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The nestmate recognition process of leaf-cutting ants (Formicidae, Myrmicinae, Attini): from behaviour to brain

O processo de reconhecimento entre companheiras de ninho de formigas cortadeiras (Formicidae, Myrmicinae, Attini): do comportamento ao cérebro

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Orientador: Prof. Dr. Fabio Santos do Nascimento

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To my beloved husband, Bruno de Paula, for the love and understanding I have received along the course of this scientific journey.

Wie jede Blüte welkt und jede Jugend
Dem Alter weicht, blüht jede Lebensstufe,
Blüht jede Weisheit auch und jede Tugend
Zu ihrer Zeit und darf nicht ewig dauern.
Es muß das Herz bei jedem Lebensrufe
Bereit zum Abschied sein und Neubeginne,
Um sich in Tapferkeit und ohne Trauern
In andre, neue Bindungen zu geben.
Und jedem Anfang wohnt ein Zauber inne,
Der uns beschützt und der uns hilft, zu leben.

Wir sollen heiter Raum um Raum durchschreiten,
An keinem wie an einer Heimat hängen,
Der Weltgeist will nicht fesseln uns und engen,
Er will uns Stuf´ um Stufe heben, weiten.
Kaum sind wir heimisch einem Lebenskreise
Und traulich eingewohnt, so droht Erschlaffen;
Nur wer bereit zu Aufbruch ist und Reise,
Mag lähmender Gewöhnung sich entraffen.

Es wird vielleicht auch noch die Todesstunde
Uns neuen Räumen jung entgegen senden,
Des Lebens Ruf an uns wird niemals enden,
Wohlan denn Herz, nimm Abschied und gesunde!

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ABSTRACT

In colonies of social insects, a major requirement is the ability of workers to discriminate nesmates from non-nestmates in order to direct altruistic acts towards closely genetically related individuals and prevent the presence of competitors. The process of nestmate recognition is categorised into three main components. The *production component* includes the process of production of chemical cues that allow individuals to recognise themselves as part of a group. The *perception component* includes the processes involved at detecting and comparison of recipient's recognition cues by the evaluator. The *action component* involves the behavioural responses triggered after perception of the recipient's cues. In my thesis, I investigated open questions in the literature on the three major components of nestmate recognition process of leaf-cutting ants, genus *Atta*. In the first chapter, I investigated the production component by asking whether the symbiotic fungus cultivated by the ant *Atta sexdens* is a source of nestmate recognition chemical cues. In the second chapter, I investigated the perception and response components of *Atta vollenweideri* by designing a controlled behavioural assay to test whether the propensity to attack an enemy is related to body size. In the third chapter, I have developed a reliable protocol to assess whether the biogenic amine serotonin (5-HT) is one possible neural substrate to regulate differences in aggressive behaviour of leaf-cutting ants. The results of the first chapter show that the chemical profile of symbiotic fungus was predominantly comprised of linear hydrocarbons, a class of chemical compounds that has been evidenced as nestmate recognition cues in social insects. Our comparative analyses revealed a great similarity between the hydrocarbon profiles of ant larva and fungus, due to the fact that both groups shared mainly highly concentrated linear hydrocarbons. As individuals progressed through developmental stages, the chemical profiles between ant and fungus became increasingly different. These findings suggest that the intimate relationship between brood and fungus might shape the hydrocarbon

profile of both species. In the second chapter, we found no relationship between body size and propensity to attack a non-nestmate. However, when exposed to an agonistic pheromone, small-sized workers showed a disproportionaly large duration of mandible opening behaviour, which is the behaviour that characterises the alarm pheromone releasing. The same behavioural pattern was observed when small-sized workers were confronted with non-nestmates. Our findings support the hypothesis previously raised which categorised small-sized workers as a morphollogicaly group specialised in defense, and show evidence against another hypothesis that predicts they are less sensitive to non-nestmate odours. In the third chapter, we found signficant differences in 5-HT levels in the optic lobe (OL) and central complex (CC) compartments of different size workers. In the OL, such differences were characterised by higher serotonin levels in intermediate body size workers (median and major workers). In CC compartments, a negative relationship between body size and serotonin level was found. As the CC is the innermost neuropil of *A. vollenweideri*, such relationship might be due to difficulty of antibodies to reach inner regions of the brain. Interestingly, intermediate workers presented a greater variability in the number and level of 5-HT in the serotonergic somata of the optic lobe cluster. According to the traditional literature, median and major workers show broader behavioural repertoires, performing tasks both inside and outside the nest, and may rely on visual cues while searching for food or returning home. Thus, the differences obtained from our protocol reflect behavioural specialisation of workers, which makes us confortable to present it as suitable for assessing differences in immunostaining intensity in brain regions of different size workers which consequently have different size neural tissues.

Key-words: Social recognition, aggressive behaviour, morphological subcastes, immunostaining quantification, serotonin, Neuroanatomy.

RESUMO

Uma exigência importante para as colônias de insetos sociais é a capacidade de seus membros em discriminar àqueles que não pertencem às suas colônias, a fim de se direcionar atos altruístas para indivíduos geneticamente relacionados e evitar a presença de competidores. O processo de reconhecimento de companheiras de ninho é categorizado em três componentes principais. *O componente de produção,* que inclui o processo de produção de pistas químicas que permitem que os indivíduos se reconheçam como parte de um grupo. O *componente de percepção,* que envolve os processos de detecção e comparação das pistas de reconhecimento entre o recipiente e o avaliador. E o *componente de ação,* que inclui as respostas comportamentais desencadeadas após a percepção das pistas do recipiente. Neste trabalho, foram investigadas questões abertas na literatura sobre os três principais componentes do processo de reconhecimento de companheiras de ninho, em formigas cortadeiras do gênero *Atta*. No primeiro capítulo, foi investigado o componente de produção ao perguntar se o fungo simbionte cultivado pela formiga *Atta sexdens* é uma possível fonte de pistas químicas que mediam o reconhecimento social. No segundo capítulo, foram investigados os componentes de percepção e ação em *Atta vollenweideri* através de um ensaio comportamental controlado a fim de testar se a propensão em atacar um inimigo está relacionada ao tamanho corporal das operárias. No terceiro capítulo, foi desenvolvido um protocolo confiável para inferir níveis da amina biogênica serotonina (5-HT) através da quantificação de imunomarcação em regiões cerebrais de indivíduos de diferentes tamanhos corporais. Através desse protocolo foi possível avaliar se a 5-HT é um possível substrato neural para regular as diferenças comportamentais relacionadas ao tamanho das operárias. Os resultados do primeiro capítulo mostram que o perfil químico do fungo simbionte foi predominantemente composto de hidrocarbonetos lineares, uma classe de compostos que podem ser utilizados como pistas de reconhecimento. Análises comparativas revelaram uma grande similaridade entre os perfis de hidrocarbonetos

das larvas de formigas e fungo, devido ao fato de que ambos os grupos compartilharam principalmente hidrocarbonetos lineares altamente concentrados. À medida que os indivíduos progrediram através dos estágios de desenvolvimento, os perfis químicos entre formiga e fungo tornaram-se cada vez mais diferentes. Estas descobertas sugerem que a relação íntima entre a prole e o fungo pode moldar o perfil de hidrocarbonetos de ambas as espécies. No segundo capítulo, não foi encontrado uma relação significativa entre o tamanho corporal das operárias e a sua propensão em atacar um inimigo. No entanto, quando expostas a um feromônio agonístico, operárias pequenas apresentaram uma duração desproporcionalmente longa do comportamento de abertura de mandíbula, que caracteriza a liberação do feromônio de alarme. Esse mesmo padrão comportamental foi observado quando operárias pequenas foram confrontadas com não companheiras de ninho. Nossos resultados apoiam a hipótese previamente levantada que caracteriza operárias pequenas como um grupo morfológico especializado em defesa, e mostram evidência contra outra hipótese que prediz que elas são menos "sensíveis" para detectar as pistas químicas de reconhecimento. No terceiro capítulo, foram encontradas diferenças significativas nos níveis de 5-HT nos compartimentos do lóbo óptico (OL) e do complexo central (CC) em operárias de diferentes tamanhos. No OL, essas diferenças foram caracterizadas por maiores níveis de 5-HT em operárias de tamanho intermediário. Nos compartimentos do CC, foi encontrada uma relação negativa entre o tamanho corporal e o nível de 5-HT no cérebro. Porém, essa relação pode ser explicada pela dificuldade dos anticorpos em atingirem o CC, que é a neurópila cerebral mais interna de *A. vollenweideri*. Interessantemente, operárias intermediárias apresentaram uma maior variabilidade no número e níveis de 5-HT nos corpos celulares serotoninérgicos do *cluster* do lóbo óptico. De acordo com a literatura tradicional, operárias intermediárias possuem repertórios comportamentais mais amplos, realizando atividades dentro e fora da colônia, e podem depender de pistas visuais enquanto forrageiam ou retornam para colônia. As

diferenças obtidas através do nosso protocolo refletem as especializações comportamentais das operárias, sendo assim confortável apresentá-lo como adequado para avaliar diferenças na intensidade de imunocoloração em regiões cerebrais de operárias de diferentes tamanhos.

Palavras-chave: Reconhecimento social, comportamento agressivo, subcasta morfológica, quantificação de imunocoloração, serotonina, Neuroanatomia.

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Introduction

Once animals live together, regardless the origins of their social arrangements, they have the potential to assist one another, as evidenced in a large range of animal´s interactions (Alcock, 2013). Eusociality, in which great part of the individuals belonging to a group reduce their own lifetime reproductive potential to raise the offspring of others, underlies the most intriguing forms of social organisation (Nowak et al, 2010). The evolution of a sterile worker caste in eusocial insects was a major problem in Evolutionary Biology until Hamilton (1964) proposed the Inclusive Fitness theory to explain that a worker caste could evolve because they were benefitted via kin selection, which is the evolutionary strategy that favours the reproductive success of an organism's relatives at a cost of the organism's own survival and reproduction (Alcock, 2013) – in other words, the abdication of its own reproduction in favour of the reproduction of a closely genetically related one could be maintained if the cost of performing such altruistic behaviour is less than the benefit generated to the beneficiary (Hamilton, 1964). In ants, eusociality in the irreversible stage is the key to their ecological success, as they are generally dominant over solitary and preeusocial competitors.

Along the course of worker caste evolution, as reproductive division of labour was established, several morphological, behavioural and physiological features have arisen to characterise workers as specialists in collecting, maintaining, and protecting colony resources. A single worker responds to simple, local information, and in a coordinate system along with other workers, they perform an array of tasks that allow the entire colony to function (Gordon, 1996). Task allocation is a broad term used to define a stable pattern of variation in behaviour among workers within a colony which allows specific workers to engage in specific tasks (Gordon, 1996; Beshers & Fewell, 2001). More precisely, each worker specialises on a subset of the complete repertoire of tasks performed by the colony, and this subset varies across individuals in the colony (revised by Beshers & Fewell, 2001). Two general patterns of task allocation are recognised in social insects: age polyethism and alloethism.

Age polyethism is well demonstrated in eusocial bees (Michener, 1974), but also in termites (Li et al, 2015), and ants (Kühbandner et al, 2014; Bernadou et al, 2015), and it has been suggested as the most common task allocation system found in eusocial insects. In age polyethism, younger workers tend to remain inside the nest developing activities related to brood and queen care. As they age, they tend to perform activities related to the outside of the colony, such as foraging and defending the colony and their resources (Beshers & Fewell, 2001). Age polyethism has been interpreted an adaptive strategy because older workers, which life expectancy is shorter than young ones, tend to perform riskier tasks when exposed to outside environmental injuries (Kuszewska & Woyciechowski, 2013).

Alloethism is commonly found in derived social species, such as ants and termites, with distinguishable morphological subcastes within the worker caste (Beshers & Fewell, 2001). Recently, alloethism has been documented in stingless bees with the description of a morphologically specialised soldier caste that improves colony defence against natural competitors (Grüter et al, 2012). Patterns of alloethism are variable, but one generalisation that appears to hold is that the more striking size variation workers have, more specialised behaviour and narrower behavioural repertoire they show (Oster & Wilson, 1978). The most common specialisations are for defence and foraging (Beshers & Fewell, 2001).

As well evidenced in foraging activities, colony context and environment have also been show to interplay with age polyethism and alloethism during task decisions in social insects (Gordon, 1996; Gordon, 2015). For example, forager´s decision on whether to search for food depends on how much food is already stored in the colony (Seeley, 1989; 1991). As colony labour demands change, workers show behavioural flexibility, either performing tasks not previously seen in their repertoires or switching from one task to another (Beshers & Fewell, 2001). Cues that determine the global situation of a colony (e.g. colony size, rate of interaction among workers, exchange of information, and pheromones) are used as task

decision cues, as evidenced in the harvest ant *Pogonomyrmex barbatus* in which the rate of active foragers increases exponentially when they detect, at the nest entrance, the cuticular hydrocarbons of a group of workers specialised in food search (Greene & Gordon, 2003). Thus, task allocation must been seen as an overall variation in behaviour among workers interplaying with individual worker behavioural flexibility and environmental context.

Since behaviour is characterised by its adaptability, it follows that the neural machinery that creates it must possess analogous features (Gall et al, 1986). As behaviour must be supported by nervous system, it's natural that plasticity in behaviour follows plasticity in the brain. Brain plasticity occurs in several levels, for example, neuromodulators can shape sensory mechanisms and synaptic transmission in order to generate behavioural flexibility (Bargmann, 2012; Cohn et al, 2015). As an organism grows, experiences, and learns, its brain continually forms new connections and also strengthens or weakens synapses connections. During this process, neurons can grow and expand their axons and dendrites, or retract and reform themselves. This may alter the shapes of the brain by increasing or decreasing volumes of certain neuropils (Gall et al, 1986).

In adult worker honeybee *Apis mellifera*, the age polyethism is associated with changes in the higher-order integration centre, the mushroom bodies. In the insects' brain morphology, odours are detected by a number of antennal sensilla equipped with olfactory receptor neurons (ORNs) that project their axons across the antennal nerve, in the region of the neuropil responsible for receiving odour information – the antennal lobes (AL). The functional units of AL are spheroidal structures called glomeruli; such units are connected by local neurons that synapse with ORNs (Schneider & Steinbrecht, 1968). The number and arrangement of glomeruli are specific to each species, in the same way that each olfactory stimulus is processed in a standard-exact timeline (revised by Gronenberg, 2008). The information processed by the glomeruli are transmitted to the projection neurons, which then

retransmit the information to the main brain centre related to the memory and learning, the mushroom bodies (Abel et al, 2001; Hansson & Anton, 2000; Yamagata et al, 2006).

In ants of tribe Attini, the relative size of mushroom bodies decreases as the colony increases in population size. Instead, workers develop larger antennal lobes, which consequently increase their olfactory capacity (Riveros et al, 2010). Despite several studies have explored the relative brain size and social behaviour in insects, focusing mainly on the structural plasticity of the mushroom bodies (Menzel & Giurfa 2001; Fahrbach, 2006; Strausfeld et al 2009), the neural mechanisms related to the different behaviour within morphological worker castes are still unknown (Kuebler et al, 2010).

Nestmate recognition

The inclusive fitness concept predicts the adaptive gain for the individual that gives up their own reproduction to promote the reproduction and survival of closely genetically related individuals (Hamilton, 1964). The benefits of such an intricate system depend upon nestmate recognition, which allows individuals to direct altruistic acts towards their siblings. Besides, the cohesion of insect societies is strongly associated with the precise ability of their members to recognise nestmates and discriminate non-nestmates. Thus, nestmate recognition can be interpreted as the first defensive line for the colony and its resources (Vander Meer & Morel, 1998).

The precision of nestmate recognition is achieved through the maintenance of a significant polymorphism in the phenotypic trait (label) used for recognition. When two individuals encounter each other, the components of the recognition system interact in synergism in order to produce the appropriate response. In this context, one individual (the evaluator) assesses the label that is present in another individual (the recipient) (Tsutsui, 2004). The evaluator then compares the recipient's label to a specific set of labels that define

the "self" – the *template* – which is a neural representation of the phenotypic trait at the level of the colony. When the recipient's label matches the evaluator's template, then the evaluator considers the recipient a nestmate. If the recipient's label mismatches the evaluator's template, then the recipient is considered a non-nestmate (Tsutsui, 2004). These characteristics of nestmate recognition (label, template, and classification) are further categorised into three main components: the *production*, *perception*, and *action* component. The production component includes the process of production of labels themselves as well as the machineries responsible for their expression and modification. The perception component includes processes involved at detecting and comparison of recipient's label by the evaluator. The action component involves the behavioural response to the match/mismatch of recipient's label (Tsutsui, 2004). Depending on how different is the label from the template, reactions can range from hostility up to extreme aggression, followed by the death of both evaluator and recipient (Starks, 2004; Tsutsui, 2004; D'Ettore & Heinze, 2005; Valadares & Nascimento, 2015).

In eusocial insects, labels are commonly chemical cues spread on the outmost layer of the cuticule, the epicuticle. Visual and tactile cues have also been shown to act in synergism with chemical cues for making up labels (Tibbetts, 2002; Tibbets & Sheehan, 2011). The epicuticle is composed of a mixture of lipids that contain hydrocarbons. Cuticular hydrocarbons (CHCs) are highlighted as the most important cues for nestmate recognition. Due to their hydrophobicity, the primary role of CHCs is to provide an external barrier against desiccation as well as to protect the insect body against parasites, a function that is likely performed by linear hydrocarbons due to their simple chemical structure do not provide much information to be used as recognition cues (Blomquist & Bagnères 2010). However, the great diversity of more complex chemical structures, such as branched CHCs, that have been identified on the insect's epicuticle combined with the exogenous and endogenous origins of

these compounds, suggests that they have evolved as chemical messengers, which codify information from intraspecific to interspecific levels (Dani et al, 2001; Blomquist & Bagnères 2010; Richard and Hunt 2013; Jandt and Gordon 2016).

In insects, biosynthesis of hydrocarbons takes place in epidermal oenocyte cells located at the internal part of the abdomen, close to the cuticle innermost layers, as evidenced in model species such as the house-fly *Musca domestica* (Dillwith & Blomquist, 1982), the fruit-fly *Drosophila* (Ferveur et al, 1997), and the German cockroach *Blattella germanica* (Gu et al, 1995). Transfer of internally synthesized hydrocarbons to the cuticle of several insect species is performed by the lipoprotein lipophorin (Schal et al, 2001). Thus, the active production of CHC depends on the metabolic activity of the organism interplaying with its genes, energy consumption and internal state (age, health condition, etc.) (revised by Châline et al, 2015). The interindividual variation within a colony is reduced by behavioural mechanisms, such as social grooming and trophallaxis, that spread CHC among nestmates and provide a uniformed, colony-specific chemical signature (Crozier & Dix 1979; Lenoir et al, 2001). This signature, together with the odours associated with the nest, is called the colony odour (Vander Meer & Morel, 1998). Since the colony odour is affected by environmental factors, such as diet, nest substrate, and colony composition, the colony odour is not constant and varies over time, which consequently requires that the template shall be updated throughout the lifetime of the individual (Liu et al, 1998; Vander Meer & Morel, 1998).

In social insects, the phenotypic traits that characterise the "self" are learned soon after emergence of adults, by a process called phenotype-matching process (revised by Hauber & Sherman, 2001). In this process, newly-emerged workers form their templates based on the perception of phenotypic traits of individuals inserted within the unambiguous social context provided within the colony (Hauber & Sherman, 2001; Bos & D'Ettore, 2012). In the carpenter ant *Camponotus floridanus*, workers that during development were removed from

their mother nest and placed in a conspecific nest to be reared by unrelated workers, become aggressive towards their sisters soon after emergence (Morel & Blum, 1988). Furthermore, social activities associated with positive reinforcement, such as trophallaxis and social grooming, could be important to the template formation, once there is evidence that the template is stored as a learned memory (Bos & D'Ettore, 2012).

Leaf-cutting ants (Formicidae: Myrmicinae: Attini: Attina)

About 30 million years ago, in the South American savannahs, ants that cultivate fungus have over evolutionary time become the leaf-cutting ants (genera *Atta* and *Acromyrmex*) (Branstetter et al, 2017). These ants reside in large subterranean nests of the Neotropical region, with galleries and fungus chambers that shelter thousands to millions of individuals, where they farm the fungus *Leucoagaricus gongylophorus*. The fungus has never been found free-living without the ants, and it is generally accepted that both organisms have co-evolved into an obligate mutualism (Schultz & Brady, 2008). For the ants, the fungus is priceless as it serves as a rearing site and unique nutritional substrate for brood development. In exchange, the ants protect the fungus against parasites and forage for fresh vegetation, which is used as substrate for fungal growth.

Leaf-cutting ants belong to the monophyletic subtribe Attina (Formicidae: Myrmicinae: Attini: Attina), a highly diverse group of approximately 250 described species restricted to the New World (Branstetter et al, 2017). Due to their co-evolution with fungi of tribe Leucocoprineae, attine ants share many biological aspects (Mehdiabadi & Schultz, 2010). Yet in the mother nest, a virgin queen stores a portion of fungus garden in her infrabuccal cavity. After the mating flight, which generally occurs in warm and sunny days during the rainy season, she finds a suitable nesting site to dig a small underground enclosure cavity where she expels the stored mycelia to raise her own fungus garden for her future

colony (Della Lucia et al, 1993). The foundation generally occurs through haplometrosis and colonies tend to remain monogynous (Fernández-Marín et al, 2004). Between 40 and 60 days after the nuptial flight, the first workers emerge and take part on the reproductive division of labour by strictly performing nonreproductive tasks, such as brood care, defence and foraging, whilst the queen is only responsible for oviposition (Forti & Boaretto, 1997; Mehdiabadi & Schultz, 2010). When foraging, most attine species search for decomposing organic matter (Schultz & Brady, 2008). However, leaf-cutting ants forage for fresh parts of plants, such as flowers, seeds, leaves, and fruits (Fernández-Marín et al, 2004; Mehdiabadi & Schultz, 2010). Mature colonies of leaf-cutting ants, also referred as to superorganisms, are known as the main herbivorous pests of the Neotropical region. Only in Brazil, the country with greater diversity of species of leaf-cutting ants, it has been identified 10 species of *Atta*, and 20 species of *Acromyrmex* (Hölldobler & Wilson 1990; Della Lucia et al 1993).

A key factor on the evolution of leaf-cutting ants is the adaptation of task allocation based on worker size variation fitted primarily to the collection and processing of fresh vegetation (Wilson, 1980). In *Atta sexdens*, the distribution of activities related to foliar substrate and fungus cultivation follows an assembly-line fashion with an overall pattern of distribution of activities among four morphological worker subcastes (Wilson, 1980). Major workers (head width around 2.2 mm) more efficiently cut and transport leaf fragments, whereas minor workers (head width around 0.8 mm) tend to remain inside the nest performing activities related to the final treatment of foliar substrate (Della-Lucia et al, 1993; Wilson, 1980). Media workers (head width around 1.6 mm) perform a wide array of tasks both outside and inside the nest. In addition, the extremely large workers, also referred as to soldiers (head width > 3.0 mm) seem virtually limited to defence, however too large to be energetically efficient foragers and presumably less effective in defence than medias and major workers (Wilson, 1980).

Objectives

In this work I aimed to investigate open questions in the literature on the three major components of nestmate recognition, the *production* component, the *perception* component, and the *action* component, using as model organisms two species of leaf-cutting ants, A*tta vollenweideri* and *Atta sexdens*. In the first chapter of my thesis, I investigated the production component by asking whether the symbiotic fungus cultivated by the ant *Atta sexdens* is a source of nestmate recognition chemical cues. In the second chapter, I investigated the perception and response components in *Atta vollenweideri* by designing a controlled behavioural assay to test whether minor workers are less sensitive to detect a non-nestmate as well as investigate whether the propensity to attack an enemy is size-related. In the third chapter, I developed a reliable protocol to investigate the differences in the serotonergic immunoreactivity in the brain of *Atta* morphological worker subcastes. This protocol will allow us to investigate whether the biogenic amine neurotransmittervserotonin (5-HT) is one possible neural substrate to regulate differences in aggressive behaviour among worker caste.

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Chapter 1

Is the symbiotic fungus a source of cuticular hydrocarbons for leaf-cutting ants?

ABSTRACT

Leaf-cutting ants live in obligate mutualism with a basidiomycete fungus that they use as a rearing site and food resource. Chemical analyses of the fungus gardens kept by these ants have revealed the presence of hydrocarbons that also occur in the epicuticle of the ants. However, whether it is the fungus or the ants which are the ultimate producers of these compounds is not yet clear. In order to shed light on the chemoecological aspects of the symbiotic relationship between ant and fungus, in the present study we aimed to characterize the changes in the cuticular chemical profiles during larval-to-adult molting of *Atta sexdens* workers, which allowed us to investigate how these changes were correlated with the chemical profile of fungal cultivars. The results show that cuticular hydrocarbon profiles of ants were comprised of linear and branched alkanes that varied significantly according to developmental stages, with several ant-specific hydrocarbons being identified as the most representative ones. The chemical profile of symbiotic fungus was predominantly comprised of linear alkanes, which also occurred in the cuticle of the ants. Chemical distances calculated with the chemical profiles of the analysed groups revealed a great similarity between the hydrocarbon profile of symbiotic fungus and those of the ants, especially at the earliest stages of ants' development, when mainly linear alkanes were identified. However, as individuals progressed through developmental stages, the chemical profiles increased in difference, due to the fact that several branched alkanes were found in great proportions in the cuticle of the ants. These findings suggest that the intimate relationship between brood and fungus might shape the hydrocarbon profile of both species, and the possible scenarios for the transference of these substances are discussed.

Key-words: Cuticular hydrocarbons, moulting cycle, *Atta sexdens, Leucoagaricus gongylophorus*.

INTRODUCTION

In insect societies, colony odor is a blend of chemical compounds either acquired by endogenous and exogenous sources associated with the nest (Vander Meer and Morel, 1998; Martin and Drijfhout, 2009; Jandt and Gordon, 2016). The biological importance of colony odor is to provide cues that allow individuals to recognize each other as part of a group; here lies the primary importance of colony odor to nestmate recognition in hymenopteran societies (Vander Meer and Morel, 1998). Among the predominant classes of compounds, cuticular hydrocarbons (CHCs) are highlighted as the most important for nestmate recognition. The main role of CHCs is to provide an external barrier to protect the insect body against parasites and to help reduce water loss; a function that is likely performed by linear hydrocarbons, which simple chemical structure do not provide much information to be used as recognition cues (Blomquist and Bagnères, 2010). However, the great diversity of branched CHCs that have been identified on the insect's epicuticle combined with the exogenous and endogenous origins of these compounds suggest that they have developed as chemical messengers, which codify information from interspecific to intraspecific levels (Dani et al, 2001; Blomquist and Bagnères, 2010; Richard and Hung, 2013; Jandt and Gordon, 2016).

It is generally accepted that leaf-cutting ants (genera *Atta* and *Acromyrmex*) and the basidiomycete fungus *Leucoagaricus gonlylophorus* have adapted into an obligate mutualism. The ants cut and process fresh vegetation to serve as a nutritional substrate for their fungal cultivars (Schultz and Brady, 2008). In exchange, the fungus serves as a rearing site and food resource for the ants. In general, diet is a great source of variation of CHC in insects (Sorvari et al, 2008; Ichinose et al, 2009; Fedina et al. 2012; Otte et al, 2015), or represents a direct source of these substances (Liang and Silverman, 2000). In leaf-cutting ants, the CHC profile is subjected to temporal variations according to the type of foliar substrate harvest by the workers (Richard et al, 2004; Lambardi et al, 2004; Valadares et al, 2015), and more recently,

differences in the CHC composition related to body size of workers have been reported (Valadares and Nascimento, 2016).

The chemical composition of fungal cultivars is comprised of amides, aldehydes, esters, acetates and linear hydrocarbons, which specificity of profiles ranges from species to colony levels (Vianna et al, 2001; Richard et al, 2007a; Richard et al, 2007b). However, whether it is the fungus or the ants which are the ultimate producers of these compounds is not yet clear (Richard et al, 2007 a; b). In order to shed light on the chemoecological aspects of the symbiotic relationship between ant and fungus, we used Gas Chromatography-mass spectrometry (GC-MS) to provide a detailed characterization of the changes on the cuticular hydrocarbon profiles during larval-to-adult molting of *Atta sexdens* workers, which allowed us to investigate how these changes were correlated with the chemical profile of symbiotic fungus.

METHODOLOGY

Colonies

We used two colonies of *A. sexdens* raised by single queens collected during mating flight in Ribeirão Preto, SP, Brazil. The colonies were kept under laboratory conditions with temperature and humidity adjusted to the preference of the species (Della-Lucia 1993). The experiment was conducted when the colonies were at the age of three years and had 3 L of fungus garden.

Preparation of extracts

The procedures for chemical extraction were adjusted from the methodology proposed by Vianna et al (2001). Before collecting the material for chemical analysis, the colonies were deprived of foliar substrate for 24 hours in order to prevent the samples from contamination by fresh pieces of leaves. Pieces of fungus garden containing brood items and adult workers were collected from fungus chambers. A total of 20 medium-sized larva (body length: 5 to 6.0 mm), 20 medium-sized pupa (body length: 5 to 6.0 mm), 20 adult workers (head width: 2.0 mm), and 400 mg of fungal mycelium (n=20) were collected for chemical analyses. Ant phenotypes and fungal mycelium were separated with the aid of small brush and fine forceps, and individually placed into glass vials and stored at -20ºC until the moment of extraction, which occurred by subjecting the samples to bathes in nonpolar solvent hexane for 2 min. Preliminary tests using equal volume of hexane for all groups did not result in well concentrated samples of brood items, which has made very difficult to determine the fragmentation pattern of hydrocarbons. For that reason, we adjusted the volume of hexane for each of the analyzed groups as followed: 40 µl of hexane for larva and pupa extractions, and 200 µl for adult workers and fungus extractions.

All samples were analyzed in a combined Gas Chromatography-Mass Spectrometer GC-MS (Shimadzu, model QP2010 plus) equipped with a silica capillary column and using helium as a carrier gas. The oven temperature was initially set to 150 °C, increasing 3 °C/min until it reached 280 °C (maintained for 10 minutes) and again 10 °C/min until it reached 300 °C (maintained for 15 min). The data were analyzed with CG-MS solution for Windows (Shimadzu Corporation), and the compounds were identified based on their mass spectra and with the aid of a standard solution with different synthetic linear hydrocarbons, as well as by the consultation of Wiley and NIST Libraries database.

Statistical Analysis

Compounds that were present in less than half of the individuals belonging to a group, and compounds contributing less than 0.5% to the total compounds were excluded from the statistical analysis. The data were standardized using square root, and a Euclidean distance resemblance matrix was calculated on normalized data. We first performed a Canonical Analysis of Principal Coordinates (CAP) to assess the classification of the samples to the groups (fungus and ant phenotypes), and we then performed a Principal coordinate (PCO) analysis to visualize similarities or dissimilarities of samples and groups. A Pearson correlation coefficient ($r < 0.7$) was introduced with vectors as variables in combination with an Analysis of Similarity (SIMPER) to identify the compounds that differed among groups. To discriminate ant phenotypes and symbiotic fungus according to their chemical profiles, we performed a permutation multivariate analysis of variance (PerMANOVA) using 9999 permutations. As a post hoc test, we ran a Pairwise test to compare the chemical profiles between groups, and mean distances between groups' centroids were calculate to estimate the chemical distances between them. These calculations were carried out using the statistical software Primer version 6 (Primer-e Ltd., 2009).

RESULTS

The bulk of cuticular compounds extracted from larvae, pupae, and adult workers of *A. sexdens* comprised of hydrocarbons ranging from 23 to 39 carbon chains, classified as linear and branched alkanes. Branched alkanes were separated into two classes of compounds: dimethylalkanes and trimethylalkanes (Table 1). The chemical composition of the symbiotic fungus *L. gongylophorus* comprised of linear alkanes ranging from 24 to 33 carbon chains. Cross validation of canonical analysis (CAP) shows that 97.5% of our samples were correctly assigned to their respective groups (ant phenotypes and symbiotic fungus), with a mis-classification error of 2.5% resulted from the assignment of two larvae to the pupae group. Principal coordinate analysis (PCO, Figure 1) shows a clearly separation of the analyzed groups based on their hydrocarbon profiles, which were significantly different overall (PerMANOVA, Pseudo-F = 44.089, $p < 0.0001$). Post hoc analysis between the chemical profiles of *A. sexdens* and that of *L. gongylophorus* were significantly different (Pairwise tests, $p < 0.001$ to all comparisons), suggesting unique chemical signatures for each of the analyzed groups. Pearson correlation vectors ($r > 0.7$) as variables (Fig 8) indicated that the most important compounds for discrimination of groups were as follows: 4,8,12-Trimethylhexatriacontane, *a,b*-Dimethylnonatriacontane, *n*-C31, n-C27, and *n*-C29.

Chemical distances (Euclidean distances) calculated between groups showed that fungus and larvae profiles were the most similar at interspecific level (Table 2). The SIMPER analysis indicated that both larva and fungus profiles shared the same odd-chained of odd-numbered linear alkanes that together made up almost 100% of total compounds (Fig 2). However, as individuals progressed through developmental stages, the chemical profiles increased in difference, due to the fact that several branched alkanes (especially trimethylalkanes) were found in great proportions in the cuticle of the ants but were not found in the chemical analysis of the fungus gardens (Table 1).

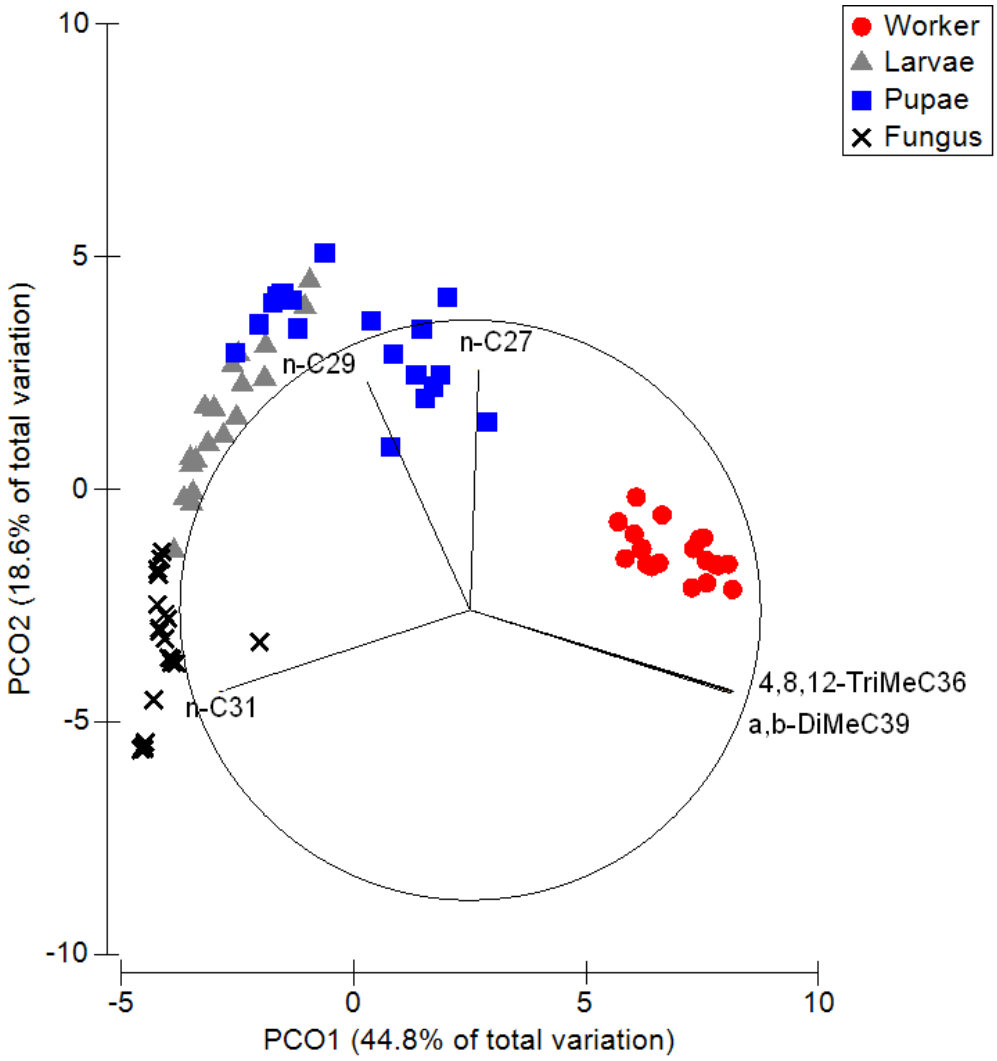

Figure 1. Principal coordinate analysis (PCO) carried out with cuticular hydrocarbons of *Atta sexdens* developmental stages and its symbiotic fungus *Leucoagaricus gongylophorus*.

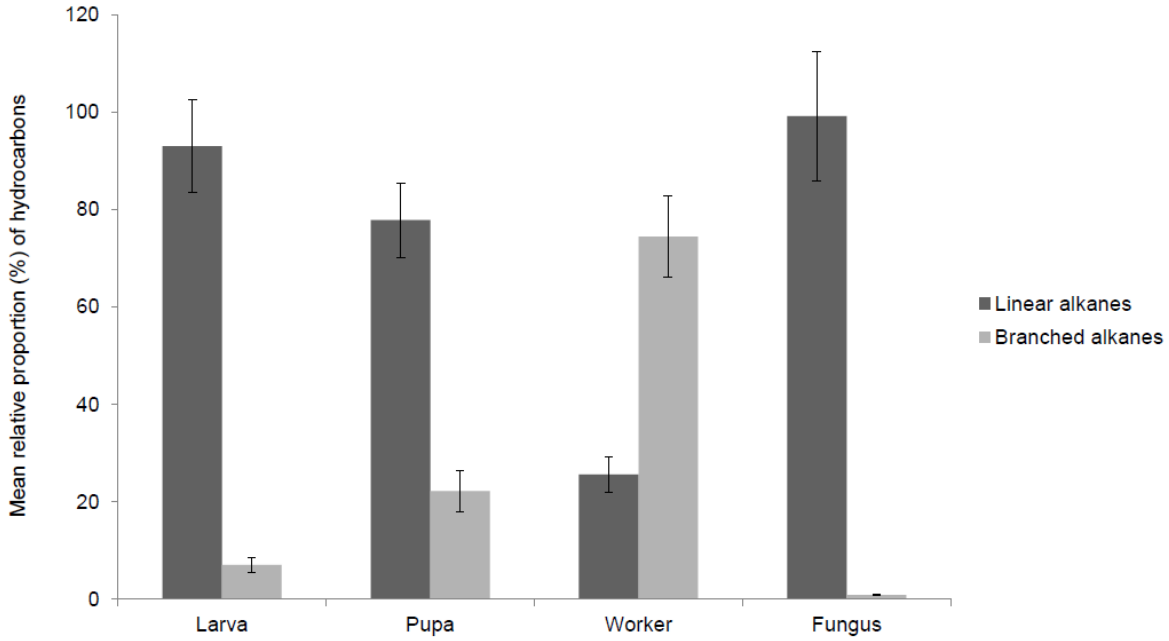

Figure 2. Mean relative proportion (%) of linear and branched cuticular hydrocarbons of *Atta sexdens* developmental stages and its symbiotic fungus *Leucoagaricus gongylophorus.* The bars represent the mean relative proportion of class of hydrocarbons and the whiskers are the standard deviation

Table 1. Chemical distance (Distance between centroids) calculated with the cuticular hydrocarbon profiles of *Atta sexdens* and its symbiotic fungus *Leucoagaricus gongylophorus.*

	Fungus	Larva	Pupa	Worker
Fungus				
Larva	5.68			
Pupa	7.79	5.09		
Worker	11.29	10.19	8.95	

Table 2. Mean and standard deviation of the relative proportion of hydrocarbons of *A. sexdens* phenotypes and the symbiotic fungus *Leucoagaricus gongylophorus.*

Kovats Index	Compound	Fungus	Larvae	Pupae	Worker
2300	Tricosane (*n*-C23)	-	-	3.6 ± 3.3	-
2400	Tetracosane (*n*-C24)	1.1 ± 2	1.9 ± 2.3	1.5 ± 2.2	-
2500	Pentacosane (*n*-C25)	1.1 ± 1.2	9.9 ± 5.7	9.5 ± 7.8	7.2 ± 2.4
2600	Hexacosane (*n*-C26)	6 ± 8	2.9 ± 2.5	2.6 ± 1	0.5 ± 0.4
2700	Heptacosane (*n*-C27)	6.7 ± 1.1	17 ± 10.6	21.9 ± 11.3	10 ± 4.6
2800	Octacosane (*n*-C28)	12.1 ± 4.5	1.7 ± 1.9	3.2 ± 3.3	-
2900	Nonacosane (*n*-C29)	6.9 ± 3.2	18 ± 3.9	17.9 ± 9.5	3.1 ± 1.8
3000	Triacontane (*n*-C30)	1.3 ± 1.9	1.7 ± 1.2	3.1 ± 2.5	-
3100	Hentriacontane (*n*-C31)	43.1 ± 8.6	28.7 ± 9.1	14 ± 7.8	2.5 ± 1.9
3165	3-Methylhentriacontane	0.5 ± 0.7	-	-	3.6 ± 1.1
3205	3,5-Dimethylhentriacontane	0.3 ± 0.5	-	-	3.9 ± 0.9
3235	3,7,11-Trimethylhentriacontane	-	4 ± 3.1	10.1 ± 13.1	8.3 ± 2.2
3298	Unknown	-	2.9 ± 1.9	9.2 ± 11.2	3.1 ± 0.9
3300	Tritriacontane (*n*-C33)	20.7 ± 15.5	10.5 ± 8.8	-	1.5 ± 1.7
3320	4,8,12-Trimethyldotriacontane	-	-	2.7 ± 3.7	1.7 ± 1.3
3437	3,7,11-Trimethyltritriacontane	-	-	-	1 ± 1
3520	4,8,12-Trimethyltretratriacontane	-	-	-	7.5 ± 2
3720	4,8,12-Trimethylhexatriacontane	-	-	-	22.8 ± 3.9
3958	*a*,*b*-Dimethylnonatriacontane	-	-	-	22 ± 4.9

DISCUSSION

In this study we were able to track the changes in the CHC composition of *A. sexdens* associated with its molting cycle. The dynamics of change was characterized by a great shift from the 'less pronounced' state of the brood's chemical profile towards a more diverse chemical profile of adult workers comprised of highly concentrated branched alkanes. Furthermore, our comparative analyses between ants and symbiotic fungus revealed a great similarity between the hydrocarbon profiles, especially at the earliest stages of ants' development, when concentrations of odd-numbered linear alkanes were found in similar proportions in both organisms. Such findings coincide with the observations made for *Acromyrmex* leaf-cutting ants (Vianna et al, 2001; Richard et al, 2007a), strengthening the evidences for the influence of the obligatory symbiosis between leaf-cutting ants and the fungus *L. gongylophorus* on their hydrocarbon profiles. However, the contexts underlying the biosynthesis and transference of hydrocarbons in both organisms are unknown, which makes it difficult to interpret the outcomes of this mutualistic relationship on their chemical profiles.

In leaf-cutting ants, the brood is always allocated inside the fungus garden, where larvae are fed on sugary and fatty substances secreted by the fungus itself (Siqueira et al, 1998; Silva et al, 2003); based on this premise, previous studies on *Acromyrmex* leaf-cutting ants raised two hypotheses to explain the presence of hydrocarbons in the chemical analyses of fungus gardens. The first considers that the fungus does not produce hydrocarbons but passively acquires them by contact with the brood (Vianna et al, 2001). More recently, however, Richard et al. (2007a) suggested that fungal cultivars might be capable of producing hydrocarbons, a prediction that has been supported by the well-known capability of fungus to degrade cellulose and synthesize hydrocarbons (Gianoulis et al, 2012; Ladygina et al, 2006; Shaw et al, 2015; Spakowicz and Strobel, 2015). In the context of the second hypothesis, the

symbiotic fungus would act as an ultimate and independent source of hydrocarbon for the ants (Richard et al. 2007a).

If we consider the resemblance between the CHC profiles of ant and fungus as a result of an active and direct transference of substances, both hypotheses coincide with our data. Fungal secretions may contain hydrocarbons that are ingested by the ants, especially at the larval stage, when ants feed more due energetic demands to the molt (Hölldobler and Wilson, 1990). The acquisition of fungus-derived hydrocarbon may be continued via nutritional interdependence among ants; the narrowness of petiole restricts adult ants to ingest only liquids, so the primary mechanism to share nutrients among nestmates is through trophallaxis. In contrast, larvae are able to ingest solid food, and due to its incomplete digestive system, they give back the faecal liquid through trophallaxis (Hunt and Napela, 1994; Cassill and Tschinkel, 1996). In leaf-cutting ant *A. sexdens*, larval faecal liquid contains proteins, glucose and amino acids, all of them considered to be important nutrients to adult ants (Schneider, 2004). Previous studies on *Atta* and *Acromyrmex* have demonstrated the influence of fungus' diet on the CHC profile of ants (Richard et al, 2004; Lambardi et al, 2004; Valadares et al, 2015), which leads to a third scenario that might be taken into consideration, once hydrocarbons originated from plant materials could also be integrated into fungal cultivars. The symbiotic fungus is a dynamic entity where foliar substrate is added constantly and the complete turnover of the fungus garden material can take a period of 6 weeks (Fisher et al, 1996).

The deposition of CHCs throughout development stages was investigated in other insects and they found that internal hydrocarbons increase drastically during instar phases, and half of them only appear on the cuticle after the molt (Dwyer et al, 1986; Guo and Blomquist, 1991; Falcón et al. 2015), which may explain why branched alkanes increased its concentration after the pupal stage in our study. The internal lipids associated with the cuticle

innermost layers, epidermal tissue, fat bodies, and especially in the haemolymph are likely associated with the deposition of CHCs on the epicuticle of insects (Blomquist, 2010). Furthermore, the pupal tissues are capable of synthesising new hydrocarbons and store them in internal tissues, with their appearance in the culticle of the adult individual (Blomquist, 2010).

CONCLUSIONS

We further conclude that there is a variation of the CHC profiles of *A. sexdens* workers associated with the molting cycle, which is characterized by an increase in the concentration of branched alkanes and a decrease in the concentration of linear alkanes as individuals progressed through developmental stages. Interestingly, the earliest stages of development (larva and pupa) showed highest concentrations of linear alkanes that matched with chemical profile of symbiotic fungus. Further investigations on the biosynthesis and transference of hydrocarbons in both organisms are required to a better understanding of the chemical ecology aspects of this co-evolutionary symbiotic relationship.

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Chapter 2

Alloethism during nestmate recognition in leaf-cutting ants

ABSTRACT

In the past 30 years, researchers have argued whether alloethism is a well-established form of task allocation during nestmate recognition process of leaf-cutting ants. So far, two hypotheses have been raised, (1) minor workers patrol the foraging trail for threats due to its large threshold response to alarm pheromone, and (2) minor workers are less sensitive to non-nestmate (NNM) odours due to brain miniaturization and consequently less space to house receptors and neurons. In order to assess these hypothesis, in this work we designed an experiment where we were able to quantify agonistic responses of morphologically worker groups of the leaf-cutting ant *Atta vollenweideri*, when they were confronted with a conspecific subject in three different contexts, *i*) stimulated with alarm pheromone, *ii*) stimulated with territory-marking pheromone, and *iii*) where no alarm stimulus was applied (control). We further hypothesised that the propensity to attack a NNM is higher for minor workers under alarm pheromone stimulation, and instead, major workers (which are efficiently foragers) would show higher propensity to attack an enemy under territory-marking pheromone stimulation. Our results show that, when detecting alarm- and territory-marking pheromone, minor workers show a disproportionaly large duration of mandible opening behaviour, which is the behaviour that characterise the alarm pheromone realising. The same pattern was observed when minor workers were confronted with a NNM. However, pre-estimulation of test subjects to alarm or territory-marking pheromone did not increase the likelihood to attack a NNM, nor a particular size group of workers were shown to be more efficient at attacking a NNM. Our findings support the hypothesis that minor workers are patrolers at foraging trails due to its prominent response to both pheromones employed during defensive strategies, and show evidence against the hypothesis that minor workers are less sensitive to NNM odours.

Key-words: alloethism, leaf-cutting ants, *Atta vollenweideri*, worker subcastes, mandible opening behaviour, alarm pheromone, territory-marking pheromone.

INTRODUCTION

Along the course of evolution, animals have adapted defensive mechanisms to cope with a broad array of threats and dangers. Most commonly, species have evolved adaptive features (e.g. morphological, physiological, and behavioural responses) that would only be employed when a given animal has an accurate assessment of the experienced agonistic context and consequently of the benefits of defence (Kavaliers & Choleris, 2001, Kleineidam et al, 2017). In hymenopteran societies, these features are generally regulated by physiological processes, e.g. honeybee's defence strategy is age-related, once behavioural and genetic evidence shows that defenders are distinct groups of older workers (Breed et al, 1990). However, in many species of derived insect societies, such as ants and termites, colonies are defended by soldiers, which are workers with enlarged body size equipped with powerful mandibles or chemical weaponry (Oster & Wilson, 1978; Hölldobler & Wilson, 1990; Grüter et al, 2012). The linkage between body size and behavioural repertoire (alloethism) has been interpreted as an adaptive feature for task allocation in social insects, since groups of workers with distinct morphological traits can display a narrower but mostly effective array of tasks (Wilson, 1980; Page et al, 2006; Gordon, 2015).

In the highly polymorphic leaf-cutting ants (genera *Atta* and *Acromyrmex*), alloethism has been well documented for tasks related to collection and processing of food resources (Wilson, 1980; Camargo et al, 2008). However, the mechanisms underlying nest defence have been overlooked and important questions remain opened. Overall, small workers are generally found inside the nest assisting the queen and brood as well as caring for the symbiotic fungus, while bigger workers are more prone to engage in foraging and nest defence (Wilson, 1980; Camargo et al, 2008). In *Atta*, however, polymorphism has evolved further than *Acromyrmex*, and workers exhibit an incredible size polymorphism of up to 200-fold difference in body mass, with a distinct soldier production in mature colonies in both laboratory and wild

conditions. The role of soldiers during task allocation is contradictory, since Wilson (1980) suggested they seem virtually limited to defence, however too large to be energetically efficient foragers and presumably less effective in defence than medias and major workers (Wilson, 1980).

If we consider that leaf-cutting ants indeed have a subcaste specialised in colony defence, and since ants are truly social insects, it is hard to think that only a subset of individuals will operate alone, but instead all size classes are part of the colony level defensive strategy. In fact, field studies have demonstrated that minor workers were more prone to react with alarm/panic behaviour to alarm pheromone stimulation under foraging context (Whitehouse & Jaffé, 1996; Hughes & Goulson, 2001). However, higher responsiveness of minor workers could be explained by the fact that, media and major workers are actually more prone to be engaged in foraging activities, and thus lack responsiveness to alarm or threat stimuli, as observed by Hughes & Goulson (2001).

Another important aspect that have been posed on the nestmate recognition system of leaf-cutting ants is the fact the minor workers were found to be less prone to attack a NNM under laboratory conditions (Larsen et al, 2014). Such finding raised the question on whether minor workers are less sensitive to NNM odours due to brain miniaturization and consequently less space to house receptors and neurons (Larsen et al, 2014).

In order to answer these question, we designed an experiment where we were able to quantify agonistic responses of morphologically worker groups of the leaf-cutting ant *Atta vollenweideri*, when they were confronted with a conspecific subject in three different contexts, *i*) stimulated with alarm pheromone, *ii*) stimulated with territory-marking pheromone, and *iii*) where no alarm stimulus was applied (control). We further hypothesised that the propensity to attack a NNM is higher for minor workers under alarm pheromone

stimulation, and instead, major workers (which are efficiently foragers) would show higher propensity to attack an enemy under territory-marking pheromone stimulation.

METHODOLOGY

Animals

Animals for behavioural experiments were obtained from two colonies of the leaf-cutting ant *Atta vollenweideri* (Forel). This species is widely distributed in the Gran Chaco region of central South America, and it is not protected under the Convention on International Trade in Endangered Species (CITES). Colonies were established at the laboratory after collection of foundress queens in 2013 at the Río Pilcomayo National Park, north of Argentina. Both queens were collected at the same area (lat. 25° 07.375', long. 58° 10.516'), one by excavation of a mature nest at an age of approximately 4 years old (Colony Regina, S. Neupert and C. Kleineidam), and the other one was collected after nuptial flight (Nov. 2013, Colony Falbala, S. Neupert and L. Kling). In the laboratory, colonies were reared in artificial nests made up of several interconnected acrylic chambers attached to an open foraging arena, where we offered leaves of *Rubus section* (Rosaceae) three times a week. At the moment of the experiments, colonies had an approximated volume of 12 L of fungus gardens, and had been kept for at least three years under controlled conditions (at 25ºC and a 12:12h LD cycle) in an insect rearing room at the University of Konstanz, Germany.

Morphological worker subcastes

Atta vollenweideri is a highly polymorphic ant species in which workers may present a 200-fold difference in body mass (Weber 1972). To classify individuals into categories based on body sizes (subcastes), we followed the traditional classification for *Atta* species in which workers are clustered into four morphological subcastes according to head widths (Wilson, 1980). Head widths (HW) were measured using as reference the longitudinal-axis distance at the level of the eyes, a procedure that represents a standard for ant taxonomy (Wilson, 1980). Prior to the experiments, we collected and measured with an ocular scale the head capsule of

25 individuals that were rank-ordered in row according to body size and we selected only those specimens with HWs around 0.8 mm, 1.4 mm, 2.2 mm, and > 3.0 mm, respectively classified as minor workers, median workers, major workers, and soldiers. These specimens were pinned with a subcaste identification label and used as reference for collecting individuals for behavioural experiments. At the end of the experiment, each test subject was photographed and had their HW measured at nearest 0.01 mm, using the image processing software ImageJ (ImageJ 1.51j8, Wayne Rasband, National Institute of Health, USA). The mean ± standard deviation of focal ants were as follow: minor workers (0.94 ± 0.11), median workers (1.55 ± 0.14), major workers (2.14 ± 0.20), and soldiers (3.45 ± 0.27).

Preparation of alarm behaviour stimuli

In ants, alarm behaviour is triggered when individuals detect the contents of specific glands that storage the so-called 'alarm pheromones'. In *Atta*, the contents of several glands was evaluated behaviourally and the mandibular glands and Dufour's gland are well known to play a role in the secretion of substances that trigger alarm behaviour (Blum, 1968; Blum, 1969; Moser et al, 1968; Hölldobler & Wilson, 1986; Salzemann et al, 1992; Hérnandez et al, 1999; Francelino et al, 2008; Norman et al, 2017). Mandibular glands are structures attached to the mandibles and are located inside the head capsule, and thus constituting part of the salivary gland system of the ants (Pavon & Mathias, 2005). Dufour's gland is found in the gaster segment of the body and it is associated with the sting apparatus (Abdalla & Cruz-Landim, 2001). The functionality of these glands is so specific that one can only present the macerated body segment where the gland is inserted to trigger the alarm response in ants. Interestingly, extracts prepared in solvent by macerating the head and gaster segments have been used as alarm behaviour stimuli across several studies (Jaffé et al, 1979; Salzemann et al, 1992; Francelino et al, 2008; Norman et al, 2017). Thus, we prepared each extract by

collecting 25 median sized workers (HW around 1.4 mm) that were killed on ice and rapidly had their head and gaster removed using a small surgical scissors. The different body segments were separated and pooled in their respective glass vial (one for head and other for gaster segments) containing 0.5 ml of methanol as solvent, where they were macerated using clean glass rods for 5 min. The macerated body segments were then removed using a clean forceps and the extracts was storage at -20ºC degrees.

Behavioural assay

Individuals for behavioural assay were collected at foraging arenas of two different colonies, and thus no effect of age polyethism is assumed since only older workers were found outside fungus chambers under laboratory conditions (Camargo et al, 2007; LV & FSN, unpubl. data). To investigate subcaste-related patterns in the agonistic behaviour of *A. vollenweideri*, we placed a single test subject (hereafter referred as to 'focal ant') in a test arena lined with filter paper and Fluon-coated wall to prevent ants from escaping. Test arenas were surrounded by a light-emitting diode (LED) strip attached to the walls of a plastic octagon, which promoted a homogenous lighting to the setup (Figure 3). For testing, each focal ant was presented to a sequence of two stimuli that are known to elicit agonistic behaviour in ants: (*i*) application of an alarm substance followed by the (*ii*) introduction of non-nestmate (NNM). In order to inhibit the behavioural response of NNMs towards the focal ant, we cut the antennae of all NNMs that were subjected to a rapid anaesthesia on ice. After recovery, NNM subjects were kept undisturbed for 20 minutes before the experiments began.

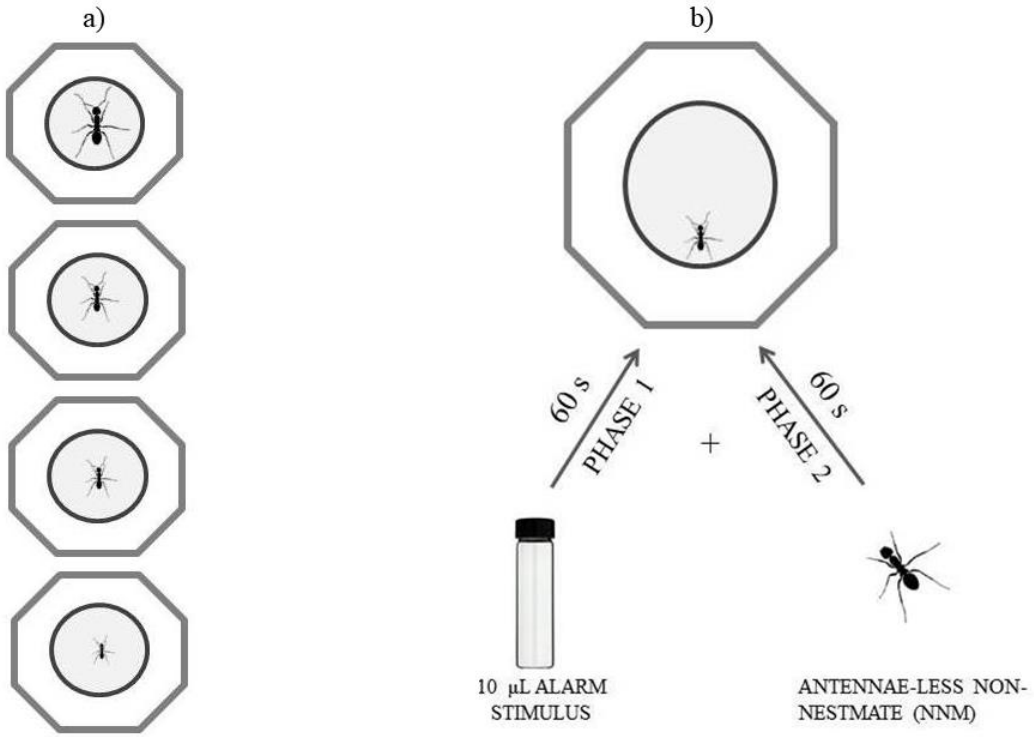

Figure 3. Testing setup. **a)** For each experimental trial, four focal ants (each one of them belonging to a size class) were placed individually into test arenas surrounded by a LED strip attached to the walls of a plastic octagon in order to promote a homogenous lighting to the setup. **b)** Each focal ant was presented to a sequence of two stimuli that are known to elicit agonistic behaviour in ants: (*i*) application of an alarm substance (alarm pheromone or territory-marking pheromone) followed by the (*ii*) introduction of an antennae-less non-nestmate (NNM).

After application of each stimulus, the behavioural responses of ants were registered for 60 s, which constitute a time period we refer as to 'Phase'. Thus, time periods of extract stimulus represents 'Phase I', and the same applies to NNM stimulus which represents 'Phase II'. At Phase I, 10 µl of either gaster or head extract (methanol was used as control) was applied onto the filter paper right in the centre of the test arena (x/y coordinates: 464/465

pixels). Phase II was characterised by the introduction of a medium-sized antennae-less NNM (HW: 1.45 mm ± 0.15) in the test arena. Each trial was videotaped using a Raspberry camera (information) at a sampling rate of 20 frames/sec for 180 s, which the first 60 s no stimulus was applied and we then refer to this time period as to 'Phase 0'. From Phase 0 to Phase II, we registered three behavioural acts of focal ants towards NNMs that are considered as part of the agonistic response of ants in general: (*i*) mandible opening, (*ii*) running, and (*iii*) biting.

To quantify mandible openings and bites, we registered the video frames where the target behavioural acts were observed, which allowed us to quantify the number of times a given ant has performed the behaviour as well as its duration. Open mandible response (MOR) is considered a first aggressive display when ants are threatened and need to defend their territories and has been considered a suitable procedure for studying chemical basis of aggression in ants (Guerrieri & d'Ettorre, 2008). To quantify running behaviour, we tracked animals using the plugin AnimalTracker (Gulyás et al, 2016) for ImageJ (ImageJ 1.51j8, Wayne Rasband, National Institute of Health, USA). This last approach allowed us to create velocity profiles of individuals and how this varies according to its size and behaviour displayed during 180 s of experimental trial.

All behaviour documentation was done by a blind observed who wasn't aware of the objectives of the study. Experimental treatments were presented in a random order and all individual data are independent from each other because subjects were killed after being tested. A total of 240 trials were recorded, which consists a sampling of 20 individuals per subcaste.

Statistical analyses

First, we measured the strength of association between body size and mandible open response by performing a bivariate Spearman correlation analysis. Next, we clustered all test subjects into four behavioural groups (subcastes) according to their head widths, and a

histogram of data distribution was plotted in order to determine the dispersion of data set. As each statistical group demonstrated a right-skewed data distribution, we performed an overdispersion test (Cameron & Trivedi, 1990) in order to find the best statistical model for our data set. As all statistical groups were overdispersed, we performed a generalised linear model (GLM) with a binomial distribution and a log-link function to assess whether there are subcaste-related differences in the number of ants that responded with MOR. Futhermore, we assessed whether there are subcaste-related differences in the duration of MOR by performing a negative binomial GLM. All statistical analyses were done using R (3.3.3) in RStudio (1.1.383).

RESULTS

Phase 1

A total of 153 out of 234 ants (65.3% of our test subjects) responded with MOR during phase 1 of the experiment, totalling 1152 s of MOR in which 722.6 s were registered in head-extract treatment, 383.9 s registered in gaster-extract treatment, and 46 s in the control (methanol). Head extracts also evoked a higher percentage of ants that responded with MOR (92.4 %, 73 out of 79 ants), when compared to gaster-extract (74.6 %, 59 out of 79 ants) and the control (27.6 %, 21 out of 76 tested ants). We found no statistical difference of duration of MOR between size classes (subcastes: minor, median, major, and soldiers), regardless of the treatment (Fig 4). Furthermore, no correlation between body size and duration of MOR were found for each treatment (Fig 6).

Phase 2

A total of 169 out of 234 ants (72.2 % of our test subjects) responded with MOR during phase 2 of the experiment, totalling 817 s of MOR in which 299.4 s were registered in gaster-extract treatment, 277.2 s registered in methanol (control), and 240.3 s registered in head-extract treatment. We found no statistical difference in the number of ants that shown MOR across subcastes, but we found that minor workers were statistically different in the duration of MOR, due to a disproportionally longer MOR when they confronted a NNM, regardless of the context that they have been previously exposed (head-extract, gaster-extract, or control). This finding was also supported by our correlation analysis, when we found a negative correlation between body size and duration of MOR only during phase-2 of the experiment (Fig 6).

Regarding biting behaviour, only 23% of our test subjects (54 out of 234 ants) have bitten the NNMs. We found no statistical difference between the percentage of ants that

shown the behaviour according to treatments (Fig 7 a), nor according to size classes (subcastes) (Fig 7 b, c, d).

Table 3. Descriptive statistics and Generalized linear model significance of MOR for each subcaste during experimental phases 1 and 2. Ns: non-significant.

		Phase 1			Phase 2		
	Subcaste	Median	Variance	GLM	Median	Variance	GLM
Control	Minor	0	7.53	ns	5.1	163.44	0.0083
	Median	0	1.02	ns	0.8	15.1	ns
	Major	0	1.3	ns	0.6	11.15	ns
	Soldier	0.05	1.74	ns	0.17	130.8	ns
Gaster extract	Minor	1.1	91.62	ns	4.85	158.45	0.0015
	Median	0.52	45.49	ns	1.7	12.75	ns
	Major	3.55	56.23	ns	1.7	6.21	ns
	Soldier	2.87	44.99	ns	0.17	38.36	ns
Head extract	Minor	13.52	157.25	ns	4.57	64.08	0.0054
	Median	6.3	196.12	ns	2.87	11.06	ns
	Major	6.8	91.11	ns	2.1	10.02	ns
	Soldier	4.2	54.06	ns	0.27	1.13	ns

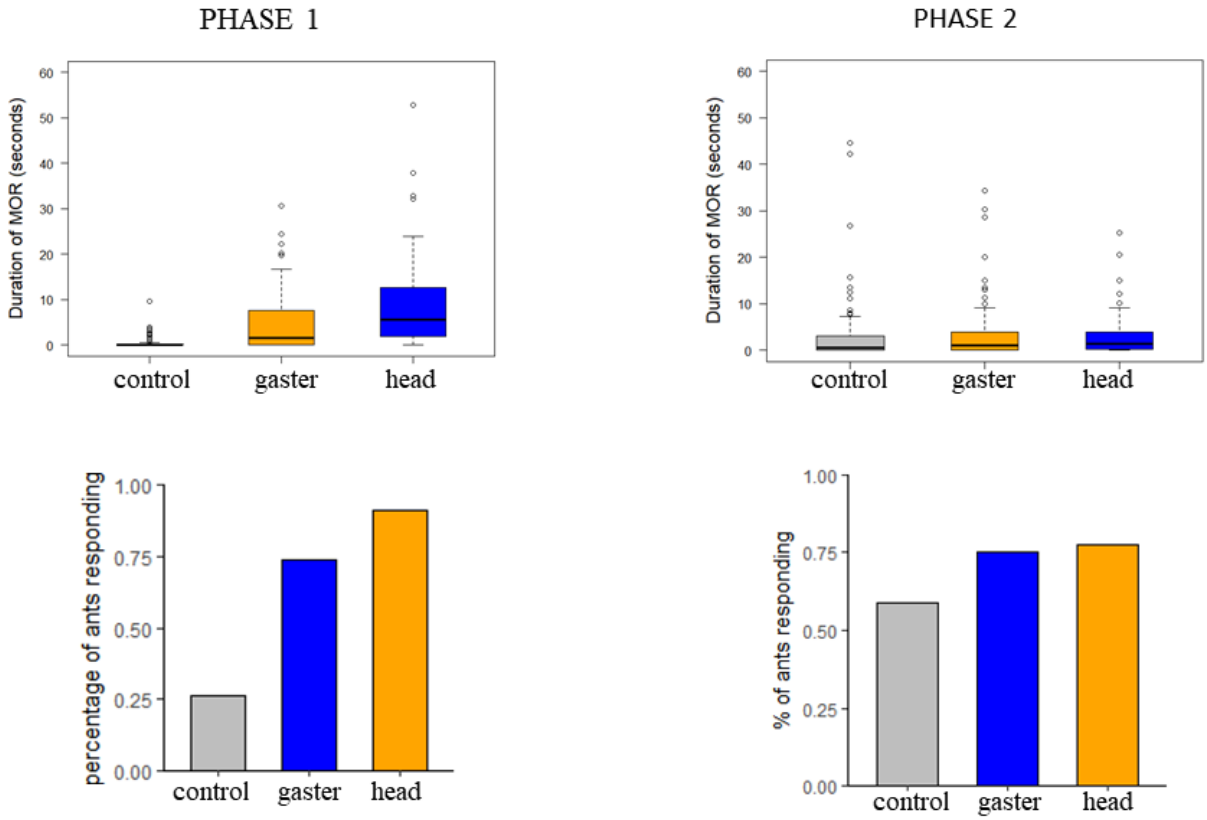

Figure 4. Mandible opening response (MOR) shown by *Atta vollenweideri* workers. Quantification of the duration of MOR during phase 1 and phase 2, and the percentage of ants responding with MOR during phase 1 and phase 2 of the experiment.

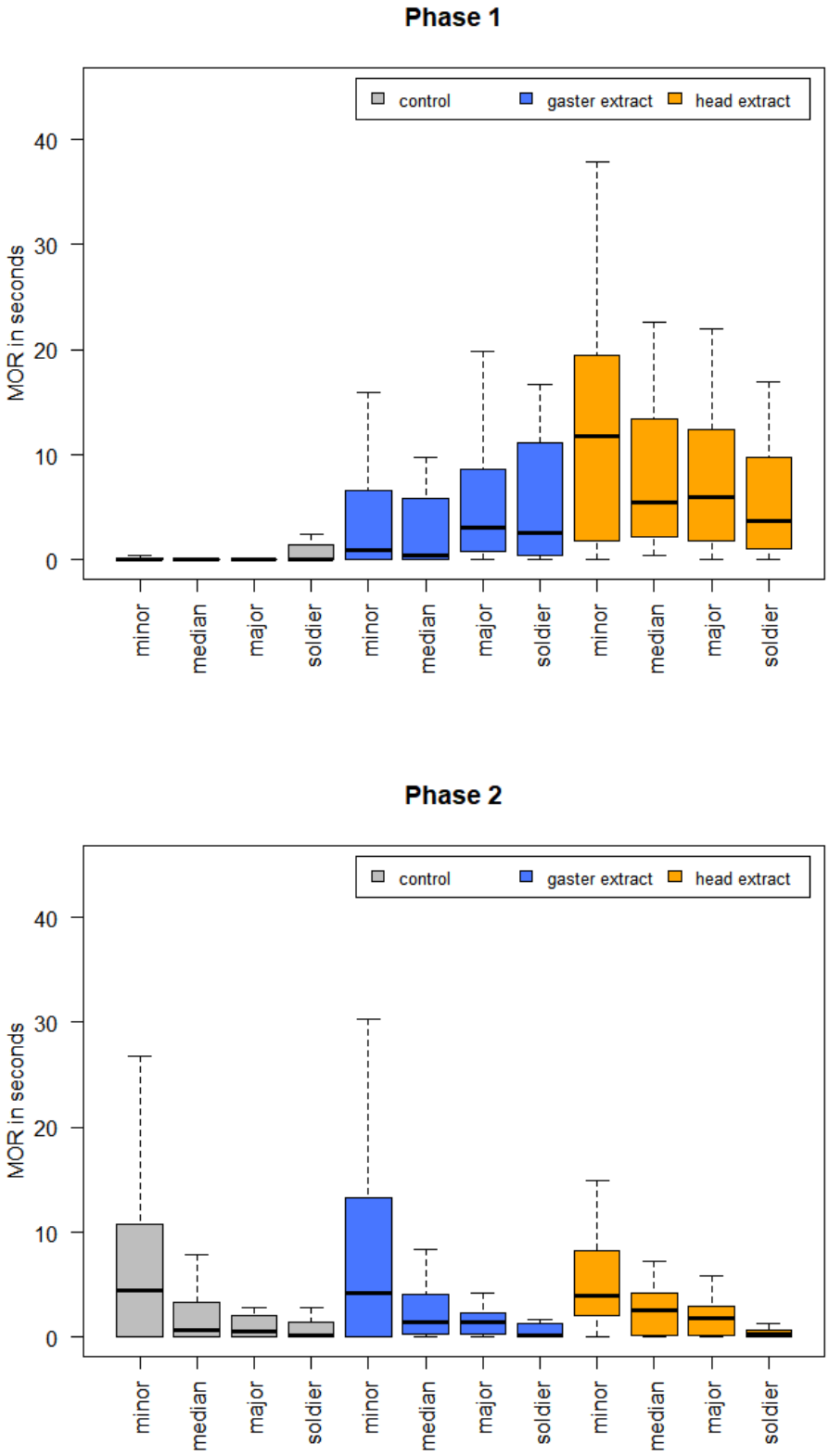

Figure 5. Boxplots of mandible opening response (MOR) of each subcastes in each treatment. Box plots show median, interquartile range and whiskers indicating the 90th and 10th percentiles.

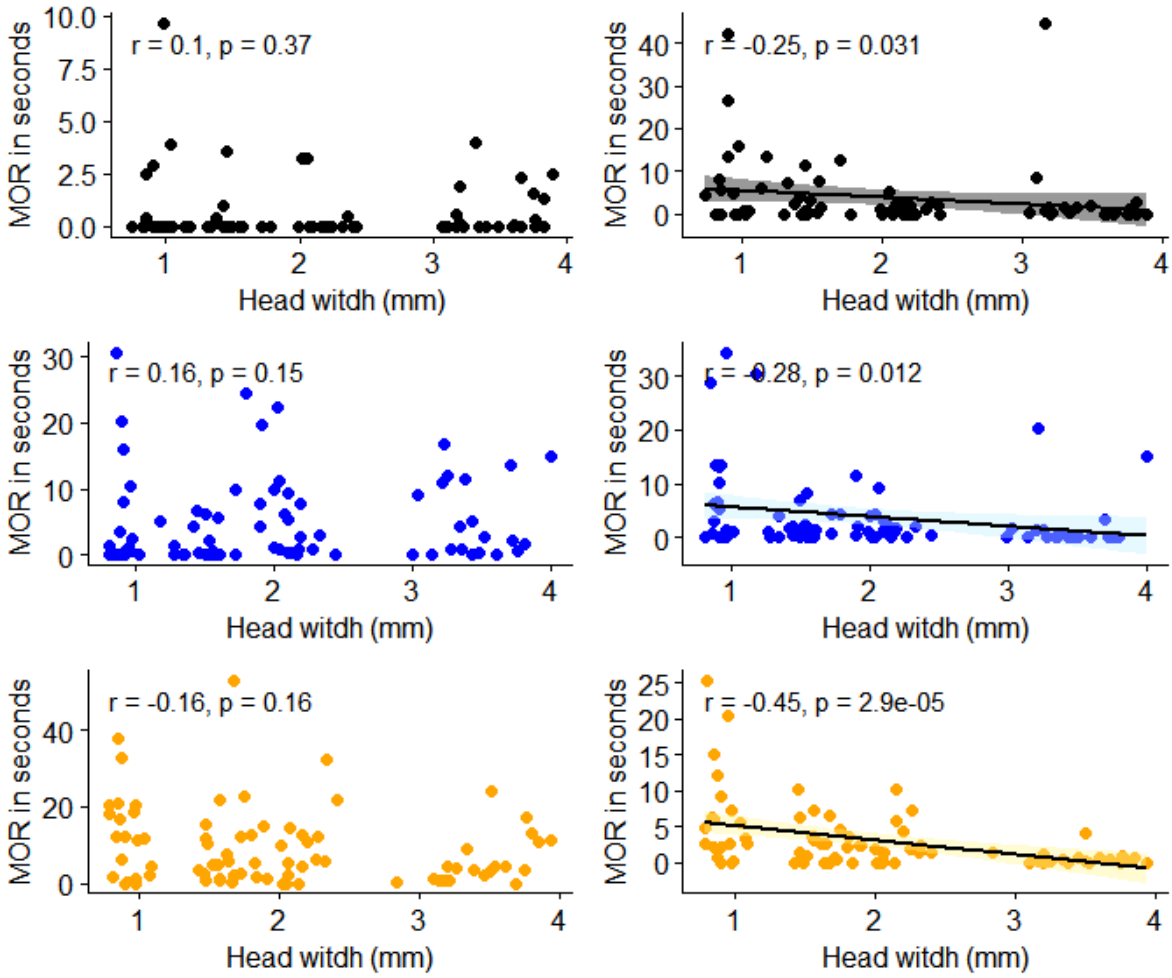

Figure 6. Pearson correlation coefficient (r) and p-value (p) between body size and mandible open response. Black, control; blue, gaster-extract treatment; yellow, head- extract treatment. Solid lines represent regression lines and shaded areas represent confidence intervals.

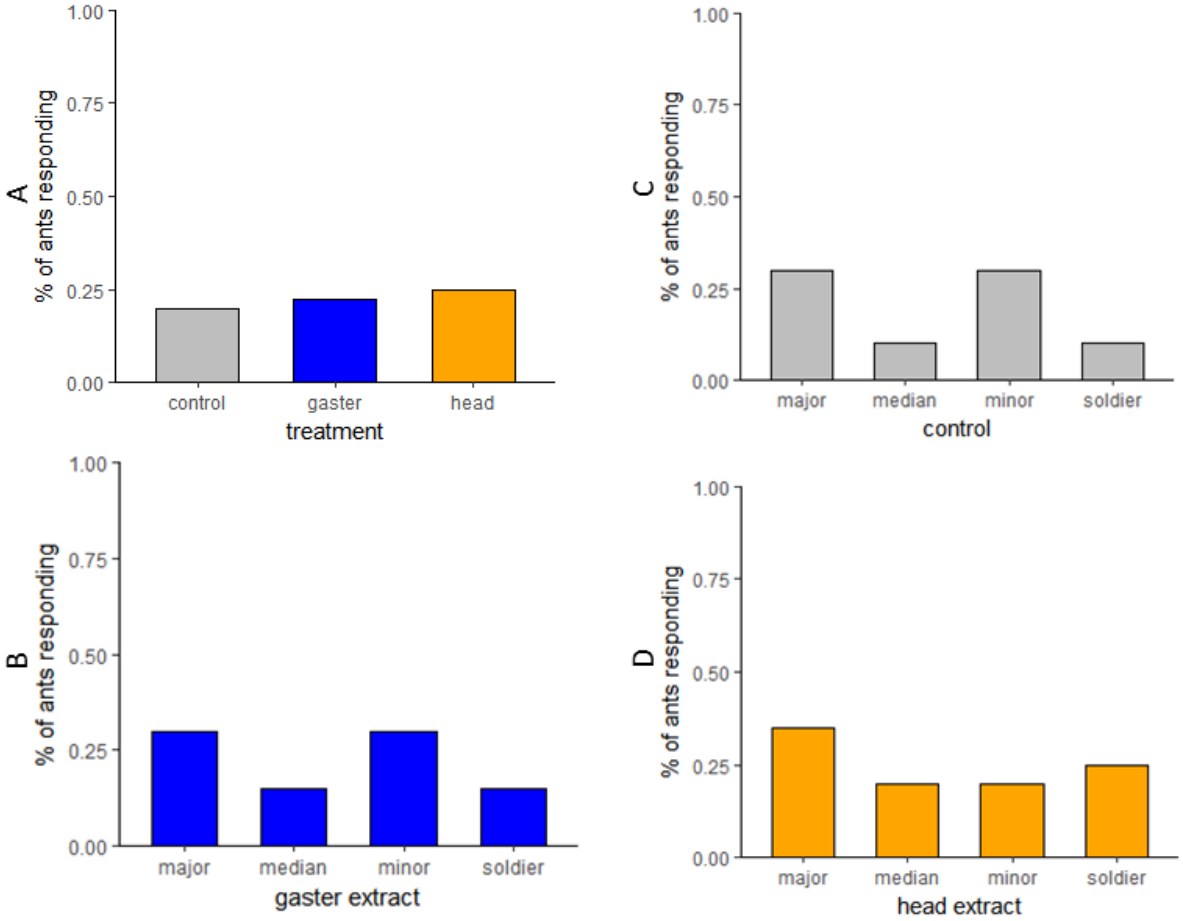

Figure 7. Biting behaviour shown by *Atta vollenweideri* workers during phase 2 of the experiment. Percentage of ants that attacked a NNM according to (a) treatment, and according to subcastes during the (b) gaster extract treatment, (c) control, and (d) head extract treatment.

DISCUSSION

In this chapter, we designed a controlled assay to measure the behavioural responsiveness of different size workers towards alarm- and territory marking pheromone. We were also able to investigate the perception and response components of nestmate recognition of *Atta vollenweideri* by testing whether minor workers are less sensitive to detect a non-nestmate, and whether the propensity to attack an enemy is size-related. Our results show that, when detecting both alarm- and territory-marking pheromones, minor workers show a disproportionaly large duration of mandible opening behaviour, which is the behaviour that characterises the alarm pheromone releasing in ants (Normann et al, 2014; Guerrieri & d'Ettorre, 2008).

Our results support previous observations made for *Atta* leaf-cutting ants when minor workers were more prone to be recruited when the colony weas threatened by conspecifics (Whitehouse & Jaffé, 1996). A higher atractiviness of minor workers to alarm pheromone sources under natural foraging conditions was also reported by Hughes & Goulson (2001). Such finding led to the hypothesis that minor workers perform an important function on foraging trails by patrolling the trail area for threats, and thus playing a key role in the alarm reaction (Hughes & Goulson, 2001). However, higher responsiveness of minor workers to alarm pheromone on foraging trails could be explained by the fact that they are seldom observed performing activities related to foraging, while media and major workers are actually more prone to be engaged in foraging activities, and thus lack responsiveness to alarm or threat stimuli, as observed by Hughes & Goulson (2001). With our work, we were able to answer this question by showing that minor workers responsiveness to alarm pheromone is in fact an intrinsic behavioural feature that strongly differs from media, major, and soldiers.

The argument to explain that the lower response of media and major workers towards alarm pheromone was a result of foraging task engagement became stronger when it was reported that odour information process during trail-following behaviour is a result of size differences among worker caste, where minor workers are less sensitive to trail-pheromone perception due to lack of an specific macroglomerulus which tunes trail-pheromone perception and response in larger workers (Kleineidam et al, 2007; Kelber et al, 2009; Kuebler et al. 2010). However, since ants are truly social insects, it is hard to think that only a subset of individuals will operate alone, but instead all size classes are part of the colony level defensive strategy. In a scenario where larger workers are energetically and behaviourally best fighters (Wilson, 1980), minor workers would fit better into the strategy of patrolling the trails and alerting the others when a threat is found. Not only minor workers disproportionally perform a longer mandible opening response as we have shown, but they also have considerably less complexity in the chemical composition of their mandibular glands than those of foragers and soldiers (Francelino et al, 2006), and are thus more efficient at eliciting an alarm reaction in nestmate workers (Francelino et al, 2008).

In our experiments, we found no statistical difference between body size and the propensity to attack a non-nestmate. A similar pattern was also observed by Norman et al (2014) working with the leaf-cutting ant *Acromyrmex echinator*. In their study, Norman et al (2014) concluded that size does not influence the likelihood to attack a NNM. A conclusion that were later on conflicted by Larsen et al (2014) that showed that size does matter in aggressive behaviour of *A. echinator*, since in their experiments larger workers were more prone to attack a NNM. Furthermore, due to lower aggressiveness shown by minor workers, Larsen et al (2014) raised the hypothesis that predicts that minor workers are less sensitive to non-nestmate odours due to miniaturisation of the brain and consequently less space to house neurons and receptors. As we have shown, minor workers showed a disproportionally large

duration of mandible opening behaviour when they were confronted in one-on-one encounters with a conspefic non-nestmate. Also, we found no statistical difference between subcastes in their proportion to display MOR, nor at the propensity to attack a NNM, which suggests that all subcastes are equally capable of discriminating and attacking a NNM.

An important difference between the methodologies employed by Norman et al (2014) and Larsen et al (2014) that is worth to be discussed is that while in the first paper they introduced the conspecific non-nestmate in one-on-one encounters, the latter designed an experiment that non-nestmates were introduced to testing arenas containing groups of ants with different body sizes. Within this later context, and assuming that the propensity to attack a non-nestmate is group-dependent, it makes sense to think that the high propensity of large workers to attack an enemy as observed by Larsen et al (2014) is a result of task allocation during nestmate recognition in leaf-cutting ants, where minor workers are efficiently good patrollers, due to a broader threshold response to alarm pheromone as well as to their capacity of secreting a mandibular gland mixture relatively more concentrated with the alarm pheromone. On the other hand, large workers would be physically more efficient at attacking an enemy than minor workers, and would rather adopt a strategy in which they are energetically more profitable.

Studies on the olfactory capacity of ants combined with threshold detection of nestmate recognition cues, as well as behavioural assays aiming to test whether mandible opening behaviour of minor workers increases the likelihood to attack a NNM are necessary to a better understanding of the mechanisms regulating differences in aggressive behaviour as well as an understading of its biological significance.

CONCLUSIONS

- Pre-stimulation of ants by alarm- and territory-marking pheromones do not affect their propensity of attacking a non-nestmate in one-on-one encounters.
- We found no correlation between body size and propensity of attacking a non-nestmante in one-on-one encounters.
- When detecting a threat, minor workers show a disproportionally long duration of mandible opening response, which is a strong evidence of alarm pheromone releasing.
- Evidence that minor workers are not less “sensitive” to non-nestmate odours lies on the fact that we did not find evidence that one subcaste is more prone to attack a non-nestmate, thus we assume they have equal olfactory capacities at discriminating non-nestmates.

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Chapter 3

SUBCASTE-RELATED PATTERNS OF SEROTONERGIC IMMUNOREACTIVITY IN THE BRAIN OF THE LEAF-CUTTING ANT *Atta vollenweideri*

ABSTRACT

Alloethism is the task allocation strategy characterised by the distribution of activities based on morphological differences of workers. In such cases, worker`s brains must have neural substrates to support the differences in behaviour among worker caste. However, the neural basis of alloethism is still unknown. One powerful candidate to regulate differences in behaviour is the biogenic amine serotonin (5-HT), which acts as neurotransmitter, neurohormone, and neuromodulator from insects to mammals. In the last chapter of my thesis, we have developed a reliable protocol from immunocytochemistry procedures to data analysis that allowed us to assess whether there are subcaste-related differences in the serotonergic immunoreactivity in the brain of the leaf-cutting ant *Atta vollenweideri*. We aimed to quantify immunostaining intensity against serotonin as a standard to assess the semi-amount of serotonin within serotonergic neurons and the neuropils where they project their axons. We found no significant differences between body size and serotonin levels in the mushroom body and antennal lobe compartments of *A. vollenweideri*. However, we found significant differences in optical lobe (OL) and central complex (CC) compartments. In the OL, such differences were characterised by an increase in serotonin level in workers with intermediate body size (median and major workers). In CC compartments, a negative relationship was found. Such relationship might be due to difficulty of antibodies to reach inner regions of the brain, once the CC is the innermost neuropil of *A. vollenweideri*. Intermediate workers also presented a greater variability in the number of serotonergic neurons in the optic lobe cluster as well as higher levels of serotonin in the somata of this cluster were found. According to the traditional literature, median workers show broader behavioural repertoires, performing tasks both inside and outside the nest, whilst major workers are traditionally considered as "foragers". Both groups may rely on visual cues while searching for food or returning home. Thus, the differences obtained from our protocol reflect behavioural specialisation of workers,

which makes us confortable to present our protocol as suitable for assessing differences in immunostaining intensity in brain regions of individuals with different body sizes and consequently different sizes of neural tissues.

Key-words: Social organisation; task allocation; alloethism; brain morphology; *Atta vollenweideri*

INTRODUCTION

The evolutionary success of bilateral symmetry must have depended on changes in the organisation of the nervous system and the distribution of sense organs (Brusca & Brusca, 2003). Behaviour is in large part a function of animal's responses to information, and a consequence of some essential internal attributes, such as its genetic machinery, amount of certain hormones, or the size of its brain neuropils (Gordon, 2015). Since behaviour is characterised by its flexibility, it follows that the neural machinery that creates it must possess analogous features (Gall et al., 1986). Thus, it is hypothesised that plasticity in behaviour follows plasticity in the brain. Brain plasticity occurs at several levels, for example, neuromodulators can shape sensory mechanisms and synaptic transmission in order to generate behavioural flexibility (Bargmann, 2012; Cohn et al., 2015). As an organism ages, experiences, and learns, its brain continually forms new connections, and also strengthens or weakens synapses connections. During this process, neurons can grow and expand their axons and dendrites, or retract and reform themselves. This may alter the shape of the brain by increasing or decreasing volumes of certain neuropils (Gall et al., 1986).

In adult worker honeybee *Apis mellifera*, the age polyethism is associated with changes in the higher-order integration centre, the mushroom bodies. In the insects' brain morphology, odours are detected by a number of antennal sensilla equipped with olfactory receptor neurons (ORNs) that project their axons across the antennal nerve, in the region of the neuropil responsible for receiving odour information – the antennal lobes (AL). The functional units of AL are spheroidal structures called glomeruli; such units are connected by local neurons that synapse with ORNs (Schneider & Steinbrecht, 1968). The number and arrangement of glomeruli are specific to each species, in the same way that each olfactory stimulus is processed in a standard-exact timeline (revised by Gronenberg, 2008). The information processed by the glomeruli are transmitted to the projection neurons, which then

retransmit the information to the main brain centre related to the memory and learning, the mushroom bodies (Abel et al, 2001; Hansson & Anton, 2000; Yamagata et al, 2006).

In ants of tribe Attini, the relative size of mushroom bodies decreases as the colony increases in population size. Instead, workers develop larger antennal lobes, which consequently increase their olfactory capacity (Riveros et al, 2010). Despite several studies have explored the relative brain size and social behaviour in insects, focusing mainly on the structural plasticity of the mushroom bodies (Menzel & Giurfa 2001; Fahrbach, 2006; Strausfeld et al 2009), the neural mechanisms related to the different behaviour within morphological worker castes are still unknown (Kuebler et al, 2010).

Alloethism is present in the highly eusocial groups; the two independent groups of ants and termites. In this system, the worker body sizes is related to their behaviour (Oster & Wilson, 1978), and the size difference is a result of the amount of food they eat while larvae (Wilson, 1980), but more recently it is known that this variation has also a genetic influence provided by the queen polyandry (Evison & Hughes, 2011). According to Wilson (1980), leafcutter ants of genus *Atta* show a classic example of alloethism, as he defines their division of labour as the extreme case of eusociality, describing a total of four morphological worker castes involved at the processing leaf substrate and the symbiotic fungus in an assembly-line fashion, with an allocation of tasks being performed according to the worker size.

Some studies have demonstrated the pivotal role of biogenic amines in the regulation of behaviour. One putative candidate to regulate the differences in behaviour is the biogenic amine serotonin (5-HT), which acts as neurotransmitter, neurohormone, and neuromodulator from insects to mammals. Biogenic amines are organic bases derived from amino acids (Downer & Hiripi, 1994). These substances are involved in a wide range of biological functions, ranging from classical neurotransmitters to neuromodulators and neurohormones distributed at the periphery and at the central level (Libersaft et al, 2004; Nouvian et al, 2018).

The most important biogenic amines found in insect brains are octopamine (OA), dopamine (DA), and serotonin (5HT) (Downer & Hipiri, 1994).

Role of biogenic amines as social modulators of aggression has been described in many species of both mammals (Lesch & Merschdorf, 2000; Miczek et al, 2002; Niederkofler et al, 2016) and arthropods (Edwards & Kravitz, 1997; Dyakonova et al, 1999; Kravitz & Huber, 2003; Dierick & Greenspan, 2007; Pedetta et al, 2010; Nouvian et al, 2018). Although the role of these molecules are thought to be conserved across animal phyla (Libersat & Pflueger, 2004), empirical studies on insects have shown that an increase of aggressiveness is correlated with an increase of biogenic amine levels, however the pattern of such correlation is not always strict. Similarities/dissimilarities between aggression and biogenic amine levels might reflect evolutionary trends and different environmental pressures for each species (Cuvillier-Hot & Lenoir, 2006). Overall, in insects high level of aggression is correlated with high level of dopamine (Sasaki et al, 2007; Penick et al, 2014; Nouvian et al, 2018), octopamine (Bloch et al, 2000; Cuvillier-Hot and Lenoir, 2006), and serotonin (Nouvian et al, 2018). Upon such premises, we have developed an experimental design based upon the following question: are there subcaste-related differences in the serotonergic immunoreactivity in the brain of the leaf-cutting ant *Atta vollenweideri*? We aimed to semi-quantify the amount of serotonin in brain regions of workers of different body sizes, in order to explore the neuronal mechanisms underlying differences in the behaviour within worker subcastes.

Objectives

To develop a protocol that allows semi-quantification of serotonin in the brain neuropils of *Atta vollenweideri*.

In regards to the four subcastes of *A. vollenweideri*, we aimed to semi-quantify and correlate:

- The distribution of serotonergic neurons
- The intensity of serotonin immunostaining of serotonergic neurons
- The absolute volume and intensity of serotonin immunostaining in four neuropiles and their respective compartments: **optic lobe**; lobula, medulla and lamina. **Antennal lobe**. **Central complex**; upper part of central complex, lower part of central complex, protocerebral bridge, and noduli. **Mushroom body**, medial lip, lateral lip, medial collar, lateral collar, medial ring, lateral ring, and pedunculus.

METHODOLOGY

Animals

Animals for immunohistochemistry procedures were collected at the foraging arena of one mature colony of the leaf-cutting ant *Atta vollenweideri* (Forel), and thus no effect of age polyethism is assumed since only older workers were found outside fungus chambers under laboratory conditions (Camargo et al, 2007; LV & FSN, unpubl. data). *A. vollenweideri* is widely distributed in the Gran Chaco region of central South America, and it is not protected under the Convention on International Trade in Endangered Species (CITES). The colony was established at the laboratory after collection of foundress queen after mating flight, by S. Neupert and L. Kling, in 2013 at the Río Pilcomayo National Park, north of Argentina. In the laboratory, colonies were reared in artificial nests made up of several interconnected acrylic chambers attached to an open foraging arena, where we offered leaves of *Rubus section* (Rosaceae) three times a week. At the moment of the experiments, the colony had an approximated volume of 14 L of fungus gardens, and had been kept for at least five years under controlled conditions (at 25ºC and a 12:12h LD cycle) in an insect rearing room at the University of Konstanz, Germany.

Brain dissections

Ants were first anaesthetised on ice and decapitated. Under a binocular microscope, heads was pinned at each side of the vertex on a 3 cm petri dish covered with a thick layer of Sylgard (184 Silicone Elastomer Kit, Dow Croning, USA) mixed to coal powder to provide a dark background to the head tissues. Head width (HW) was taken at nearest 0.01 mm, using an ocular calibrated with 1 cm scale. HWs were measured using as reference the longitudinal-axis distance at the level of the eyes, a procedure that represents a standard for ant taxonomy (Wilson, 1980). Next, we cut off the antenna and poured ice-cold fixative solution (4%

Paraformaldehyde in Phosphate-buffered saline solution PBS pH ~7.3) into the petri dish until the head and the pins were completely covered. With the aid of a sharp razor blade, we opened a "window" on the ant's head (Fig 8) by cutting the cuticle at the margins of the clypeus, and alongside the left to the right eye, following the dorsal edges of the head. The cuticle was pulled out and detached from the head capsule to allow the fixative to penetrate the tissues. To prevent the brain from floating over the fixative solution, we removed the air in the trachea by sucking it with a fine tip plastic pipette. Next, most muscles and trachea were removed, and the fibres connecting the optic lobes to the eyes, and the antennal lobes to the antennae were cut off using surgical scissors. Using a fine forceps, the brain was grabbed ventrally at the subesophageal ganglion and pulled out from the head capsule. Next, brains were placed individually into wells of cell culture plates filled with fresh fixative solution, and a label containing the date of dissection and brain identification was created and added to the data base for further identification of the specimen. The brains were kept in fixative solution for 2 h, at room temperature, and care was taken to maintain all samples within the same fixation time. As brains were dissected straight in fixative solution, the fixation time count started as soon as the "window" was opened on the head and the solution was allowed to bath the tissue.

Immunocytochemistry procedure

After fixation time, brains were extensively washed with gentle agitation in washing solution made up of PBS with 0.2% Triton X-100 (PBST 0.2%), at room temperature. The PBST 0.2% was changed several times during 24 h. Brains were blocked overnight in an Antibody Blocking Solution (ABDB, 2% bovine serum albumin and 0.04% sodium azide (NaN_3) in 0.1 M PBS diluted 1:10 in PBST 0.2%). Next, brains were incubated for 17 days in two primary antibodies, one against serotonin (α-serotonin, reference S5545, Sigma, Japan) at

concentration 1:3000 diluted in ABDB, and another one against synapsin (α-Synorft1, DSHB, USA) diluted in ABDB at concentration 1:30.

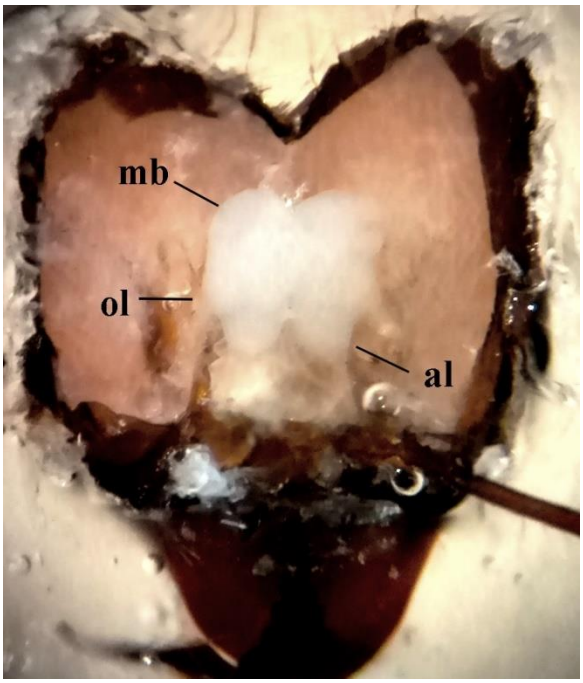

Figure 8. Brain dissection of a media worker (head width, 1.6 mm) of *A. vollenweideri*. A "window" was opened on the ant's head by detaching the cuticle at the margins of the clypeus and alongside the eyes, following the dorsal edges of the head. **mb**, mushroom body; **ol**, optic lobe; **al**, antennal lobe.

After primary incubation, brains were washed in washing solution, on gentle agitation for 24 h. Brains were then incubated for 17 days, on a shaker and at a dark room, in two secondary antibodies, one biding to the primary antibody against serotonin (Invitrogen Alexa Fluor 488 F(ab')2 Fragment of Goat Anti-Rabbit, Thermo Fisher, EUA) diluted at 1:300 in ABDB, and another one biding to the primary antibody against synapsin (Invitrogen Alexa Fluor 546 F(ab')2 Fragment of Goat Anti-Mouse IgG(H+L, Thermo Fisher, EUA), diluted at 1:300 in ABDB. After the second incubation, brains were washed in PBST 0.2% for 24 h and then dehydrated in an ascending series of ethanol (1x 50%, 70%, 80%, 90%, 2x 98%, and

100%). The brains were embedded in methyl-salicylate as mounting medium, and were mounted in aluminium slides.

Image acquisition

Image acquisition of whole-mounted brains was obtained using a laser scanning confocal microscope (LSM 700, Zeiss, Germany), located at the Bioimaging Center (BIC) of the University of Konstanz, Germany. A confocal microscope illuminates and detects, once at the time, multiple diffraction-limited points in the specimen across a focal plane (Paddock, 2000). Each focal point is focused on the specimen using a magnification objective which projects beams of lasers onto a focal point.

In a confocal microscope, the laser beam passes through the *illumination pinhole* and hits the focal point in the specimen. The reflected light is deviated by a dichroic mirror, while the emitted fluorescent light passes through the mirror in the direction of the photodetector. Right in front of the photodetector, there is a second pinhole, the *detector pinhole*. These two pinholes ensure that only information from the focal plane reaches the photodetector, eliminating the "out-of-focus" fluorescence (Reynaud et al, 2001). The great advantage of confocal microscopy over conventional microscopy is the elimination of the "out-of-focus" light (Reynaud et al, 2001), which promotes a sharp and fine visualisation of neural structures. A set of focal points along the x,y plane forms an optical section. Each optical section is collected in the form of electrical signals from the detector, which is further digitalised by proper computer software to form a pixel-based image representing the optical section in itself (Reynaud et al, 2001). To get from one focal point to the other, a moving device is coupled to the stage where the specimen is placed and allows the movement of the specimen across the x,y plane. To gain depth into the specimen, this procedure is repeated at different z-positions, creating multiple optical sections. Collection of a multiple optical

sections/slices forms a “stack” of images from different depths, and allows a three dimension reconstruction of the brain (Reynauld et al, 2001).

In our experiment, we first put the specimen under the microscope using an external stage coupled to the original stage of the microscope. This external stage allowed us to properly orientate the sample on x,y planes in order to have the mushroom bodies pointed superiorly and the optical lobes pointed inferiorly. Also, brains were always positioned lying on the slide with the anterior portion facing towards the objective, which in the LSM 700 is positioned inversely. This allowed us to scan all brains from anterior to posterior portion, and each brain was scanned individually.

First, we took overview scans to identify which brain showed strongest immunostaining intensity measured by the number of brightest pixels within the brain. Overview scans were taken with a 10x water objective (C-Apochromat, Carl Zeiss, Germany) with a numerical aperture of 0.45. Low quality scanning parameters were chosen, such as low laser power intensity and thick optical sections, to avoid photo bleaching of the dyes in the specimens. The data depth was set to 16 bits, frame size to 1024 x 1024, and scan zoom at 0.5. Two lasers were selected, creating thus two channels that were simultaneously scanned throughout the samples, the *green channel* with laser at wavelength of 488 nm that excites the fluorophores of secondary antibody labelling serotonin, and the *red channel* with laser at wavelength of 555 nm that excites the fluorophores of secondary antibody labelling synapsins. Lasers were used with power intensity of 0.2 % and 0.4 % of its capacity. The length of the lasers’ wavelengths was adjusted to prevent cross-talk between the two channels.

After overview scans were taken, we used the software LSM Browser (Zeiss, Germany) to subset each stack into anterior and posterior part of the brain. Such subset were achieved by dividing the total number of optical slices with a stack by 2. We then created projection views of both subsets. Projection views allow overlapping and merging of multiple

optical sections into a single pixel-based image. Projection views were created in greyscale, which converts the value of each pixel into a focal plane representing only an amount of light that carries the intensity information. In other words, image of this kind are composed of shades of grey varying from black as the weakest intensity (areas in the optical section representing the background or weak immunostaining intensity) to white at the strongest (areas in the brain with strongest immunostaining intensity). Each projection view was then copied to the clipboard with the option 1:1 pixel mapping, that preserves the original number of pixels of the image, and we then copied it to a canvas in the software Adobe Photoshop (Adobe System, EUA). To a better visualisation of the brightest pixels, in Adobe Photoshop we used the colour inversion mode, which in this case represented the brightest pixels as black pixels. Each brain with its two projection views (anterior and posterior portions) was then sorted at the canvas according to head widths of ants, and a catalogue was generated. This procedure allowed us to better detect which brain was the brightest by manually estimating the number of black pixels within the brain.

Once the brightest brain was detected, the scanning parameters for the high-resolution scans were adjusted. High-resolution scans were taken for qualitative and quantitative purposes. With the green channel (serotonin), we aimed for immunostaining quantification of serotonin as well as mapping and 3D reconstruction of serotonergic somata. In the red channel, 3D reconstructions of neuropils as well as its volumetric data was extracted. To achieve that, only the left side of the brain was scanned due to time constrains as well as to the range limitation of the microscope to frame the entire brain. High-resolution scans were taken with the same parameters of the overview scan's channels (Table XX), except for small changes to increase image resolution and optical scanning time, e.g. we used a 25x multi-immersion objective (LD LCI Plan-Apochromat, Zeiss, Germany) with a numerical aperture of 0.8, set to oil immersion. The data depth was set to 16 bits, frame size to 2048 x 2048, and

scan zoom at 0.5. Since our aim is quantification of three-dimension structures, and the smallest unit of a three-dimensional object is a voxel made up of pixels, from now on we will refer to “voxel brightness” instead of “pixel brightness”.

Table 04. Scanning parameters of overview scans and high-resolution scans

	Overview scans	**High-resolution scans**
Scaling X	1.25 µm	0.25 µm
Scaling Y	1.25 µm	0.25 µm
Scaling Z	4.75 µm	1.22 µm
Size X	1280.35 µm	512.14 µm
Size Y	1280.35 µm	512.14 µm
Size Z	346.73 µm	499.66 µm
Scan zoom	0.5	0.5
Pixel time	1.58 µs	0.79 µs
Avagare	1	1
Detector Gain Ch 1	596	530
Detector Gain Ch 2	477	450
Amplifier Gain Ch 1	1	1
Amplifier Gain Ch 2	1	1
Amplifer Offset Ch 1	1427	1470
Amplifer Offset Ch 2	1477	1530
Pinhole Ch 1	33 µm	33 µm
Pinhole Ch 2	33 µm	33 µm
Filter Ch 1	SP 555	SP 555
Filter Ch 2	LP 560	LP 560
Wavelength Ch 1	555 nm 0.2%	555 nm 0.2%
Wavelength Ch 2	488 nm 0.4 %	488 nm 0.2 %

Data Analysis

The data was processed and analysed using Amira 6.0.1 (Termo Fisher, USA) and R (3.3.3) in R Studio (R Development Core, 2014), following the methodology proposed by Kümmerlen (2017).

The stacks of high-resolution scans were imported to Amira. When a given stack was too big to be loaded by the software, we subset it using the software Zen (Zeiss, Germany),

that preserves the original bit depth of optical slices. For geometric segmentation of regions of interest (ROI), we worked with the Segmentation menu in Amira. For neuropils, segmentation was performed under the red channel (specific for neuropils). Neuropils were manually outlined, once at a time, with the brush tool every five slices from the first anterior slice to the last posterior slice within the neuropil. This means that neuropils that contains up to 250 optical slices, such as the pedunculus, outlining was repeated up to 50 times per brain. We then applied the function *interpolate*, under the selection panel, to mark all voxels within the ROI of non-outlined slices between the marked ones. After all voxels have been selected, we assigned them to a specific material to be used as reference later on. This procedure allows marking all voxels within a given neuropil, which further allows 3D reconstruction as well as further data extraction.

To segment roundish structures such as somata, a different approach took place. Under the green channel (specific for serotonin), the contrast and brightness of the stack were adjusted to a better visualisation of serotonergic somata. When we localised the first soma from anterior to posterior direction within the brain, we placed in the centre of the cell an intersection between the x,y-plane, which allows to project that region to other planes of the 3D structure, the x,z-plane and the y,z-plane. With the brush tool, we outlined the entire soma and a little bit of background around it in two random planes. We then applied the *wrap function* under the selection menu to mark the pixels of the third non-outline plane, creating thus an outlined three dimension structure. After all voxels have been selected, we assigned them to a specific material to be used as reference later on. This procedure was repeated until all somata were segmented from anterior to posterior direction within the brain.

After all ROI were segmented and assigned, the brightness values of their voxels were exported with the histogram function. This function computes a histogram of the data set with the count of voxels plotted against their corresponding brightness values (Kümmerlen, 2017).

For both somata and neuropils, the brightness intensity range of voxels was set from 0 to 4095, which are further divided into 273 bins for better visualisation of plots. This means that each bin contains 15 brightness values, and all 273 bins covers the 4096 possible brightness values. In addition to the histograms of brightness values, the *material statistics* were also exported. The material statistics contain, among other things, the absolute number of all voxels in one material, the absolute volume of the material, the lowest and the brightest value and their coordinates (Kümmerlen, 2017). The exported data sets were then processed with R studio.

RESULTS

This is the first work, to the best of our knowledge, to use immunostaining intensity as a standard for inferring semi-quantification of serotonin across individuals of different body sizes. Serotonergic immunostaining intensities as a standard for semi-quantification of serotonin was first developed by Kümmerlen (2017) and Cremer (2017), when they found that quantification of brightness intensity of voxels imaged under serotonin immunoreactivity in honeybee workers of different ages and developmental stages shown similar patterns of amount of serotonin than those observed through High Performance Liquid Chromatography (HPLC) (Fuchs et al, 1989; Taylor et al, 1992), which is the standard method for assessing the amount of serotonin in the brain.

The distribution and immunostaining intensity of serotonergic neurons

Somata were organised in two "clusters"; the *optic lobe cluster*, varying between 0 to 19 somata, which project their axons to the optic lobes, and the *posterior protocerebral cluster*, varying from 4 to 13 somata (Table 05). We defined as cluster a group of cells that were conservatively dispersed in a delimited region across brains. A close up look at the neurites in the antennal lobes revealed fine projections surrounding glomeruli. Two additional somata with no cluster formation were found, one close to the anterior lobe of the pedunculus and another one lying at the superior portion of the antennal lobe (Figure 10-11). Due to the difficulty on following fine fibre structures within the brain, at the resolution the scans were taken, we were not able to identify where these somata project their axons to. In the optic lobe cluster, we found a greater number of somata in major workers (16 ± 3.0), median workers (12 ± 4.0), soldiers (7.2 ± 4.8), and minor workers (3.8 ± 3.2). In the protocerebrum cluster, we found a greater number of somata in median workers (9.6 ± 2.3), soldier (7.2 ± 2.8), minor workers (7 ± 4.7), and major workers (6 ± 1). A clear and statistically significant difference

on brightness intensity of somata across different size workers were found (optic lobe somata, GLM, DF = 190, $p < 0.0001$; protocerebrum cluster, GLM, DF = 146, $p < 0.0001$), wherein brains of minor workers and soldiers, which are located at the both extremes of the range of body size, generally showing weaker stained cells when compared to media and major workers (Figure 14-15). In the optic lobes, higher immunostaining intensities were found respectively in majors, medians, soldiers, and minors. On the other hand, in the protocerebrum cluster, we found higher immunostaining intensities respectively in medians, soldiers, minors, and majors.

Immunostaining intensity and volume of selected neuropil compartments

Serotonergic immunoreactivity was detected in the four neuropils that we were able to mark using synapsin antibody (Figures 16-19). Significant differences in the intensity of serotonergic imunostaining were found in two compartments of optic lobes (medulla, GLM, DF =3, $p = 0.043$; lamina, GLM, DF = 3, $p < 0.0001$), and in all four compartments of central complex (Upper part of central complex, GLM, DF =3, $p < 0.0001$; Lower part of central complex, GLM, DF =3, $p < 0.0001$; Protocerebral bridge, GLM, DF = 3, $p < 0.0001$; Noduli, GLM, DF = 3, $p = 0.01243$). Most of our comparisons between body size and volume of neuropils showed a strong strength of association between the two variables, however, the compartments of optic lobes showed the strongest comparisons (figure 22). On the other hand, compartments of central complex showed the weakest strength of association between body size and volume of compartment (figure 23).

Table 05. Number of somata per cluster across subcastes. OL Cluster: optic lobe cluster; PT Cluster: posterior part of protocerebrum cluster**;** SD: standard deviation.

	OL CLUSTER			PT CLUSTER		
	Total	Mean	SD	Total	Mean	SD
MINOR	19	3.8	3.2	35	7	4.7
MEDIAN	60	12	4	48	9.6	2.3
MAJOR	80	16	3.0	30	6	1
SOLDIER	36	7.2	4.8	36	7.2	2.8

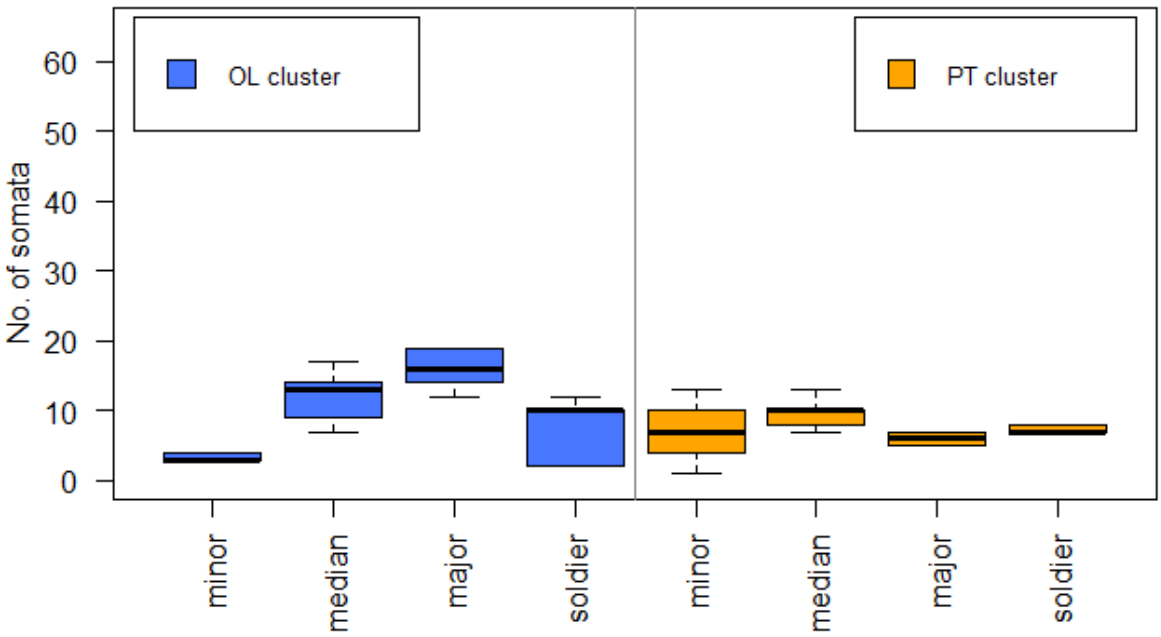

Figure 9 - Number of somata cluster across subcastes. OL, optic lobe cluster; PT, posterior part of protocerebrum cluster

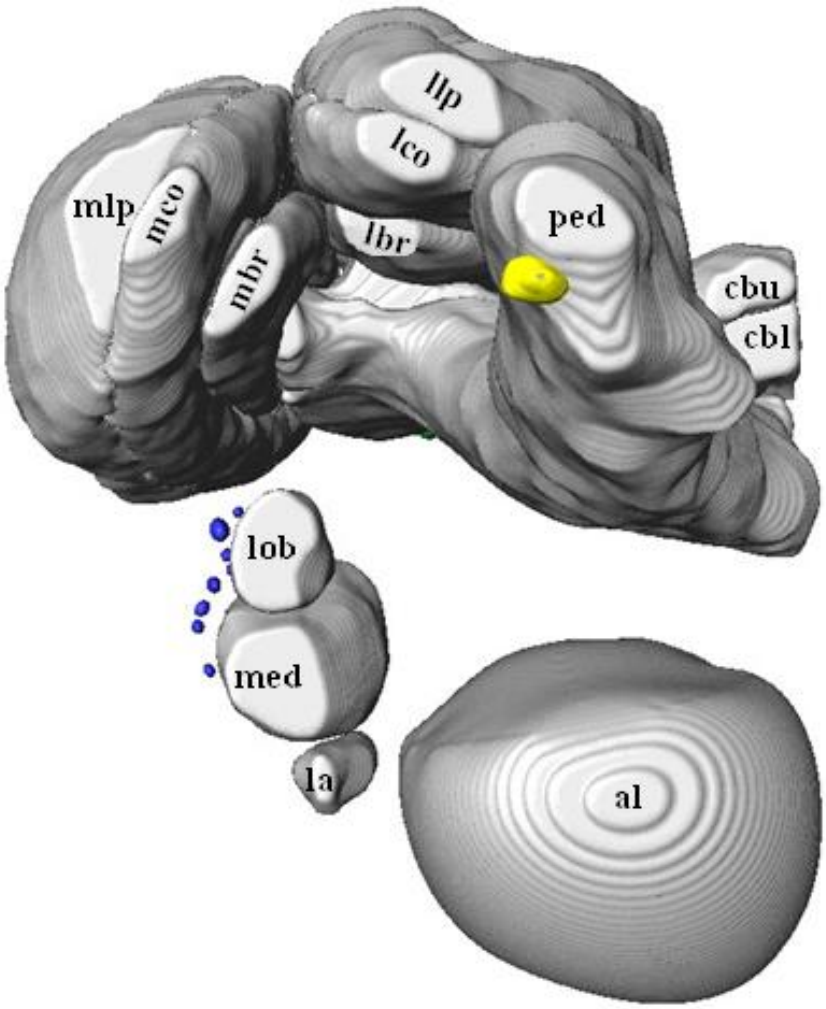

Figure 10. Anterior view of a 3D reconstruction of the serotonergic somata on the left side of an ant brain, *Atta vollenweideri*. Blue, serotonergic somata cluster near the optic lobe; yellow, serotonergic soma near the pedunculus. Neuropils in shades of grey: al (antennal lobe), la (lamina), med (medulla), lob (lobula), mbr (medial basal ring), mco (medial collar), mlp (medial lip), lbr (lateralx basal ring), lco (lateral collar), llp (lateral lip), cbu (upper part of the central body), cbl (lower part of the central body), ptb (protocerebral bridge).

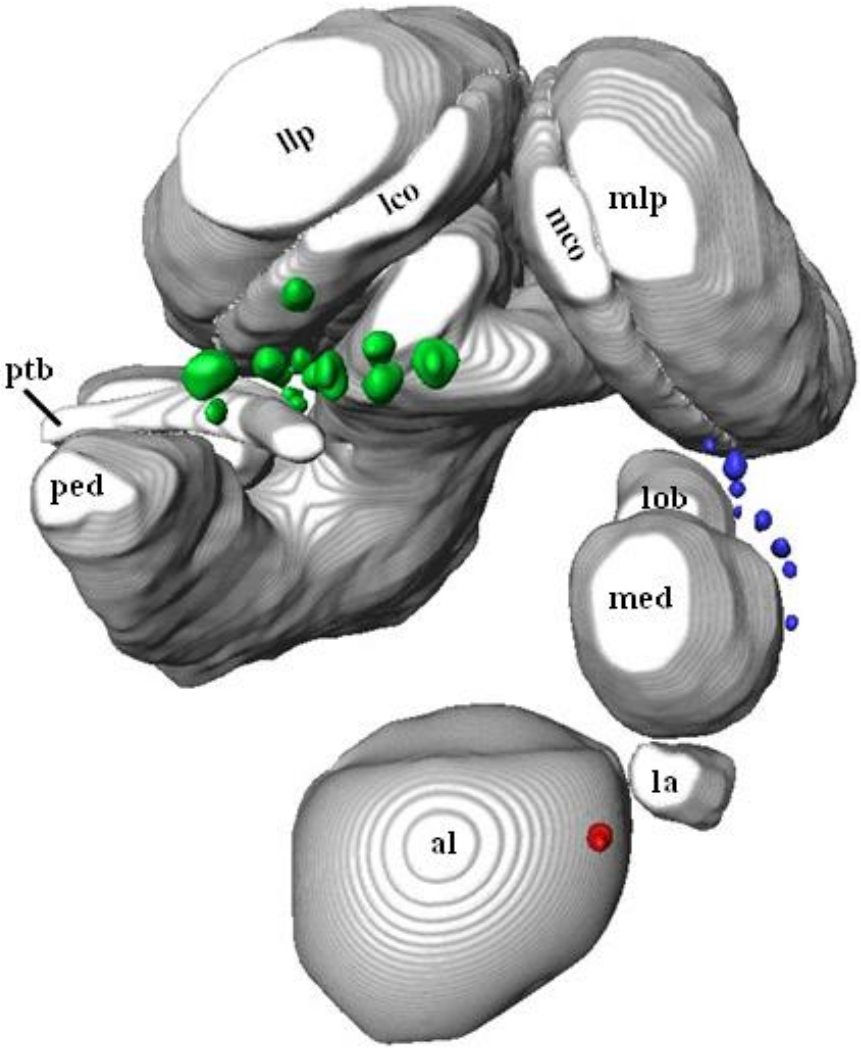

Figure 11. Posterior view of a 3D reconstruction of the serotonergic somata on the left side of an ant brain, *Atta vollenweideri*. Blue, serotonergic somata cluster near the optic lobe; Green, somata cluster located at the posterior part of the protocerebrum (not shown); red, serotonergic soma near the left antennal lobe. Neuropils in shades of grey: al (antennal lobe), la (lamina), med (medulla), lob (lobula), mbr (medial basal ring), mco (medial collar), mlp (medial lip), lbr (lateralx basal ring), lco (lateral collar), llp (lateral lip), cbu (upper part of the central body), cbl (lower part of the central body), ptb (protocerebral bridge).

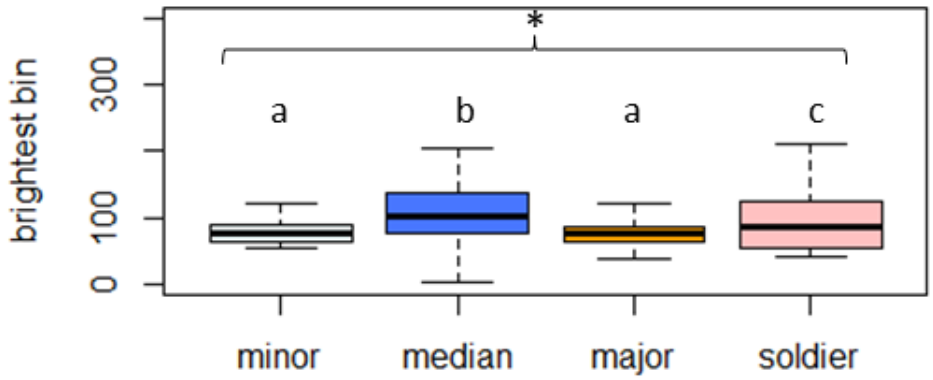

Figure 12. Immunostaining intensity against serotonin of neurons located near the protocerebrum (PT cluster). Groups with the same letter are not statistically significant (Tukey test, $p < 0.0001$)

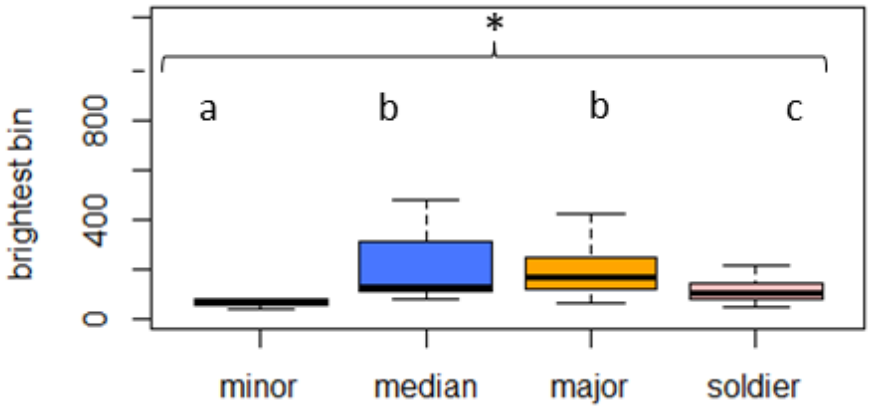

Figure 13. Immunostaining intensity against serotonin of neurons located near the optic lobe (OL cluster). Groups with the same letter are not statistically significant (Tukey test, $p < 0.0001$)

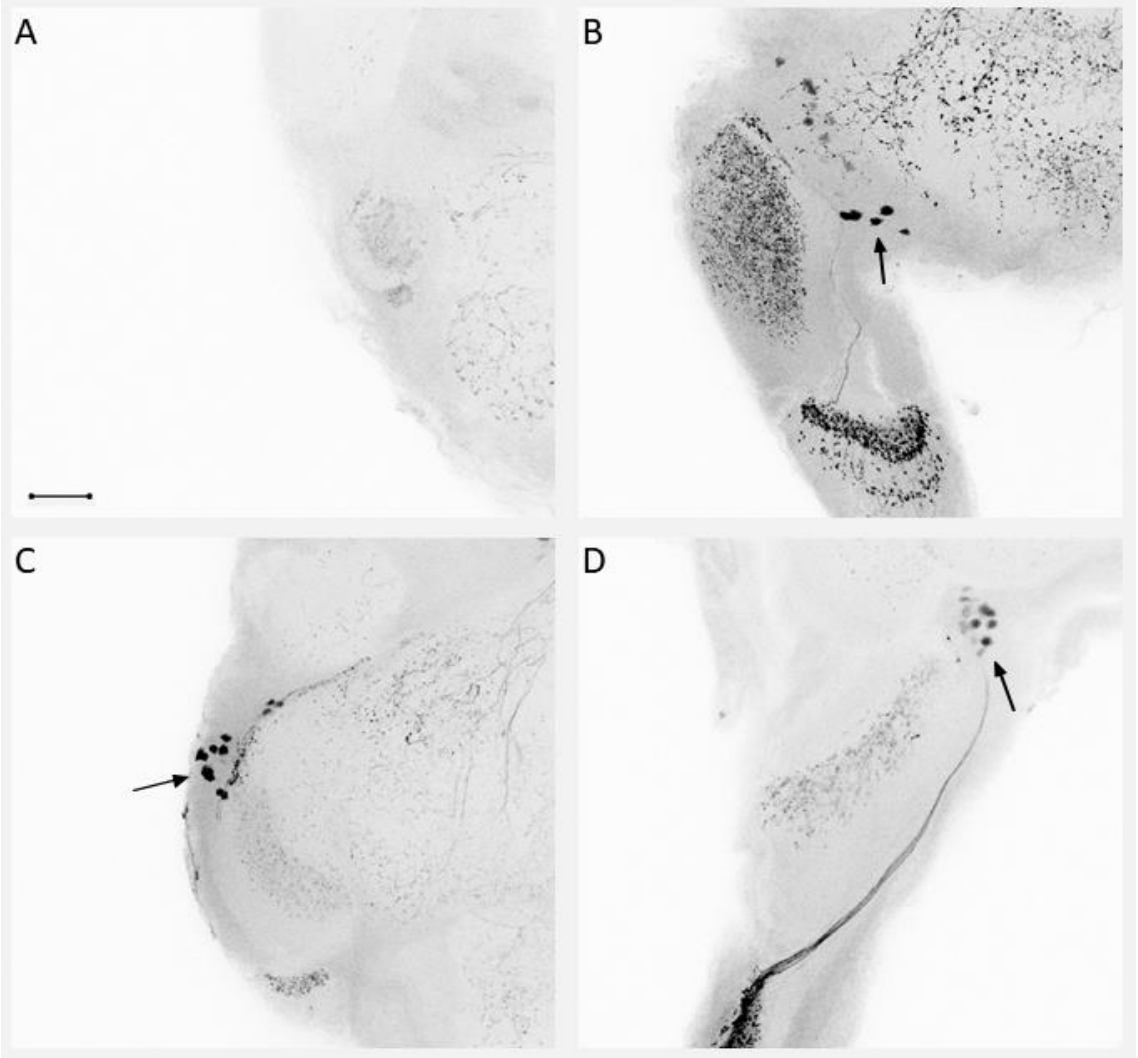

Figure 14. Projection view focused on the somata cluster near the optic lobe (arrows) of four worker's brains of the ant A. vollenweideri. (a) brain of a minor worker (HW: 0.56 mm), (b) brain of a media worker (HW: 1.3 mm), (c) brain of a major worker (HW: 2.7 mm), and (d) brain of a soldier (HW: 3.84 mm). Scale bar (a) indicates 100 μm.

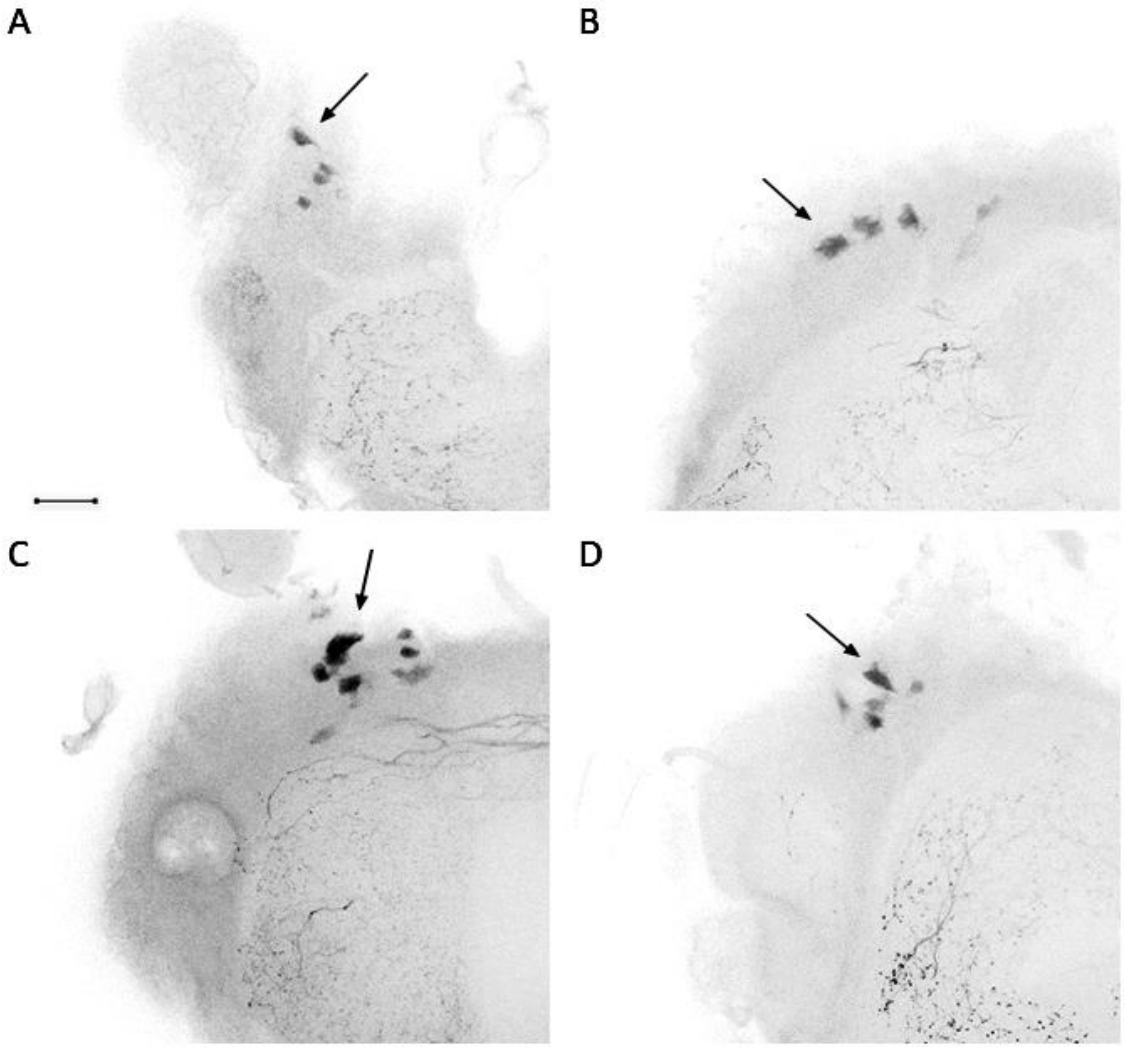

Figure 15. Projection view focused on the somata cluster (arrows) located at the posterior part of the protocerebrum of four worker's brains of the ant *A. vollenweideri.* (a) brain of a minor worker (HW: 0.56 mm), (b) brain of a media worker (HW: 1.3 mm), (c) brain of a major worker (HW: 2.7 mm), and (d) brain of a soldier (HW: 3.84 mm). Scale bar (a) indicates 100 μm.

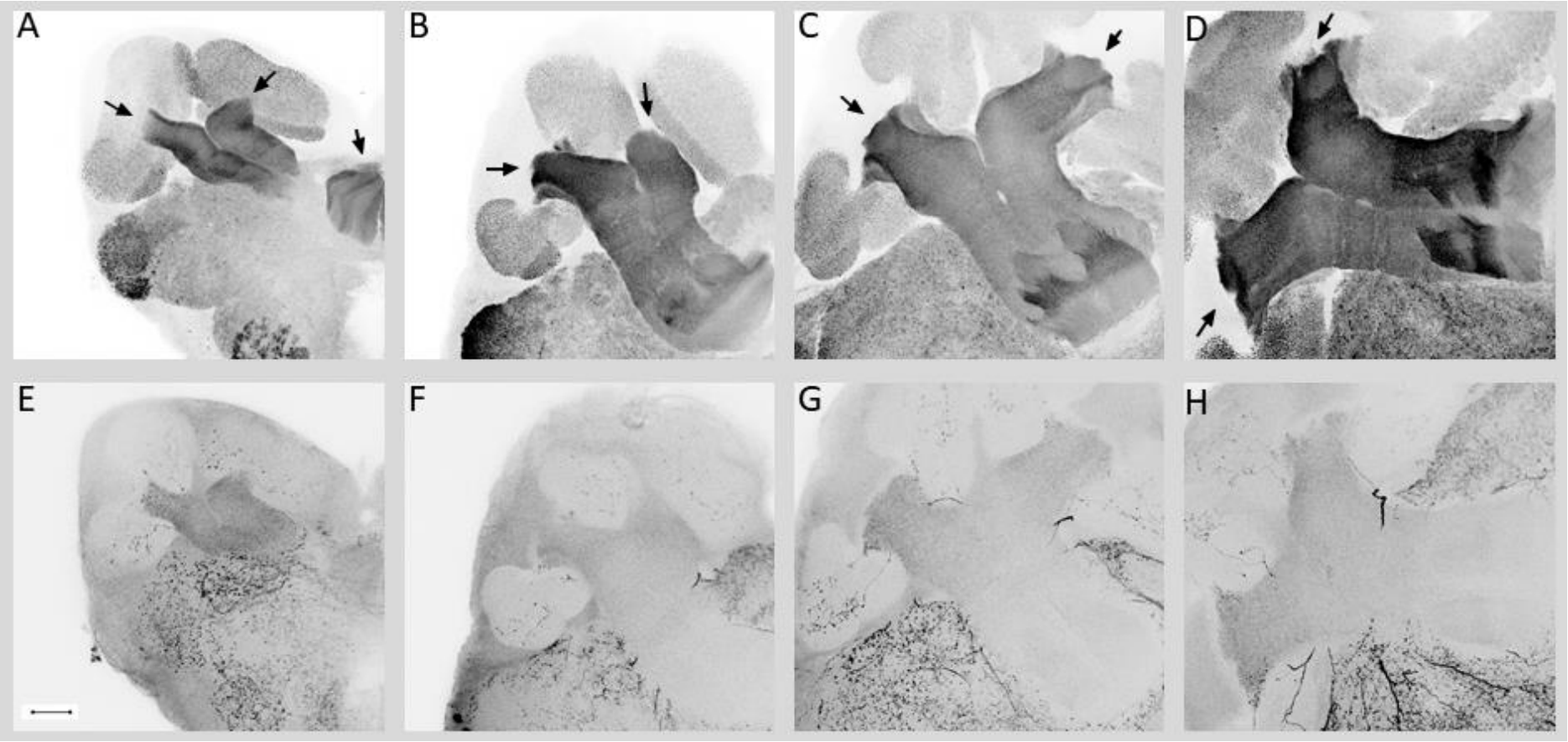

Figure 16. Anterior projection view focused on the left Pedunculus (arrows) of four brains. The darkest areas/structures within the brains show the spots with higher immunostaining intensity. The figures in the upper panel (**a-d**) show the synapsin immunostaining which allows visualisation of neuropils. The figures in the lower panel (**e-h**) show immunostaining against serotonin (5-HT). (**a,e**) brain of a minor worker (HW: 0.56 mm), (**b,f**) brain of a media worker (HW: 1.3 mm), (**C,G**) brain of a major worker (HW: 2.7 mm), and (**d,h**) brain of a soldier (HW: 3.84 mm). Scale bar (**e**) indicates 100 μm.

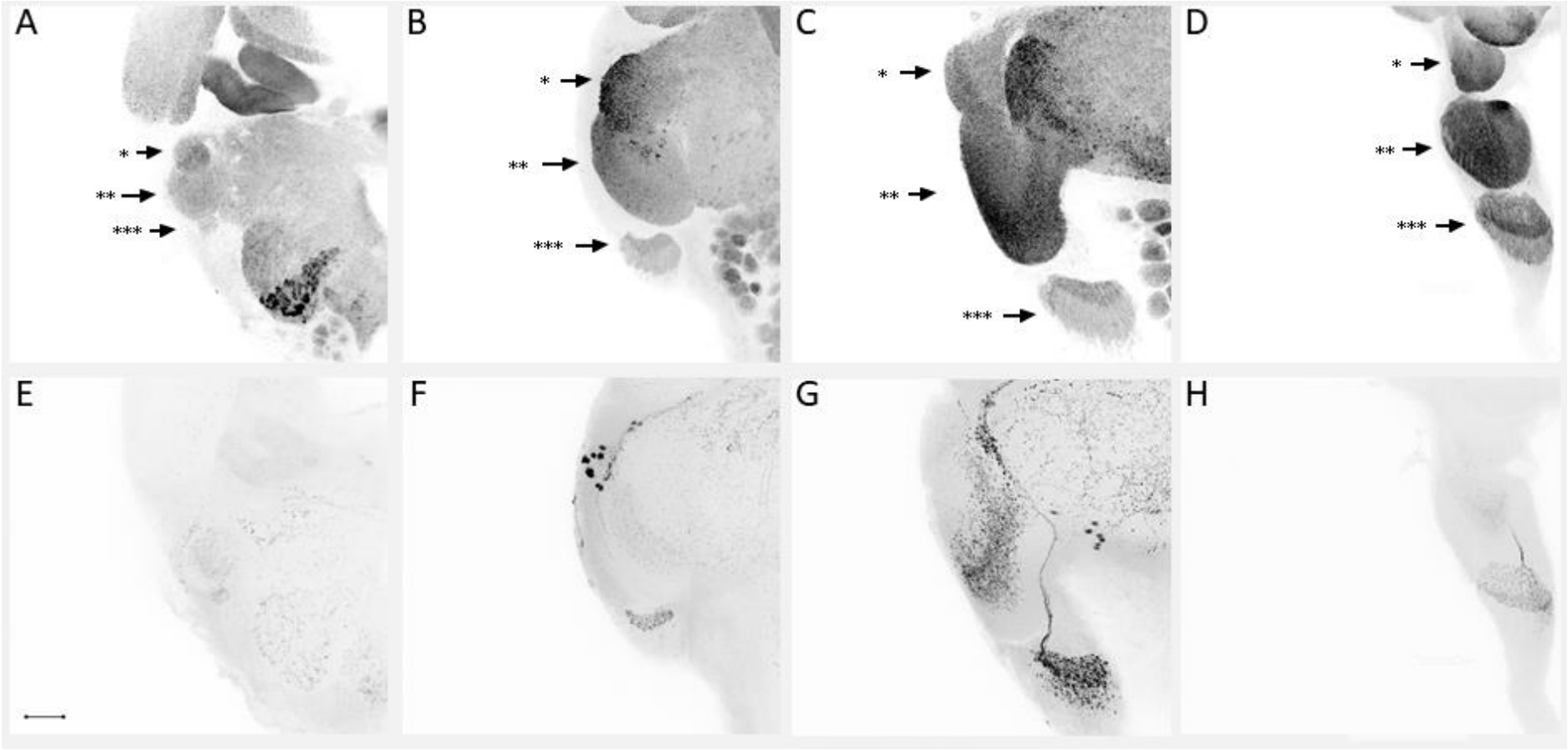

Figure 17. Anterior projection view focused on the left optic lobe of four brains. Arrows indicate the three compartments of the optic lobe (*, lobula; **, medulla; ***, lamina). The darkest areas/structures within the brains show the spots with higher immunostaining intensity. The figures in the upper panel (**a-d**) show synapsin immunostaining which allows visualisation of neuropils. The figures in the lower panel (**e-h**) show immunostaining against serotonin (5-HT). (**a,e**) brain of a minor worker (HW: 0.56 mm), (**B,F**) brain of a media worker (HW: 1.3 mm), (**c,g**) brain of a major worker (HW: 2.7 mm), and (**d,h**) brain of a soldier (HW: 3.84 mm). Scale bar (**e**) indicates 100 μm.

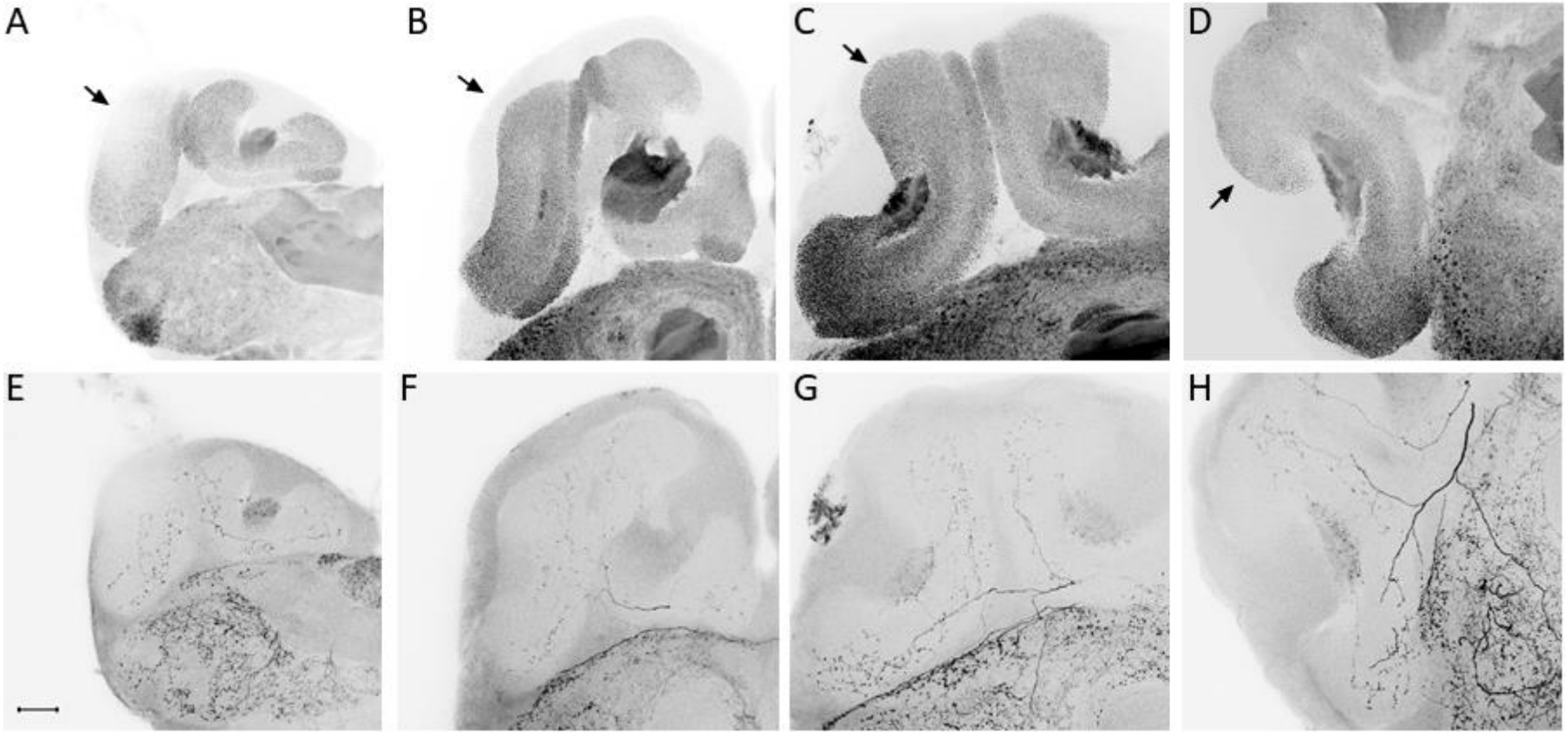

Figure 18. Anterior projection view focused on the left calyx (arrows) of four brains. The darkest areas/structures within the brains show the spots with higher immunostaining intensity. The figures in the upper panel (**a-d**) show the synapsin immunostaining which allows visualisation of neuropils. The figures in the lower panel (**e-h**) show immunostaining against serotonin (5-HT). (**a,e**) brain of a minor worker (HW: 0.56 mm), (**b,f**) brain of a media worker (HW: 1.3 mm), (**C,G**) brain of a major worker (HW: 2.7 mm), and (**d,h**) brain of a soldier (HW: 3.84 mm). Scale bar (**e**) indicates 100 μm.

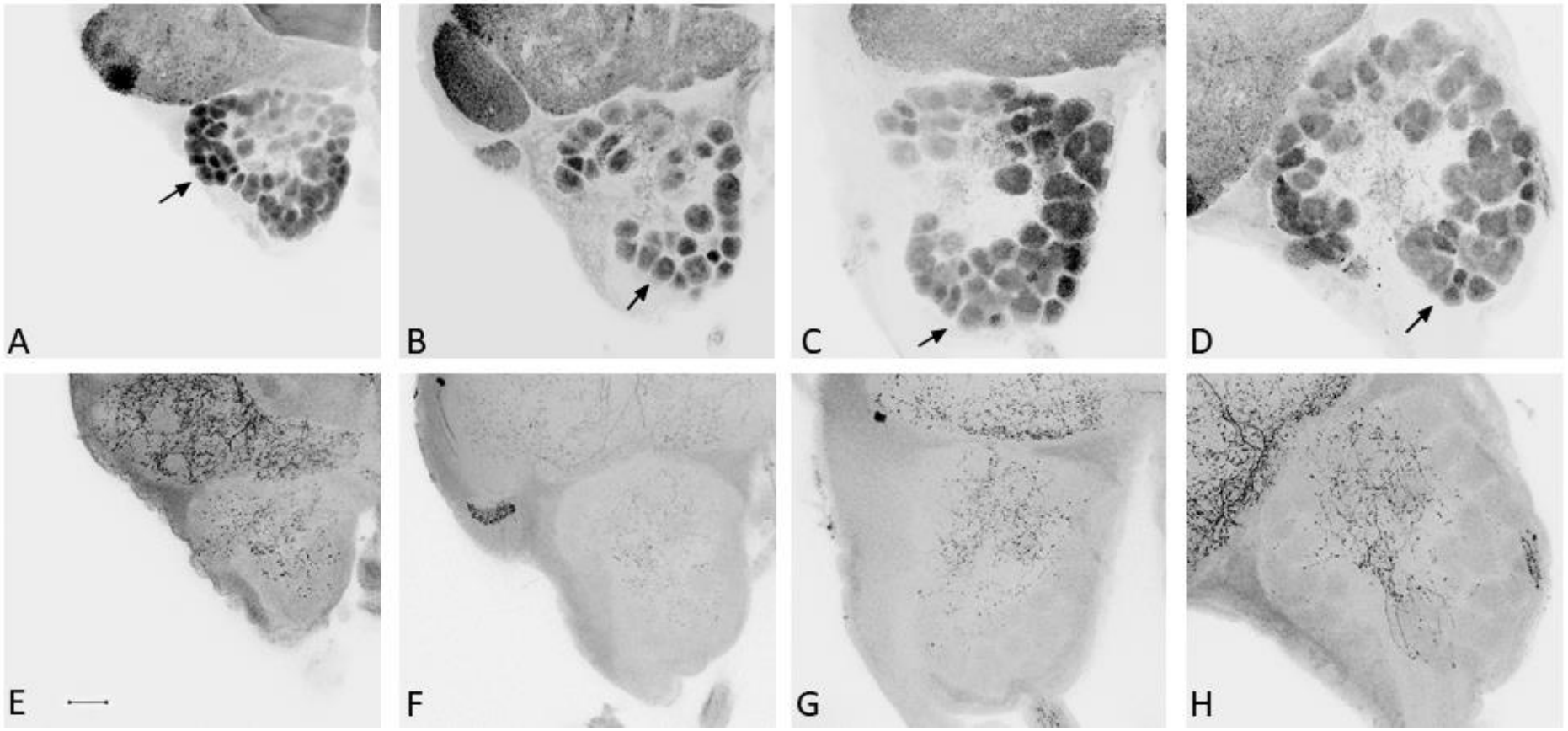

Figure 19. Anterior projection view focused on the left antennal lobe of four brains. The arrow in each figure indicates a single glomerulus. A set of glomeruli represents the functional unit of the antennal lobe. The darkest areas/structures within the brains show the spots with higher immunostaining intensity. The figures in the upper panel (**a-d**) show the synapsin immunostaining which allows visualisation of neuropils. The figures in the lower panel (**e-h**) show immunostaining against serotonin (5-HT). (**a,e**) brain of a minor worker (HW: 0.56 mm), (**b,f**) brain of a media worker (HW: 1.3 mm), (**C,G**) brain of a major worker (HW: 2.7 mm), and (**d,h**) brain of a soldier (HW: 3.84 mm). Scale bar (**e**) indicates 100 μm.

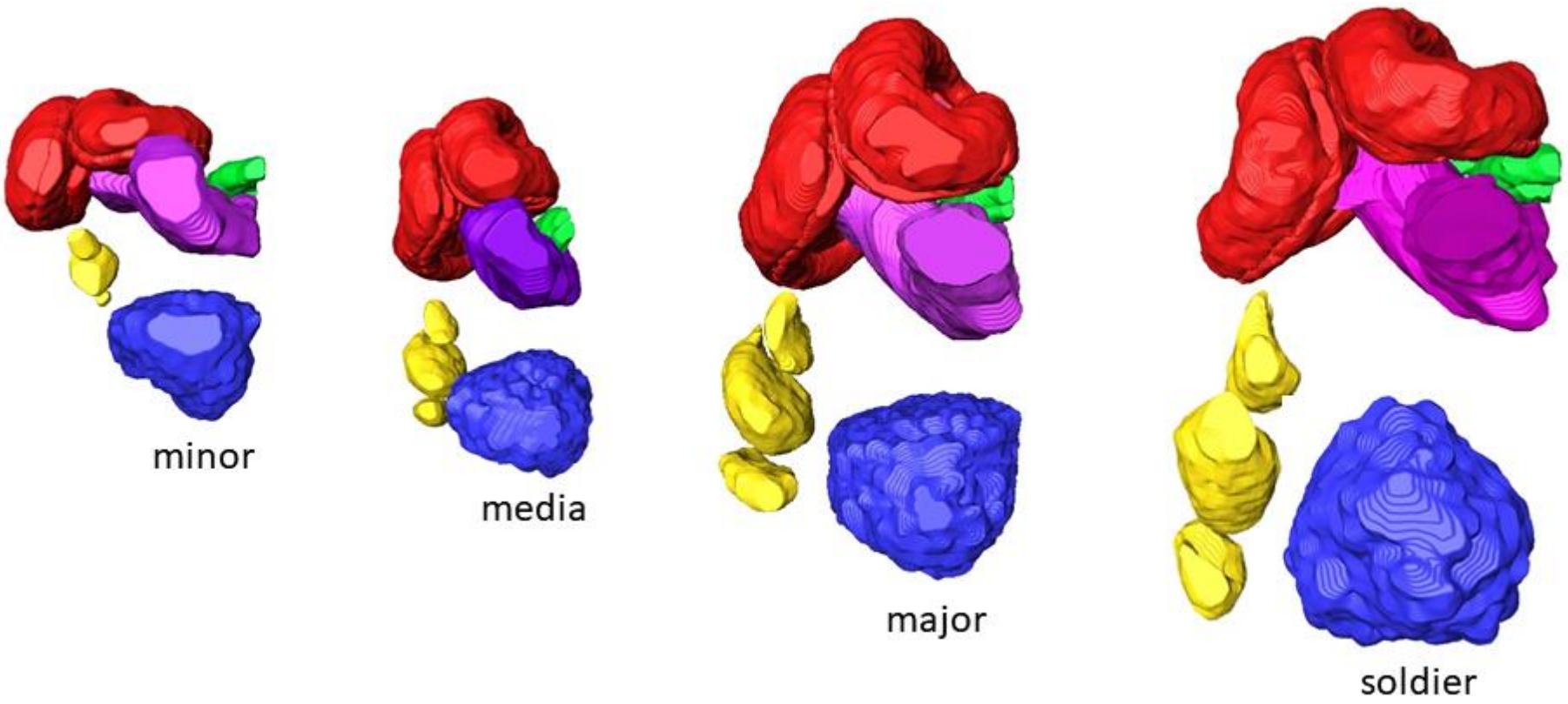

Figure 20. Three dimension reconstructions of the left side of four worker's brains of the ant *A. vollenweideri*. Blue, antennal lobe; yellow, optic lobe; magenta, pedunculus; green, central complex; red, calyces

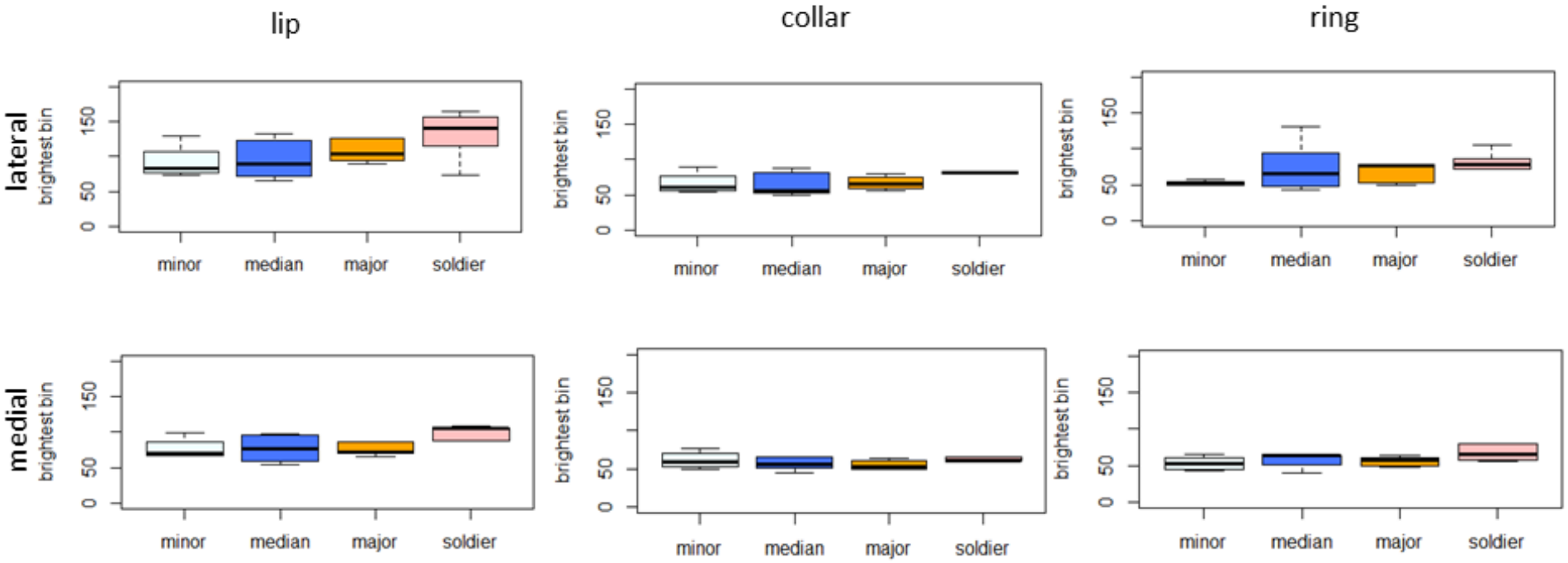

Figure 21. Boxplots showing distribution of 1000 brightest voxels of three compartments of calyx: lip, collar, and ring of A. vollenweideri worker subcastes.

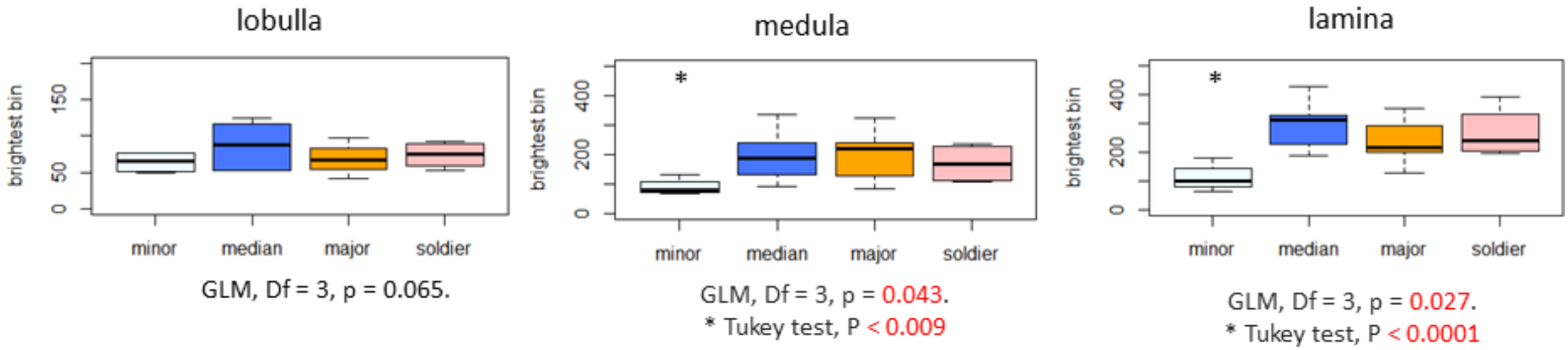

Figure 22. Boxplots showing distribution of 1000 brightest voxels of three compartments of optic lobe: lobulla, medula, and lamina of *A. vollenweideri* worker subcastes.

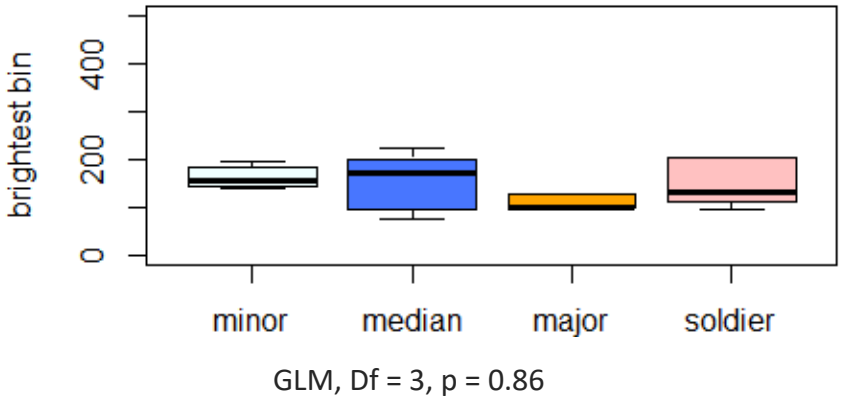

Figure X. Boxplots showing distribution of 1000 brightest voxels of the left antennal lobes of *A. vollenweideri* worker subcastes.

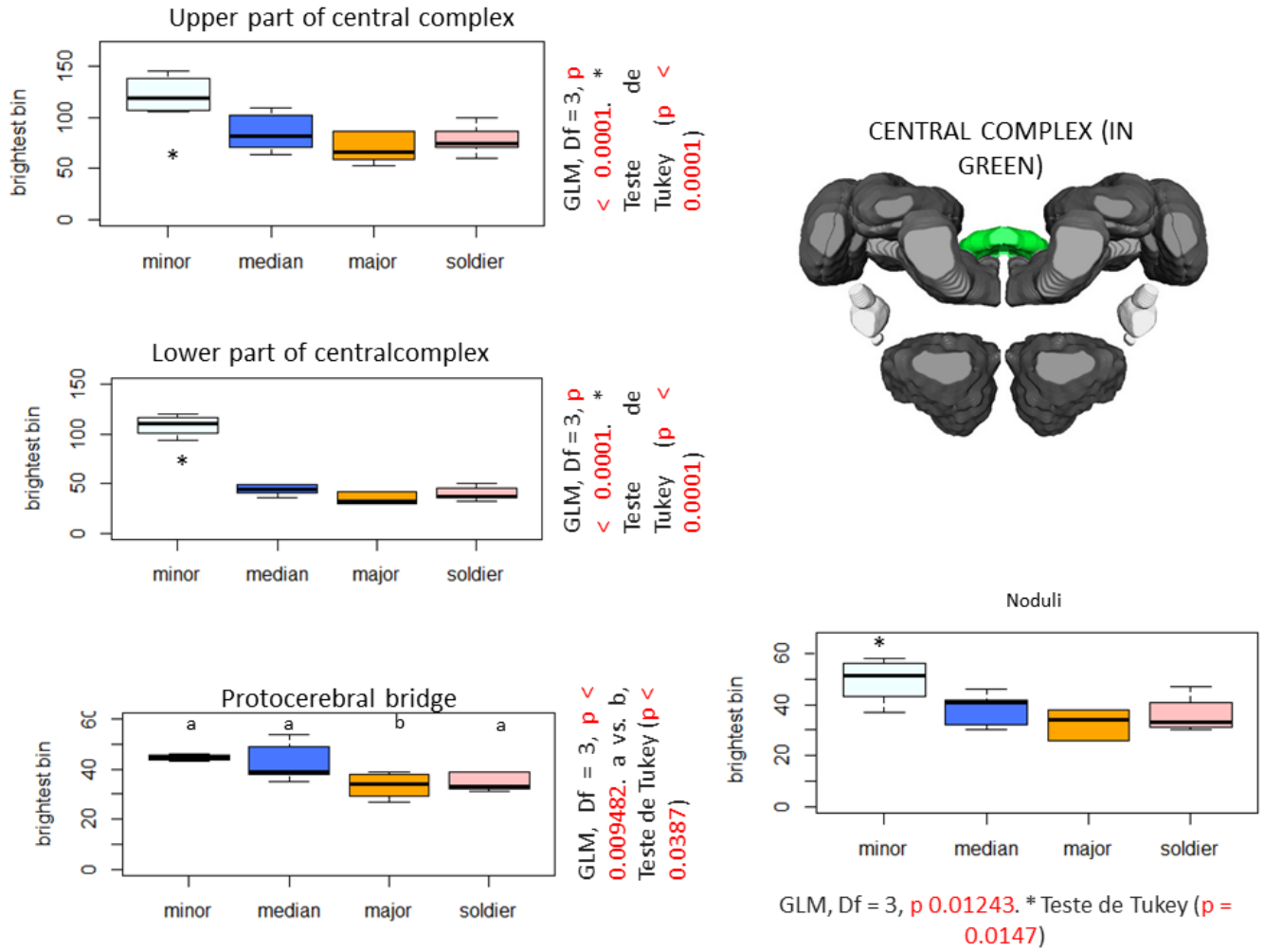

Figure 23. Boxplots showing distribution of 1000 brightest voxels of four compartments of central complex of *A. vollenweideri* worker subcastes.

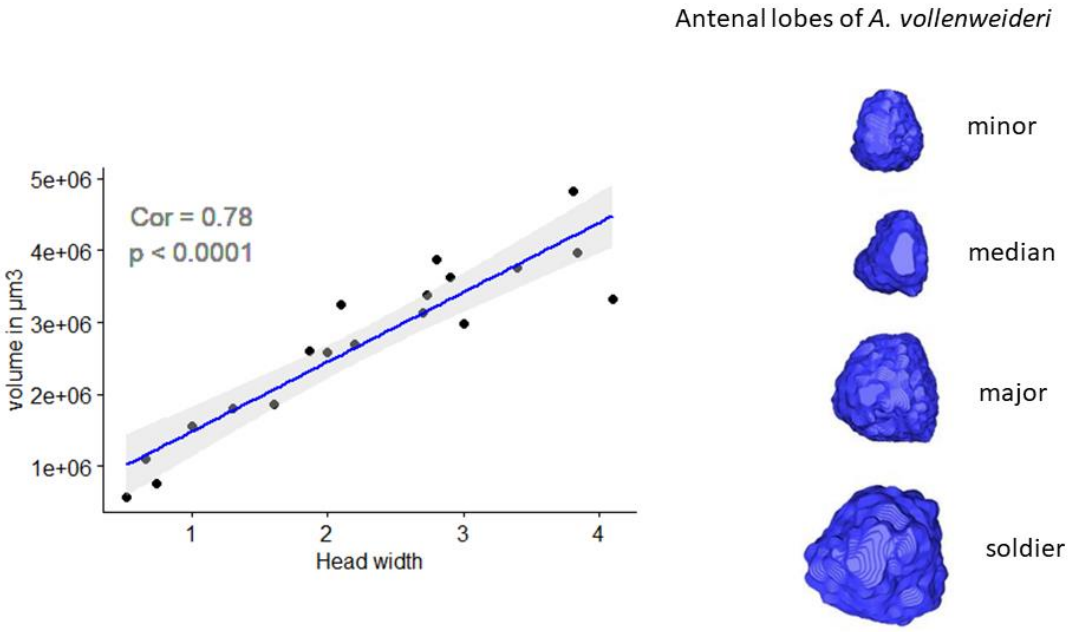

Figure 24. Kendall rank correlation coefficient between antennal lobe volume and body size of ants

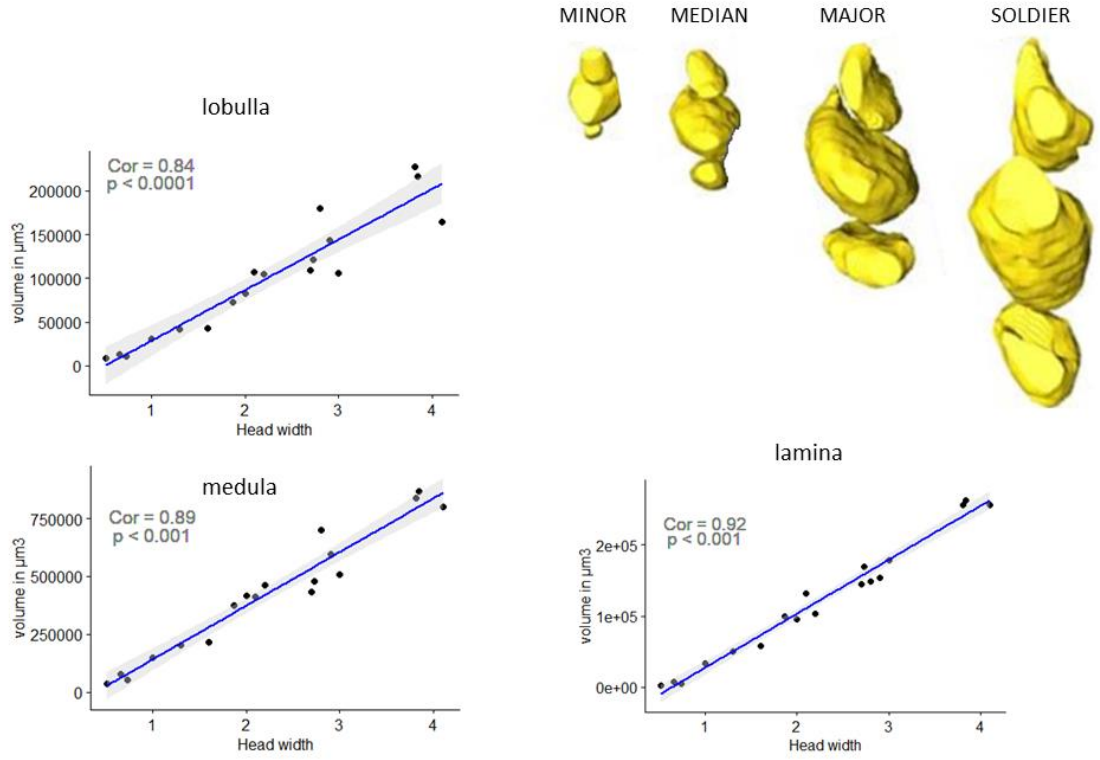

Figure 25. Kendall rank correlation coefficient between antennal lobe volume and body size of ants

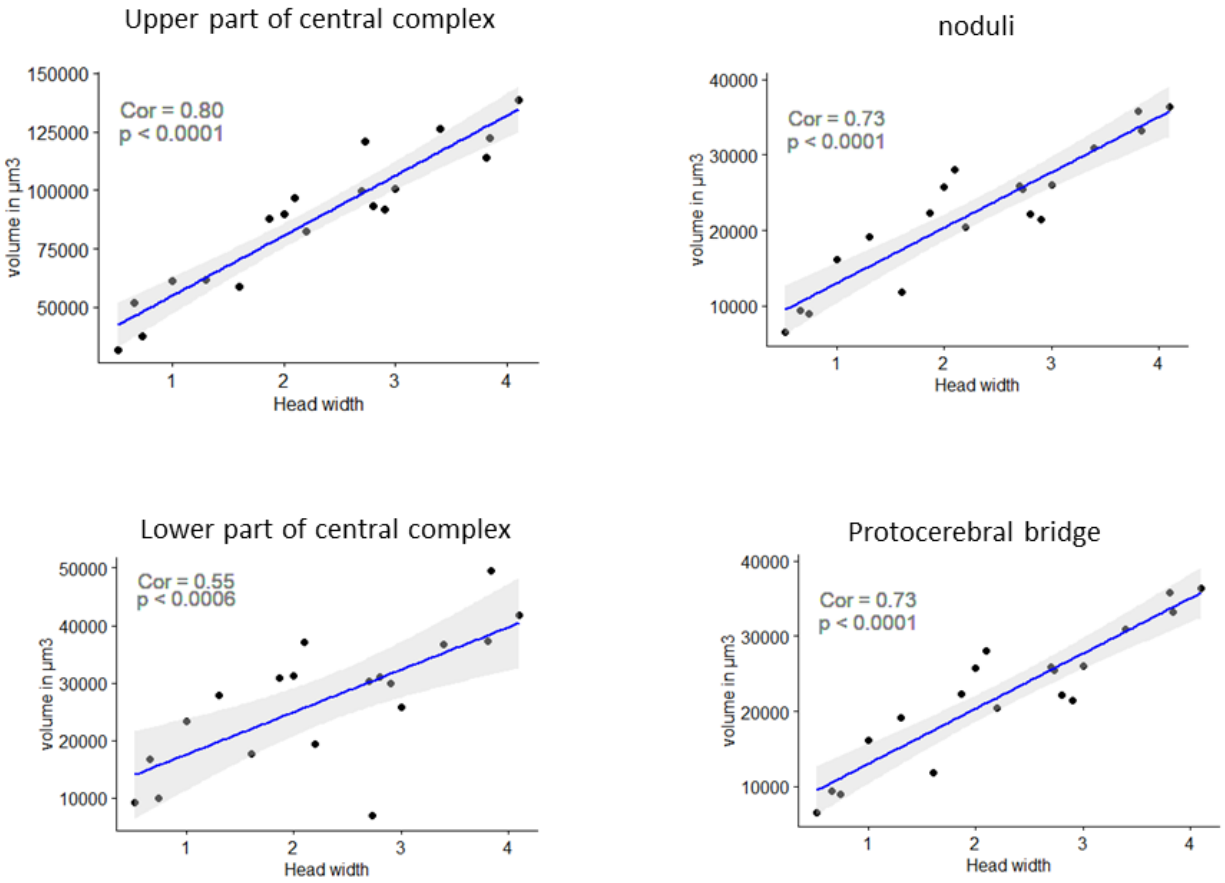

Figure 26. Kendall rank correlation coefficient between volume of four compartments of left side of central complex and body size of *A. vollenweideri*

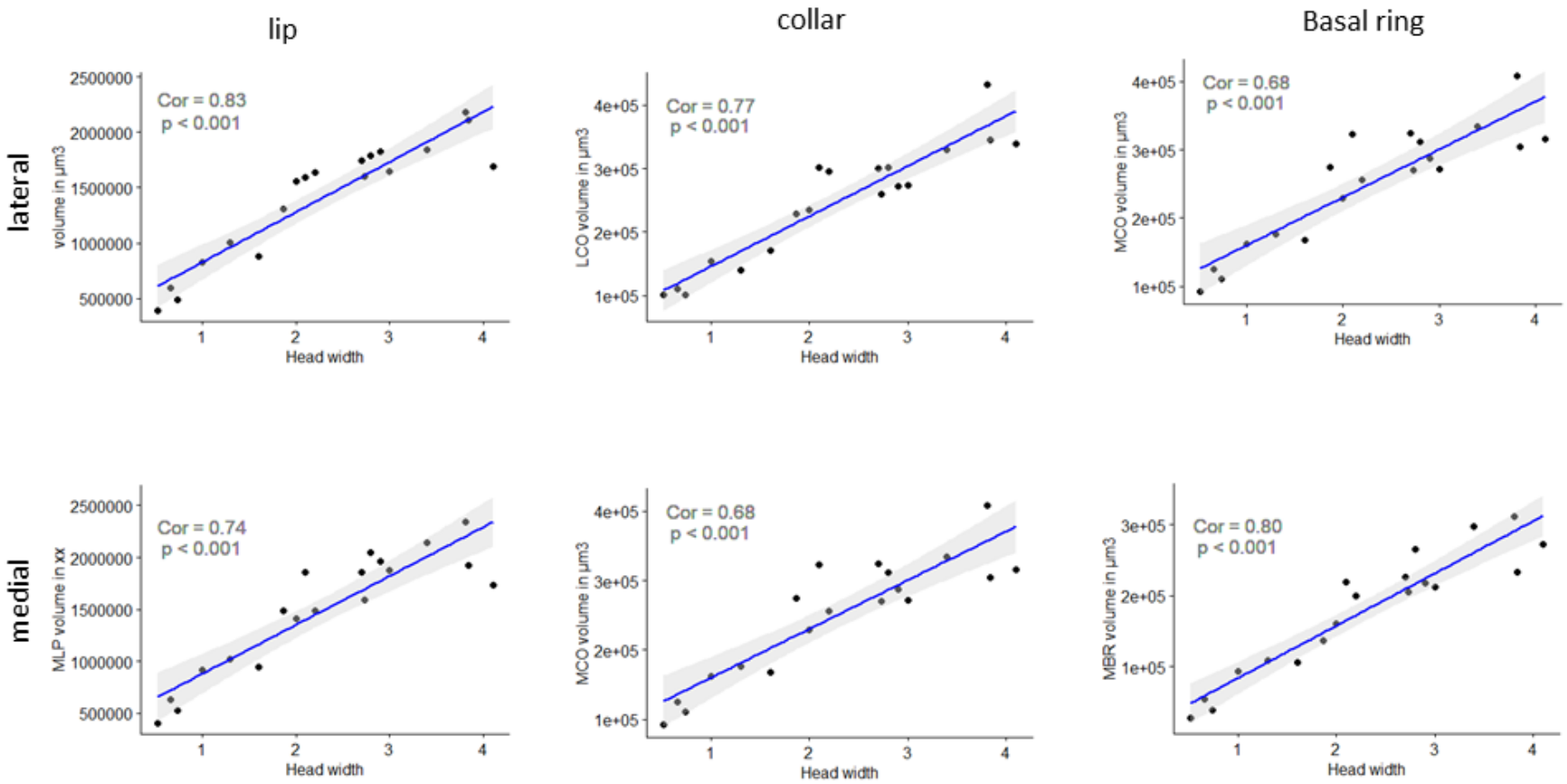

Figure 27. Kendall rank correlation coefficient between volume of three compartments (lip, collar and basal ring) of left calyx and body size of *A. vollenweideri*

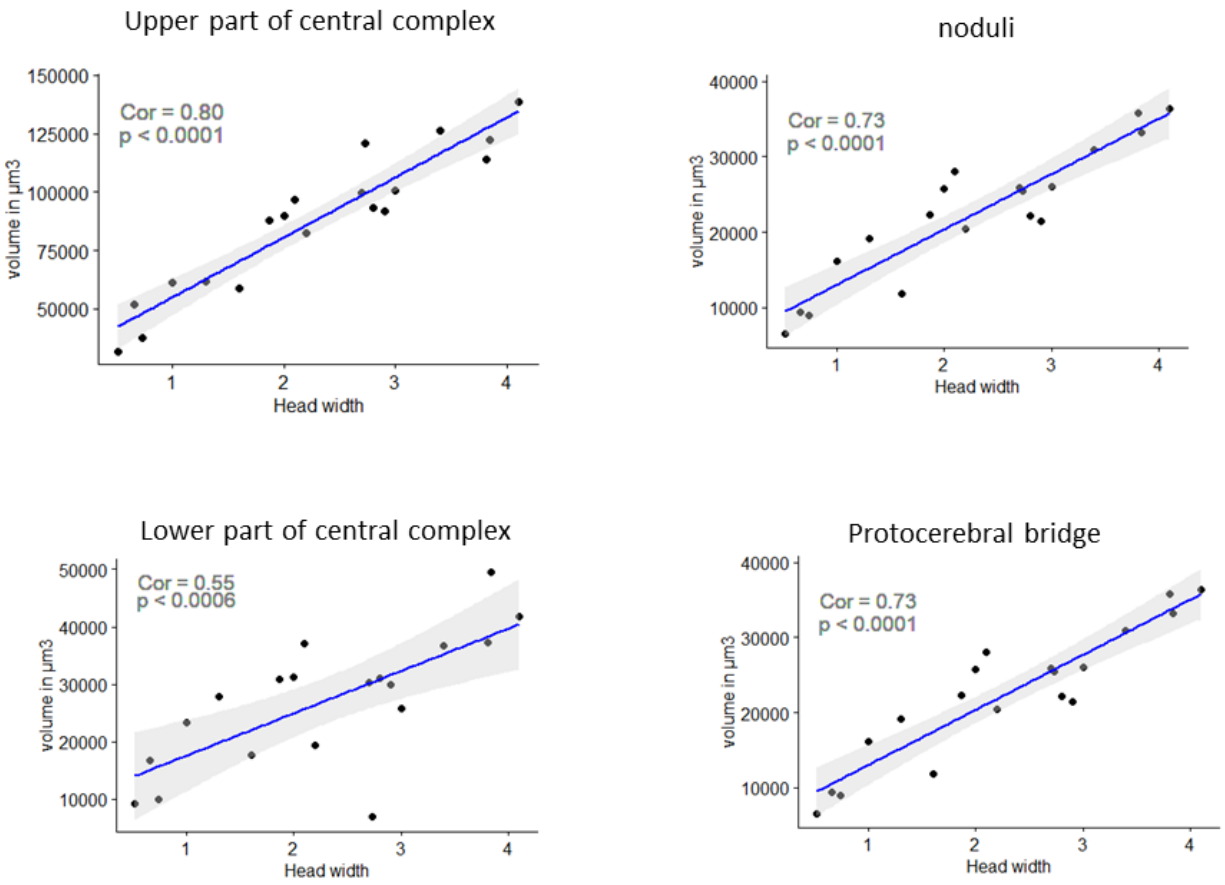

Figure 28. Kendall rank correlation coefficient between volume of four compartments of left side of central complex and body size of *A. vollenweideri*

DISCUSSION

One of the reasons that motivated us to develop this protocol was to assess whether differences in immunostaining intensities across individuals of different body sizes would be an artefact created due to difficulty of antibodies to reach inner tissues of larger individuals that consequently have larger brains. Such artefacts appeared not to be true since neuropils of the smallest ants were not the strongest stained ones. Serotonergic immunostaining intensities as a standard for semi-quantification of serotonin was first developed by Kümmerlen (2017) and Cremer (2017), when they found that quantification of brightness intensity of voxels imaged under serotonin immunoreactivity in honeybee workers of different ages and developmental stages shown similar patterns of amount of serotonin than those observed through High Performance Liquid Chromatography (HPLC) (Fuchs et al, 1989; Taylor et al, 1992).

In fact, the absolute number of brightest voxels increases as body size increases until the limit between major workers and soldiers, with the latter showing a decrease in the number of brightest voxels of selected neuropils. We found a tendency of increasing the number of serotonergic somata as body size increases until the limit between major workers and soldiers, with the latter showing a decrease in the number of serotonergic somata. Furthermore, a clear difference on brightness intensity of somata across different size workers is visible, with brains of minor workers and soldiers, which are located at the both extremes of the range of body size, showing weaker stained cells when compared to median and major workers. Such differences in brightness of somata and neuropils might not be due to difficulty of antibodies to reach inner structures in larger brains, since somata clusters are superficially located within the brain. No significant differences were found between body size and serotonin levels in the mushroom body and antennal lobe compartments of *A. vollenweideri*. However, we found significant differences in optical lobe and central complex compartments. In the optic lobe, such differences were characterised by an increase in serotonin level in

workers with intermediate body size (median and major workers). In central complex compartments, we found a negative relationship between ant body size and serotonin levels. Such negative relationship might be due to difficulty of antibodies to reach inner regions of the brain, once the central complex is the innermost neuropil of *A. vollenweideri*.

According to the traditional literature (Wilson, 1980), median workers show broader behavioural repertoires, performing tasks both inside and outside the nest. Major workers are traditionally referred as to “foragers”, since they are generally involved in activities related to search and collecting of food resources (Wilson, 1980). Thus, these two groups of workers may rely on visual cues while searching for food, defending territory, or returning home. Serotonin in optic lobes is known to entrain circadian rhythms to environmental cues (Chen et al., 1999; Tomioka, 1999). The increases in serotonergic activity in foragers may create or strengthen the coupling of circadian rhythms to day and night (Seid et al, 2008). Similar patterns of increase of 5-HT levels in ant foragers were reported in the fire ant *Pheidole dentata* (Seid et al, 2008). Thus, the differences obtained from our protocol reflect behavioural specialisation of workers, which makes us confortable to present it as suitable for assessing differences in immunostaining intensity in brain regions of different size workers which consequently have different size neural tissues.

CONCLUSIONS

No significant differences were found between body size and serotonin levels in the mushroom body and antennal lobe compartments of *A. vollenweideri*. However, we found significant differences in optical lobe and central complex compartments. In the optic lobe, such differences were characterised by an increase in serotonin level in workers with intermediate body size (median and major workers). In central complex compartments, we found a negative relationship between ant body size and serotonin levels. Such negative relationship might be due to difficulty of antibodies to reach inner regions of the brain, once the central complex is the innermost neuropil of *A. vollenweideri*. Intermediate workers also presented a greater variability in the number of serotonergic neurons in the optic lobe cluster as well as higher levels of serotonin in the somata of this cluster were found. According to the traditional literature, median workers show broader behavioural repertoires, performing tasks both inside and outside the nest, whilst major workers are traditionally considered as "foragers". Both groups may rely on visual cues while searching for food or returning home. Thus, the differences obtained from our protocol reflect behavioural specialisation of workers, which makes us confortable to present our protocol as suitable for assessing differences in immunostaining intensity in brain regions of individuals with different body sizes and consequently different sizes of neural tissues.

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