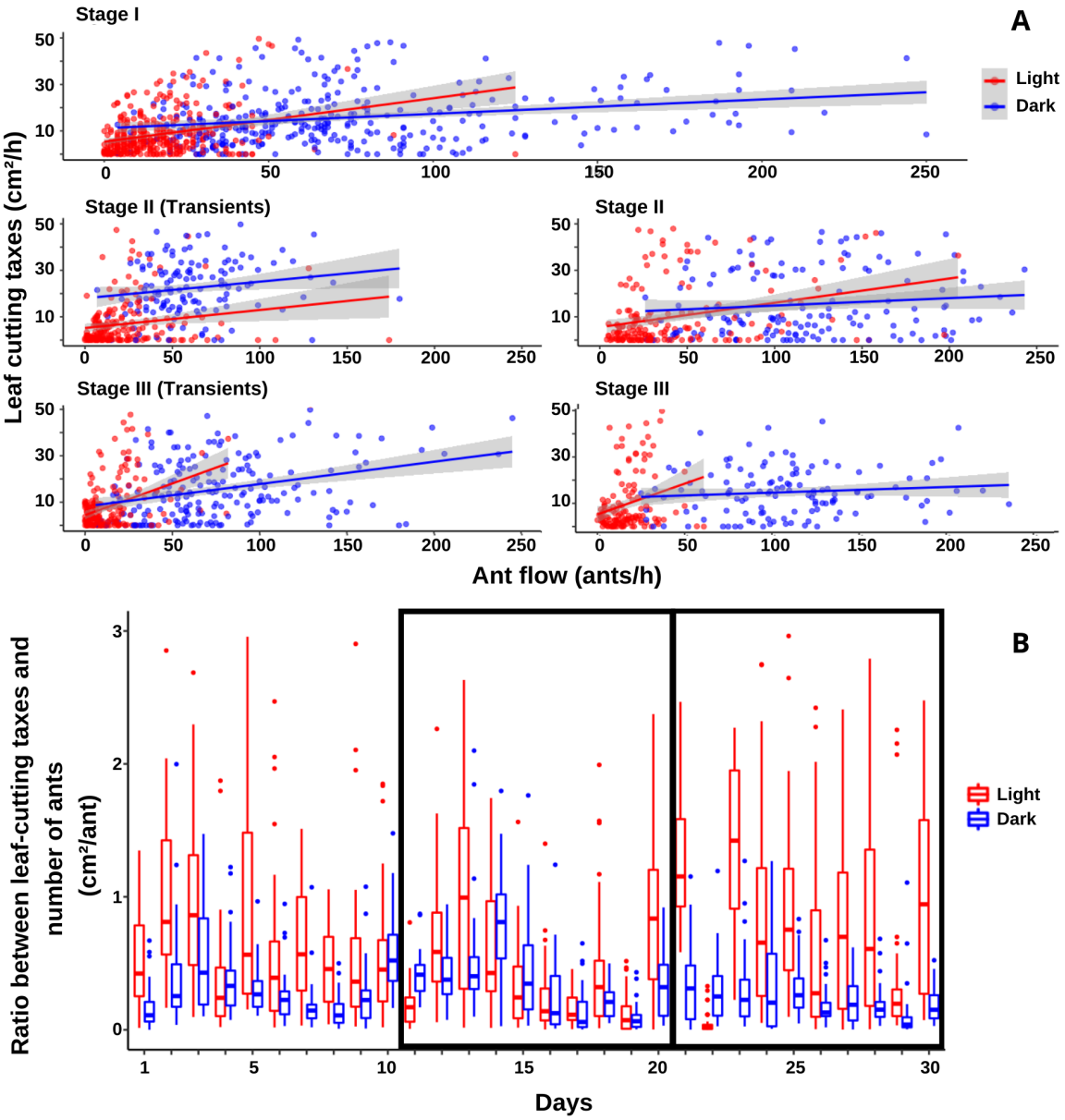


Figure 6: Relationship between leaf-cutting taxes and the number of ants. A) Relationship between ant flow and leaf cut taxes for stages I, II, and III. Stages II and III are divided into possible transients and synchronized days. For Stage II, we considered the first five days after the phase shift (days 11 to 15) as transients. For Stage III, days 21 to 26 were considered transient days. **B)** Ratio between leaf cut taxes and number of ants during both light (red) and dark (blue) phases. Boxes indicate the stages in which phase shifts of the LD cycles occurred.

DISCUSSION

Our experiment showed clearly that daily and synchronized activity rhythms at the colony level could be reproduced in a colony maintained under artificial LD cycles and constant temperatures under laboratory conditions. The colony presents foraging and leaf-cutting activities throughout the day. Nevertheless, most of them occur in the dark phase (Figure 3). For that reason, the colony was considered “nocturnal.” Analysis of the acute responses to leaf replenishments shows that the colony is particularly sensitive to leaf replenishment events near 18h when the transition between light and dark phases occurs and near 0h when colony foraging rhythm reaches its peak (Figure 4).

A shift in the LD cycle was then applied to verify its effects on the daily rhythms. Our results indicated that daily rhythmicity at the colony level could respond to its internal temporal order perturbations. Phase shifts of the LD cycle cause phase shifts of daily rhythms, but resynchronization usually occurs after several transient days, in a process similar to “jet-lag” in humans (Waterhouse *et al*., 2007). It has also been observed that the rate of resynchronization is different between phase delays and phase advances (Wever, 1966). We applied a 6h phase delay of the LD cycle and then a 6h phase advance, assuming that a 10 day interval between these perturbations was sufficient for resynchronization.

Effect of LD perturbation on daily leaf intake

After the phase shifts, both during Stages II and III, we observe an increase in daily food intake (Figure 5). It is possible that as a system, the colony is trying to optimize the consumption of the food source (Dussutour et al., 2007; Wirth *et al*., 2003). Nonetheless, another possible option is that during this stressful condition, the colony basal foraging activity is higher, as more ants could be engaged in the task of searching for new food sources throughout the day. Considering an individual scale, the resynchronization process may increase food intake, as we see in many species (Kalsbeek et al., 2011, 2014; McHill and Wright, 2017). An essential component in the

foragers' diet is the chemical compounds (especially carbohydrates) present in the plant sap (Littledyke and Cherrett, 1976); therefore, foraging may also increase due to energetic individual needs.

Our experimental protocol could not monitor ants individually, so we were unable to identify precisely the processes involving the inversion between the proportional daily leaf intake in light and dark phases (Figure 5B). Previous research from our laboratory indicates that leaf-cutter ant foragers divide their work into temporal shifts (Constantino *et al*., 2021). It is possible that during the assumed transient days, ants use the strategy of recruiting other ants to communicate the environmental temporal cues to the whole colony, allowing synchronization at the colony level. However, ants may be individually "jet-lagged," requiring energetic resources and social interaction to attain full synchronization since social cycles constitute a significant *zeitgeber* for the social insects (Bloch *et al*., 2013). We remind that most of the research regarding daily rhythms in ants is performed in individuals or small groups. Ants' survival is compromised when isolated from the colony context, even when an adequate environment and food sources are provided (Sharma *et al*., 2004). Considering the inversion between the proportional leaf intake between dark and light phases in the last days from Stage II and III, it is conceivable that the 10 days of our protocol allocated for these stages have not been long enough for a full resynchronization to the new LD phases. Task allocation is a major process in the colony organization (Ingram *et al*., 2016; Gordon, 2021), which dynamically confers the colony's ability to distribute activities between ants according to the system requirements. Also, it provides a remarkable energetic supply to the ant colony, as a high amount of the ants are not engaging in any evident activity most of the time (Hasegawa *et al*., 2016). The possibility of quickly recruiting a high number of ants when facing challenging conditions is an astonishing evolutionary advantage to ant colonies (Gordon, 2019). Considering all these aspects, we hypothesize that the inversion between the preferential phase on which most of the leaf intake occurs may be due to these fine recruitment adjustments in the colony that could make available high numbers of ants from distinct work shifts. This

internal reorganization might also be an example of the alternative stable states described in other ecological systems (Fukami and Nakajima, 2011).

Under natural conditions, a significant group of *Atta* ants lives under constant darkness, as their fungus chambers can be located up to 7m underground (Moreira *et al*., 2004). Even though 6h phase shifts in the LD cycle are entirely artificial and do not represent natural challenges in the evolutionary history of leaf-cutter ants, these experiments allow us to explore how resynchronization arises in the colony and also hypothesize how these processes occur in other systems.

Since the formal study of biological rhythms recently emerged, we have observed that phase shift experiments induce a less active state in most animal models (Wever, 1966) usually associated with hyperphagia behavior (Chavan *et al*., 2017). Phase shifted rodents present higher chances to develop diseases such as obesity and diabetes (Escobar *et al*., 2011). For human societies, a vast amount of research trying to understand the effects of work shifts identified risks of developing metabolic syndrome, cardiovascular disease, gastrointestinal disorders, breast cancer, and psychological disorders (McHill and Wright, 2017, Azmi *et al*., 2020). Considering all these pieces of evidence, it seems undeniable the evolutionary importance of preserving the internal temporal order and living under synchronized conditions since they appear to provide an optimal energetical state for the organism's physiology. In other words, it is possible to investigate the effects of phase shifts, considering them as disturbances in the system's homeostasis.

Effect of LD perturbation on the relationship between the number of ants and leaf-cutting tax

Here, for further discussions, we define the optimal energetical organization state and its changes according to temporal synchronization as the system efficiency. We propose that the relationship between leaf-cutting taxes and the number of ants foraging may be an exciting measure of system efficiency for the leaf-cutting ant colony. The colony's survival relies on finding

and acquiring food sources, such as leaves, on feeding its symbiont fungus (Ronque *et al*., 2019), which can grow according to it. Also, maintaining high numbers of ants foraging throughout the day, as described in *Atta* ants, involves a high energy expenditure (Wilson, 1980). Therefore, in our particular experiment, since the fungus size remained constant, we proposed that the leaf cut taxes represent the energetic input to the colony system and that the number of foragers active in the trail represents the energetic output. Thus, the fewer ant numbers required to obtain the minimal energetic needs are considered more efficient.

Results in Figure 6 show distinguished patterns of efficiency for light and dark phases over Stages I, II, and III. Similar to the results found by Constantino *et al*., 2021, although colony foraging and leaf-cutting are much higher in the dark phase, the relative increase in the number of ants resulting in higher leaf-cutting taxes is associated with the light phase. Foragers whose activity is located in the dark phase represent a higher absolute amount of leaf cut, but individually a great part of them is not necessarily engaging in leaf-cutting or leaf-carrying (Constantino *et al*., 2021). In this manner, light-phase foragers tend to be more efficient (Figure 6B). Nonetheless, when we consider the colony scale, it is undeniable that most of its energetic input is provided by dark phase foragers.

In Figure 6, we divided Stage II into the first and last 5 days, assuming that the first 5 days are transients and that the ants may or not be resynchronized in the last 5 days. For Stage III, we assumed 6 and 4 days, respectively. Transients from Stage II (Figure 6A) present a distinct efficiency pattern for light and dark phases. This result agrees with the expectations after a perturbation in the internal temporal order of the colony. Here we observe that foragers from the dark phase are proportionally cutting more leaves than in Stage I. That pattern probably results in most daily leaf intake occurring in the dark phase (Figure 5B).

After the phase advance, foragers from the dark phase are again less efficient than those in the light phase, yet leaf-cutting taxes on both light and dark phases seem to reach higher measures. In the last 4 days of Stage III,

we see that high ant frequencies from the light are again not related to high leaf-cutting taxes, similar to the pattern described in Stage I.

Given all of this, it is vital to acknowledge that discussing efficiency aspects in ant colonies involves distinct strategies when considering individual and collective scales. Foragers may be more efficient when they cut smaller leaf fragments, and the relationship between distance to the nest and ant speed is energetically balanced (Wilson, 1980; Burd, 1996). By all means, when considering a colony as a superorganism, many individual efficient foraging strategies may be involved, as they confer to the colony the ability to cope with various environmental challenges. Individual strategies considered less efficient may even have a high adaptive value when efficient at the colony level, where natural selection can act (Korb and Heinze, 2004).

Considering biological rhythms in this context, it is conceivable that multicellular organisms facing disruption on their internal temporal order might have similar adaptive strategies to reestablish their homeostasis, with distinct processes and patterns occurring at the cellular (individual) and organismal (colony) levels. In this way, we may observe synchronization at a higher level before it appears completely at all smaller scales such as cells and tissues, according to their oscillations' particular aspects. The maintenance of long-term strategies plays a relevant role in dynamic complex systems such as ant colonies (Hasegawa *et al*., 2016). Therefore, synchronization at higher levels might be a priority as it quickly allows living systems to face temporal disturbances. Nonetheless, synchronization at smaller levels is energetically more efficient at a long-term strategy as it saves energy, which can be allocated to different tasks performed and challenges faced by organisms.

Leaf-cutter ant colonies complex organization is present in spatial and temporal aspects since several records show how their temporal organization strategies vary according to distinct environments and seasonality (Gamboa, 1976; Nickele *et al*., 2016; Wirth *et al*., 1997; Caldato, 2014). Therefore, we believe they are an extraordinary model to answer further questions of the dynamics involved in the adaptive strategies regarding temporal challenges and biological rhythms.

REFERENCES

Azmi, N. A. S. M., Juliana, N., Teng, N. I. M. F., Azmani, S., Das, S., and Effendy, N. (2020). **Consequences of circadian disruption in shift workers on chrononutrition and their psychosocial well-being.** International Journal of Environmental Research and Public Health, 17(6). https://doi.org/10.3390/ijerph17062043

Bloch, G., Robinson, G.E., 2001. **Chronobiology: Reversal of honeybee behavioural rhythms.** Nature. 410, 1048.

Bloch G, Toma DP, Robinson GE (2001) **Behavioural rhythmicity, age, division of labor, and period expression in the honeybee brain.** J Biol Rhythms 16:444–456.

Bloch, G., Herzog, E. D., Levine, J. D., & Schwartz, W. J. (2013). **Socially synchronized circadian oscillators.** Proceedings of the Royal Society B: Biological Sciences, 280(1765). https://doi.org/10.1098/rspb.2013.0035

Burd, M. (2016). **Foraging Performance by Atta colombica , a Leaf-Cutting Ant.** The American Naturalist , Vol . 148 , No . 4 (Oct ., 1996), pp . 597-612 Published by : The University of Chicago Press for The American Society of Naturalist. 148(4), 597–612.

Caldato, N. (2014). **Ontogenia das trilhas físicas em Atta capiguara Gonçalves 1944 (Hymenoptera: Formicidae.** Tese (Doutorado). Universidade Estadual Paulista, Faculdade de Ciências Agronômicas, Botucatu, 124 f.

Chavan, R., Albrecht, U. and Okabe, T. (2017) **Circadian Clocks, Metabolism, and Food-Entrained Rhythms.** Kumar, Vinod. Markers of Circadian Rhythms in Humans In Vivo. Springer. https://doi.org/DOI 10.1007/978-81-322-3688-7_13

Constantino, P. B., Valentinuzzi, V. S., Helene, A. F. (2021). **Division of labor in work shifts by leaf-cutting ants.** Scientific Reports, (1), 1–18. https://doi.org/10.1038/s41598-021-88005-0

Crailsheim K, Hrassnigg N, Stabentheiner A. (1996). **Diurnal behavioral differences in forager and nurse honey bees (*Apis mellifera* carnica**

Pollm). Apidologie 27:235–244.

Das, Biplabendu and de bekker, Charissa. (2021). **Time-course RNASeq of Camponotus floridanus forager and nurse ant brains indicate links between plasticity in the biological clock and behavioral division of labor.** 10.1101/2021.03.27.433505.

Dussutour, A., Beshers, S., Deneubourg, J., and Fourcassié, V. (2007).**Crowding increases foraging efficiency in the leaf-cutting ant *Atta colombica*.** Insectes Sociaux 54, 158–165. https://doi.org/10.1007/s00040-007-0926-9

Escobar, C., Salgado-Delgado, R., Gonzalez-Guerra, E., Tapia Osorio, A., Angeles-Castellanos, M., and Buijs, R. M. (2011). **Circadian Disruption Leads to Loss of Homeostasis and Disease.** Sleep Disorders, 2011, 1–8. https://doi.org/10.1155/2011/964510

Frisch B, Koeniger N. (1994). **Social synchronization of the activity rhythms of honey bees within a colony.** Behav. Ecol. Sociobiol. 35:91–98.

Fuchikawa, T., Fujioka, H., Shimada, M., Tsuji, K., Okada, Y., and Abe, M. S. (2017). **Ant circadian activity associated with brood care type.** Biology Letters, 13(2), 20160743. https://doi.org/10.1098/rsbl.2016.0743

Fukami, T., and Nakajima, M. (2011). **Community assembly: Alternative stable states or alternative transient states?** Ecology Letters, 14(10), 973–984. https://doi.org/10.1111/j.1461-0248.2011.01663.x

Gamboa, G.J. (1976). **Effects of temperature on the surface activity of the desert leaf-cutter ant, Acromyrmex versicolor versicolor (Pergande) (Hymenoptera: Formicidae).** American Midland Naturalist, 95: 485-491

Gordon, D. M. (2019). **The ecology of collective behavior in ants.** Annual Review of Entomology, 64(September 2018), 35–50. https://doi.org/10.1146/annurev-ento-011118-111923

Gordon, D. M. (2021). **Movement, Encounter Rate, and Collective Behavior in Ant Colonies.** Annals of the Entomological Society of America, 114(5), 541–546. https://doi.org/10.1093/aesa/saaa036

Hasegawa, E., Ishii, Y., Tada, K., Kobayashi, K., and Yoshimura, J. (2016). **Lazy workers are necessary for long-term sustainability in insect societies.** Scientific Reports, 6, 1–9. https://doi.org/10.1038/srep20846

Ingram, K.K., Krummey, S., LeRoux, M. (2009) **Expression patterns of a circadian clock gene are associated with age-related polyethism in harvester ants, *Pogonomyrmex occidentalis*.** BMC Ecology 9:7

Ingram, K. K., Gordon, D. M., Friedman, D. A., Greene, M., Kahler, J., and Peteru, S. (2016). **Context-dependent expression of the foraging gene in field colonies of ants: The interacting roles of age, environment and task.** *Proceedings of the Royal Society B: Biological Sciences*, *283*(1837). https://doi.org/10.1098/rspb.2016.0841

Kalsbeek, A., Scheer, F. A., Perreau-Lenz, S., La Fleur, S. E., Yi, C. X., Fliers, E., and Buijs, R. M. (2011). **Circadian disruption and SCN control of energy metabolism.** FEBS Letters, 585(10), 1412–1426. https://doi.org/10.1016/j.febslet.2011.03.021

Kalsbeek, A., La Fleur, S., and Fliers, E. (2014). **Circadian control of glucose metabolism.** Molecular Metabolism, 3(4), 372–383. https://doi.org/10.1016/j.molmet.2014.03.002

Kay, J., Menegazzi, P., Mildner, S., Roces, F., and Helfrich-Förster, C. (2018). **The Circadian Clock of the Ant *Camponotus floridanus* Is Localized in Dorsal and Lateral Neurons of the Brain.** Journal of Biological Rhythms, 33(3), 255–271.

Korb, J., and Heinze, J. (2004). **Multilevel selection and social evolution of insect societies.** Naturwissenschaften, 91(6), 291–304. https://doi.org/10.1007/s00114-004-0529-5

Littledyke, M., and Cherrett, J. M. (1976). **Direct ingestion of plant sap from cut leaves by the leaf-cutting ants *Atta cephalotes* (L.) and *Acromyrmex octospinosus* (reich) (Formicidae, Attini).** Bulletin of Entomological Research, 66(2), 205–217. https://doi.org/10.1017/S0007485300006647

McCluskey ES (1958) **Daily rhythms in Male Harvester and argentine ants.** Science 128: 536-537.

McCluskey ES (1965) **Circadian rhythms in male ants of five diverse species.** Science 150: 1037-1039.

McCluskey ES (1967) **Circadian rhythms in female ants, and loss after mating flight.** Comp. Biochem. Physiol 23:665-677.

McHill, A. W., and Wright, K. P. (2017). **Role of sleep and circadian**

disruption on energy expenditure and in metabolic predisposition to human obesity and metabolic disease. Obesity Reviews, 18(February), 15–24. https://doi.org/10.1111/obr.12503

Moore D (2001). **Honey bee circadian clocks: behavioral control from individual workers to whole colony rhythms.** J. Insect Physiol. 47:843–857.

Moore D, Angel JE, Cheeseman IM, Fahrbach SE, Robinson GE. (1998). **Timekeeping in the honeybee colony: integration of circadian rhythms and division of labor.** Behav. Ecol. Sociobiol. 43:147–160.

Moreira, A. A., Forti, L. C., Andrade, A. P. P., Boaretto, M. A. C., and Lopes, J. F. S. (2004). **Nest architecture of Atta laevigata (F. Smith, 1858) (Hymenoptera: Formicidae).** Studies on Neotropical Fauna and Environment, 39(2), 109–116. https://doi.org/10.1080/01650520412331333756

Moritz, R.F.A., Sakofski, F., 1991. **The role of the queen in circadian rhythms of honeybees (*Apis mellifera* L.).** Behav. Ecol. Sociobiol. 29, 361–365.

North RD (1987) **Circadian rhythm of locomotor activity in individual workers of the wood ant *Formica rufa*.** Physiol Entomol 12:445–454

North RD (1993) **Entrainment of the circadian rhythms of locomotor activity in wood ants by temperature.** Anim Behav 45:393–397

Nickele, M. A., Filho, W. R., Pie, M. R., and Penteado, S. R. C. (2016). **Daily foraging activity of Acromyrmex (Hymenoptera: Formicidae) leaf-cutting ants.** Sociobiology, 63(1), 645–650. https://doi.org/10.13102/sociobiology.v63i1.902

Oda, G.A., Bellusci, S., Marques, M.D., 2007. **Daily Rhythms Related to Distinct Social Tasks Inside an Eusocial Bee Colony.** Chronobiol. Int. 24, 845-858.

Roces F, Nunez JA (1996) **A circadian rhythm of thermal preference in the ant *Camponotus mus*: masking and entrainment by temperature cycles.** Physiol. Entomol. 21:138-142

Ronque, M. U. V., Feitosa, R. M., and Oliveira, P. S. (2019). **Natural**

history and ecology of fungus-farming ants: a field study in Atlantic rainforest. Insectes Sociaux, 66(3), 375–387. https://doi.org/10.1007/s00040-019-00695-y

Sharma VK, Lone SR, Goel A, Chandrashekaran MK (2004a) **Possible evidence for shift work schedules in the media workers of the ant species *Camponotus compressus*.** Chronobiol Int 21:297–308.

Sharma VK, Lone SR, Goel A, Chandrashekaran MK (2004b) **Circadian consequences of social organization in the ant species *Camponotus compressus*.** Naturwissenschaften 91:386–390.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). **Fiji: An open-source platform for biological-image analysis.** *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Southwick EE, Moritz RFA. (1987). **Social synchronization of circadian rhythms of metabolism in honeybees (*Apis mellifera*).** Physiol. Entomol. 12:209–221.

Toma DP, Bloch G, Moore D, Robinson GE (2000) **Changes in period mRNA levels in the brain and division of labor in honeybee colonies.** Proc Natl Acad Sci USA 97:6914–6919.

Waterhouse, J., Reilly, T., Atkinson, G. and Edwards, B. (2007). **Jet lag: trends and coping strategies.** Lancet, vol. 369, no. 9567, pp. 1117–1129.

Wever, R. (1966). **The duration of re-entrainment of circadian rhythms after phase shifts of the Zeitgeber A theoretical investigation.** Journal of Theoretical Biology, 13(C), 187–201. https://doi.org/10.1016/0022-5193(66)90016-6

Wilson, E.O. (1980). **Caste and Division of Labor in Leaf-Cutter Ants (Hymenoptera: *Formicidae: Atta*) II. Ther Ergonomic Optimization of Leaf Cutting.** Behavioral Ecology and Sociobiology, v. 7, n. 2, p. 143-156.

Wirth, R., Beyschlag, W., Ryelt, R. J. and Hölldobler, B. (1997). **Annual foraging of the leaf-cutting ant Atta colombica in a semideciduous rain forest in Panama.** Journal of Tropical Ecology, 13: 741-757.

Wirth R., Beyschlag W.,Herz H.,Ryel R.J. and Holldobler B. 2003. **The**

Herbivory of Leaf-Cutting Ants. A Case Study on *Atta colombica* in the Tropical Rainforest of Panama. Ecological Studies 164. Heidelberg, Springer Verlag, 230 pp

Zhu, X., Lyu, S., Wang, X., & Zhao, Q. (2021). ***TPH-YOLOv5: Improved YOLOv5 Based on Transformer Prediction Head for Object Detection on Drone-captured Scenarios*.** 2778–2788. https://doi.org/10.1109/iccvw54120.2021.0031

Final Conclusions

Considering all the results and ideas discussed previously, we would like to emphasize that an ant colony can be considered a "superorganism" (Wilson and Sober, 1989). Results in this project show that ant colonies can provide an interesting analogy for how synchronization processes occur in multicellular organisms, as ants' individual rhythmicity may be compared to cellular rhythmicity. Through social synchronization, we can observe the coupling process between multiple oscillators. Many are the physiological processes that allow communication and integration between different cells and tissues, and this high degree of cohesion and interdependence are some of the main aspects of multicellular organisms. Although ants individually are also multicellular organisms, social insects' colony context also involves many physiological and behavioral processes that allow its existence in such a high level of eusociality complexity.

Considering the more intimately related aspects to the photic synchronization of multicellular organisms, not all their cells are sensitive to temporal cues obtained from light-dark cycles. In humans, for example, photoentrainment is only possible due to photosensitive cells located in the retina. After perceiving these environmental time cues, this information is processed in the mammalian s4uprachiasmatic nucleus (SCN). Only then, SCN transmits its information to other organism cells, starting cell synchronization processes, coupling mechanisms, and, finally, the organism's full synchronization (Floessner and Hut*.,* 2017).

Similarly, we believe temporal information from light/dark cycles flows in an ant colony and other social insects colonies. Only a group of ants is exposed to light and darkness in natural conditions, and through social interactions and other physiological processes, this information flows throughout the colony. However, many are the questions that can still be asked to understand synchronization in this social system, and those studies

might be able to expand our knowledge about the complexity and the evolutionary importance of living under synchronized conditions. For example, we know the role played by SCN in humans, but ant colonies organize themselves without central control. Is it possible that the colony possesses some component that plays a similar role to SCN? Individually, ants' brains contain a group of neurons responsible for their control of rhythmicity (Kay *et al*., 2018). May the queen play a part in this process? Even under constant darkness, the ant queen could receive temporal information and somehow regulate the ants' individual clocks at a molecular level while it similarly lays eggs that regulate many aspects of the colony reproduction (Dolezal, 2019). Does the fungus play any role in the colony's temporal synchronization? Could it be a combination of both the fungus and the queen? Many fungi species present daily rhythms of growth and spore release (Bell-Pedersen *et al.,* 1996). Also, the interdependence between a leafcutter ant colony and its fungus is very high (Schultz and Brady, 2008).

Atta ant's communication occurs mainly through chemical components. Is there a chemical component that plays a similar role to melatonin, allowing this superorganism synchronization? Several studies show that each cell has its own period of oscillation in mammals, and those periods are similar for cells in the same tissue. Ants seem to have a similar relationship when we think about the temporal division of labor between subcastes.

Considering all the remaining questions about colony rhythmicity, we would like to emphasize the complexity of discussing efficiency in these systems, not only regarding rhythmic aspects. As environmental challenges change, colonies' behavioral response changes, and the efficiency measures and the concept of efficiency also require flexibility. An instantly efficient strategy may not be efficient in the long term. This is valid not only for ant colonies and other social insect systems but also for other organisms in their own particular aspects and natural history. Meanwhile, as biological rhythms are present in all groups of living organisms and play an essential role in the organisms' survival by modulating their behavior and physiology, they are likely a major component of efficient strategies over different species.

The present work has made some progress regarding the questions involving biological rhythms in ant colonies, especially as it proposes a new tool to proceed with these studies. We are aware that not all researchers will be able to use the video-tracking software we developed, as it involves its particular challenges. However, we believe that as technology's advances happen more quickly every day, soon better and more accessible versions of this software will be able to promote many breakthroughs and new insights in behavioral biology.

REFERENCES

Bell-Pedersen, D., Garceau, N., and Loros, J. J. (1996). **Circadian rhythms in fungi.** Journal of Genetics, 75(3), 387–401. https://doi.org/10.1007/bf02966317

Floessner, T. and Hut, R. A. (2017). Basic Principles Underlying Biological Oscillations and Their Entrainment. **Markers of Circadian Rhythms in Humans In Vivo.** https://doi.org/DOI 10.1007/978-81-322-3688-7_13

Dolezal, A. G. (2019). **Caste determination in arthropods.** In Encyclopedia of Animal Behavior (Second Edi, Vol. 4, Issue May 2018). Elsevier. https://doi.org/10.1016/B978-0-12-809633-8.20815-7

Schultz, T. R., and Brady, S. G. (2008). **Major evolutionary transitions in ant agriculture.** *Proceedings of the National Academy of Sciences*, *105*(14), 5435–5440. https://doi.org/10.1073/pnas.0711024105

Wilson, D. S., and Sober, E. (1989). **Reviving the superorganism.** Journal of Theoretical Biology, 136(3), 337–356. https://doi.org/10.1016/S0022-5193(89)80169-9

SUPPLEMENTARY MATERIAL

Automatic Counts Tutorial

SYSTEM REQUIREMENTS:

This tutorial was elaborated for Ubuntu 20.04 LTS.

HARDWARE REQUIREMENTS:
GPU (NVIDIA GTX 1060 in our case)
16GB RAM (we used 32GB)

SOFTWARE REQUIREMENTS:

All codes and Readme.md files are available on GitHub labcog/Contador repository.

BASIC USAGE (= local copy)###
git tutorial: http://rogerdudler.github.io/git-guide/

1. Download the current version:
* if you are downloading it for the first time, you must clone the repository:

```
#!git
$ git clone
https://bitbucket.org/toddy757/labcog/branch/contador
PS: PRECISA AJUSTAR COMO VAMOS DISPONIBILIZAR O REPOSITÓRIO
(Não me recordo se ele fica fechado para edição para todo
mundo que não faz parte do repo do labcog. E que eu me lembre
precisavamos mover ele pra master ao invés de branch, certo?


#!git
$ git pull origin master
 All the alterations that you do will only be saved locally.


2. If you want to delete your modifications and download the
current version again:
```

```
#!git
$ git fetch --all
$ git reset --hard origin/master


# yolo
v5: requirements.txt from repositor ultralytics/yolov5
v3: opencv (gpu e módulo dnn linkado
pandas

## dataRRANSAC
opencv-python
matplotlib
pyaml
numpy

# dataCountline
numpy
pyaml

# optimization (we do not talk about the optimizers in this
tutorial, but some information is available on the repository
contador)
bayesian-optimization
motmetrics
scipy
```

Figure 1: Flowchart of all the steps involved in our video-tracking software development. Blue box involves data acquisition (experiments). Red boxes show all steps involved in the software validation stage. Green and yellow boxes show respectively the deep learning train model stage and running our model.

1. **Data acquisition**
2. **Software validation**
3. **Data annotation**
4. **Training model**
5. **Testing the object detection model**
6. **Tracking Tool**
7. **Counting Tool**
8. **Analyzing your data:**
    a. **Organizing the data**
2. **Running Object Detection Model for all videos**

3. **Running Tracking tool for all videos**
4. **Running Counting tool for all videos**
5. **Some tips and reading suggestions**

1. **Data Acquisition:**

If you plan on using video-tracking tools to analyze your behavioral data, you must record your data in a way that maximizes the probability of the software's success. That means that video quality, recording angle, recording distance, and focus are all essential features. They should not change across your video data. We strongly recommend recording your video data from an angle that does not include unnecessary objects in the images (for example, shadows, reflections, and other objects that will not be analyzed).

Video tracking tools usually contrast the background and the object of interest to identify the object and track it. For behavioral research, that means: if your animal model has light colors, you should use darker backgrounds; if it has dark colors, you should use lighter backgrounds. Also, it would help if you thought about recording time: the longer your videos are, the longer is the time required to analyze them. Also, longer videos usually occupy a lot of memory storage. If you need to compress the video data to save it, you will lose image quality, which can be a problem. We also recommend checking the video formats available to your recordings (we prefer .mp4 or .mkv). Also, the more objects you need to track, the harder it is to obtain a high accuracy from most video-tracking tools. All this information and tips are important because video-tracking programs perform poorly when analyzing video recordings containing noisy images.

Using iSpy for scheduling recordings:

- Download iSpy: https://www.ispyconnect.com/download.aspx (it runs only on Windows. Agent DVR is another tool from the same group that runs on other operating systems, but we have not tested it. CHECK IF YOUR VERSION SHOULD BE 32bits or 64bits)
- Install it.
- Run iSpy and add all your recording devices. By right-clicking, you should set all the recording features to that camera and schedule your recordings. Pay attention to which directory your data is being saved.

- iSpy user guide: https://www.ispyconnect.com/userguide.aspx

2. Software Validation:

- From all your video data, randomly select a group of videos for software validation (20-50) videos. If you have different recording conditions (in our case, we had videos recorded under light and darkness conditions), make sure that your selected recordings represent all your possible conditions.
- In our case, we manually counted the number of ants in each of these selected videos using the software **contadorManual.py**

contadorManual.py

```
This program opens the video with a blue line drawn in the
middle of the screen and allows manual counting as follows:
The count takes place when the ant crosses the line.
If it crosses the line in the top direction of the video: UP
If it crosses the line in the bottom direction of the videos:
DOWN
or
If it crosses the line in the right direction of the video:
RIGHT
If it crosses the line in the left direction of the videos:
LEFT

To run it (at the directory that contains it): python
contadorManual.py
Parameters:
-video (required)
[- h]: optional (help)
- line [LINE LINE LINE]: allows you to set 4 parameters to
adjust the position of the line (x,y, and x,y coordinates)

COMMANDS:
SHIFT: Play/pause
W: Count Up
S: Count Down
+: accelerate video
-: Decreases the video speed
```

(you can also set different keys for each command in the software code).

It will save a .txt file with the results.

EXAMPLE:
```
$ python contadorManual.py -video
/PATH_TO_VIDEO/VIDEONAME.mp4
```

- Run contadorManual.py for all your select videos for validation (we recommend saving them in a single directory).
- Organize all .txt result files into a single file (.csv)
- Here we stop the validation process and proceed with the Data Annotation. We will return to it after training and testing the model.

3. Data Annotation:

- Select the videos manually counted with the highest number of ants (10.000 video frames are the minimum required. It is crucial to select videos from all your experiment conditions. In our case, we selected four videos, two from the dark phase and two from the light phase).
- We ran all our data annotations in https://cvat.org/.
- After creating our profile, we created a project, defined the object class for annotation (ants and ants+leaf), and created a task for each video. You can organize the data in other ways.

NOTE: Data annotation is probably the most exhaustive part of this tutorial, but it is also one of the most important. We recommend CVAT because it allows you to have help during annotations and stop and continue your annotations. We took almost a month annotating the data working about 8-10 hours per day, so plan your time accordingly.

CVAT TUTORIAL

CVAT (Computer Vision Annotation Tool) is a tool developed by Intel co. and is open source. In it, you can upload images for annotation and do the annotation directly on video, using one or more classes and with various annotation features. It

allows you to download these image annotations in different formats, depending on which one you will use to train your network. CVAT also allows you to start and stop a video annotation at any time.

CVAT can be run in two ways: via the website (cvat.org), and the other is installation via GitHub repository, running through docker with port forwarding. The difference is that the one via the website has an upload limit of 500mb per user.

Note: CVAT via GitHub has access to openVino, an automatic annotation tool, and its docker images occupy a lot of storage(you will need at least 15GB of free disk space).

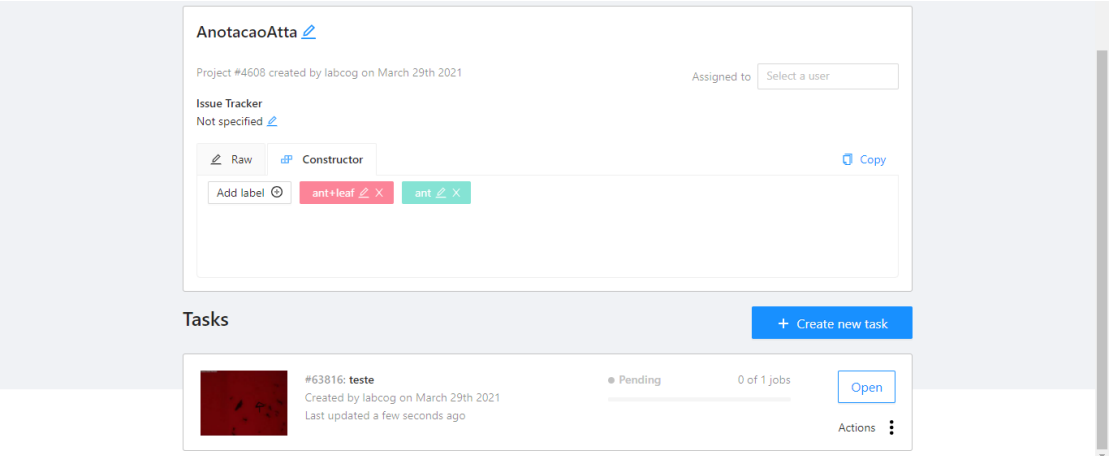
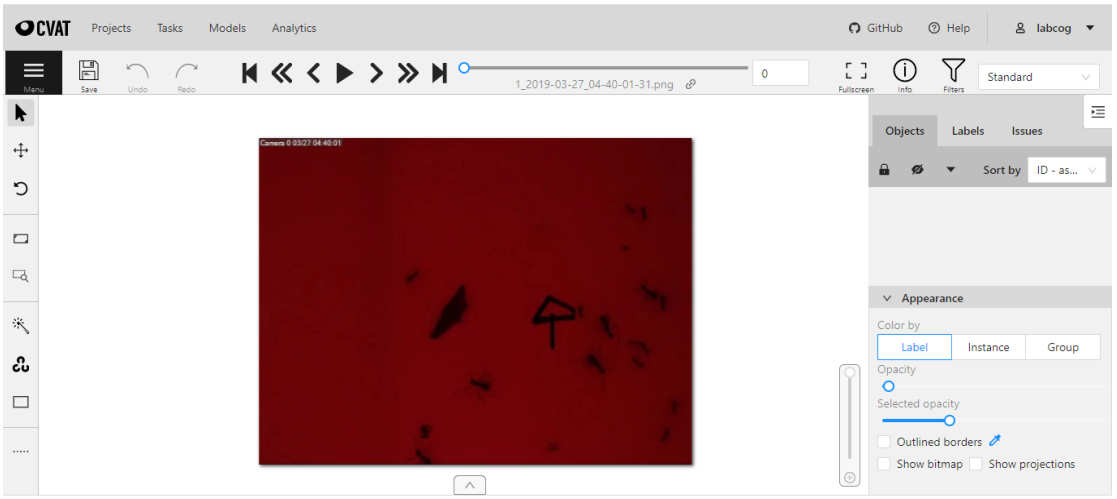


Figure 2: CVAT online.

How to annotate images on CVAT:

- Click **Open the task** and then click **Job #NUMBER**
- The annotation tool should open with the images associated with that task.

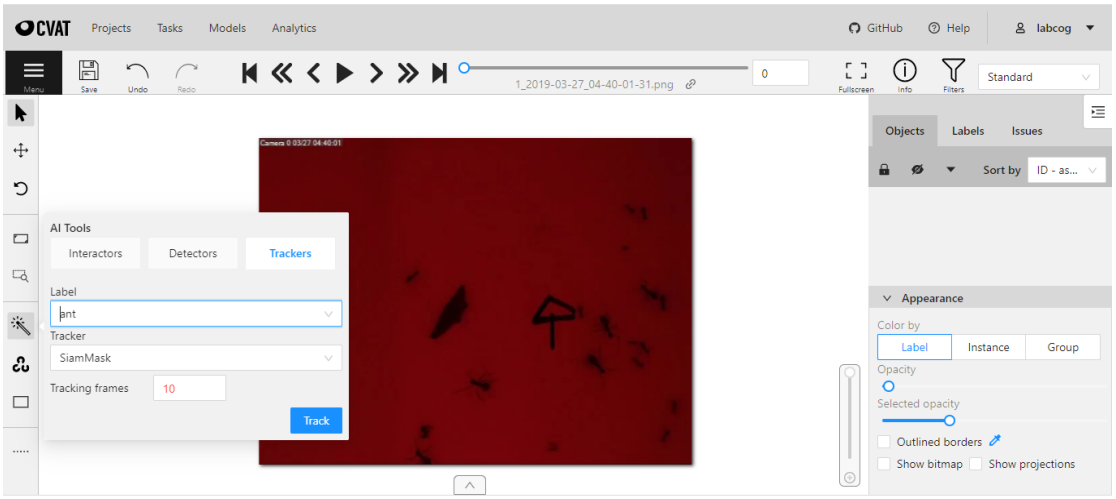


- There are many ways to annotate the images, and the options are in the left corner of the screen. In the right-hand corner, you will see the objects you have annotated in the "Objects" section as you do so. Here we have the automatic annotation recommendation.
    - Using annotation with the tracker function.

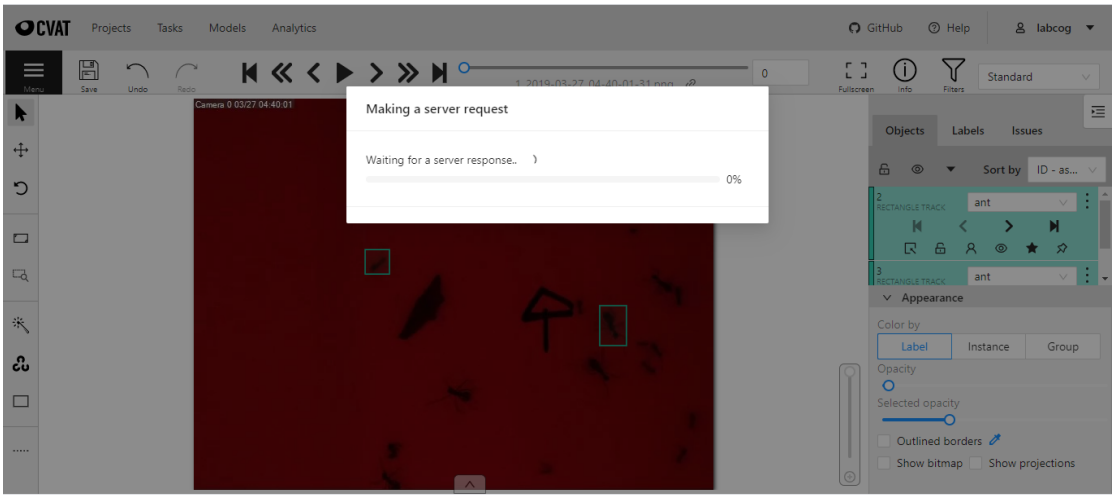
This function is intended to annotate objects every n frames automatically. To use it, you will annotate the object using the cross-like manual annotation and wait while the system cycles through the following frames and annotates the position of that object as it changes in the video. This process may take a while.

To use this function, click on the "**magic wand**" button and select the "**tracker function**":

Select the class you want to annotate, change 10 to 4 frames (it will be faster to process and probably contain fewer errors), and click "**track**".



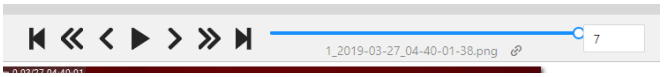
To make an annotation, using a cross on the tip of the mouse, you will click on the image and select the area around the ant.
IMPORTANT: SELECT THE WHOLE ANT WITHOUT LEAVING TOO MUCH SPACE OUTSIDE.



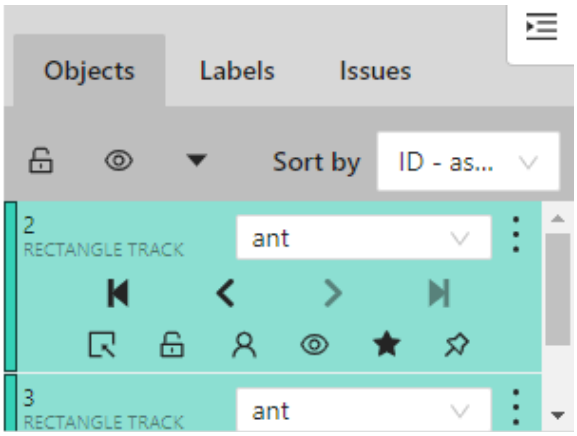
You will see that it has automatically annotated that ant in the subsequent four frames when you finish. Annotate all the ants in that frame.

Go through the following image frames correcting the annotation if there are any errors (probably depending on the ant's position, the rectangle may be of inadequate size, so it is worth adjusting or moving it if the ant has moved). Each ant gets an id number (right side of the screen).
To make a new annotation, press the "n" key on your keyboard, and you will see the marking cross on the tip of your mouse again.

These little arrows allow you to navigate between the frames of the video.



In the first frame that an ant enters, you should start the annotation, even if it is just partially in the image.
As soon as the ant disappears from the image, you should click on the box (see the object's id), go to the Objects section to find the object with that id, and click on the square with a small arrow ("Switch Outside Property"). This function indicates to the image that that object has left the video. If you delete the box, it will understand that that object NEVER EXISTED and delete all annotations for that object.



Note: Remember that the id number says that the object is unique (same ant). If the same ant is not accurately identified (more than one id number for the same object or the same id number corresponding to a different object), the tracking will not work.
Note: Ants interact a lot. Use the zoom mode to make sure where your annotations are accurate.
Note: Save often. I usually save after using the tracker for each new ant and every ten frames by correcting/adjusting the boxes (there is also an option on CVAT that saves your annotations automatically, but I also recommend manually saving it to be safe).

4. Training Model:

- We used YOLOv5 (a state-of-the-art real-time object detector system) to train the object detection model.
- Download all the annotations from CVAT in the format YOLO to train the YOLOv5 network.
- You will also need to download all the annotations in the CVAT video (.xml) format for adjustments in the tracking mode (RRANSAC - will be treated later).
- All the following steps will require GPU to run. Our graphic card model was NVIDIA GTX 1060
- Install the NVIDIA driver **(some of these steps might change according to your NVIDIA model)**

```
$ wget -c
https://us.download.nvidia.com/XFree86/Linux-x86_64/450.80.02
/NVIDIA-Linux-x86_64-450.80.02.run

$ wget -c
https://developer.nvidia.com/compute/cuda/10.0/Prod/local_ins
tallers/cuda_10.0.130_410.48_linux
   1) $ chmod +x for the first file
   2) $ ls Downloads
   3) Run: sudo ./NVIDIA..(name) --dkms
   4) Follow the instructions on the screen
        a) If an error occurs:
            i)   $ more /etc/X11/default-display-manager (it
                 returned -gdm3, but it could other display
                 manager)
                 $ sudo systemctl stop gdm3
                 $ sudo ./NVIDIA-Linux-x86_64-450.80.02.run
                 --uninstall
        b) Reboot
        c) Repeat the installation process
   5) Check GPU status
$ nvidia-smi
It should return something like this if everything is
working.

mila@mila-System-Product-Name:~$ nvidia-smi
Wed Jan 15 11:55:05 2020
+-----------------------------------------------------------------
---------------+
| NVIDIA-SMI 435.21    Driver Version: 435.21      CUDA
Version: 10.1    |
|-------------------------------+----------------------+------
---------------+
| GPU  Name        Persistence-M| Bus-Id        Disp.A | Volatile
Uncorr. ECC |
| Fan  Temp  Perf  Pwr:Usage/Cap|         Memory-Usage | GPU-Util
Compute M. |
|===============================+======================+======
```

```
================|
|   0  GeForce GTX 106...  Off  | 00000000:01:00.0 Off |
      N/A |
|  0%   47CP5    7W / 120W |169MiB /  3019MiB |    2%
Default |
+-------------------------------+----------------------+------
---------------+


+-----------------------------------------------------------------
---------------+
| Processes:
      GPU Memory |
|  GPU      PID   Type   Process name
Usage      |
|=================================================================
===============|
|     0     2223       G   /usr/lib/xorg/Xorg
      96MiB |
|     0     2411       G   /usr/bin/gnome-shell
      70MiB |
+-----------------------------------------------------------------
---------------+
```

- You might also need to install the Cuda toolkit:

```
Installation (item 3.8 Ubuntu):
https://docs.nvidia.com/cuda/cuda-quick-start-guide/index.html#ubuntu-x86_64
$ sudo sh cuda_<version>_linux.run
   1. Create a file at /etc/modprobe.d/blacklist-nouveau.conf
      with the following contents:

      blacklist-nouveau
      options nouveau modset=0

   2. Regenarate the kernel initramfs:

      $ sudo update-initramfs -u
   3. Reboot
```

- YOLOv5 Repository (please check the Readme.md file for instructions): https://github.com/ultralytics/yolov5
- 10000 training cycles are sufficient for small data sets, but the ideal, especially for larger data sets, is 100000 cycles.

- You can find all the scripts described here in: https://drive.google.com/drive/folders/1PBiS48w303iVhtvE3M0aBI7ZgWXO6c9s?usp=sharing
- Run the exp.sh
    - It is a bash script that generates all the other files

  In this case, it takes images in 4 folders (each one corresponds to an annotated video), mixes the images, and separates them between training and validation so that all videos are represented in both the training and the validation set. In the end, it generates a list of images and labels with this training set and another one with the test set.
  Output of exp.sh: shows for each folder (video) how many images are used in training and in validation

```
##exp.sh##

#!/bin/bash
DATAPATH="/home/marcelo.arruda/Projetos/M/datasets/videosComplicados/"
#DATAPATH=""
V1="1_2019-03-27_04-40-01"
V2="1_2019-04-04_23-00-00"
V3="1_2019-04-05_06-40-00"
V4="1_2019-04-08_12-00-01"

echo "
# ReTreinar Yolo em VideosComplicados
"

echo "
## Dados
    1_2019-03-27_04-40-01 (treino)
    1_2019-04-04_23-00-00 (validação e teste)
    1_2019-04-05_06-40-00 (validação e teste)
    1_2019-04-08_12-00-01 (treino)
"

printVideoDSInfo () {
    video=$1
    datapath=$2
    echo "- ${video}:
    - images: $(ls "${datapath}${video}"/*.png | wc -l)
        $(ls "${datapath}${video}"/*.png | head -1)
    - txts:   $(ls "${datapath}${video}"/[0-9]*[0-9].txt | wc -l)
        $(ls "${datapath}${video}"/[0-9]*[0-9].txt | head -1)
```

```
    - list:   $(cat
"${datapath}${video}/list_${video}.txt" | wc -l) lines
        $(cat "${datapath}${video}/list_${video}.txt" |
head -1)
        $(cat "${datapath}${video}/list_${video}.txt" |
tail -1)
    "
}

printVideoDSInfo $V1 $DATAPATH
printVideoDSInfo $V2 $DATAPATH
printVideoDSInfo $V3 $DATAPATH
printVideoDSInfo $V4 $DATAPATH


echo "
## Treino:
    ${V1}: $(cat "${DATAPATH}${V1}/list_${V1}.txt" | wc
-l) +
    ${V4}: $(cat "${DATAPATH}${V4}/list_${V4}.txt" | wc
-l) "
cat "${DATAPATH}${V1}/list_${V1}.txt"
"${DATAPATH}${V4}/list_${V4}.txt" > list_train.txt
echo "    -> list_train.txt $(cat list_train.txt | wc
-l)"


echo "
## Validação e teste:
    ${V2}: $(cat "$DATAPATH""${V2}/list_${V2}.txt" | wc
-l) +
    ${V3}: $(cat "$DATAPATH""${V3}/list_${V3}.txt" | wc
-l) "

cat "${DATAPATH}${V2}/list_${V2}.txt"
"${DATAPATH}${V3}/list_${V3}.txt" > list_testval.txt
shuf list_testval.txt -o list_testval.txt
head -n 1000 list_testval.txt > list_validation.txt
tail -n +1001 list_testval.txt > list_test.txt
echo "    -> list_validation.txt $(cat list_validation
```

- Run the fullTrainingVComplicados.sh
    - YOLO training script on the lists listTrain.txt and listVal.txt. The execution of this script will actually produce a trained model.

```
#fullTrainingVComplicados.sh#
```

```
rush=/usr/local/bin/rush

find "$1" -name "*[0-9].txt" | $rush "python
multilabelsYoloAnnotation.py {} {}"
```

- fullTrainingVComplicados.yaml: contains all especifications from the YOLO model

5. Testing the object detection model

- Install docker: https://docs.docker.com/v17.09/engine/installation/linux/docker-ce/ubuntu/
- Building the dockerfile and open a new image: (tutorial https://www.youtube.com/watch?v=LQjaJINkQ)

```
$ make cudnn_tensorflow_opencv-10.2_2.2.0_4.3.0
$docker images

output example:

REPOSITORY                                TAG                                 IMAGE ID            CREATED             SIZE
datamachines/cudnn_tensorflow_opencv      10.2_2.2.0_4.3.0-20200615           d58af4a715a4        14 hours ago        5.86GB
nvidia/cuda                               10.2-cudnn7-devel-ubuntu18.04       2b8bb5f68029        6 days ago          3.82GB
$ xhost +local:docker
$ docker run -ti --rm --gpus all -e DISPLAY=$DISPLAY -v
/tmp/.X11-unix/:/tmp/.X11-unix --ipc host --device /dev/nvidia0:/dev/nvidia0
--device /dev/nvidiactl:/dev/nvidiactl --device /dev/nvidia-uvm:/dev/nvidia-uvm
-v /home/:/home/ d58af4a715a4 /bin/bash (the image id varies according to your
computer)


if an error occurs:
           “docker: Error response from daemon: could not select device driver ""
with capabilities: [[gpu]].”
                $ sudo apt install nvidia-container-runtime


PS: you might need to install some packages inside the docker container:


$ apt-get update
$ apt-get upgrade
$ apt install python3
$ apt install python3-pip`
$ python3 -m pip install --upgrade pip
$ python3 -m pip install numpy`
```

```
$ python3 -m pip install opencv-python
$ python3 -m pip install opencv-python==3.2.0.8 (this version may change)
$ python3 -m pip install pyaml
```

PS: --GPU is the option that makes the computer run using the GPU instead of the CPU. You can check the GPU and CPU usage while yolov5Detect.py is running to verify "how much it is taking from the computer." If you do not use the --GPU parameter, yolov5Detect.py will run using only the CPU, which will be slower and require a lot of computer power.

to check CPU usage on Ubuntu:

`$ htop` (shows CPU usage, if the CPU memory usage increases a lot while you run yolov5Detect.py, you are probably using only the CPU.

`$ nvidia-smi` (if the GPU memory usage does not change while you run yolov5Detect.py, you are probably using only the CPU)

- Inside the container:

Choose some videos to test the YOLO network
Run a test:

```
$ python3 yolov5Detect.py --source
../../yolov5/1_2019-04-04_23-00-00.mp4 --weights
../../yolov5/weights/yolov5l_best_50.pt --conf-thres
0.1 --view-img --nosave --yml teste.yml --yolov5_path
../../yolov5
```

ps: check all the filenames. The command above is an example.

```
$ xhost -local:docker
```

6. Tracking tool

- For tracking the ants, we run our implementation of the RRANSAC, which reads the .yml files generated by the yoloVideoDetect.py and creates a new .yml file with the tracks. For more information see the Readme.md file on /labcog/contador/dataRRANSAC/.

```
Options:
- data: .yml input file
- out: .yml output file with the generated tracks
- outfw: .yml output file with the generated tracks
separated by vectors (frames, ids, position)
- display: shows the video while it runs (optional - it takes
more time to run)
- RRANSAC params: (must have)
  -M          (int) Number of stored models
  -U,         (float) Merge threshold
  -tau_rho    (float) Good model quality threshold
  -tau_T,     (int) Minimum number of time steps needed for a
good model
  -Nw,        (int) Measurement window size
  -ell,       (int) Number of RANSAC iterations
  -tauR,      (int) Inlier threshold (multiplier)
  -Qm,        (float) Q multiplier
  -Rmx,       (float) Rx multiplier
  -Rmy        (float) Ry multiplier


$ ./dataRRANSAC -data exemploInput.yml -M 50 -U 5 -tau_rho
0.1 -tau_T 3 -Nw 100 -ell 200 -tauR 2 -Qm 2 -Rmx 5 -Rmy 17
-display PATH_TO_VIDEO -out exemploInput_RRANSAC.yml

All these parameters were manually adjusted. They change
according to the YOLO model quality.
```

7. Counting tool

- For counting the ants, we run the COUNTLINE, which reads the .yml files generated by the dataRRANSAC and creates a .txt file containing all the counts. For more information see the Readme.md file on /labcog/contador/dataCOUNTLINE/.

```
$ python3 dataCountline.py -yml {}  -cline 0 239 640 241 >
{^_RRANSAC.yml}_count.txt'


PS: -cline defines where the line is positioned on the video
frame. The counts occur when the object crosses that line.
```

8. **Analyzing your data:**
    a. **Organizing the data**

- If everything is working well, you should proceed to analyze all your data. However, it is crucial to organize all the data and programs well. We organized all the output files for each step (video data, detection, tracking, and counting) on different directories. Also, as we had more than 2000 videos to analyze, we divided them into "days," allowing us to run all the steps for every day recorded. That is a good option because all the steps will run faster, and it makes it easier to find errors (basically, if the number of output files is different between the directories, an error occurred, and you should run that step for the missing file again).
- Do not move the programs from their original paths! Many programs used here access other program files to run, and if you change the directories, they will not find them, returning errors.
- Example of organization:
    - Directory Day_1:
        - DATA: 72 video files (.mp4)
        - YOLO: 72 .yml files
        - RRANSAC: 72 _RRANSAC.yml files
        - COUNTS: 72 .txt files + Day1allcounts.txt file (will be discussed later)

9. **Running Object Detection Model for all videos:**

- You will use a cross-platform command-line tool for executing jobs in parallel to run the detection for all the videos (as well as for the tracking and counting steps) called RUSH.
- Install RUSH: https://github.com/shenwei356/rush
- You must run this command for every directory containing the video data. That step will take much time according to the number of files that will be analyzed and the computer settings.

```
# 1. run the YOLO detector
find /PATH_TO_DIRECTORY_CONTAINING_THE_VIDEOS_FROM_THAT_DAY
-name "*.mp4" | rush "python3 yolov5Detect.py --source
../../yolov5/1_2019-04-04_23-00-00.mp4 --weights
```

```
../../yolov5/weights/yolov5l_best_50.pt --conf-thres 0.1
--view-img --nosave --yolov5_path ../../yolov5 -out
{^.mp4}.yml"
```

10. Running Tracking tool for all videos:

- You must run this command for every directory containing the YOLO detections.

```
# 2. run dataRRANSAC
find
/PATH_TO_DIRECTORY_CONTAINING_THE_DETECTIONS_FROM_THAT_DAY
-name "*.yml" | rush "./dataRRANSAC -data {} -M 50 -U 5
-tau_rho 0.1 -tau_T 3 -Nw 100 -ell 200 -tauR 2 -Qm 2 -Rmx 5
-Rmy 17 -out {^.yml}_RRANSAC.yml"
```

11. Running Counting tool for all videos:

- You must run this command for every directory containing the YOLO detections.

```
# 3. run Countline
find
/PATH_TO_DIRECTORY_CONTAINING_THE_TRACKINGS_FROM_THAT_DAY
-name '*_RRANSAC.yml' | rush 'python3 dataCountline.py -yml
{}  -cline 0 239 640 241 > {^_RRANSAC.yml}_count.txt'

cd  /PATH_TO_DIRECTORY_CONTAINING_THE_COUNTS_FROM_THAT_DAY
# 4. join all counts
tail $(find
/PATH_TO_DIRECTORY_CONTAINING_THE_COUNTS_FROM_THAT_DAY -name
"*_count.txt")  | grep -Po "^==>\s+(\S+)|(\d+)$" | tr '\n'
';' | tr -d '=' | tr '>' '\n' >> Day1allcounts.txt
```

- **PS: PAY ATTENTION TO FILE NAMES AND PATHS. You might need to change something from our command lines. Remember that if you do not change the name of the output files, they will be overwritten.**
- **After running these steps for all data, we recommend organizing all the .txt files containing the counts for each day on a single file (.csv, .xml, or .txt)**

12. Some tips and reading suggestions

YOLO:
https://pjreddie.com/darknet/yolo/
https://towardsdatascience.com/yolo-v5-is-here-b668ce2a4908
https://medium.com/deelvin-machine-learning/yolov4-vs-yolov5-db1e0ac7962b
https://blog.roboflow.com/yolov5-improvements-and-evaluation/
DOCKER:
https://www.youtube.com/watch?v=sYr4frA_1d8
https://djangostars.com/blog/what-is-docker-and-how-to-use-it-with-python/
NVIDIA-DRIVER:
https://medium.com/@rosdyanakusuma/how-to-install-nvidia-driver-in-ubuntu-18-04-307c25f73259

If something returns an error, do not hesitate to google the error. StackOverflow and other online forums contain many different solutions for almost every error.