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Study on leaf-cutting ants: from laboratory maintenance to applied personality studies with behavioral and molecular approaches

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Study on leaf-cutting ants: from laboratory maintenance to applied personality studies with behavioral and molecular approaches

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Supervisor: Dr. Odair Correa Bueno
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In memory of those who were at the beginning of this journey…

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O único comando que a natureza expressa é "Observe. Ouça. Fique atento"

(LEWIS, 2009)

ABSTRACT

Silva, J.P. (2022). Study on leaf-cutting ants: from laboratory maintenance to applied personality studies with behavioral and molecular approaches (Doctoral Thesis). Institute of Psychology of University of São Paulo, São Paulo, Brazil.

Atta sexdens, popularly known as leaf-cutting ant, is a species of biological and economic importance. Although these leaf-cutting ants have been studied for decades, there are still unanswered questions. An alternative that helps the development of research difficult to be executed in field is the maintenance of colonies under controlled conditions, such as in a laboratory. Tests realized in a laboratory environment can assess different lines of research, such as behavior and genetics. Considering the advantages of maintaining colonies of leaf-cutting ants in the laboratory, this thesis addresses the relationship between the species and research, covering the initial stages of creation until its application in studies in different areas. The thesis is divided into chapters. Chapter 1 introduces the topics explored and outlines of the thesis. Chapter 2 is composed by the protocol for collecting and maintaining leaf-cutting ants in the laboratory, followed by results exemplifying research and emphasizing the educational importance of ants. In Chapter 3, behavioral tests were performed to assess the existence of personality and behavioral syndrome in leaf-cutting ants. The results indicate that both exist in multilevels (colony and caste), in addition to some traits being related to worker size and subcastes. Chapter 4 (under development) investigated the reproduction system and genetic diversity associated with the personality of leaf-cutting ants. Preliminary results indicate that polyandry level is higher than literature recorded, but increasing the genetic variability of the colony. And low paternity skew within colonies gives evidence of uniform use of sperm. But only with the conclusion of the analysis will it be possible to clarify the genetic relationship within the colony with the personality of the workers.

Keywords: *Atta sexdens*; behavior; genetic; behavior syndrome; protocol

RESUMO

Silva, J.P. (2022). Estudo de formigas-cortadeiras: da criação em laboratório aos estudos aplicados de personalidade com abordagens comportamentais e moleculares (Tese de Doutorado). Instituto de Psicologia da Universidade de São Paulo, São Paulo, Brasil.

A *Atta sexdens*, popularmente conhecida como formiga cortadeira, é uma espécie de importância biológica e econômica. Embora essas formigas-cortadeiras tenham sido estudadas por décadas, ainda existem perguntas sem resposta. Uma alternativa que auxilia o desenvolvimento de pesquisas difíceis de serem realizadas em campo, é a criação de colônias em condições controladas, como em laboratório. Testes realizados em ambiente laboratorial podem avaliar diferentes linhas de pesquisa, como comportamento e genética. Considerando as vantagens de manter colônias de formigas-cortadeiras em laboratório, essa tese aborda a relação da espécie com a pesquisa, abordando as etapas iniciais de criação até a aplicação em estudos de diversas áreas. A tese está dividida em capítulos. O Capítulo 1 introduz os temas abordados e linhas gerais da tese. O Capítulo 2 é composto pelo protocolo de coleta e manutenção de formigas-cortadeiras em laboratório, seguido de resultados exemplificando pesquisas e salientando a importância educacional das formigas. No Capítulo 3 foram realizados testes comportamentais para avaliar a existência de personalidade e síndrome comportamental em formigas-cortadeiras. Os resultados indicam que ambos existem em multiníveis (colônia e casta), além de alguns traços estarem relacionados com o tamanho das operárias e as subcastas. O capítulo 4 (em desenvolvimento) investigou o sistema de reprodução e diversidade genética associado a personalidade das formigas-cortadeiras. Resultados preliminares indicam que poliandria na espécie é maior do que os registros na literatura, aumentando a variabilidade genética da colônia. E o baixo desvio de paternidade dentro das colônias evidencia o uso uniforme de esperma. Mas apenas com a conclusão das análises será possível esclarecer a relação genética dentro colônia com a personalidade das operárias.

Palavras-chave: *Atta sexdens*; comportamento; genética; síndrome comportamental; protocolo

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CHAPTER 1

1. GENERAL INTRODUCTION

Thesis description

The doctoral thesis presented here is the result of a long academic journey, which began long before the project was approved. It contains overcoming individual and global obstacles, as half of its journey was covered during the COVID-19 pandemic that devastated planet Earth. As a result, much of what was proposed in its initial project has changed. However, its objective remains the same: to contribute to the scientific advance of research on eusocial insects, especially in the object of study, leaf-cutting ants.

This thesis is divided into chapters. It starts with (1) a general introduction to the study topics; followed by (2, 3 and 4) three chapters in scientific article format; and (5) final remarks.

(1) As articles are more succinct texts, I make use of the general introduction to develop the subjects presented throughout the thesis freely;

(2) The first article explores protocols for collecting and breeding leaf-cutting ants in the laboratory for scientific and educational purposes.

(3) The second article assesses the existence of personality and/or behavioral syndrome in leaf-cutting ants through behavioral tests;

(4) The third article combines behavior and molecular to investigate the heritability of personality in leaf-cutting ants from maternal and paternal lineages;

(5) Finally, the final considerations unite the results of the topics addressed.

Study of leaf-cutting ants in the laboratory

The study of the behavior of leaf-cutting ants in Brazil began with the pioneering work of prof. Walter Hugo de Andrade Cunha, who in the 1960s invited researchers to study naturalistic animal behavior (CUNHA, 1965). The first leaf-cutting ant nests were built in the Animal Psychology laboratory at the University of São Paulo (FUCHS, 1995; OTTA; RIBEIRO; BUSSAB, 2013). Reports from the time denote the difficulty of breeding in the laboratory due to the complexity to keep the colonies alive and constant escapes. Records from the time report efforts to maintain the different stages of colonies, from newly collected queens to maintaining adult colonies (FUCHS, 1995). Obstacles overcome, the colonies were ready to be used in teaching and scientific research, influencing generations of future researchers (FUCHS, 1995; OTTA; RIBEIRO; BUSSAB, 2013).

Decades later, the study of leaf-cutting ants in the laboratory continues to be of great relevance in Brazil. In addition to behavior, leaf-cutting ants are the subject of research in several areas, such as ecology (NUNES, 2021), biogeography (BARRERA et al., 2022), pest control (RODRIGUES PEREIRA, 2021), among others. Its role in education continues to be important, connecting students at all school levels to science in a natural and even playful way (CANEDO-JÚNIOR; SILVA; KORASAKI, 2021).

Animal Personality

A long time ago, observers attentive to animal behavior noticed the existence of individual differences in the same situation. Questions like "why do some individuals always run away while others always attack their predators?" and many other questions have motivated an increase in research on these behaviors in recent decades.

Terms such as personality (DALL; HOUSTON; MCNAMARA, 2004; GOSLING, 2001), temperament (RÉALE et al., 2007), and behavioral syndromes have been used by researchers of animal behavior to explain consistent individual differences in behavior. Behavior of animals across time and context. The terminology found in the literature has varied definitions (see Table 1) and even Réale and collaborators (2007) took turns using personality and temperament. However, they chose personality after the term was consolidated in the literature (RÉALE et al., 2010; RÉALE; DINGEMANSE, 2012; SIH et al., 2015). In consideration of the already consolidated literature, we will distinguish the terminology of this work considering personality as a consistent individual behavioral difference across time and/or situations that, when correlated in a population, form behavioral syndromes (RÉALE; DINGEMANSE, 2012).

Inspired by the Big Five Personality Dimensions of the psychology of individual differences, which has a long tradition of studying humans (JOHN; SRIVASTAVA, 1999; MCADAMS; PALS, 2006), animal personality traits are generally studied along five axes. Behaviors proposed by Réale et al. (2007) (Table 2). The inherent characteristics of these behavioral axes allow for a wide range of research covering both vertebrates and invertebrates.

Table 1 – Definitions of the concepts "Personality", "Temperament" and "Behavioral Syndrome".

DEFINITION	REFERENCE
Personality: can be defined as those individual characteristics that describe consistent patterns of feeling, thinking, and behavior.	Gosling, 2001
Personalidade: Between-individual differences in behavior that persist through time.	Carter et al., 2013
Personality: Consistent individual differences in behavior across time and/or across contexts, for both humans and nonhuman animals.	Dall, Houston, & Mcnamara, 2004
Temperament is considered a construct closely related to personality, and has been defined as the inherited, early-appearing tendencies that continue throughout life and serve as the foundation for personality.	Gosling & Mehta, 2013
Temperament, personality and individuality describe the phenomenon in which individual behavioral differences are consistent across time and/or situations. (...) Since personality and temperament are often distinguished on arbitrary grounds, both are treated here as synonyms.	Réale *et al.*, 2007
(...) repeatability of a single behavior detects the animal personality or the correlation between multiple behaviors detects behavioral syndromes.	Garamszegi; Herczeg, 2012
In evolutionary ecology, correlated character sets are commonly referred to as syndromes, therefore, we refer to sets of correlated behaviors as behavioral syndromes.	Sih *et al.*, 2004
Behavioral syndrome: correlation between two characters or between measurements of a character in two different environmental conditions.	Réale *et al.*, 2007
Behavioral syndromes exist when the average phenotypes of individuals in one context/situation are correlated with the average phenotypes of the same individuals in a different context/situation such that populations harbor consistent individual variation in suites of correlated behaviors.	Dingemanse, Dochtermann, & Nakagawa, 2012
Behavioral syndromes: Correlations between different behaviors in a population.	Herczeg & Garamszegi, 2012
The behavioral syndrome refers to the correlation between differences in rank order between individuals over time and/or between situations and is therefore a property of a population.	Bell, 2007

Table 2 – Description of personality traits studied in animals, according to Réale et al. (2007).

PERSONALITY	DEFINITION
Boldness-Shyness	An individual's response to a risky situation
Exploration-Avoidance	An individual's response to a new situation, including a new environment, new food, or new object
Activity	The individual's general activity level in a risk-free home environment
Sociality	An individual's response to the presence or absence of conspecifics. Sociable individuals seek the company of conspecifics, while antisocial individuals avoid them
Aggressiveness	Agonistic response against a conspecific

Behavioral syndrome

In behavioral and evolutionary ecology, the term behavioral syndrome is used to designate correlations between individual differences in behavioral patterns (BELL, 2007; RÉALE et al., 2007; SIH et al., 2004; Table 1). These correlations may be the result of internal constraint, such as genetic inheritance, or adaptation by natural selection (BELL, 2005; BOUCHARD; LOEHLIN, 2001).

Research in this area has addressed the role of syndromes in relation to the ecological and evolutionary relevance of correlations between populations. Wray, Mattila and Seeley (2011) describe how syndromes can affect productivity in bee hives (*Apis mellifera*) and, in the long term, the survival of bees. According to the authors, the studied hives showed excitability correlated with the defensive response or the defensive response correlated with foraging activity. In the first case, the hives responded more sensitively (excitable, nervous) to external disturbances (e.g., beekeepers or predators), but were less willing to repair destroyed areas of the hive. In the second, they had a propensity to adjust to changes in the environment, whether challenges or new opportunities, which affected food production, their availability to feed their offspring and the subsistence of the entire hive. Therefore, different behavioral syndromes may have important ecological implications for species, affecting their population or ecological dynamics (SIH et al., 2012).

In several studies, behavioral syndromes are reported in social contexts, given that group living is a frequent strategy in several species (KRAUSE; RUXTON, 2002;

MAGNHAGEN; BUNNEFELD, 2009). Social contexts are observed both in temporary encounters (e.g. reproductive season; BROMMER et al., 2014) and in stable and lifelong social groups (GARAI et al., 2016).

Personality and behavioral syndrome in social insects

Compared with vertebrates, the characterization of behavioral syndromes in groups that exhibit collective behaviors, such as social insects, has been even less studied (see reviews by CARTER et al., 2013; JANDT et al., 2014). In eusocial insects, the fitness of the group is related to the fitness of its individuals, due to collective behavior. If, for example, a colony has individuals with more active and exploitative traits, who tend to roam a larger area in search of food, one possible outcome is that they find a food resource of high value to the colony. Not only will the individuals that forage recover the energy expended, but the nestmates will benefit from the food, increasing the overall fitness of the colony (RUEL; CERDÁ; BOULAY, 2012; WRAY; MATTILA; SEELEY, 2011).

Behavioral traits can be evaluated in a species from several types of groupings. Some studies separate organisms by sex (males versus females; GARAI et al., 2016), life stage (before and after maturation, adulthood; ECCARD; HERDE, 2013), physiology (metabolic rate; CAREAU et al., 2008) and morphology (small versus large; WRIGHT; KEISER; PRUITT, 2015). The advantage of studying social insects is that it is possible to evaluate their behavior at different levels: genetic, individual, between castes, colonies and even species (KELLER et al., 2011). Behavioral differences can be present, given that many of them have groups specialized in performing tasks (JANDT et al., 2014). In species with age polyethism (e.g., young workers versus older workers; SULLIVAN et al., 2000) or morphological (e.g., nurses versus soldiers; WILSON, 1976), behavioral differences may be associated with groups (such as castes) that constitute them or even among their own individuals. Such specializations are seen to have adaptive value at the colony level, usually given to the level of efficiency during task performance (BESHERS; FEWELL, 2001; SEELEY, 1982) and, in some cases, due to extremely specific function (e.g. bees Tetragonisca angustula, GRUTER et al., 2012; polymorphism in soldiers: ants *Cephalotes* spp., POWELL, 2008).

In recent decades, research has sought associations between social groups and personality traits (see reviews JANDT et al., 2014; WRIGHT et al., 2019). Social groups can differ from each other based on different “collective personalities” (STEWART; BARRICK; RYAN, 2003), which influence the survival of the group. In colonies of eusocial insects, in which the colony interacts as a "superorganism" (HÖLLDOBLER; WILSON, 2009), it is possible to verify these traits, since selective pressures act more on differences between colonies than between the individuals that constitute them (BERGMÜLLER et al., 2007; KORB; HEINZE, 2004). Therefore, the study of personality at the colony level is relevant to understand the evolution of these groups.

According to the review by Jandt et al. (2014), the most studied behavioral traits in social insects are divided into classical (behavioral syndromes), cognitive (psychological and learning) and social (social insects). Among the popularly studied classic traits are aggressiveness, exploration, and daring-hesitation. In the case of *Myrmica* spp., the monomorphic castes presented syndromes related to task allocation, with patrollers more daring, aggressive and active than foragers and caretakers of the offspring (CHAPMAN et al., 2011).

Cognitive traits are represented by sensory bias, learning, speed and accuracy in decision making. In *Bombus terrestris dalmatinus* bees, it was found that foragers have learning capacity at different levels and from multiple factors, such as chemosensory substances and the presence of experienced foragers (MOLET; CHITTKA; RAINE, 2009). In this case, the best learning performances occurred when the new substance was not only released into the environment, but ingested with nectar, and when it was introduced into the hive by successful foragers, indicating that learning can be developed from stimuli and situations.

Social traits include cooperation, communication, hygienic behavior, house-hunting, foraging, and defense. An example of cooperative behavior is observed in the wasp *Poliste dominulus*, which builds the nest together with other wasps, related or not (adopted) (STARKS, 2001). However, the females adopted by the colony spend less time and energy cooperating for its maintenance than the founders.

Genetic and personality in social insects

Genetic factors are potentially one of the proximal mechanisms associated with animal personality, given that the ground for defining personality is behavioral consistency and repeatability (LEMANSKI et al., 2019; WALSH; GARNIER; LINKSVAYER, 2020; WATT; SHUKER, 2010; ZAYED; ROBINSON, 2012). Studies that address the genetic basis of behavior help to answer several questions, both in an ecological and evolutionary context (DRENT et al., 2005). In fact, to understand whether personality evolves by natural selection, it is first necessary to confirm one of its main premises, heritability.

Heritability is defined as the relative amount of genetic variation in relation to phenotypic variation (FALCONER; MACKAY, 1996). The proportion of variance due to additive genetic components allows tracking evolutionary changes and calculating the genetic heritability of traits.

The personality of a social group may be related to its genetic variability, since several studies indicate that behavioral traits can be inherited (DRENT et al., 2005; OERS; MUELLER, 2010; PENKE; DENISSEN; MILLER, 2007). In social insects, the reproductive caste is responsible for the genetic structure of the colony. Breeders are represented by the queen and male with which they mate. However, reproductive strategies vary between taxa, as well as the genetic variability that makes up the colony. For example, a single queen can have multiple mates (polyandry) and/or multiple reproductive queens can stay in the same colony (polygyny) (BOOMSMA; KRONAUER; PEDERSEN, 2009; BOURKE; FRANKS, 2019; CROZIER; PAMILO, 1996). The genetic variability of the colony increases according to the amount of genetic material of the breeders, being able to generate offspring with different degrees of kinship and, consequently, different behavioral traits (CARERE et al., 2014; COLE, 1983; STRASSMANN, 2001; WRIGHT et al., 2019). In bees, genetic variation among workers altered behaviors associated with learning (CHANDRA; HOSLER; SMITH, 2000), division of work (PAGE; ROBINSON, 1991) and defensive behavior (BREED; GUZMÁN-NOVOA; HUNT, 2004).

The possibility of working with intracolonial genetic diversity becomes an advantage for personality researchers, as it allows research on the effect of genetic inheritance at the individual level, between castes and colonies.

Ants

Ants are an example of a eusocial group, organized into colonies with different levels of organization (individual, caste and colony) and division of tasks (reproductive or not), which guarantee the integrity of the group (HÖLLDOBLER; WILSON, 1990). Tasks can be divided among ants by age, physiological or morphological polyethism (ROBINSON, 2009; WILSON, 1976). Due to the varied nature of the tasks performed by ants, it is possible that some individuals be better suited to performing a task than others, indicating a relationship between task allocation and the ant's personality. In fact, ants of the genus *Myrmica* spp. Exhibit behavioral syndromes at individual, caste and colony levels (CHAPMAN et al., 2011). The pioneering work of Chapman et al. (2011) raised the possibility that other species present behavioral syndromes at different levels, as proposed by this research project with ants of the species *Atta sexdens*.

Leaf-cutting ants

The subfamily Myrmicinae, the most diverse among the Formicidae, has 140 genera and six tribes (Baccaro et al., 2015). The Attina subtribe is composed exclusively of leaf-cutting ants or basidiomycetes fungus cultivators, and its main representatives are *Atta* (saúva) and *Acromyrmex* (quenquéns), the most derived genera within the tribe (BARRERA et al., 2022; HÖLLDOBLER; WILSON, 1990; SCHULTZ; BRADY, 2008). Its distribution encompasses the entire Neotropical region, from the southern United States to southern South America (MARICONI, 1970). According to Hölldobler and Wilson (1990), these ants are the main consumers of vegetation, more than any other taxonomic group, including mammals and other invertebrates. The collected vegetation, composed of leaves, fruits, flowers, seeds and branches, is used to cultivate the Basideomycete fungus garden, with which the ants have a mutualistic relationship. Because the fungus is one of the main sources of food, it is essential for the ants that the fungus is constantly fed and cared for, preventing the proliferation of other microorganisms (such as pathogenic fungi) that could harm it (LACERDA et al., 2014). One of the ways to keep the fungus healthy is in underground chambers with temperature and humidity favorable to its development (WEBER, 1972). The depth and quantity of underground chambers, used for fungus cultivation or garbage disposal, depend on the species, containing from a few units to hundreds and being distributed superficially or deeply (up to eight meters deep; DELLA LUCIA,; OLIVEIRA, 1993; MOREIRA et al., 2004; VERZA et al., 2007).

Despite being known as one of the main agricultural pests in the country, in general, leaf-cutters have an important ecological role and contribute significantly to the ecosystem in which they are found. By collecting plant material to feed their symbiotic fungus, the cutters clean seeds and relocate the pulped ones in the soil in different places from the origin, contributing to the germination and dispersion of several plant species. (CHRISTIANINI; MAYHÉ-NUNES; OLIVEIRA, 2007; CHRISTIANINI; OLIVEIRA, 2010).

A important members of Attina is the leaf-cutting ant, *Atta sexdens*, one of the first ant species described in Brazil, present in the book "*Systema Naturae*" by Linnaeus

(1758). *A. sexdens* nest are large distributed in Brazil, high populated and present in agricultural and anthropic areas (Figure 1; SUGUITURU et al., 2015).

Figure 1. Distribution and occurrence map (green) of Atta sexdens.

Fonte: Antmaps (GUÉNARD et al., 2017).

The reproductive forms of the species are popularly known as *tanajuras* or *içá* (queens) and *bitús* (males), drawing attention at nuptial flight, due to the number of individuals and the nutritional value of the queens, which end up becoming the main ingredient at regional culinary (ROMEIRO; OLIVEIRA; CARVALHO, 2015). During the flight, the queen can copulate and be fertilized by more than one male, since the males produce less sperm than the queen can store in the spermatheca (*Atta* genera: FJERDINGSTAD; BOOMSMA; THORÉN, 1998; MARINHO et al., 2011). Subsequently, the queen will begin excavating the soil to form the chamber in which she will deposit the piece of fungus and begin the oviposition of the workers, a process described in detail by Cunha (1968) and Ribeiro (1995).

Workers are responsible for non-reproductive tasks. Divided into subcastes, consisting of ants that present morphological polyethism, they can be classified according to taske as: gardeners, take care of fungal hyphae and are one of the smallest ants in the colony (cephalic capsule between 0.6 to 1.2 mm); nurses, actively interact with the immature and, like the overalls, are small (cephalic capsule between 0.6 and 1.2 mm); foragers, explore the external area of the nest, cut and transport food (cephalic capsule between 1.7 and 2.4 mm); waste remover, transport and handle garbage, such as food not incorporated, and fungus and workers dead (cephalic capsule between 1.4 to 2.4 mm); and soldiers, responsible for the defense of the nest (cephalic capsule larger than 3.0 mm) (Figure 2; CARVALHO, 1972; HART; RATNIEKS, 2001; WILSON, 1971).

Figure 2 – Morphological polyethism of *Atta sexdens*.

Source: author.

Intraspecific morphological polyethism divides workers into different tasks and also allocates them to different locations in the nest. In the case of workers that perform tasks outside the fungus pot, such as foragers and garbage cans, there is a considerable risk of contamination by pathogens in relation to the internal castes, due to the environment in which they perform their tasks. Therefore, it is important that the workers responsible for the maintenance of the fungus, the planters, have the ability to recognize risk factors for the transmission of pathogens. One form of recognition is through chemical cues (CHÂLINE et al., 2015). To verify how planters would react to foragers and waste remover contaminated with spores of the fungus *Escovopsis webweri*, Lacerda and colab. (2014) designed an experiment in which ants, contaminated or not, were placed under the fungus garden. The result showed that planters did not behaviorally discriminate contaminated workers or not, nor did they show aggressive behavior. However, dumpsters were more sanitized, inspected and immobilized than foragers, which indicates that gardeners have the ability to discriminate average workers by task.

Another type of recurrent interaction between leaf-cutting ant workers in nature is interspecific. Jutsum (1979) observed intra and interspecific dyadic encounters (*Atta cephalotes* and *Acromyrmex octospinosus*) in the laboratory and found differences in recognition behaviors (stop, antennae, turn to the other ant), antennation duration and aggressiveness, indicating that there were differences in recognition nestmates of other leaf-cutting species. Although the laboratory experiments connecting colonies were carried out without the morphological description of the ants that interacted aggressively, there are indications that the interactions occurred between medium and/or large workers.

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CHAPTER 2

2. Leaf-cutting ants maintenance in laboratory conditions

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2.1.SUMMARY:

Here is described a protocol to successfully collect, and maintain healthy *Atta* (Hymenoptera: Formicidae) ant colonies for long periods of time in laboratory conditions. Additionally, different nest types and configurations are detailed together with possible experimental procedures.

2.2.ABSTRACT:

Ants are one of the most biodiverse groups on the planet and inhabit different environments. Maintaining ant colonies in laboratory conditions makes it possible to better understand their biology, enhance population control methods and, consequently, reduce economic damage caused by certain species such as those of *Atta* genus. These leaf-cutting ants are considered agricultural pests widely distributed throughout the American continent. They are highly socially organized and inhabit elaborated underground nests composed of a variety of chambers. Their maintenance on a controlled environment, where external humidity, temperature, and luminosity are artificially regulated, also depends on a daily routine of several procedures and frequent care that are described here. It initiates with the collection of queens during the reproductive season

(i.e., nuptial flight), which are individually transferred to plastic containers. Due to the high mortality rate of queens, a second collection can be carried out six months after the nuptial flight, when incipient nests with developed fungi wad are excavated, hand-picked, and placed in plastic containers. In the laboratory, leaves are daily offered to established colonies, and ants-produced waste is weekly removed along with remaining dry plant material. As the fungi garden keeps growing, colonies are transferred to different types of nests according to experimental purpose. Following a pattern, artificial leaf-cutting ant nests generally have a three interconnected transparent container configuration, representing fungi, waste, and foraging natural chambers. This nest design is ideal to monitor factors such as waste amount, fungus garden health, and the behavior of workers and queens. Tests carried out in a laboratory environment can assess the effect of toxicity, behavioral and physiological changes. Facilitated data collection and more detailed observations are considered the greatest advantage of keeping ant colonies in controlled conditions, which is impractical in field tests.

2.3.INTRODUCTION:

Ants compose a diverse group of individuals that presents high influence on most terrestrial environments. They act as efficient predators, dispersers, and environment architects, highlighting their importance and ecological success on natural ecosystems. All ant species are classified as eusocial insects; however, their social organization varies greatly among different species groups, i.e., labor division systems, functional groups, communication among individuals, forage organization, colony foundation and reproduction process[1]. As a highly diversified group, they resort to several food resources and specialized feeding behaviors. As a matter of fact, agriculture was not only a huge step for human civilization, but also for ant species. Approximately 55 to 65 Ma ago[2], attine ants began to culture fungi and incorporate them into an almost exclusive diet. They became so specialized that they developed strict, dependent, and obligatory interactions classified as symbiosis, where one individual does not survive without the other.

Lower fungus-growing ants collect and process dead organic matter, as fragments of rotting leaf, to grow their mutualistic fungi, while higher fungus-growing ants harvest fresh plant material, composing one of the most successful symbiotic natural systems[3]. This highly specialized agriculture technique allowed them to seize a new niche. The higher attine ants comprise the leaf-cutting ants, a monophyletic group that arouses between 19 Ma (15–24 Ma) and 18 Ma (14–22 Ma)[4–6] consisting of four valid genera:

Atta Fabricius, *Acromyrmex* Mayr, *Amoimyrmex* Cristiano, and *Pseudoatta* Gallardo. The leaf-cutting "higher" agriculture performed by the genera *Atta* and *Acromyrmex*, evolved from derived agriculture systems, but characteristically it exploits exclusively one mutualistic fungus species, marking a significant evolutionary transition. The fungal cultivars are transmitted vertically, from original nests to offspring, suggesting that they are clonally propagated.

Remarkably, *Atta* societies developed a complex organizational structure of enormous importance in their environment and great interest to myrmecologists. Their population can be composed of millions of individuals, most of them sterile female workers that display an accentuated polymorphism, i.e., distinct size and anatomical morphology. The population is distinguished by castes according to age, physiological state, morphological type, behaviors, and specialized activities in the colony[7]. Workers can be discriminated into gardeners and nurses, within-nest generalists, foragers and excavators, and defenders or soldiers[7]. This organization allows the performance of tasks in cooperation and a self-organizing system that can produce highly structured collective behaviors, allowing them to respond efficiently to environmental disturbances.

The role of population renewal is played by a single queen, for as long as she lives, composing the permanent reproductive caste. *Atta* queens are known to live more than 20 years, laying eggs throughout their lifespan. As the queen is irreplaceable, its endurance is crucial for colony survival. However, thousands of reproductive females and males can be found in the nest during breeding seasons, but none stays in the original nest, forming a temporary caste. New *Atta* colonies are established through haplometrosis, where a single queen commences a new nest. It occurs when a colony reaches sexual maturity, approximately 38 months from its implementation, and is repeated annually ever since until it is extinguished[8,9]. In *Atta sexdens* colonies, winged reproductive females and males are produced, nearly 3.000 and 14.000 individuals of each, respectively[9]. When environmental conditions are favorable, they leave the underground nest to begin the nuptial flights. The period of its occurrence is arranged by region, differing along the year throughout Brazilian territory depending on the species. However, the event seems to be preceded by rainfalls and humidity elevation[10], which can be related to excavation facilitation due to soil moisture[11]. Frequently, one to five weeks before the nuptial flight, nest entrances and channels are widened to facilitate the reproductives depart. Before leaving their mother colonies, the females collect a portion of the mutualistic fungus *Leucoagaricus gongylophorus* and store it in an infrabuccal cavity. Multiple copulations are performed mid-flight, and it is calculated that one queen can be inseminated by three to eight males (i.e., polyandry) in some species, ensuring genetic variability [12]. Afterward, the queens proceed to the soil, giving preference to locations with no or few vegetation [9], where they remove their wings and excavate their first nest chamber. This is the only period where queens can be seen outside the nest and it is unknown any successful nuptial flights in laboratory conditions[13].

The initial nest construction corresponds to the most crucial period of the colony, which can last six to eight hours[8,9]. At this moment, the queen cloisters herself in the initial chamber, and in a matter of days, oviposition begins. The first eggs are fed to the mycelial wad the queen regurgitates, marking the start of the colony's fungus garden. The first larvae appear in approximately 25 days[11], and about the end of the first month, the colony consists of a mat of proliferating fungus where eggs, larvae, and pupae are nested, and also the queen, that raises her initial offspring in isolation[8]. Eggs are also the food resource of the first larvae and are highly consumed by the queen[3]. Additionally, the queen sustains herself with fat-body reserves and catabolizing wing muscles with no use. The initial fungus culture is not spent, as the colony survival depends on its development, and during this period, the queen fertilizes it with fecal fluid [3]. Days after emerging, the first workers open the nest entrance and begin a foraging activity in the immediate area of the nest. They incorporate the material collected to the substrate of the fungus garden, which is now serving as food for the workers. Before being added to the fungal culture, the plant material carried in by the workers is cut into tiny pieces and moistened with fecal liquid. The ants manipulate fungus inoculum to increase and control its growth, which will serve for partitioning big soil excavated chambers, specialized in conditioning the garden.

Six months after the nuptial flight, *A. sexdens* nests contain a fungus chamber and a few channels. The great specialization in the construction of leaf-cutting ant nests works as a defense mechanism against natural enemies and unfavorable environmental factors[11]. Leaf-cutting ants are known to fragment the fungus garden and transpose it to chambers with high humidity when chambers start to dry out [3]. Thus, despite the excavation of the nest having a considerable energy cost, the energy invested is reversed in benefits for the colony itself [11]. With a few exceptions, *Atta* species also make specialized chambers for the colony's waste, made mostly of depleted fungi substrate and bodies of dead ants, isolating it from the rest of the nest, and establishing an important social immunity strategy [14]. In addition, a distinct group of workers manipulate the refuse directly, to avoid the contamination of other individuals. Workers constantly forage to nurture the fungi, which is the main nutritional resource of the colony. However, they can feed on plant sap while cutting fragments. Plant material is carefully selected regarding the fungus garden maintenance and influenced by many factors such as leaves traits and properties of the ecosystem [3].

The foraging strategy of leaf-cutting ants to obtain fresh material is highly complex, and combining it with the high harvest demand of established colonies, results in considerable economic loss to agricultural producers and jeopardizes forest restoration areas [11,15]. Therefore, these ants can be categorized as pests in most areas where may be encountered, from southern United States, to north-eastern Argentina and western Uruguay in South America. The extinguishing of problematic colonies is almost impossible since it depends on the queen mortality, the most protected individual of the colony. Hence, there are population control strategies mainly resorting to man-made chemical agents formulated within baits [16]. However, as leaf-cutting ants efficiently spot and eliminate potentially harmful substances to both fungi and colony individuals, new natural compounds and alternatives of control are constantly being tested[17–19]. As experiment results are hardly able to be monitored on field-tested colonies, preliminary essays are conducted in laboratory-controlled environment.

Thus, experimental protocols must be adapted corresponding to groups of interest considering the heterogeneous lifestyles of ants. Thereby, it is possible to efficiently study general biology on a species level, accounting colonies as operational units, where one single ant is only an element of a complex superorganism[1]. The reports gathered until now concerning the economically and evolutionarily important *Atta* genus allowed to successfully collect and maintain colonies in laboratory conditions and acknowledge their basic needs and general functioning. Based on their natural processes such as reproduction, colony founding, and feeding behaviors, a routine of practices has been developed that permits the long-term establishment of colonies in different types of nests. In the laboratory, it is possible to monitor fungi growth, colony and individual activity with distinct experimentation purposes and expand the knowledge in areas such as basic biology, behavior, genetics, microbiology, histology, and morphology. Here, it is described the procedure protocol applied to maintain leaf-cutting ants in laboratory for general research, and educational expositions, activities that can difficultly be performed in the field.

2.4.PROTOCOL:

1 Queens collection

1.1 Search in the literature for the period of *Atta* reproductive season in the region of interest.

NOTE: The period of reproductive season occurrence, frequency and day time of nuptial flights, varies according to regions climate conditions, thus this information must be gathered for the location where collections intend to occur.

1.2 Identify and mark locations with *Atta* nests considered as possible areas for queens and colonies collection.

NOTE: During nuptial flights queens are dispersed around nest locations, therefore areas with a great amount of colonies have higher chances to have queens landing spots where they initiate new nests excavation.

1.3 Check the areas selected previously for signs of nuptial flight during the reproductive season of *Atta* ants.

NOTE: During the reproductive season of *Atta* ants be aware of distinctive environment conditions of nuptial flight days, such as hot and rainfall weather. Identify leaf-cutting ant nests of the areas selected previously and look for external features that indicate the upcoming departure of winged reproductives. Nest features include: tunnel entrances widened, increased flow of workers showing more aggressive behavior towards possible predators, and winged reproductives appearing on tunnel entrances.

1.4 Prepare plastic lidded containers with a bottom plaster layer to retain the queens individually.

NOTE: Containers should be approximately 200 mL. The plaster layer at the bottom should have around 1 cm in height and be highly absorbent to perform humidity control purposes.

1.5 Prepare an environment with constant temperature and humidity.

NOTE: To correspond to the natural environment of leaf-cutting ants, individuals must be placed in controlled areas with constant temperature of 23±1ºC and approximately 70±10% of relative humidity. Fluctuations on these specifications can cause water condensation or moisture loss and compromise the fungi garden. The location should not hold intense activities and a high flow of people to avoid vibrations and disturbance. The cleaning products used in the area must be of neutral fragrance to prevent any interference in ants behavior.

1.6 During the nuptial flight, collect wingless queens that initiated nest excavation and carefully place them individually in the plastic containers prepared with a plaster layer. Avoid skin touching the queens using latex gloves or entomological tweezers.
NOTE: Wings removal and soil excavation behavior indicate female reproductives that already copulated, and therefore, are able to found a new colony.

1.7 Move the queens to the controlled environment designated.
NOTE: Queens transportation must be with ultimate caution, avoiding too much disturbance and maintaining a minimum temperature constancy.

1.8 Do not manipulate or move the queens for approximately 3 days after collection to refrain from stress.

2 Queens maintenance

2.1 Initially, water the plaster layer of the recipient every two days with the help of a needle syringe.
NOTE: It is recommended to use a needle syringe to water the recipients holding the queens to avoid disturbance due to manipulation. The recipient lid must be punctured with the needle and the same hole should be used during the period. The water amount should not let the plaster layer soaked. The water flow must avoid the queen, the initial fungi sponge and any immatures. For as long as the fungi garden exhibits a dry aspect with the absence of water, the recipient should be irrigated.

2.2 After a week, check if the fungus has been regurgitated by the queens. If there is no fungus, transfer approximately 2 grams of fungus obtained from another colony. The process can also be done if the fungus does not develop.
NOTE: To transfer fungus, it is necessary to collect healthy fungus from an established colony and remove all ants that are in it. Use tablespoon, entomological tweezers and latex gloves to manipulate the fungus.

2.3 After the appearance of the firsts workers begin offering fragments of young and thin leaves regularly, according to the cut activity of the colony.
NOTE: As the first workers initiate the leaves foraging, plant material must be offered after their appearance. The leaves offered must be healthy and the plants should not have been treated with insecticides or other chemical substances. The offering frequency depends on the agility with which the workers incorporate the plant material on the fungi, but can range to 2-3 days a week. At early stages, the leaf fragments should not be over 3 cm. Oat flakes and corn flakes can also be offered, but should be intercalated with leaves to avoid fungi dryness.

2.4 Remove the leaf fragments unused and other kinds of waste when offering new leaves.
NOTE: Avoid the use of perfumes, moisturizers, creams or any substance with a strong odor when manipulating the queens. Additionally, latex gloves should be used during all the processes.

2.5 Follow the development of the fungi and the offspring and when it is stable enough transfer to artificial perdurable nests.

3 Young colonies collection

3.1 Acquire plastic containers of approximattely 500 ml.

3.2 Identify indicative granulated soil mounds of incipient *Atta* nests six months after the nuptial flight on the locations previously marked with *Atta* nests.
NOTE: Six months after nuptial flight, the nests of young colonies are estimated to be up to 1 meter deep in the soil. A new collection is indicated at this period to achieve higher chances of successful colonies. Incipient nests mounds of most *Atta* species have a tower shape, with granulated soil particles.

3.3 With a garden hoe, excavate the nest entrance until reach the young colony.

3.4 Collect the queen, fungi garden, immatures and young workers, and place them in the plastic containers.

NOTE: The collection should be as gentle as possible. Naturally, a great amount of soil will be collected too and ought to be removed gradually in future maintenance procedures in the laboratory.

3.5 Move the colonies to the controlled environment designated.
NOTE: Young colonies transportation must be with ultimate caution, avoiding too much disturbance and maintaining a minimum temperature constancy.

3.6 The colonies must not be manipulated or moved for approximately 3 days to avoid stress.

4. Young colonies maintenance

4.1 Provide thin young leaves 3 times a week.
NOTE: The leaves offered must be healthy and the plants should not have been treated with insecticides or other chemical substances. The offering frequency depends on the agility with which the workers incorporate the plant material on the fungi. With the cut activity being intense, the offering can happen 2 times a day 3 times a week. At this stage, the leaf fragments should be at least 6 cm. Oat flakes and corn flakes can also be offered, but should be intercalated with leaves to avoid fungi dryness.

4.2 When offering new leaves remove colony waste, including soil remnants, with the help of a spoon.
NOTE: Workers themselves separate the soil from the fungus. When manipulating the young colonies avoid the use of perfumes, moisturizers, creams or any substance with a strong odor. Additionally, latex gloves should be used during all the processes.

4.3 Follow the development of the fungi and the offspring and when it is well-founded/stable/established enough transfer to artificial perdurable nests.

5 Perdurable artificial nests

5.1 Three plastic containers configuration

NOTE: Cloistered nests configuration should always have different recipients to separately pose as fungi garden chamber, waste disposal chamber and foraging chamber. The nests may vary in material and size according to their experimental purpose. The containers must be without openings, or the ants will escape. It can be used for general research.

5.1.1 Prepare a transparent lidded container of approximately 1 L with a plaster base.

5.1.2 Select two transparent lidded containers of approximately 500 mL.

5.1.3 Perforate and connect the containers with a transparent tube or a hose. The plaster base container should be on the middle and the other containers on opposite sides.
NOTE: The fungi chamber should always have a plaster base. The ants will choose the waste disposal chamber and the foraging chamber, and after that, they should not be interchanged.

5.1.4 Carefully transfer the fungi sponge of established colonies along with the queen, workers and immatures to the plaster base container.
NOTE: Before the transference, make sure the plaster base is watered. Use latex gloves.

5.2 Horizontal configuration.
NOTE: Nests with horizontal configuration allow close observation of the fungi garden and workers activities on it, immatures and the queen. It can be used for behavioral focusing research.

5.2.1 Acquire a transparent lidded container.
NOTE: Here it is used a custom-made lidded glass aquarium with a 31 cm x 21 cm x 4.5 cm. In this case, the little space between the lid and aquarium is closed with masking tape to avoid the ant escape. Two opposite faces must have a hole to allow the connection with other containers.

5.2.2 Add a plaster base of approximately 1 cm to the container.

5.2.3 Select two transparent lidded containers of approximately 500 mL.

5.2.4 Connect the plaster base container with the two lidded transparent containers on the opposite sides with a transparent tube or a hose.

5.2.5 Carefully transfer the fungi sponge of established colonies along with the queen, workers and immatures to the plaster base container.
NOTE: Before the transference, make sure the plaster base is watered. Use latex gloves.

5.3 Open arenas
NOTE: Open arenas nests allow the collection of ants without great disturbance and a better analysis of foraging behaviors. They can also provide a reliable representation of a colony found in nature for environmental education purposes.

5.3.1 Select lidded transparent containers to maintain the fungi garden and add a plaster base of approximately 1 cm.
NOTE: The size of the containers must be proportional to the size of the colony wanted, but it is recommended to start with 1 L containers and gradually increase to containers with higher volumes.

5.3.2 Select an open arena.
NOTE: The size of the arena can vary according to study purposes. If selected a large arena, the containers holding the fungi garden can be placed in its interior. And in the case of a small arena, it can be connected to the fungi garden containers with a transparent hose or tube. The arena will serve as a foraging chamber. Because the ants could also dispose of waste on it, the arena should not be too small.

5.3.3 Add one layer of polytetrafluoroethylene liquid (CAUTION) to the arena border to contain the ants.
NOTE: The polytetrafluoroethylene liquid must be applied in a single movement with the help of a soaked cotton and a nitrile glove.

5.3.4 The arena must be cleaned whenever food is offered.

6. Developed colonies maintenance

6.1 Daily offer at least one leaf per colony with 1 L fungi garden.

NOTE: The number of leaves varies according to the ants cut activity. If the fungus is dry, leaves should be pre-moistened to provide extra humidity. Here it is collected leaves from plants species such as mulberry (*Morus nigra*), mango (*Mangifera indica*), eucalyptus (*Eucalyptus* sp.), jambolan (*Syzygium cumini*), hibiscus (*Hibiscus* sp.), acalypha (*Acalypha wilkesiana*), and ligustrum (*Ligustrum lucidum*). Oat flakes and corn flakes should intercalate with the leaves. To colonies in open arenas, offer fruits such as orange, apple and blackberry once per week, also oat and corn flakes daily.

6.2 Remove all the content of the waste chamber every two weeks from all colonies.

NOTE: Workers should also be removed for population control purposes. If the workers transfer healthy fungi to the waste chamber it should also be removed, but it must be paid attention if the queen is on it. Bigger colonies should have their waste and workers removed once per week.

6.3 Remove dry leaves from the foraging chamber whenever offering new leaves.

NOTE: If the workers transfer healthy fungi to the foraging chamber it should be disturbed, the container lid left opened and neutral talcum powder applied to the chamber surface. This procedure must be done if there is still space on the fungi chamber, this way the workers will transfer the fungi back to the middle container without losing any immatures. Supposing that more fungus garden is wanted, another plastered bottom container should be added.

2.5.REPRESENTATIVE RESULTS:

Here it is shown some of the results that could be obtained by using the protocol of collection, maintenance and nest configurations described previously.

1. Influence of reproductive status in the immune response of leaf-cutting (*Atta laevigata)* ant queens

Queens of different species of ants have a long-life span and are therefore more likely to be exposed to the same pathogen more than once. By initiating a new colony, queens are isolated and a trade-off between reproduction and immunity can reduce investment in the immune defenses of these founding ants. Therefore, their resistance to disease may be related to their reproductive status. It was verified that the encapsulation cellular defense in *Atta laevigata* ants can vary with the reproductive status and the time elapsed after the mating, but it does not alter with weight and head height. By collecting queens in different moments of their early life cycle, and introducing a nylon thread into the gaster of the individuals, it was possible to evaluate individual pathogen resistance by measuring encapsulation rate. Six months mated queens presented the darkest nylon filament, thus, the higher encapsulation rate and more efficient immune defense (Figure 1). However, newly mated queens collected just after the nuptial flight, and virgin queens collected before nuptial flight shared approximately the same encapsulation rate among them (Figura 1). About a hundred queens were collected in total using the protocols previously described, and it allowed to better understand the investment of queens in immune response at early stages of the life cycle, the cost of reproduction, and the support provided to the queen by workers and an initial fungus garden. General studies on the cellular defense mechanisms of leaf-cutting ants can contribute to the clarification of the immune responses of insects and to the improvement of current control methods.

2. Leaf-cutting ants preference to sub products used in baits

Currently, the most applied method to control the population of leaf-cutting is through offering toxic baits associated with an attractive vegetal origin compound, being the citric pulp the most used. However, it is known that workers associate the bait toxicity with the attractive compound after prior contact and reject it, making the method inefficient. Therefore, the use of alternative attractive compounds such as soybean (100%), soybean plus citric pulp (50%/50%), and cashew plus citrus pulp (50%/50%) are proposed for a possible rotation between baits. These compounds were selected after preference experiments with *A. sexdens* and *A. laevigata* colonies. Three container nests were attached with glass arenas with divisions and insecticide-free baits with different compositions were offered. In total, 32 one-year-old colonies were used with approximately 1 L of fungi garden. The colonies were previously collected and maintained using the protocols described. The most loaded treatments of *A. sexdens* were citric pulp, soybean (100%), and soybean plus citric pulp (50%/50%) (Figures 2 and 3). And similarly, for *A. laevigata*: citric pulp, soybean plus citric pulp (50%/50%) and cashew plus citric pulp (50%/50%) (Figures 2 and 3). The results obtained for the two species showed that the soy and cashew sub products were the most attractives after citric pulp, suggesting that other baits composition could be used for chemical population control in leaf-cutting ants.

3. Environmental education

During the years 2016 to 2019, approximately 2,020 students from about 34 schools were able to visit the Laboratory of Leaf-cutting Ants (LAFC) and observe the ant colonies collected and maintained using the protocols described previously. The excursions had different aims, from general biology to social behavior learning. Aside from the knowing importance of insects such as ants, the complex physical structures built by them in nature represented by artificial nests in laboratory conditions are an example of collective effort and work organization that have educational interest. To each educational level (Figure 4) it was given a different detailed presentation, in some cases according to the approach asked by the teachers responsible for the students. Two colonies with the open arena type of nest are maintained for such purposes, each one has approximately 66 chambers with 177 L of fungi garden. Unfortunately, the excursions were unable to be made during the pandemic. However, it is possible to see a rise in the number of students visiting during the last four years reported (Figure 5). Nonetheless, to maintain scientific dissemination during the pandemic, virtual visits were made through video sharing platforms. The video[20] showing how a nest of leaf-cutting ants is inside, made in partnership with "Manual do Mundo", a channel with 16 million subscribers, had 1.9 million views and almost 3 thousand comments in less than 1 year. There, the biology and creation of leaf-cutting ants in the laboratory were approached.

2.6.FIGURE AND TABLE LEGENDS:

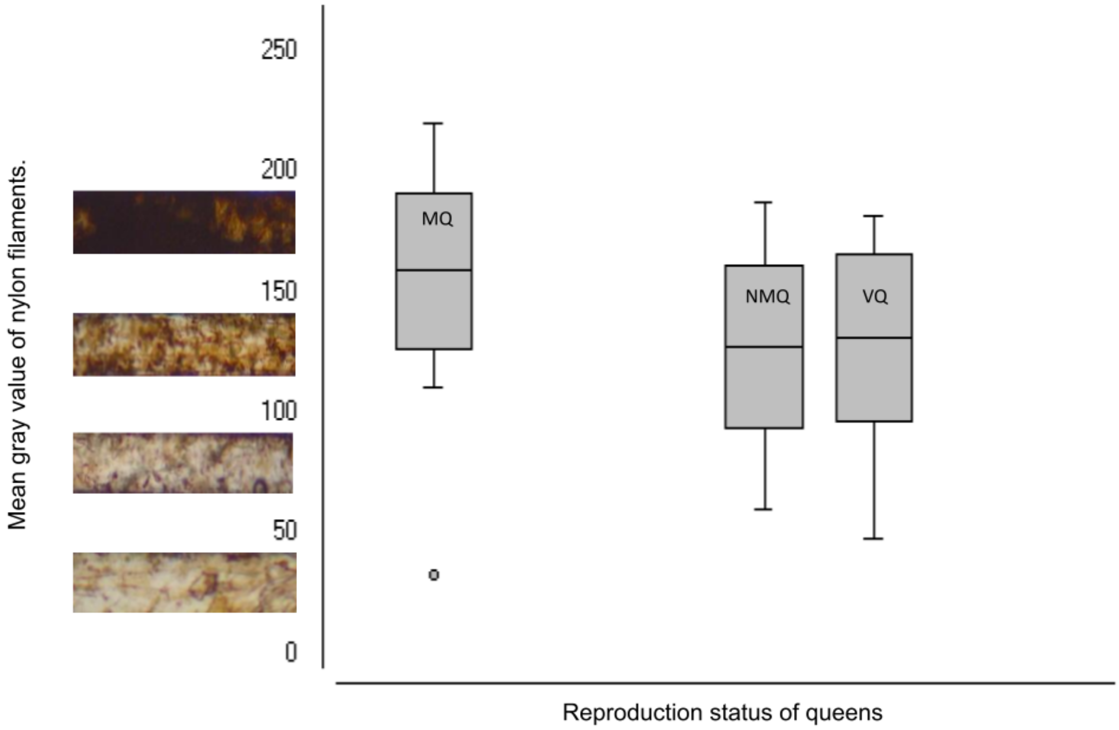

Figure 1. Comparative analysis between the encapsulation rate of queens at different reproductive status. Encapsulation levels were evaluated through the mean gray value from images of the nylon filaments inserted into the gaster of the individuals. Thus, the darkest threads were considered to represent an efficient cellular defense because it was assumed that more overlapping hemocytes were lining the target, as illustrated in the figure above. Mated queens (MQ) showed the highest level of encapsulation ($p<0.0001$), hence an effective individual immunity defense. It was observed that the newly mated queens (NMQ), and virgin queens (VQ) presented, similarly, lower rates of encapsulation, and consequently a less effective individual immunity defense. The results obtained suggest that mating and nest establishment events have effect on the cellular immune responses of *Atta laevigata* queens.

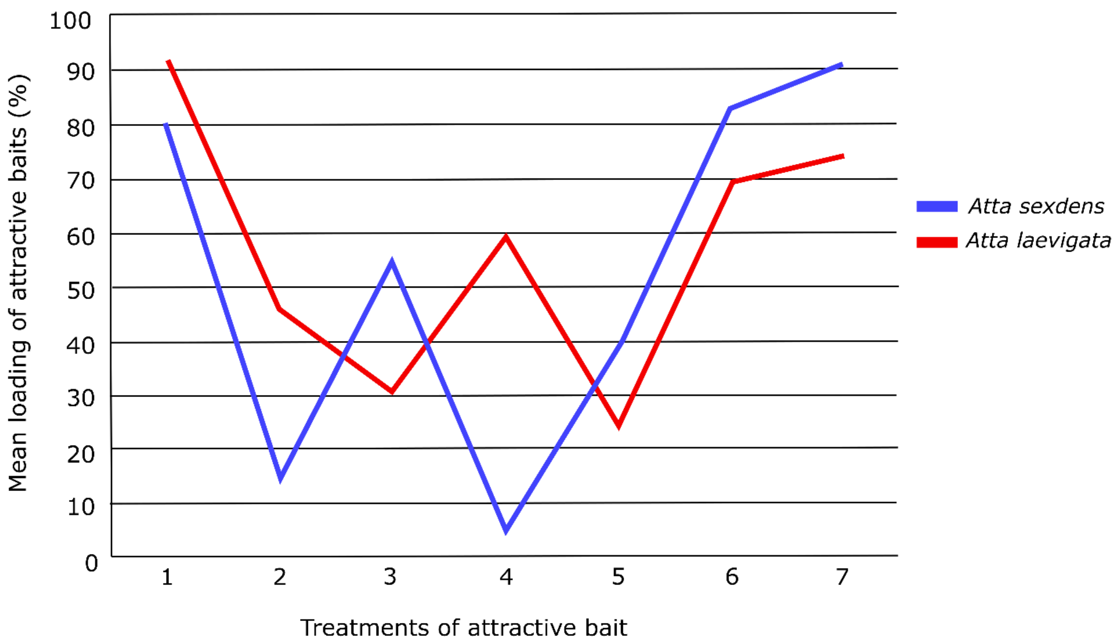

Figure 2. Comparison of the loading activity of *Atta sexdens* and *Atta laevigata* workers to attractive baits. Similarly, the citric pulp (100%), soybean (100%) and soybean plus citric pulp (50%/50%) treatments were the most loaded for both species. However, the least loaded treatment for *A. sexdens* (Cashew plus citric pulp), was 60% loaded by *A. laevigata* workers. Legend: 1. Citric pulp (100%); 2. Cashew (100%); 3. Cashew plus citric pulp (50%/50%); 4. Cashew plus citric pulp (2%/98%); 5. Mango plus citric pulp (50%/50%); 6. Soybean (100%); and 7. Soybean plus citric pulp (50%/ 50%).

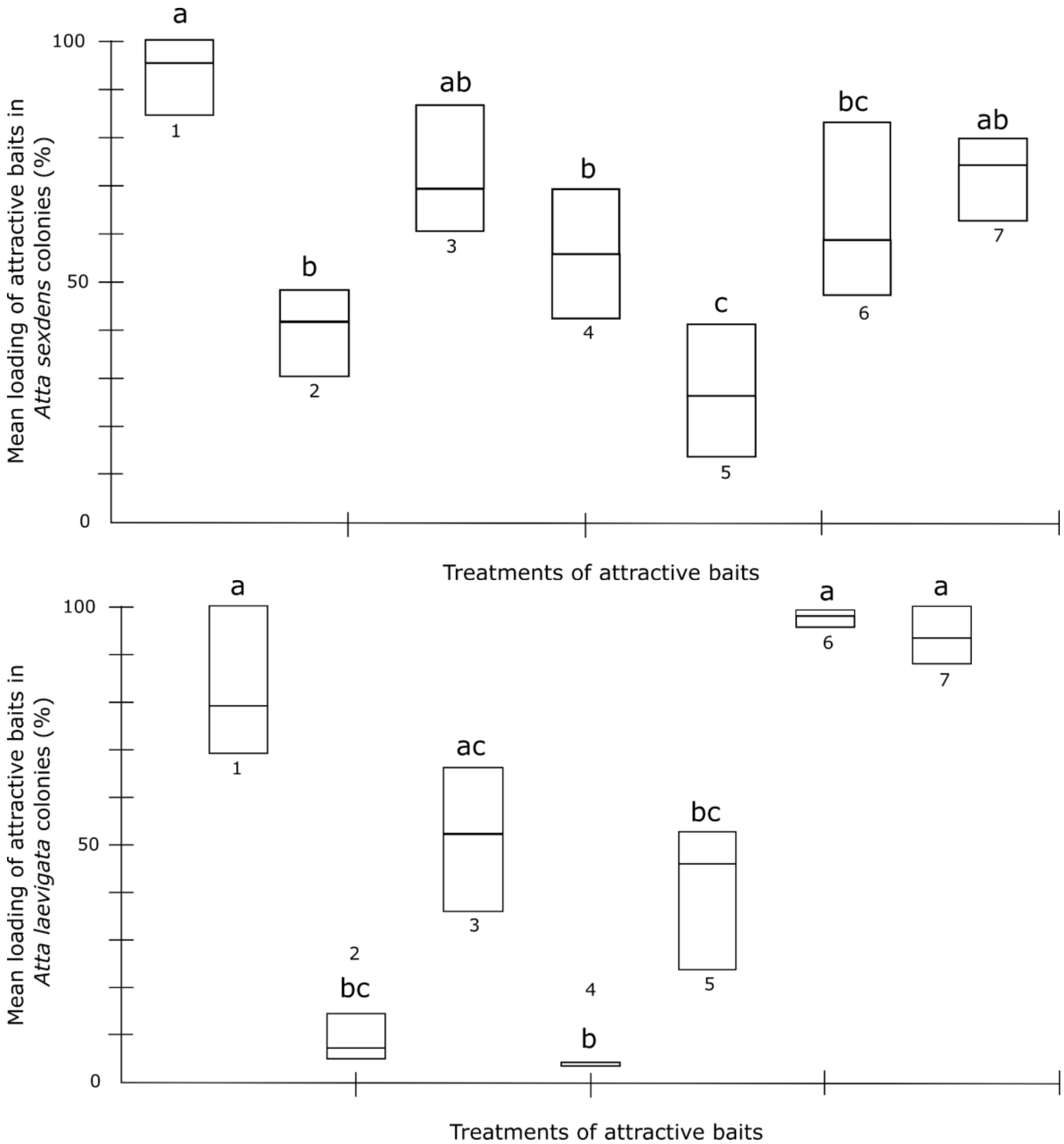

Figure 3. Loading preference to attractive baits in *Atta sexdens* and *Atta laevigata* colonies with the levels of significance (p<0,0001). Legend (left to right): 1. Citric pulp (100%); 2. Cashew (100%); 3. Cashew plus citric pulp (50%/50%); 4. Cashew plus citric pulp (2%/98%); 5. Mango plus citric pulp (50%/50%); 6. Soybean (100%); and 7. Soybean plus citric pulp (50%/ 50%).

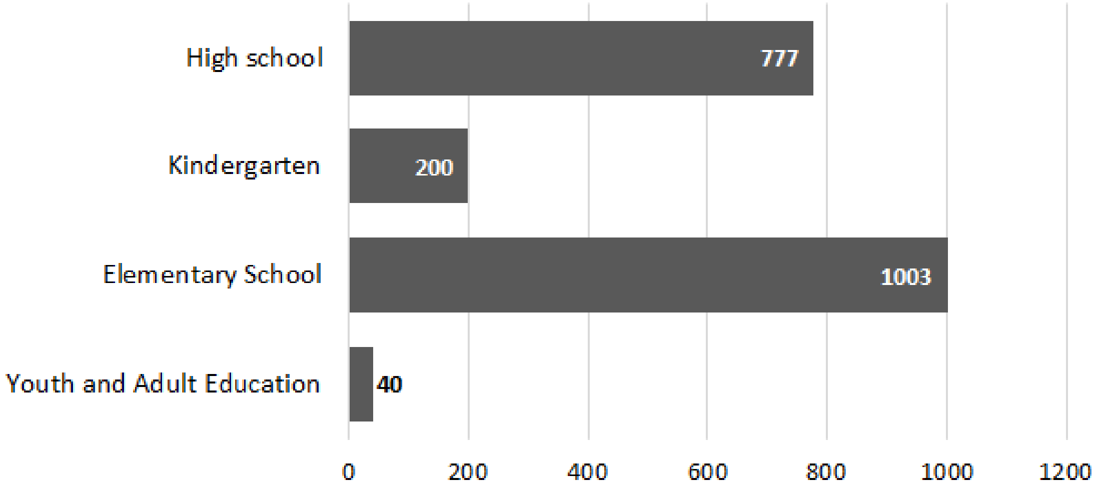

Figure 4. Number of students according to the level of education that were present in excursions during the period of the years 2016-2019. The excursions aimed different approaches involving leaf-cutting ant colonies that are maintained in the Laboratory of Leaf-cutting Ants.

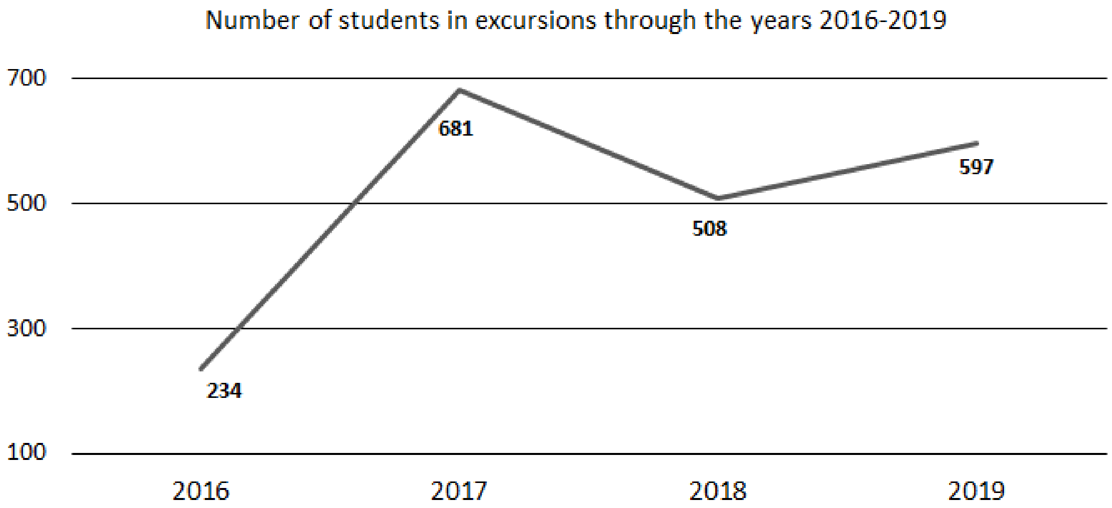

Figure 5. Number of students that were present in excursions to the Laboratory of Leaf-cutting Ants during the period of the years 2016-2019. It is possible to see an increase in the interest of educational organizations to exhibitions of live insects, such as ants, that allow a close observation by the students.

2.7.DISCUSSION:

The protocols described here can be used to maintain leaf-cutting ant colonies through an assertive and replicable method, which allows the development of research that would be limited by field conditions. Their maintenance in the laboratory includes several procedures described here, which include site preparation under favorable abiotic conditions, collection of queens and young colonies, and continued care. Thereby, healthy ants and colonies become available for research in several areas such as comparative morphological, toxicological, histological and pesticide action studies [21,22], microbiome studies [23,24], or research with attractive and repellent plants [25] at individual and colony level. However, it should be emphasized that some steps are crucial for the success of leaf-cutting ants maintained in controlled conditions. Colonies in the early stages need distinguished care, and following the initial procedures earlier detailed can guarantee their survival. For example, it is necessary to identify the place where the species are found and the period when the winged reproducers initiate the nuptial flight. Currently, the possibility of captivity breeding is extremely low, as it is extremely rare to record a nuptial flight in the laboratory [13]. Additionally, leaf-cutting ant colonies in the fertile phase are large and difficult to excavate, and given the nest architecture, it is a challenge to locate and collect the queen [26]. For instance, *Atta laevigata* nests can be up to 67 m^2 of nest area, and occupy more than 563 m^2 of internal surface area divided into 7,864 chambers in 7 m depth [27].

The advantage of collecting queens promptly after the nuptial flight is to guarantee a greater number of reproductive forms in a short period of time. Similarly, collecting young nests a few months after the nuptial flight ensures that the queen's chamber is not deep into the surface. In this phase, only the queens with more fitness remain, which survived the food shortage phase and were able to take care of the fungus and the first workers during the claustral phase [28–31]. In laboratory conditions, queens survival rate can reach 14,5% in the initial months, even with healthy fungus transferred to 90% of queens (data unpublished). This can be due to the lack of fungus development or natural contamination with entomopathogenic fungi [32].

The implementation of the protocols addressed here can be applied to other species of scientific interest, such as other fungus growing ant like the genus *Acromyrmex* (Mayr). These leaf-cutting ants are also considered agricultural pests in particular regions of America, and have recently attracted more interest in early stages colony development [33] and toxic baits interaction [34] focused research. Although this work focuses on leaf-cutting ants, especially for highlighting fungus garden care, the maintenance protocol and the different types of nests described are also applicable to other groups of ants. Each section of procedures outline fundamental ant necessities that must be taken into consideration when preserving them in controlled conditions, such as: identifying the most appropriate diet; offering food at regular intervals in a foraging chamber or specific area; providing a high humidity level chamber with plaster base to keep the queen and immatures; avoiding ants escape through contingency substances added to the structures of the artificial nests; and transferring the colonies to another artificial nest when necessary.

Over the past decade, universities and researchers have been dedicated to being more inclusive, inviting the society to be part of the laboratories and their research [35a]. Embracing with great determination the educational purpose, the ant laboratories become tools to attract people's curiosity and connect the academic world with common knowledge. In many cases, students can observe demonstrations of the natural ant colonies seen on a daily basis through school visits that address basic biology, colony maintenance, fun facts and questions that can be answered through scientific research. On a more expressive level, through extension projects such as “First Steps in Science” [36] the students can actually be part of research teams Consequently, ant studies with educational purposes benefits student development as it enhances investigation and experiences while encouraging greater interest in scientific research.

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CAPES, CNPq, and FUNDUNESP

2.9.DISCLOSURES:

The authors have nothing to disclose.

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CHAPTER 3

3. Personality and behavior syndromes at multiple levels on leaf-cutting ants

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3.1.INTRODUCTION

For decades, researchers have noted the existence of individual differences in behavior of animals facing the same situation or during long periods. Over time, there has been an increase in research on consistent individual differences in the behavior traits of animals across time and context, considered as animal personality (DALL; HOUSTON; MCNAMARA, 2004; DINGEMANSE; WOLF, 2010; GOSLING, 2001; RÉALE et al., 2007). Personality traits compose the axes of animal personality, which vary along a particular dimensions such as Exploration-Avoidance and Boldness-Shyness (BELL; HANKISON; LASKOWSKI, 2009; RÉALE et al., 2007; SMITH; BLUMSTEIN, 2008). When two or more personality traits present correlation in a population is designated as behavioral syndrome (BELL, 2007; RÉALE et al., 2007; SIH et al., 2004).

Evidence confirms the occurrence of personality traits in several species (BEEKMAN; JORDAN, 2017; CARTER et al., 2013; DE AZEVEDO; YOUNG, 2021; GOSLING, 2001; MODLMEIER et al., 2015; ROCHE; CAREAU; BINNING, 2016; WEISS, 2018; WILSON et al., 2019). But most researches have focused on the personality development of unitary organisms. For example, comparing sex (male vs female; SCHUETT; TREGENZA; DALL, 2010), age/life stages (CABRERA; NILSSON; GRIFFEN, 2021), and different locations (STUBER; CARLSON; JESMER, 2022). However, personality is also verified at group or colony levels when behavioral differences are temporally consistent between distinct social groups, also known as collective personality (BENGSTON; JANDT, 2014; JANDT; GORDON, 2016; WRIGHT et al., 2019). Collective personality is a relevant factor for the development and survival of highly structured groups, such as eusocial insects.

In eusocial insects, personality emerges at the group level (colony and caste) in addition to the individual (DALL et al., 2012). Many studies support the presence of collective behavior traits in colonies. For instance, the Argentine ant (*Linepithema humile*) present different levels of aggressiveness when subjected to the same treatment according to the region where the colony was collected (BUCZKOWSKI; SILVERMAN, 2006). Inter-colonial aggressiveness in honey bees (*Apis mellifera*) varied during summer and winter seasons, and was correlated with juvenile hormone (PEARCE; HUANG; BREED, 2001). Social wasp colonies (*Vespula vulgaris* and *V. germanica*) were proved consistently aggressive according to different disturbances levels during short and long periods of time (JANDT et al., 2020). Termite (*Nasutitermes corniger*) colonies do not behave differently toward neighbors and stranger colonies, but aggression probability increases accordingly to intra-colony relatedness between colonies (ADAMS; ATKINSON; BULMER, 2007).

Colony behavior can emerge from within and among castes, also inter-colony interactions can affect intra-colony behavior by feedback (JANDT et al., 2014). Intra-colony variation is an important component of their organization, affecting division of labor among workers (MODLMEIER; LIEBMANN; FOITZIK, 2012). In fact, this research area has focused on behavioral differences among individual workers and task allocation associated with behavioral specialization or different forms of division of labor (BENGSTON; DORNHAUS, 2014; LOFTUS; PEREZ; SIH, 2021). Typical temporal polyethism in social insects predicts changes in worker behavior with age (ROBINSON; JANDT, 2021). Usually, young workers developed their tasks inside the nest, while older workers task allocation varieties (TRIPET; NONACS, 2004). In *Leptothorax acervorum* ants, behavioral syndromes are associated with task allocation and physiological traits in workers from the same age (KÜHBANDNER; MODLMEIER; FOITZIK, 2014). Younger workers are less aggressive and active than older ones with well-developed ovaries. Also, it was observed that aggression in common wasp *Vespula vulgaris* increases with age (SANTORO et al., 2015). Morphological differences can be or not an important factor at division of labor. The monomorphic ants, *Myrmica rubra* and *Myrmica ruginodis*, boldness-aggressiveness syndrome was correlated with the task performed by the workers (CHAPMAN et al., 2011). In these ants, recruiter to foraging and nurse castes were less aggressive, active and bolder than patroller caste. At polymorphic workers, such as *Acromyrmex echinatior* and *Oecophylla smaragdina* ants, smaller workers tend to be less aggressive than larger or major workers (KAMHI et al., 2015; LARSEN et al., 2014).

While studies have highlighted the relative importance of colony or caste individually (PORTER; JORGENSEN, 1981; POWELL, 2008; POWELL; FRANKS, 2005), little has been done to explore the importance of behavioral variation observed encompassing both levels simultaneously, especially in polymorphic species. Here we tested if personality traits and behavioral syndrome are present in the leaf-cutting ant *Atta sexdens* (Hymenoptera: Formicidae) at multiple levels, colony and caste. Leaf-cutting ants are known as one of the economically most important South American agricultural pests (DE BRITTO et al., 2016; WEBER, 1966). Generally, colonies consist of reproductive and sterile castes (DIJKSTRA; NASH; BOOMSMA, 2005). A single reproductive queen (i.e., monogynic) lays the eggs and workers take care of the brood, nest, fungus garden, foraging and defense (DIJKSTRA; NASH; BOOMSMA, 2005; WILSON, 1971, 1980). Leaf-cutting workers exhibit one of the most complex forms of morphologically worker castes, with a head-width size range from 0.5 to 4.5 mm (SCHOFIELD et al., 2011; WILSON, 1980). For decades studies demonstrated the relation between workers' morphology and division of labor (SCHOFIELD et al., 2011; WILSON, 1980). Usually, smallest workers worked within chambers at fungus and brood care, while medium-size workers are foragers and generalists, and largest workers are defenders (DELLA LUCIA,; OLIVEIRA, 1993; WILSON, 1980).

In the present study, we investigated the hypothesis that leaf-cutter ants present personality at multiple levels and are associated with caste morphology. We reason that group composition is an important factor to colony personality, so we expect colony-level differences to correspond to workers size and collective behavior. We predict that if caste-level personalities are linked to worker size, probably smallest and largest ants are going to be at opposite personality ranges. Since there are almost no previous tests about waste remover behavior, we anticipate that medium workers in general will behave similarly. To address these hypotheses, we divided workers into five subcaste according to task: nurse, gardener, forager, waste remover and soldier. The same colonies and castes were tested in five assays to measured four behavior traits: Exploration-Avoidance, Boldness-Shyness, Sociability, and Aggressiveness.

3.2.METHODS

Nine colonies of *Atta sexdens* were maintained in the laboratory before the behavioral assays at controlled temperature (24°C±2°C), humidity (70%±10%) and light/dark cycle (12h). We used 10 ants per caste (nurse, forager, gardener, waste remover and soldier) individually. Both colony, caste and ant test orders were randomly determined. Ants were selected while performing the task corresponding to their caste: nurse in physical contact with eggs, larvae or pupae; forager transporting food; gardener incorporating vegetal material to the fungus; waste remover carrying remains of dead fungus or corpse in the garbage disposal; soldier were selected not engaging at the tasks described above and by the largest cephalic capsule size.

Each ant performed the five behavioral assays (adapted from Chapman et al., 2011) described below three times on the same day to verify the agreement between repeated test measures, known as consistency or repeatability (CARTER et al., 2013). After performing the tests, ants were dead by freezing and preserved in 90% ethanol.

Morphologic measure – Ant size

Each leaf-cutting ant was measured to have an indication of its size. For this purpose, workers size was measured as head-width, which is the maximal distance (in mm) between the eyes in full-face view. This measurement is considered a reliable index of body size in leaf-cutting ant (FJERDINGSTAD; BOOMSMA, 1997). The cephalic capsule was photographed and measured with the aid of stereomicroscope (Leica MZ APO) and ImageJ software (National Institutes of Health, v. 1.8.0).

Behavioral assay

To test leaf-cutting ants' behavior, we conducted five behavior assays to measure the correspondent personality trait: (1) open field to *Exploration-Avoidance;* response to (2) alarm pheromone and (3) garbage assays to *Boldness-Shyness*; (4) social assay to *Sociability*; and (5) heterospecific to *Aggressiveness*.

All tests were conducted in a glass arena (Ø18 cm) coated with Fluon (Polytetrafluoroethylene) to prevent ants from escaping. A paper on the base was changed after each test to avoid pheromone demarcation. Tests were recorded from above using a webcam (Logitech 1080p full HD) and analyzed by behavioral categorization software BORIS (Behavioural Observation Research Interactive Software, v. 7.7.3) according to each personality trait measure.

• Open field assay: the focal ant was placed in the arena under a shelter (plastic cup, Ø5 cm) with an opening (1 cm^2) for exit and observed during 300 s. We registered: "*Emerge*", defined as time latency to leave the shelter; and "*Return*", the total time inside the shelter after emergence. "*Exploration*" trait was subtracted from: total time (300 s) less "Emerge" and "Return" times.

• Response to alarm pheromone assay: alarm pheromone was extracted by crushing a nestmate head (BLUM, 1969; HERNÁNDEZ; CABRERA; JAFFE, 1999; MOSER; BROWNLEE; SILVERSTEIN, 1968) in a piece of absorbent paper (1 cm^2). The focal ant was placed in the arena and left to acclimatize for 60 s. Then, the stimulus was placed 1 cm away from the focal ant for another 60 s. The time remaining close (≤ 1 cm) to the stimulus was measured and considered as "*Boldness-Shyness"*.

• Response to garbage assay: garbage (1 g) composed of discarded fungus without ant corpses was collected from the garbage disposal and placed on paper (1 cm^2). The focal ant was placed in the arena and left to acclimatize for 60 s. Then, the stimulus was placed 1 cm away from the focal ant for another 60 s. The time remaining close (≤ 1 cm) to the stimulus was measured and considered as "*Boldness-Shyness"*.

• Social alignment assay: a nestmate of the same caste and similar size was immobilized with carbon dioxide, to prevent its behavior from influencing the observed ant response. The focal ant was placed in the arena and left to acclimatize for 60 s. Stimulus was placed 1 cm away from the focal ant for another 60 s. The time remaining close (≤ 1 cm) to the stimulus was measured and considered as "*Sociability*".

• Heterospecific assay: firstly, the focal ant was placed in the arena and, afterward, a heterospecific leaf-cutting ant of *Atta laevigata* was placed 1 cm away. The heterospecific ant had similar size and was previously immobilized with carbon dioxide to exclude potential effects of the opponent behavior. The first three focal interactions were registered during 180 s. "*Aggressiveness*" was scored on a 0-5 scale according to the level of agonistic response (adapted from Kamhi et al., 2015): 0 – non-aggressive behavior such as reversing direction and walking away from the heterospecific ant or not altering behavior when located at the arena; 1 – walking by or above heterospecific ant; 2 – open mandible in the direction of the heterospecific ant without physical contact; 3 – Olfactory evaluation, marked by antennation in the direction and/or touching the heterospecific ant; 4 – biting the heterospecific ant; and 5 – transporting the heterospecific ant in the mandibles.

Repeatability

Consistency for each behavioral trait was calculated by the measures of time (exploration-avoidance, boldness-shyness, sociability) or score (aggressiveness) among the three replicates of each leaf-cutting ant using Intraclass Correlation Coefficient (ICC). ICC is considered a standard measure of repeatability (LESSELLS; BOAG, 1987) and is primordial to ensure that a behavior conforms to the definition of personality (BELL; HANKISON; LASKOWSKI, 2009). Its values are absolute Pearson's correlation coefficients assumed as indices of interrater reliability of quantitative data. The rejection of the null hypothesis corresponds to consistency between measurements.

Multilevel effect

Adjusted repeatability for each behavioral trait was measured using the average value of the three replicates by Repeatability estimation using the Linear Mixed Model (LMM) method. This analysis measures the effect of caste and colony (Fixed Effects- FE) in the behavioral traits (Dependent Variable – DV). Value of $R>0.05$ indicates that FE should be considered in the explanation of DV (BUDAEV, 1997; NAKAGAWA; SCHIELZETH, 2010). When the test result indicated the effect of one of the levels, the information was considered and explored in the following analysis.

Then GLMM (Linear Mixed Model – REML) was applied to determine what may be driving the behavior variations of the leaf-cutting ants. The analysis was applied only to the FE that had an effect on the DV, as indicated by repeatability estimate of LMM method. For this reason, two separate models were used to test multilevel, colony and caste. (1) To colony analysis, colony ID and size were considered as FE and caste as a random effect with ID of each ant and VD for repeated measures. Hierarchical control was given by ant size and ID inside Caste. (2) At caste analysis, caste was considered as a FE and colony as a random effect with ant ID and VD for repeated measures. Hierarchical control was given by ant ID inside Colony. The analyses include Analysis of Variance (ANOVA) to obtain the variance of the components and test *Post hoc* Tukey pairs to control for multiple comparisons.

Behavioral syndromes

The existence of common dimensions underlying the personality traits, known as behavioral syndromes, was tested for all behavior trats in two analyses.

Firstly, Principal Component Analysis (PCA) was performed by entering the value of each leaf-cutting ant grouped by caste. PCA is commonly chosen in analysis of behavioral data because it explains the difference between measured variables by building composite axes to maximize differences observed (BUDAEV, 2010). Here the dimensions with eigenvalues greater than one were considered as valid.

Then, Canonical Correlation Analysis (CCA) was applied to the average of three replicates. Personality traits and castes were analyzed separately to complete and highlight PCA results. CCA describes data with fewer variables, points out which original variables are most important, and tries to find the linear combination that best expresses the correlation between sets of variables.

Data analysis

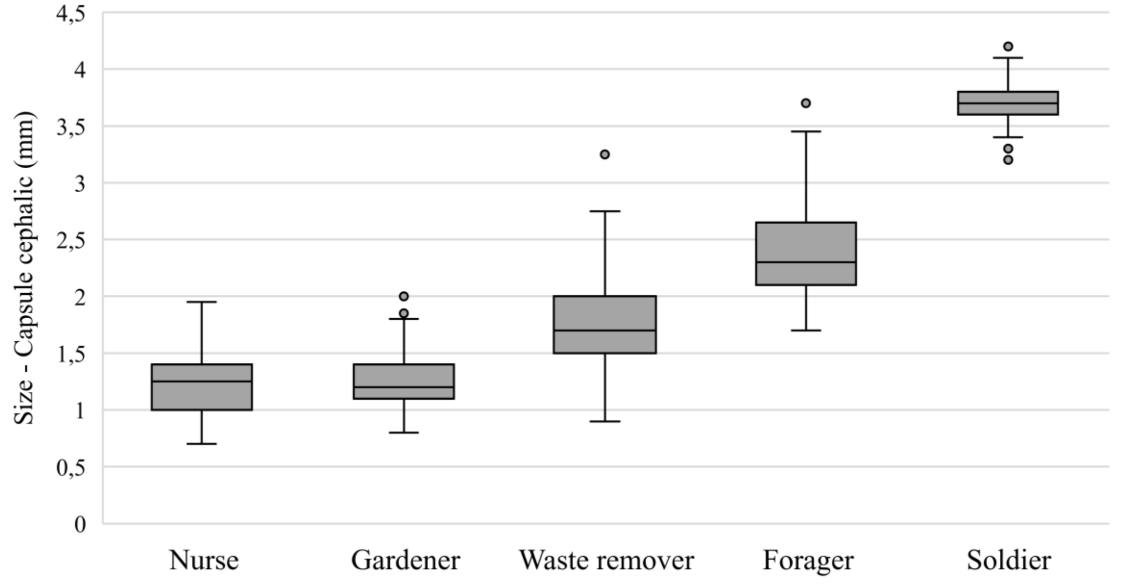
All statistics were performed using R (version 4.1.2, R Core Team, 2019) and the packages: irr (icc command) to ICC; rptR (rpt command) to Repeatability estimation using the (LMM) method; lmerModLmerTest to GLMM; Factoshiny (PCAshiny command) to PCA; and ltm (rcor.test command) to CCA.

3.3.RESULTS

Morphologic measure – Ant size

Workers of leaf-cutting ants collected during the performance of their activities (N=450) showed a wide gradient of cephalic capsule size, ranging from 0.7 mm to 4.2 mm (Figure 1). The smallest castes, nurse and gardener, both workers from fungus chambers, had similar sizes (N=90, 1.24±0.25 mm; N=90, 1.27±28 mm, respectively). Medium-size workers were forager and waste remover (N=90, 2.41±0.42 mm; N=90, 1.74±0.42 mm) and soldiers were the largest caste (N=90, 3.69± 22 mm).

Figure. 1. Cephalic capsule size of worker of leaf-cutting ants (N=450) organize by caste.



Consistency over trials

Repeatability analysis of all data (N= 1350) showed a consistent behavior response across trials (N= 3) in leaf-cutting ants (N= 450) for all personality traits. Despite the consistency of all traits being significant (p<0.001), ICC test shows a range from 0.449 to 0.779, indicating that "Boldness-Shyness (Garbage)" and "Sociability" traits were less consistent than "Aggressiveness" in this study (Supplementary Material 1).

Effects of confounding factors

Repeatability estimation using the LMM method revealed the significance of fixed effects, which was included in the next analysis. Caste (N= 5) had more effect on personality traits than colony (N= 9; Table 1).

Table 1. Results of Repeatability estimation using the LMM method indicating the effect of the fixed effects, caste and colony, on personality traits.

Behavioral traits	R values	
	Caste	**Colony**
Exploration	**0.245**	0.0367
Boldness-Shyness (Pheromone)	**0.193**	0.0347
Boldness-Shyness (Garbage)	**0.0457**	**0.12**
Sociability	**0.118**	0.0348
Aggressiveness	**0.352**	**0.0459**

Differences among colonies

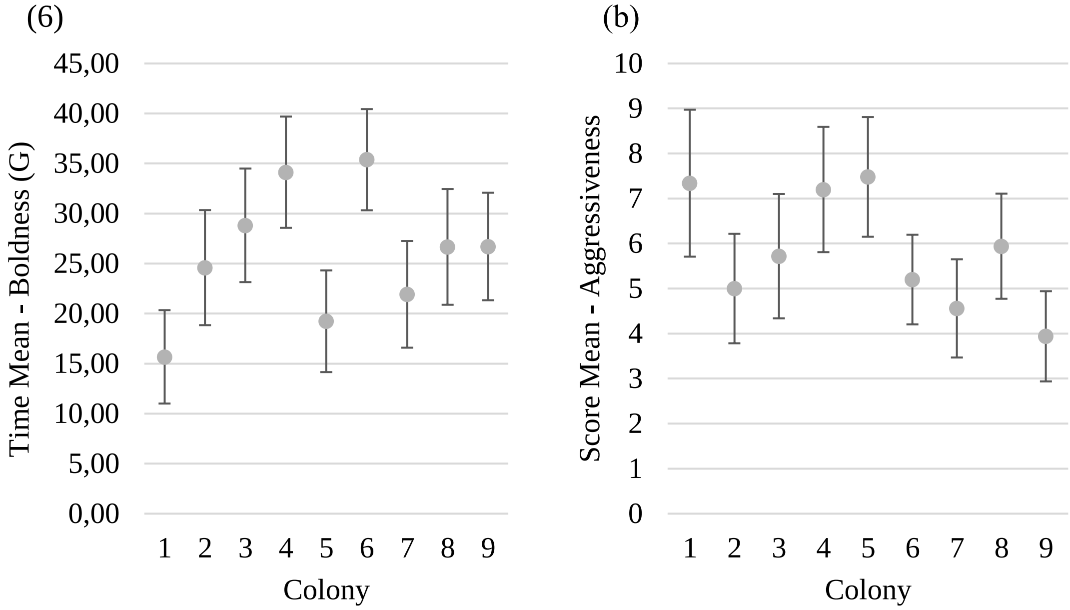
The results of the GLMM analysis to verify the traits “Boldness-Shyness (Garbage)” and “Aggressiveness” indicates significant behavior variances between colonies ($p<0.001$; Table 2). “Aggressiveness” also had size effect ($p<0.001$; Table 2).

Table 2 – Analysis of Variance (ANOVA) of fixed effects, colony and size, from the five personality traits.

	Colony				Size			
	F	**Df**	**Df. Res**	**p-value**	**F**	**Df**	**Df. Res**	**p-value**
Boldness-Shyness (Garbage)	84.425	8	437.32	**<0.001**	39.773	1	13.91	0.0661
Aggressiveness	43.455	8	437.08	**<0.001**	389.649	1	43.69	**<0.001**

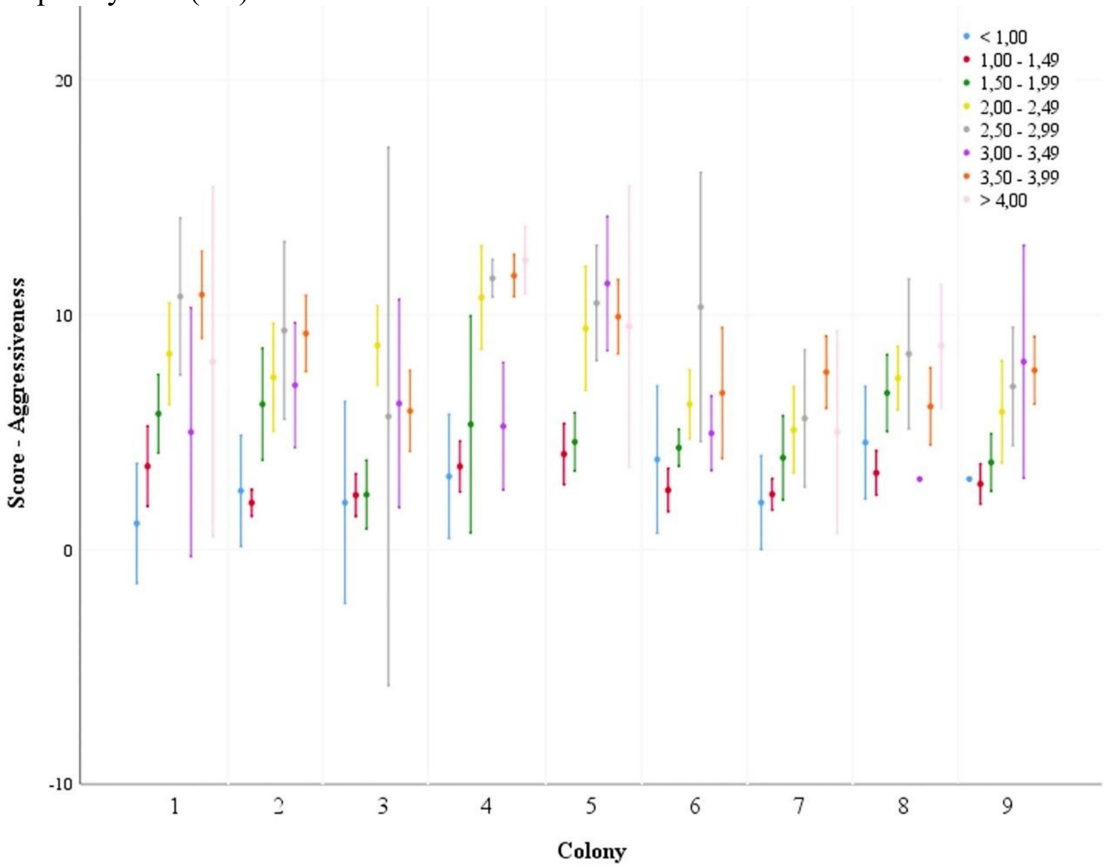
To “Boldness-Shyness (Garbage)” trait, the significant differences involved all colonies. The highest Boldness colony mean (32.09±13.35 seconds; Mean±Standard Error) was two times greater compared to the more Shyness colony (14.76±10.55 seconds) (Figure 2a; Supplementary Material 2).

Figure 2. Average measures of the nine colonies of leaf-cutting ants by personality traits: (a) Boldness-Shyness (Garbage) and; (b) Aggressiveness. 95% confidence interval.



Aggressiveness score differed between leaf-cutting colonies and size. Colonies 1, 4 and 5 were the most aggressive (Figure 2b). Colonies have progressive increase in aggressiveness mean up to 2.99 mm (colonies 1, 2, 3 and 7), 3.99 mm (colonies 5 and 9), more than 4.00 mm (colony 4; Figure 3). For data of each colony aggressiveness and size, see Supplementary Material 3.

Figure 3. Average measures of the aggressiveness score from colonies of leaf-cutting ants grouped by size (cm). 95% confidence interval.

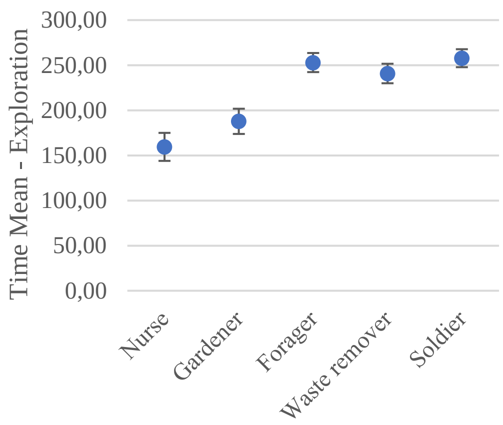


Differences among castes

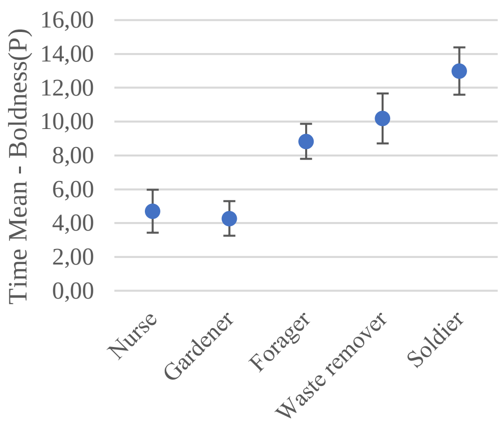
Results from GLMM caste analysis of all personality traits showed significant behavior variations between castes (Supplementary Material – 4) grouping workers with similarly size. Nurses and gardeners differ significantly from at least one caste in all traits. Both castes were mostly "Shyness (Pheromone)" and "Avoidance" than "Exploration", and presented as less "Sociability" and "Aggressiveness". Foragers and waste remover castes were more at "Exploration" and "Aggressiveness" than nurses and gardeners, but differ from itself in two traits: foragers present more "Sociability"; and waste removers were the most "Boldness (Garbage)" between all castes. Soldiers had similar responses to forager as more "Shyness (Garbage)", "Sociability", "Aggressiveness" and "Exploration"; but differ from each other at "Boldness-Shyness (Pheromone)" (Figure 4).

Figure 4. Average measures of the castes by personality traits: (a) Exploration-Avoidance, (b) Boldness-Shyness (Pheromone), (c) Boldness-Shyness (Garbage), (d) Sociability, and (e) Aggressiveness.

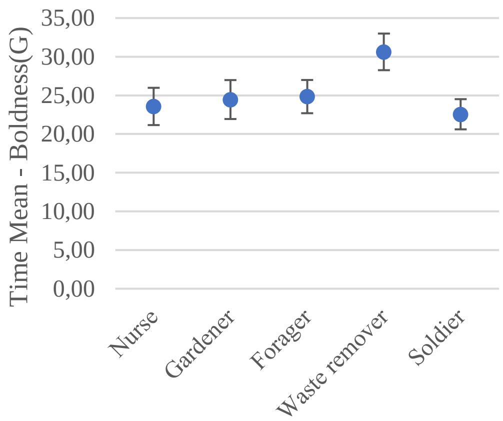
(a)



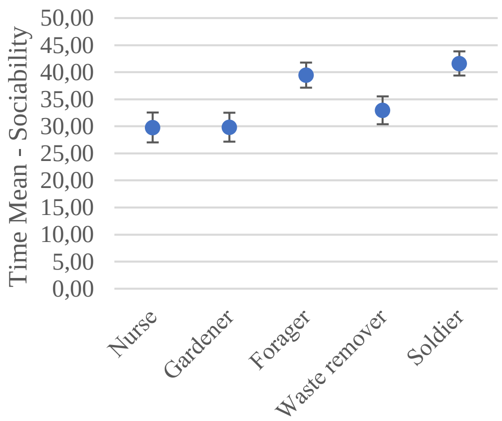
(b)



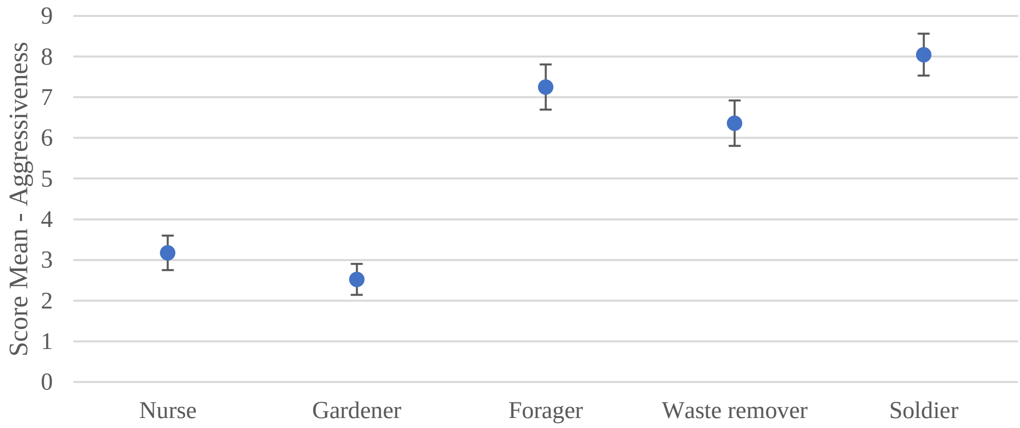
(c)



(d)



(e)



Behavioral syndrome

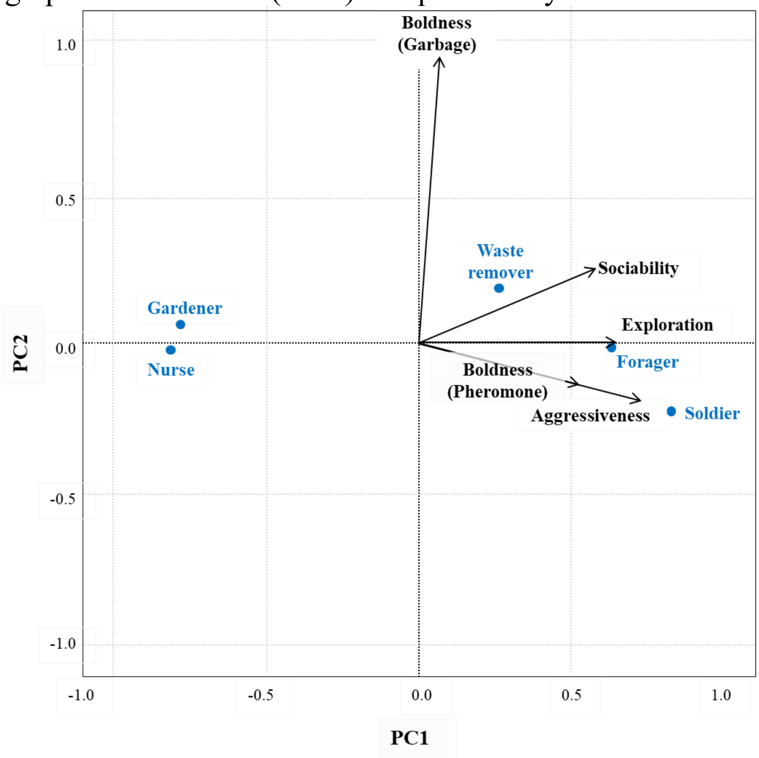
Principal Component Analysis (PCA) with all personality traits confirmed behavior syndromes and effect of castes. The procedure generated two behavior dimensions which explain 70.81% of the total dataset (Table 3). And Wilks test (p=<0.001) indicates that caste explains the distance between individuals.

Table 3. Principal component analysis (PCA) Eigenvalues values of the five dimensions.

	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
Eigenvalue	1.60	1.02	0.91	0.82	0.64
Percentage of variance	32.06	20.47	18.28	16.41	12.78
Cumulative % of variance	32.06	52.53	70.81	87.22	100.00

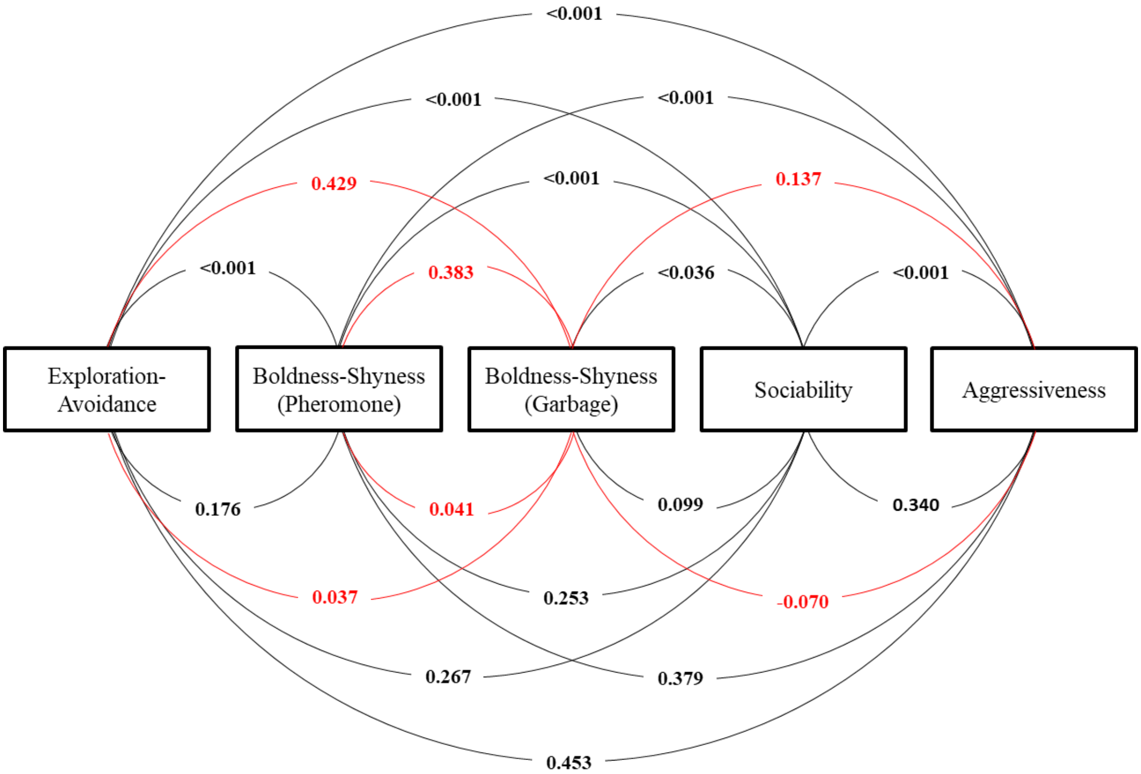
The first behavior dimension (PC1) was explained by Aggressiveness, Exploration, Sociability and Boldness (Pheromone) (by order of magnitude). Two castes were highly but opposite correlated with this dimension, nurse and soldier (respectively, -0.81 and 0.83). While the second dimension (PC2) was explained only by Boldness-Shyness (Garbage) without association with castes (Figure 5). To see how much each factor (personality trait and caste) contributed to dimensions, see Supplementary Material – 5 and 6.

Figure 5. PCA graph of individuals (caste) and personality traits.



Canonical correlation analysis (CCA) confirmed behavior syndromes in leaf-cutting ants. Boldness-Shyness (Pheromone), Exploration-Avoidance, Sociability and Aggressiveness traits were highly correlated. While Boldness-Shyness (Garbage) was only correlated with Sociability. All castes show correlation between personality traits (Figure 6). For analyze results of personality traits and castes, see Supplementary Material – 7 and 8.

Figure 6. Canonical Correlation Analysis (CCA) results from workers of leaf-cutting ants. Values on the top side contain p-values, and the underside shows Pearson correlation coefficient I. Black line indicates significant correlations between traits ($p<0.05$), while red lines are not correlated.



3.4.DISCUSSION

Our study confirmed the hypotheses that leaf-cutting ant, *Atta sexdens*, present personality and behavioral syndrome at multiple levels (colony and caste) associated with worker morphological size. Behavioral variation was markedly higher at caste level than at colony level. In fact, group composition was an important factor and workers were grouped by size with minor castes differing from major castes in all personality traits.

Behavioral studies on social insects composed of many individuals tend to focus on high levels, such as colonies (BENGSTON; DORNHAUS, 2014; PLANAS-SITJÀ et al., 2015). In part, this is due to the difficulty of locating and testing the same individuals more than once. Therefore, it is often necessary to adjust protocols to solve the problem.

Our study raises the debate that some adaptation is necessary to explore other levels and reality of species such as leaf-cutting ants. The replication of behavioral tests on the same day is an example.

Personality in social insects is not only colony-base, as result of intra-colony variation be one determinant factor for behavioral characteristics of leaf-cutting ants (GANDRA et al., 2021; KRALJ-FIŠER; SCHUETT, 2014; UDINO et al., 2017). Aggressiveness and boldness in the face of garbage are important personality dimensions for colonies. Colonies had a positive correlation between aggressiveness and worker size, indicating that the larger the colony's workers, the more aggressive it was. Previous studies with social insects showed variations between nest-guarding behavior and aggressiveness were associated with the colony, especially the queen (WRIGHT et al., 2017). Considering that leaf-cutting ants colonies, *A. sexdens*, are monogynic, we cannot rule out the possibility that the queen had some effect on the result, since leaf-cutting worker size variation is in part determined genetically (DOCHTERMANN; SCHWAB; SIH, 2014; HUGHES et al., 2003).

Behavioral variation on castes highlights how within colony variation drives colony behavior. In this study, differences grouped castes by size and task. Nurses and gardeners, both workers from fungus chambers, had similar behavioral responses and differed from ants from medium and large castes, who performed tasks outside or in the waste chamber. The behavioral association between task and personality has been explored in the literature (LOFTUS; PEREZ; SIH, 2021), however our study contributed for the first time with results for all leaf-cutting ant castes.

In leaf-cutting ants, caste function is associated with colony success. The personality of nurses and gardeners is in line with the behavioral response observed in risk situations. When there is a sign of danger in the nest, the lesser castes avoid and flee with the immature, while soldiers attack the invaders. This response is associated with both aggressiveness and the presence of daring in response to alarm pheromone recruitment, sociability and exploration of a new situation. Since division of labor in eusocial societies increases colony efficiency and contributed to the ecological success of social insects (GORDON, 2016; KELLER et al., 2011), the behavior syndrome we documented is likely to be a crucial component in the functioning and success of the social group.

We expect to find evidence of caste and group-level behavioral syndromes in other species, with appropriate protocol adjustments. Research in this area is in its childhood, compared to the literature on groups. Many issues related to personality differences between groups still need to be explored to clarify the factors that influence social insects, such as genetics, ontogeny, physiology, among others.

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CHAPTER 4

4. Analysis of breeding system, genetic diversity and personality of leaf-cutting

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Manuscript in preparation

4.1.INTRODUCTION

Animals of different taxa show consistent behavior across time and context, a characteristic known as animal personality (DALL; HOUSTON; MCNAMARA, 2004; DINGEMANSE; DOCHTERMANN; WRIGHT, 2010; GOSLING, 2001; RÉALE et al., 2007). This phenomenon has been widely explored by behavioral ecologists and evolutionary researchers, aiming to understand one of its main premises, the heritability (CARTER et al., 2013; GARTLAND et al., 2021; RÉALE et al., 2007).

Previous studies have suggested that genetic composition is an important mechanism underlying behavioral traits, mainly in Eusocial Hymenoptera (ants, bees, wasps) (LEMANSKI et al., 2019; WALSH; GARNIER; LINKSVAYER, 2020; WATT; SHUKER, 2010; ZAYED; ROBINSON, 2012). Typically, colony genetic structure is determined by the reproductive caste, which is composed by the queen and the male she copulated with. Such genetic makeup may vary with the reproductive strategy: multiple mates by a single queen (polyandry) and multiple reproductive queens in the same colony (polygyny) (BOOMSMA; KRONAUER; PEDERSEN, 2009; BOURKE; FRANKS, 2019; CROZIER; PAMILO, 1996).

In colonies with polyandry, an increase in intra-colony genetic variation may lead to a variety of behaviors. Behavioral diversity can be beneficial as they favor adaptation to changes in the colony's internal and external needs (BENGSTON; JANDT, 2014). For instance, in bees it can influence food preference (PAGE JR; ERBER; FONDRK, 1998), learning (CHANDRA; HOSLER; SMITH, 2000) and task allocation (AMDAM; PAGE JR, 2010; PAGE JR; ERBER; FONDRK, 1998). Despite limited research, there are evidences that male's genotype contributes to offspring behavior, and may be an indicative of colony behavior (PAGE; FONDRK; RUEPPELL, 2012). However, conflicts can arise in colonies with several patri- or matrilines. In several species of social insects, workers with reproductive capacity can cause worker-policing (FOSTER; RATNIEKS, 2001, 2000; HALLING et al., 2001; OLDROYD et al., 2001; RATNIEKS; VISSCHER, 1989) and even aggressive behaviors to prevent them from laying eggs which would develop into males (IWANISHI; HASEGAWA; OHKAWARA, 2003; MONNIN; RATNIEKS, 2001). In this context, reproductive strategy has a strong effect on the genetic and behavior diversity in the colony.

Genetic studies in social insects also show heritable differences may emerge in behaviors from reproductive and sterile castes, and at both individual and collective levels. For instance, courtship behaviors of hybrids male wasp from *Nasonia* genera (*N. vitripennis* and *N. longicornis*) were more variable than non-hybrids, presenting an intermediate behavior between the parental species (BEUKEBOOM; ASSEM, 2001). Collective behaviors, such as foraging and defensive responses, are influenced by a gene network and allelic differences in bees (HUNT et al., 2007). Furthermore, foraging alterations were observed in colonies of the leaf-cutting ants *Acromyrmex versicolor* with distinct matrilines (FRIEDMAN; GORDON, 2016).

Among ants, one of the most complex social structures is observed on leaf-cutting ants. The most derived group of the Attina subtribe (Formicidae:Attini), leaf-cutting ants are endemic to the Americas and considered dominant herbivores of the Neotropics (BARRERA et al., 2022; HOLLDOBLER; WILSON, 2011; MARICONI, 1970; WILSON, 2019). The taxon is widely distributed, spanning from southern Argentina to the southern United States (FERNÁNDEZ; SENDOYA, 2004; HÖLLDOBLER; WILSON, 1990; HOLLDOBLER; WILSON, 2011; KEMPF, 1972; MARICONI, 1970). Leaf-cutting ants occupy a wide range of environments (FORTI et al., 2020; SIQUEIRA et al., 2018) and are considered pests in agricultural systems because of their ability to modify the environment, causing a major impact on vegetation (DELLA LUCIA,; OLIVEIRA, 1993; FOWLER et al., 1989; GARCIA et al., 2003; MARICONI, 1970; VINHA et al., 2020).

In the case of *Atta sexdens* (Linnaeus), the colony is formed by reproductive and sterile castes (DIJKSTRA; NASH; BOOMSMA, 2005). Winged males and females remain temporarily in the nest, while fertilized female (founding queen of the colony) and sterile workers are permanent castes (WILSON, 1971, 1980).

Reproductive female, or queen, only mate during a single nuptial flight. While the queen can mate with multiple males (polyandry), the males die just few days after copulation (ARMITAGE; BOOMSMA; BAER, 2010; BOOMSMA; BAER; HEINZE, 2005; FJERDINGSTAD; BOOMSMA, 2000). After copulation, the queen excavates the first chamber of her nest in the soil and stays underground, laying eggs, for as long as she lives (MARICONI, 1970).

Workers of *A. sexdens* present morphological polyethism, with division of labor determined primarily by morphological distinctions. The sub castes composed of smallest workers are responsible for the fungus and immatures. Medium-size workers forage for food and maintain the nest (e.g. discarding garbage). The largest workers are usually associated with protection of the nest (DELLA LUCIA,; OLIVEIRA, 1993; WILSON, 1980). With such highly developed social organization, populous adult colonies can reach 5 to 8 million individuals (FOWLER et al., 2019; WEBER, 1972).

Previous works on polyandry in *A. sexdens* showed that queens mate with two to six males (ARMITAGE; BOOMSMA; BAER, 2010; FJERDINGSTAD; BOOMSMA, 2000). While studies keep investigating reproductive strategies, worker lineages and personality separately (DAHAN et al., 2022; EVISON; HUGHES, 2011; GANDRA et al., 2021; VILLESEN; GERTSCH; BOOMSMA, 2002), one group in which heritability and personality has been little investigated is the leaf-cutting ants.

Several studies evaluated heritability of behavior in social insects, which usually present low to moderate heritability (i.e. heritability between 0.2 and 0.5; DRENT *et al.*, 2005; MEFFERT; REGAN, 2002; OERS; MUELLER, 2010; PENKE; DENISSEN; MILLER, 2007). However, few studies have developed the topic in ants (ANDRAS et al., 2020; BOCKOVEN; WILDER; EUBANKS, 2015; WALSH; GARNIER; LINKSVAYER, 2020). Indeed, to the authors' knowledge, this is the first study that evaluates the contribution of both patri- and matrilines to personality traits in leaf-cutting ants. Here, we aimed to identify paternal lineages and queen mating frequency variances and covariances of five personality traits – exploration-avoidance, boldness-shyness, sociability, and aggressiveness – in laboratory colonies of *A. sexdens*. We hypothesized that if personality has an association with genetic composition, the degree of relatedness between workers should influence their behavior. The same can be expected between the kinship between males that copulated with the same queen. The more genetically distinct the males, the more likely that workers will exhibit different behaviors.

4.2.METHODS

DNA extraction

The DNA was extracted individually from 10 workers (two workers per caste categorized in five castes: “nurse”, “forager”, “gardener”, “waste remover” and “soldier”) from nine colonies, in a total of 90 workers. The tissues were macerated and incubated in a lysis solution TNES (100 mM Tris, pH 9.1, 100 mM NaCl, 50 mM EDTA, 0.5% SDS) at 55°C for 3 h with proteinase K. Subsequently, the protein residues were precipitated with 5 M NaCl, and the remaining mixture containing the DNA was transferred to a new tube containing 1.5 mL of 100% isopropanol and then washed with 70% ethanol. The pellet was dried and the DNA was resuspended in TE (10 mM Tris, 1 mM EDTA, pH 8) (RAMALHO et al., 2016). For the extraction process we used the whole-body ants.

Microsatellite analysis

Four microsatellite loci were analyzed following conditions PCR (Table 1). For each forward primer 5’ end a M13 tail (5′- CACGACGTTGTAAAACGAC - 3′) was added (SCHUELKE, 2000). The Liz 600 size standard (ThermoFisher Scientific, Waltham, MA) was added to amplified microsatellite fragments. The four labeling with different fluorescents (6-FAM, VIC, NED and PET; Applied Biosystems) were used to optimize the genotyping process. Samples were genotyped by capillary electrophoresis in an ABI 3130 automatic sequencer (Applied Biosystems TM) and analyzed using the Genemarker® software (HULCE et al., 2011).

Table 1. Basic information on the primers used for genetic analysis of *Atta sexdens* colonies.

Primers	Primer sequences 5'- 3'	Reference	TM °C	Fluorescence
ANT1343	F-TCGGTCCCGTGCCTTCGATT R-GRGGGCGCGTCAAATTTGCT	BUTLER *et al.*, 2014	58.2	NED
ANT3993	F-TGATCCGCTCTTAAAATTAGATGGA R-ACTTTCCGCRGCATTAAACATTTTCTT	BUTLER *et al.*, 2014	53	VIC
ANT575	F-TCAGGTTCGACACATGTGCC R-TCAAGATCGTTTGTCAGGCTGA	BUTLER *et al.*, 2014	54.8	PET
ETTA1-2TF	F-GTATTGTTCGATGAGAAATAGAGC R-CGGCTGACGTGTTGAATC	FJERDINGSTAD; BOOMSMA, 2000	55 - 50	6-FAM

Descriptive microsatellite loci analyses

We evaluated microsatellite loci for stuttering and reduced amplification of large fragments using Micro-Checker (VAN OOSTERHOUT et al., 2004). We characterized each locus for allelic richness, expected (H_E) and observed heterozygosity (H_O) using the Microsatellites Toolkit supplement in Excel (PARK, 2008). We used FreeNA software (CHAPUIS; ESTOUP, 2007) to estimate locus null allele frequency. Loci adherence to Hardy–Weinberg equilibrium (HWE) were tested using Genepop 4.7.5 (ROUSSET, 2008) and linkage disequilibrium (LD) between all pairs of loci was estimated using FSTAT 2.9.4 (GOUDET, 1995). The significance value of 0.05 was corrected for multiple comparisons for both HWE and LD analyses.

Paternal lineage identification and queen mating frequency

Using offspring genotypes, we inferred the paternal lineages intra-colonies using the software MateSoft (MOILANEN; SUNDSTROEM; PEDERSEN, 2004). This analysis was carried out in three steps, following author´s recommendation. First, putative queen genotypes were estimated. Then, putative genotypes of male mates were inferred, and offspring was assigned to patrilines. Finally, queen mating frequency was estimated as the effective number of male mates (PEDERSEN; BOOMSMA, 1999). Matesoft reconstruct all putative genotypes of queen and male mates. Thus, we considered estimates from queen and male mates with the highest genotype probability. In case of more than one putative queen genotypes presented the same probability, we chose one based on the following parsimonious criteria: (i) mating with fewer males and (ii) presenting less ambiguous paternity. Workers with ambiguous parentage were discarded in the upcoming analyses.

Behavior assay

Nine colonies of *A. sexdens* were maintained in the laboratory at controlled temperature (24°C±2°C), humidity (70%±10%) and light/dark cycle (12h). We used 4 ants per caste (nurse, forager, gardener, waste remover and soldier) from each colony. Behavior ssay to determine personality traits - exploration-avoidance, boldness-shyness, sociability, and aggressiveness - were performed according to Silva and col. (unpublish).

The existence of common dimensions from behavior traits was explored by Principal Component Analysis (PCA), using the value of behavior assays and indicating patri- and matrilines of each leaf-cutting ant. PCA is commonly chosen in analysis of behavioral data due to explaining the difference between measured variables by building composite axes to maximize differences observed (BUDAEV, 2010). Here the dimensions with eigenvalues greater than one were considered as valid. Then, Hierarchical Clustering on Principal Components (HCPC) approach was applied to the same dataset. Its combine principal components and clustering methods to create hierarchical and partitioning clustering of the data (HUSSON; JOSSE; PAGES, 2010).

Statistics were performed using R (version 4.1.2, R Core Team, 2019) and the packages: Factoshiny (PCAshiny command) to PCA; FactoMineR (HCPC command) to HCPC and factoextra (ggplot2-based command) to data visualization.

4.3.RESULTS

Descriptive microsatellite loci analyses

We recorded 3 to 20 alleles per loci. H_E ranged from 0.44 to 0.921, with an average of 0.682, whereas H_E ranged from 0.45 to 0.919 (mean of 0.657). The frequency of null alleles was low, with a mean of 0.022. We did not find evidence of stuttering and reduced amplification of large fragments for all loci. The four microssatelite loci were under expectation of HWE and we did not detect LD between all pairs of loci. Detailed loci characterization can be found in Table 2.

Table 2. Characterization of four microsatellite loci amplified for *Atta sexdens.*

Locus	Reference	A	H_E	H_O	freqNull
ANT1343	BUTLER *et al.*, 2014	15	0.9067	0.9189	0
ANT3993	BUTLER *et al.*, 2014	3	0.4395	0.45	0.00001
ANT575	BUTLER *et al.*, 2014	6	0.4611	0.4028	0.05065
ETTA1-2TF	FJERDINGSTAD; BOOMSMA, 2000	20	0.9215	0.8588	0.0391

A: allelic richness; H_E: expected heterozygosity; H_O: observed heterozygosity; freqNull: frequency of null alleles.

Paternal lineage identification and queen mating frequency

We found a high mating frequency in the nine colonies of *A. sexdens*, ranging from 3 to 8 male mates by queen (Table e). Effective number of mates ranged from 3 to 37.3 per queen (Table 3). We also found a very low paternity skew intra-colonies, with a mean ranging from 1 to 3 workers per putative male mate.

Table 3. Number of genotyped workers, queen mating frequency, number of workers with unambiguous paternity identified and mean number of workers per male per colony of *Atta sexdens*.

Colony ID	**Number of genotyped workers**	**Estimated Mating frequency**	**Effective Mating Frequency**	**Number of workers with unambiguous paternity**	**Mean number of workers per male**
C04	10	8	18.4	9	1.125
C05	9	8	36.0	9	1.125
C07	8	7	37.3	7	1
C08	10	7	13.1	9	1.286
C09	10	6	11.2	10	1.667
B02	10	3	3.0	9	3
B04	10	4	3.5	10	2.5
B09	10	6	10.0	6	1
B10	10	6	9.6	9	1.5

Behavior assay

To behavioral assay, 10 individuals from 9 colonies, totaling 270 tests, were analyze in five behavior assay (Table 3).

Ants without identified paternal lineage (N= 12) were excluded from the analysis (PCA and HCPC).

Exploratory data analysis by PCA procedure generated two behavior dimensions which explain 51.31% of the total dataset (Table 4). The first behavior dimension (Dim 1) was explained by “Aggressiveness” followed by “Exploration”, “Sociability” and “Boldness (Pheromone)” (by order of magnitude). While the second dimension (Dim 2) was better explained by “Boldness-Shyness (Garbage)” (Fig. 1).

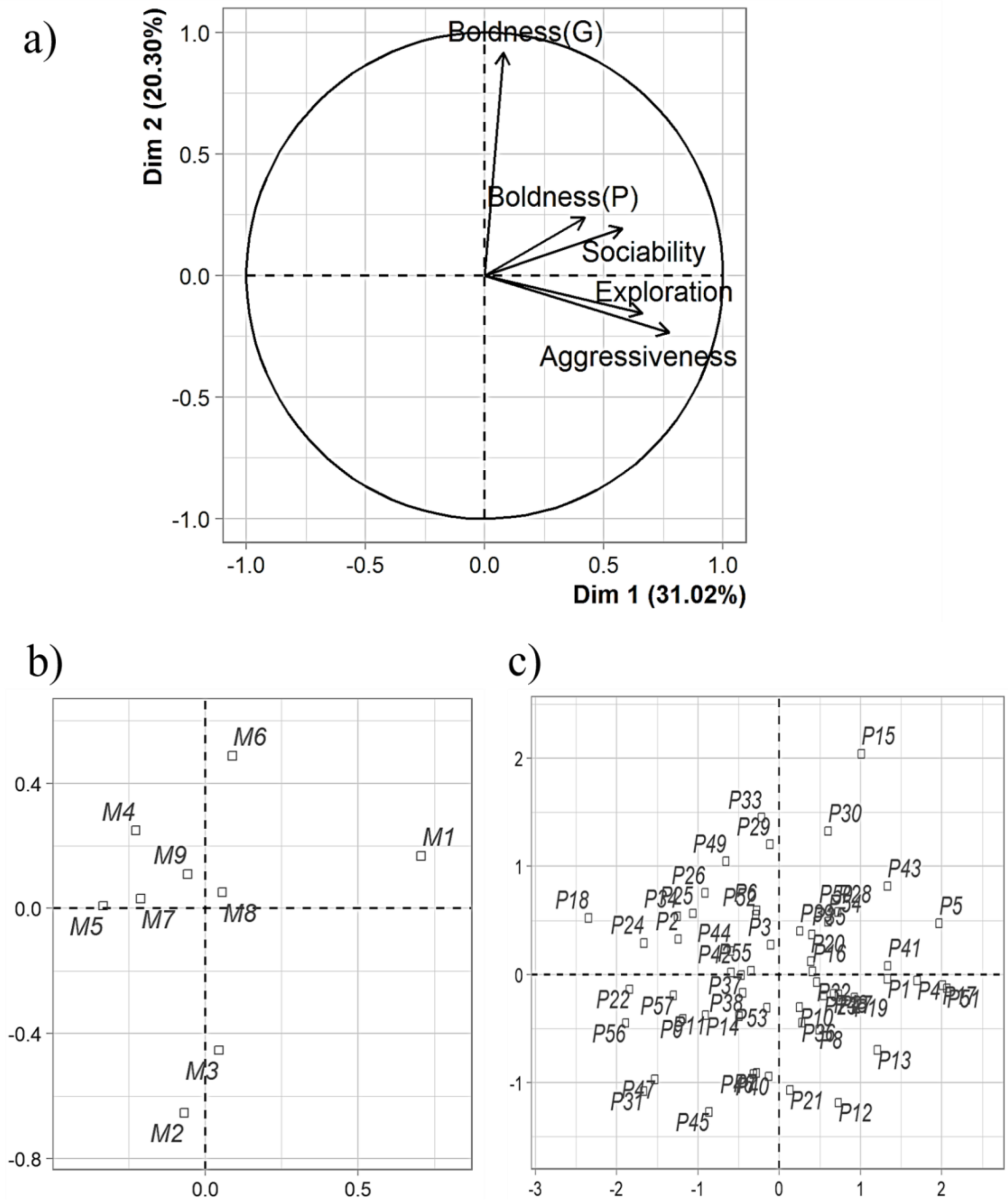
Table 3. Mean result of the five behavior assay replicated 3 times by each worker (N= 90) from 9 colonies (N=270) of *Atta sexdens*.

	Boldness (P)		**Boldness (G)**		**Sociability**		**Aggressiveness**		**Exploration**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
C4	5.38	4.98	26.48	17.03	26.55	23.11	4.27	4.95	211.19	114.22
C5	5.61	7.81	35.06	18.80	39.05	21.14	4.33	3.33	234.35	108.16
C7	9.07	8.81	24.29	16.08	29.13	19.86	3.30	3.58	229.44	93.72
C8	14.58	17.16	21.13	20.54	35.56	23.30	4.50	3.73	191.28	126.10
C9	4.95	5.68	28.98	19.49	32.62	23.87	5.50	3.93	208.43	115.08
B2	6.40	13.52	13.45	14.39	27.87	22.56	5.17	5.57	236.31	90.23
B4	8.28	12.58	17.33	15.73	27.08	20.13	5.17	5.10	247.95	79.62
B9	9.25	12.09	29.31	22.88	28.14	23.74	4.77	4.50	186.38	127.78
B10	7.86	6.68	32.28	20.10	36.79	20.77	8.07	5.09	253.72	77.89

Table 4. Principal component analysis (PCA) Eigenvalues values of the five dimensions.

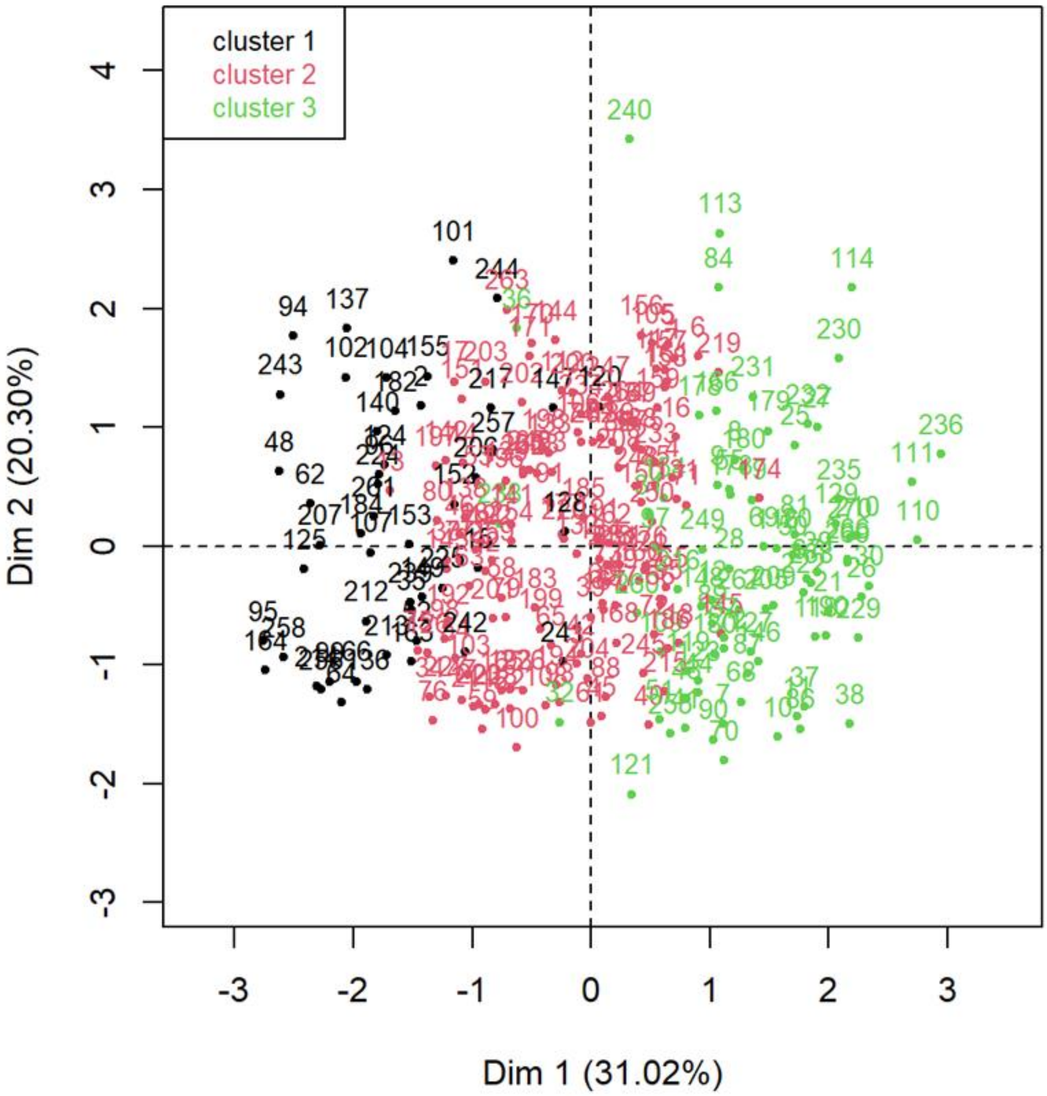
	Dim.1	**Dim.2**	**Dim.3**	**Dim.4**	**Dim.5**
Variance	1.551	1.015	0.981	0.848	0.606
% of variance	31.017	20.296	19.612	16.952	12.122
Cumulative % of variance	31.017	51.313	70.925	87.878	100.000

Figure 1. PCA graphs of: a) behavior traits; b) matriline; and c) patriline of leaf-cutting ants, *Atta sexdens*.



HCPC classification grouped individuals in 3 clusters (Fig. 2). The cluster 1 is made of individuals sharing low values of Exploration, Aggressiveness and Boldness (P) (variables are sorted from the weakest). The cluster 2 is composed of individuals with high values of Exploration and Boldness (G) (variables are sorted from the strongest), and low values for the variables Aggressiveness, Sociability and Boldness (P) (variables are sorted from the weakest). And cluster 3 is characterized by high values of Aggressiveness, Boldness (P), Sociability and Exploration (variables are sorted from the strongest).

Figure 2. Factor map indicating clusters from leaf-cutting ants, *Atta sexdens*.



4.4.DISCUSSION

Preliminary results record for the first time the use of primers ANT1343, ANT3653, ANT575 in leaf-cutting ants. This output demonstrate that conserved microsatellite loci and designed primers for PCR amplification can be successfully used across genera within the same ant subfamily (e.g. Myrmicinae; BUTLER *et al.*, 2014).

A high mating frequency was determinate to *A. sexdens* colonies, ranging from 3 to 8 male mates by queen. In the past, polyandry was considered as a rare event (STRASSMANN, 2001), but it occurs in several species of leaf-cutting ants (DAHAN et al., 2022; MIKHEYEV, 2004). In fact, *Acromyrmex* sp typically mate with nine or 10 males (MUÑOZ-VALENCIA et al., 2020; ORTIUS-LECHNER et al., 2003; SUMNER et al., 2004).

Low paternity skew within colonies in *A. sexdens* suggest that offspring is almost equally distributed among males. Uniform use of sperm reduced intra-colony genetic similarity and was recorded in species with highly polyandrous queens (JAFFÉ et al., 2012) and large colonies (LOOPE; CHIEN; JUHL, 2014). Genetic diversity in leaf-cutting ants is considered advantageous in different situations, such as enhanced disease resistance (BOOMSMA; FJERDINGSTAD; FRYDENBERG, 1999; HUGHES; BOOMSMA, 2004), but it still is necessary to know if exist a relationship with behavior.

At this stage, behavior assay suggests variations between *A. sexdens* colonies. Even though the distribution of patri- and matrilines between dimensions (Figure 1, a and b.), it is early to assume connections among lineage and behavior. PCA analyses indicates a common dimension (possible behavior syndrome) between aggressiveness, exploration, sociability and boldness to pheromone, isolating boldness from garbage. Similar cluster was obtained at HCPC analyses, which also clustered works with low values of exploration and aggressiveness with shyness to pheromone, and high values of boldness to garbage with exploration (Figure 2).

In conclusion, future analyzes of this work should clarify if genetic diversity intra-colony is a factor to be considered regarding the personality of the workers.

4.5.FUTURE STEPS AND ANALYSIS

To finalize this work, we are going to performed the following steps and analyses before doctoral defense:

- Add the genotyping of the 90 ants from preliminary results to five microsatellites (ANT2936, ANT3653, ANT8424, ATCO12 and ATCO37) using GeneMarker software;
- Add the genotyping of 90 workers (10 from the 9 colony already tested) to nine microsatellites (ANT1343, ANT3653, ANT575, ETTA1-2TF, ANT2936, ANT3653, ANT8424, ATCO12 and ATCO37) using GeneMarker software;
- Analyze the relatedness between workers using RELATEDNESS software;
- Analyze the relatedness between males (output matrix) also using RELATEDNESS software;
- Verify genetic clusters between intra-colonies workers using Clusters analysis (e.g. Bayesian) by Structure Software;

- Evaluate the correlations between relatedness of workers and personality traits, and male mates and workers personality using two separate models of Generalized Linear Mixed Model (GLMM) from R package lmerModLmerTest;
- Verify heritability and genetic correlation between factors using animal-model approach from R package MCMCglmm.

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5. CONCLUSION

- Leaf-cutting ants, such as *Atta sexdens,* are important organisms of study in several areas. Maintaining ant colonies in laboratory conditions makes it possible to better understand parts of their biology that are impractical in the field;
- The development of protocols to standardize collection and maintenance of leaf-cutting ants increases the chances of survival of colonies in the laboratory;
- Different artificial models of leaf-cutting ant nests facilitate data collection during experiments;
- Artificial nests can have educational purposes and bring society closer to universities and researchers;
- Leaf-cutting ants exhibit the personality traits Exploration-Avoidance, Boldness-Shyness (Pheromone), Boldness-Shyness (Garbage), Sociability, and Aggressiveness;
- Colonies have different levels of boldness-shyness and aggression. In fact, aggressiveness is related to the size of the ant;
- As expected, gardener and nurse, the small subcastes, had similar behavioral responses but opposite to the larger castes on most tests. Avoidance, shyness, less sociability and aggressiveness behavioral sets the smallest subcastes apart from the rest. Workers who spend most of their time in the fungus garden, the smaller subcastes tend to avoid confrontation and, in danger, move to another location;
- "Waste remover" behavior was similar to foragers and soldiers. However, they were bolder in the presence of garbage, indicating a relationship between personality and subcaste specialization;
- Foragers responded similarly to "waste remover", but were more sociable. The exchange of information between workers during foraging is one of the factors associated with the success of the task;
- Soldiers were more responsive in the presence of an alarm pheromone, a warning of danger communicated among workers. Its behavior reinforces the association of the defense function widely studied in this subcaste;
- Leaf-cutting ant behavioral syndrome indicates that daring in the face of garbage is an isolated dimension from the other traits;
- Preliminary results prove that primers developed from conserved microsatellite loci can be applied to other ant species;

- Polyandry queens of *Atta sexdens* can mate with up to 8 males, a figure previously only recorded for *Acromyrmex* sp.;
- Despite the genetic diversity among workers, low paternity skew within colonies gives evidence of uniform use of sperm;

6. SUPPLEMENTARY MATERIAL

Supplementary Material - 1. Intraclass Correlation Coefficient from all data (N= 1350) of leaf-cutting ants (N= 450) behavioral repeatability of the five personality traits over tree trials.

	ICC	**F**	**Df/ Df. Res**	**p-value**
Exploration	0.654	2.89	449,898	**<0,0001**
Boldness-Shyness (Pheromone)	0.576	2.36	449,898	**<0,0001**
Boldness-Shyness (Garbage)	0.449	1.82	449,898	**<0,0001**
Sociability	0.499	1.99	449,898	**<0,0001**
Aggressiveness	0.779	4.52	449,898	**<0,0001**

Supplementary Material - 2. Descriptive data of "Boldness-Shyness (garbage)" and "Aggressiveness" traits by colony.

	Boldness-Shyness (Garbage) (time)					**Aggressiveness (score)**				
Colony	**Min.**	**Max.**	**Mean**	**SD**	**Total N**	**Min.**	**Max.**	**Mean**	**SD**	**Total N**
B2	1,69	52,88	14,76	10,55	50	0,00	11,67	5,38	3,90	50
B4	0,00	50,44	21,68	12,52	50	0,00	12,33	3,82	3,40	50
B9	0,00	59,59	28,10	12,85	50	0,00	11,67	3,89	3,47	50
B10	0,00	59,08	29,15	12,73	50	0,00	11,33	5,34	3,72	50
C4	0,00	58,05	23,89	12,53	50	0,00	11,00	4,91	3,39	50
C5	3,89	58,81	32,09	13,35	50	0,00	9,67	3,49	2,14	50
C7	0,28	47,32	24,94	10,88	50	0,00	8,67	3,18	2,30	50
C8	1,26	57,50	25,56	14,30	50	0,00	11,00	4,55	2,68	50
C9	0,56	57,04	26,75	12,86	50	0,00	10,00	3,91	2,71	50

Supplementary Material - 3. Descriptive data of colonies aggressiveness by cephalic capsule size (mm).

Colony	Size (mm)	Min.	Max.	Mean	Standard Deviation	Total N
1	< 1,00	0	0	0	0	3
	1,00 - 1,49	0	16	4	5	11
	1,50 - 1,99	0	16	6	6	14
	2,00 - 2,49	0	16	10	5	9
	2,50 - 2,99	12	14	13	1	3
	3,00 - 3,49	0	12	6	8	2
	3,50 - 3,99	7	14	11	3	7
	> 4,00	8	8	8		1
2	< 1,00	0	3	2	2	2
	1,00 - 1,49	0	9	3	2	23
	1,50 - 1,99	0	12	6	6	7
	2,00 - 2,49	5	13	9	4	3
	2,50 - 2,99	5	14	11	5	3
	3,00 - 3,49	0	8	5	3	4
	3,50 - 3,99	2	14	7	5	8
3	< 1,00	3	3	3		1
	1,00 - 1,49	0	14	4	4	15
	1,50 - 1,99	0	11	3	4	7
	2,00 - 2,49	0	14	9	5	13
	2,50 - 2,99	11	11	11		1
	3,00 - 3,49	0	13	7	7	3
	3,50 - 3,99	0	12	6	5	10
4	< 1,00	0	8	5	5	3
	1,00 - 1,49	0	13	4	4	20
	1,50 - 1,99	3	12	8	6	2
	2,00 - 2,49	3	13	10	4	5
	2,50 - 2,99	8	14	11	2	6
	3,00 - 3,49	0	12	6	5	4
	3,50 - 3,99	10	13	12	1	9
	> 4,00	12	12	12		1
5	1,00 - 1,49	0	12	7	5	15
	1,50 - 1,99	0	13	5	4	16
	2,00 - 2,49	0	14	9	6	4
	2,50 - 2,99	9	13	11	2	4
	3,00 - 3,49	12	12	12		1
	3,50 - 3,99	5	12	11	2	8
	> 4,00	5	14	10	6	2
6	< 1,00	5	9	7	3	2
	1,00 - 1,49	0	9	3	3	12
	1,50 - 1,99	0	10	5	3	16
	2,00 - 2,49	3	14	7	4	9
	2,50 - 2,99	9	9	9		1
	3,00 - 3,49	0	12	5	4	7
	3,50 - 3,99	5	12	7	4	3
7	< 1,00	0	3	2	2	3

	1,00 - 1,49	0	6	2	2	19
	1,50 - 1,99	2	14	5	4	7
	2,00 - 2,49	0	9	5	3	7
	2,50 - 2,99	6	12	11	3	4
	3,50 - 3,99	0	12	7	4	9
	> 4,00	6	6	6		1
8	< 1,00	6	11	8	3	3
	1,00 - 1,49	0	11	3	3	14
	1,50 - 1,99	0	12	7	5	9
	2,00 - 2,49	0	10	7	3	10
	2,50 - 2,99	3	12	8	5	3
	3,00 - 3,49	3	3	3		1
	3,50 - 3,99	1	12	7	4	7
	> 4,00	3	12	8	5	3
9	< 1,00	3	3	3		1
	1,00 - 1,49	0	9	3	3	19
	1,50 - 1,99	0	6	3	2	8
	2,00 - 2,49	0	10	5	4	5
	2,50 - 2,99	0	12	6	5	6
	3,00 - 3,49	6	6	6		1
	3,50 - 3,99	0	12	6	4	10

Supplementary Material - 4. Analysis of Variance (ANOVA) of the caste from the five personality traits.

	F	Df	Df. Res	Pr(>F)
Exploration-Avoidance	31.763	4	437	<0.001
Boldness-Shyness (Pheromone)	23.556	4	437	<0.001
Boldness-Shyness (Garbage)	5.9258	4	437	0.0001
Sociability	13.511	4	437	<0.001
Aggressiveness	55.964	4	437	<0.001

Supplementary Material - 5. Principal Component Analysis (PCA) of the five personality traits.

	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5
Boldness(P)	0.53	-0.13	0.75	0.23	0.27
Boldness(G)	0.07	0.95	0.08	0.27	-0.10
Sociability	0.59	0.25	0.05	-0.77	0.04
Aggressiveness	0.75	-0.19	-0.09	0.20	-0.60
Exploration	0.64	0.01	-0.57	0.25	0.44

Supplementary Material - 6. Principal Component Analysis (PCA) of *Atta sexdens* castes.

	Dim.1	**Dim.2**	**Dim.3**	**Dim.4**	**Dim.5**
Forager	0.50	-0.04	-0.16	-0.01	-0.09
Gardener	-0.78	0.07	-0.06	-0.13	0.18
Nurse	-0.81	-0.01	0.10	-0.17	-0.04
Soldier	0.83	-0.21	0.09	0.01	-0.04
Waste remover	0.26	0.18	0.03	0.30	-0.01

Supplementary Material –7. CCA results of the five personality traits. The upper diagonal part contains Pearson correlation coefficient (***r***) and lower diagonal part contains corresponding p-values.

	Boldness (P)	**Boldness (G)**	**Sociability**	**Exploration - Avoidance**	**Aggressiveness**
Boldness (P)	*****	0.041	0.253	0.176	0.379
Boldness (G)	0.383	*****	0.099	0.037	-0.070
Sociability	<0.001	0.036	*****	0.267	0.340
Exploration-Avoidance	<0.001	0.429	<0.001	*****	0.453
Aggressiveness	<0.001	0.137	<0.001	<0.001	*****

Supplementary Material – 8. CCA results of *Atta sexdens* castes. The upper diagonal part contains Pearson correlation coefficient (***r***) and lower diagonal part contains corresponding p-values.

Nurse					
Boldness (P)	*****	0.110	0.102	0.016	0.008
Boldness (G)	0.300	*****	0.120	0.041	0.074
Sociability	0.337	0.261	*****	0.261	0.049
Exploration-Avoidance	0.884	0.700	0.013	*****	0.336
Aggressiveness	0.942	0.486	0.643	0.001	*****
Gardener					
Boldness (P)	*****	0.092	0.006	-0.271	-0.010
Boldness (G)	0.386	*****	0.169	-0.078	0.072
Sociability	0.952	0.112	*****	0.061	0.175
Exploration-Avoidance	0.010	0.465	0.567	*****	0.116
Aggressiveness	0.928	0.499	0.100	0.277	*****
Forager					
Boldness (P)	*****	0.072	0.220	0.170	0.102
Boldness (G)	0.500	*****	0.090	0.002	-0.059
Sociability	0.037	0.399	*****	0.102	0.254
Exploration-Avoidance	0.109	0.982	0.336	*****	0.313
Aggressiveness	0.337	0.581	0.016	0.003	*****
Waste remover					
Boldness (P)	*****	0.006	0.350	0.243	0.496
Boldness (G)	0.954	*****	0.013	0.222	-0.228
Sociability	0.001	0.903	*****	0.275	0.362
Exploration-Avoidance	0.021	0.036	0.009	*****	0.321
Aggressiveness	<0.001	0.031	<0.001	0.002	*****
Soldier					
Boldness (P)	*****	-0.074	0.081	-0.190	0.132
Boldness (G)	0.491	*****	0.262	-0.089	-0.278
Sociability	0.447	0.013	*****	0.090	0.243
Exploration-Avoidance	0.072	0.405	0.398	*****	0.410
Aggressiveness	0.216	0.008	0.021	<0.001	*****