LARA OLIVEIRA CLEMENTE

TERMITE INQUILINISM: PROXIMATE MECHANISMS MEDIATING COEXISTENCE

Thesis submitted to the Ecology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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To nature, to its diversity and power.

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Nature is queer.

ABSTRACT

Clemente, Lara Oliveira, D.Sc., Universidade Federal de Viçosa, October, 2023. **Termite Inquilinism: proximate mechanisms mediating coexistence**. Adviser: Og Francisco Fonseca de Souza. Co-advisers: Judith Korb and Rebecca Rosengaus

Symbiosis, the long-term intimate relationship between different organisms, is ubiquitous. Understanding the proximate mechanisms that enable symbiosis can advance understanding of the evolutionary history of species and origins of biodiversity. The symbiotic system composed of the host termite species *Constrictotermes spp* and the inquiline termite species *Inquilinitermes spp* is a good working model for such studies, since several patterns and mechanisms mediating this cohabitation are already known. However, the invasion stage is less understood and interesting questions remain open. In this work we investigated the context of host nest invasion by inquilines from two approaches: mechanical and physiological. The results show correlations between the ontogeny of hosts and the positioning of their nests with the presence of inquilines, reinforcing the diversity of mechanisms mediating this symbiotic relationship.

Keywords: Symbiosis, Isoptera, Physiology, Social Insects, Cohabitation

RESUMO

Clemente, Lara Oliveira, D.Sc., Universidade Federal de Viçosa, outubro de 2023. **Inquilinismo em cupins: mecanismos proximais mediando coexistência**. Orientador: Og Francisco Fonseca de Souza. Coorientadores: Judith Korb e Rebecca Rosengaus

A simbiose, a relação íntima de longo prazo entre organismos diferentes, é onipresente. A compreensão dos mecanismos próximos que permitem a simbiose pode promover a compreensão da história evolutiva das espécies e das origens da biodiversidade. O sistema simbiótico composto pelas espécies do cupim hospedeiro *Constrictotermes spp* e pela espécie de cupins inquilinos *Inquilinitermes spp* é um bom modelo de trabalho para tais estudos, já que diversos padrões e mecanismos mediando essa coabitação já são conhecidos. Contudo, a etapa de invasão é menos compreendida e perguntas interessantes permanecem em aberto. Nesse trabalho investigamos o contexto da invasão do ninho hospedeiros pelos inquilinos sob duas abordagens: mecânica e fisiológica. Os resultados mostram correlações entre a ontogenia dos hospedeiros, local de construção do ninho e a presença de inquilinos, reforçando a diversidade de mecanismos mediando essa relação simbiótica.

Palavras-chave: Simbiose, Isoptera, Fisiologia, Insetos Sociais, Coabitação

Contents

Chapter 1

Nest arboreality impairs inquilinism in termites

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Contents

1 Highlights

- Inquiline termites are more likely to invade arboreal host termitaria positioned closer to the soil surface.
- We provide quantitative evidence for previous hypothetical claims that nest arboreality provide adaptive advantages by reducing predation and parasitism.
- The likelihood of an arboreal host termite nest to house inquiline termites can be estimated by an equation including nest distance to the soil surface, nest volume, and host colony ontogeny.
- The use of such an equation will allow sampling optimization, speeding research efforts
- Sampling optimization, in its turn, will avoid unnecessary removal and destruction of nests that are unlikely to attend to experimental aims.

Abstract

Termite nests (i.e., termitaria) provide habitat for hundreds of cohabitants, which use a variety of strategies to gain entry therein. Likewise, it seems expectable from nest builders to adopt strategies to limit guest entrance in their nest. Nesting in sites that minimize guest access to the nest, such as trees, can be one of such strategies to impair invaders. *Constrictotermes* spp. termites build arboreal nests attached to tree trunks at varying distances from the soil surface. These host nests frequently house a single colony of obligatory inquiline termites (either *Inquilinitermes microcerus* or *Inquilinitermes fur*) and no other inquiline species. Here we show that inquiline load in arboreal termitaria is negatively affected by the nest distance to the soil surface. This helps to explain the low diversity of inquilines normally found in *Constrictotermes* spp. nests as opposed to other termite hosts such as *Cornitermes cumulans* which build nests on the soil surface (so called "epigeous termitaria"). Additionally, it provides sampling optimization, speeding up research efforts at the same time that it avoids unnecessary disturbance or destruction of nests that are unlikely to attend experimental needs.

Resumo

Os ninhos construídos por cupins fornecem habitat para centenas de coabitantes, que usam uma variedade de estratégias para invadir o ninho. Da mesma forma, é provável que espécies de cupins hospedeiros, construtores de ninhos, adotem estratégias para limitar a entrada de coabitantes em seu ninho. Ninho em locais que minimizem o acesso de coabitantes, como árvores, pode ser uma das estratégias para controlar a presença de coabitantes. Os cupins da espécie *Constrictotermes spp.* constroem ninhos arbóreos presos a troncos de árvores a distâncias variadas da superfície do solo. Esses ninhos frequentemente abrigam uma única colônia de cupins inquilinos obrigatórios das espécies (*Inquilinitermes microcerus* ou *Inquilinitermes fur*), raramente abrigando outras espécies. Aqui mostramos que presence de inquilinos em ninhos arbóreos é afetada negativamente pela distância entre o ninho e a superfície do solo. Isto ajuda a explicar o baixa diversidade de inquilinos normalmente encontrados em ninhos de *Constrictotermes spp.*, em oposição a outros hospedeiros, como *Cornitermes cumulans* que constroem ninhos na superfície do solo. Além disso, esse resulta possibilita otimizar amostragens em campo, acelerando os esforços de investigação ao mesmo tempo que evita perturbações desnecessárias ou destruição de ninhos que provavelmente não atenderão às necessidades experimentais.

2 Introduction

The conspicuous ecological importance of social insects nests (Wilson et al., 1990; Noirot and Darlington, 2000; Korb, 2011) is partially due to their potential to serve as an habitat for many other species (Redford, 1984). Hundreds of organisms, specially arthropods, can be found cohabiting a termite nest along with its builder (Costa et al., 2009; Parmentier, 2021). Frequently, among such cohabitants we can find even other termite species, the so-called "inquilines" (Mathews, 1977; Costa et al., 2009, 2019).

Host termite species, *i.e.*, those who build the nest, are expected to employ strategies that filter and impair nest invasion by cohabitants. After all, even when invaders coexist peacefully with their host (*e.g.*, Florencio et al., 2013; Hugo et al., 2020) they impose, at least, the cost of occupying a space which was not originally built for them. Among the strategies employed by the host termite to impair nest invasion, thicker nest walls (Mathews, 1977) and active defense by soldiers (DeSouza et al., 2016) are normally considered effective impairments for cohabitants. Additionally, morphological adaptations such as falciform mandibles, are also known to help host workers to contend inquilines (Clemente et al., 2021).

Selecting a strategic nest location may also help to reduce risks in general and cohabitant load in particular (Hughes et al., 2008; McGlynn, 2012; Grüter et al., 2018). After all, building nests in the arboreal stratum (i.e., arboreality) is often thought of as a defense mechanism because it reduces contact to terrestrial predators, pathogens and parasites, not only in social insects (Boomsma et al., 2005) but in chimpanzees as well (Pruetz et al., 2008).

As with several animal species, termites are also able to build arboreal nests. Termite nest arboreality, however, has not yet been considered as an impairment for inquilinism in termites, despite all evidence. After all, being termites themselves, inquiline founding reproductives are unable to perform directional flights (Costa-Leonardo and Haifig, 2013). and that may impose severe costs to their search for a nesting place. That is, providing that an undirectional flight will drastically reduce the chances of stumbling upon a host nest, inquiline reproductives will most likely land on the soil. From there, differently from other termite founding pairs, inquilines will have to find the right tree bearing the rigth host nest where to found their own colony. That is to say, arboreality implies that inquiline termites must search for the host nest in a three-dimensional space scale, instead of the two-dimensional soil-surface based scale. In itself, this is already a costly scenario, that may aggravate the higher the host nest is posed in the tree.

Interesting, this seems to be evidenced by the fact that permanent epigeal nests, such as those built by *Cornitermes* termites, can house up to twenty six inquilines species simultaneously (Costa et al., 2009). Opposingly, arboreal nests, such as those build by *Constrictotermes*, usually host only one inquiline termite species. In extreme cases, such as with damaged/unhealthy nests, one might find up to three inquilines species simultaneously cohabiting in those nests (Costa et al., 2019). Similarly, *Microcerotermes exiguus* nests can house only up to three inquiline species (Campbell et al. (2016), Appendix S1).

Here we test the hypothesis that arboreality of termite nests is protective against the invasion of these nest by other termite species. That is to say, we hypothesize that arboreality impairs inquilinism in termite nests. This hypothesis would be *falsified* if host arboreal nests positioned closer to the soil *would not* house inquilines more frequently than nests positioned farther from the soil surface. On the contrary, our hypothesis would find support if the likelihood of housing inquilines would decrease as the distance between an arboreal nest and soil surface increases. Here we bring evidence corroborating this hypothesis in the inquilinism between the host *C. cyphergaster* and the obligatory inquilines *Inquilinitermes microcerus*.

3 Materials and Methods

3.1 Terms definition

- Colony is a group of closely-related termite individuals that live together in the same nest.
- Nest, or termitarium (plural: termitaria), is the physical structure a colony builds to house itself.
- Builder species are those termites who build and keep the nest they live in, without the help of their cohabitants (but see Miura and Matsumoto, 1997, for one exception).
- Host is a termite builder species whose nest houses inquilines.
- Inquiline termite species are those who live in a nest built by another termite species.
- Inquilinism is the cohabitation of different termite species in one nest.
- Epigeal nests are termitaria positioned on the soil surface or upon rocks with no significant hypogeal (underground) part.
- Arboreal nests are termitaria attached to tree trunks, positioned at different heights relatively to the soil surface.

3.2 Sampling

The study area was located in the district of São José das Lajes, within the municipality of Cordisburgo (19° 07’ 30” S 44° 19’ 15”), Minas Gerais state, Brazil. The vegetation in the site is Cerrado *stricto sensu* (Brazilian savanna), composed of arboreal and shrub-herbaceous strata. Trees are small to medium heights with irregular branches and thick barks (Graeff, 2015). The soil is classified as dark-red latosol, presenting a rocky/sand appearance. The climate is tropical Aw according to Köppens classification, presenting a dry winter and rainy summer. The mean annual precipitation is 1200mm and the mean temperature is 21.5°C. These nests varied between an oval and spherical form which can be visually split into hemispherical caps and cylinders to facilitate nest volume inference (Cristaldo et al., 2012).

In the study area, *Constrictotermes cyphergaster* can be found living in arboreal nests, attached at different heights on tree trunks and commonly housing colonies of *Inquilinitermes* spp. It is not possible to affirm whether these nests were found and built directly on tree trunks or they were found in soils migrating afterwards to the tree. Epigeal nests of *Constrictotermes cyphergaster*, however, were not seen.

A total of 28 *Constrictotermes cyphergaster* wild nests were collect in February (15 nests), May (8 nests), and July (5 nests) of 2021. For each of these nests we recorded (i) volume, (ii) presence of alates/pre-alates, (iii) presence of inquilines, and (iv) distance between the bottom of the nest and the soil surface. Nest shape varied between an oval and spherical form, which can be visually split into hemispherical caps and stacked cylinders to ease nest volume estimate (following Cristaldo et al., 2012). Nest volume is the sum of the volume of all of its composing nest cylinders and hemispherical caps. Volumes of composing parts of the nest were estimated from measurements of their circumference and height, which were obtained in the field using a measuring tape.

We measured the shortest vertical distance between two reference points: (i) the bottom the nest and (ii) the soil surface right below it. The reference point for the nest was set on the bottommost external surface of the nest itself, disregarding any tunnel-like structures emerging from the nest towards the ground. The reference point in the ground was the soil surface after any plant litter was removed. Later in the lab, collected nests were opened and inspected for inquiline colonies and host alates and pre-alates.

3.3 Modelling

To properly challenge our hypothesis, we built a global model containing not only our variable of interest, but also two covariates which are known to affect inquiline presence in arboreal nests. In doing so, we would allow these covariates to compete for explanatory power with our variable of interest, increasing the chances to falsify our hypothesis. This procedure is in strict accordance with recomendations by Chamberlin (1965) and Popper (1968) regarding tests of hypotheses.

This global model was:

$$log_n(p/q) \sim dist + vol + reprod \tag{1}$$

where $log_n(p/q)$ is the binary response variable informing the presence ($log_n(p/q) = 1$) or absence ($log_n(p/q) = 0$) of inquilines in a given nest. Our variable of interest, $dist$, is a continuous variable informing the distance in metres from the arboreal nest to the soil surface. In its turn, vol is a continuous variable informing the nest volume in litres. Nest volume has been already reported by Cristaldo et al. (2012) to predict the invasion of termitophiles and inquilines in *C. cyphergaster* arboreal nests. As for $reprod$, it is a categorical variable informing the host colony reproductive status, taking the value of $reprod = 1$ when nymphs or alates are present or $reprod = 0$ in their absence. Earlier experimental (Rodrigues et al., 2018) and analytical (Rodrigues et al., 2021) results strongly evidenced the importance of this variable in determining the propensity of arboreal termitaria in housing invaders.

Modeling proceeded by model selection based on Akaike's Information Criterion (AIC) (Burnham and Anderson, 2002). This procedure starts creating all possible models out of all the combinations of the variables in the global model. Then, the AIC of each model is calculated according to the trade-off between the likelihood of the model and the number of parameters the model contains. The model presenting the lowest AIC is assigned as the *best* model and all the others are ranked according to the difference between their own AIC and the AIC of the best model. Models whose AIC differs by 2 or less units from the best model's AIC are considered equally probable, forming a subset of models from which one could derive biological inference. For convenience, this subset can be summarized into a single model built from a weighted average across the set of the equally probable models.

We used this averaged model for graphical purposes (see section 4.1.1 below). Analyses were performed in R (RStudio Team, 2020), using package MuMIn (Barton and Barton, 2015).

4 Results

4.1 AIC

A total of five candidate models were considered equally probable (Table 1). The explanatory variables (pre)alates presence, nest volume and nest-soil distance were included as important predictors of inquilinism occurrence. The variables nest volume ("vol") and host (pre)alates presence ("alates") were positively correlated to the presence of inquilines ("inqc"). Nest-soil distance ("dist") showed a negative correlation to the presence of inquilines ("inqc"). Hence, inquilines are more likely to be found cohabiting with (i) reproductive mature colonies, (ii) in bigger nests and (iii) nests positioned closer to the ground.

4.1.1 AIC table

Table 1: Models with $\Delta \leq 2$ derived from AIC analysis. Models are based on 28 observations. Abbreviations: df = degrees of freedom used by the model, Loglik = log-likelihood, AIC = Akaike information criterion, Δ = difference between the model under concern and the best model, Weight = Akaike weight, that is, the likelihood of that model compared against other models in the set. Global model: Probability of housing inquilines $\sim$ nest volume (vol) + distance between nest and ground (dist) + reprodutive status of the host colony (reprod). Values below each continuous variable inform their coefficient in the model. The + symbol informs the presence of that categorical variable in the model.

	(Intercept)	reprod	dist	vol	df	logLik	AIC	delta	weight
2	-0.69	+			2	-16.5	36.9	0.0	0.50
6	-1.23	+		0.05	3	-16.1	38.1	1.2	0.27
4	-0.38	+	-0.01		3	-16.2	38.4	1.5	0.23

5 Discussion

As predicted by our hypothesis, arboreal nests positioned farther from the soil surface are less likely to house inquilines. We then in position to state that our results support the hypothesis that host arboreality impairs inquilinism in termite nests. Arboreal nest distance from the soil, however, was not the only variable explaining inquiline load in these termitaria. As in previous works (Cristaldo et al., 2012; Rodrigues et al., 2018), the volume of the nest and the reproductive status of its building colony also predicted inquiline load (Table 1, Fig. 1), correlating positively with it. Combined, these results shed intriguing light on the biology of invasion of *C. cyphergaster* nests by *I. microcerus* and, maybe, on termite inquilism in general.

Initially it is worth noting that while host nest arboreality was an impairment to inquilines, enlarged host nests which reached maturity welcomed inquilines in it. In other words, while arboreality seems to add defensive power to the termite host nest, its physical and ontogenetic development may turn the nest more vulnerable to invasions. It is tempting to speculate that these three traits are, in fact, interwined. It is now well known that *C. cyphergaster* starts its nest in the soil, migrating it to trees as the colony develops in size and maturity (Silva et al., 2021). It is also known that inquilines enter these nests *only* after they attain a critical volume (*c.a.* 13 L, according to Cristaldo et al., 2012). Interestingly, at this points, these nests are entering reproduction (Rodrigues et al., 2018) and are arboreal! It seems therefore, that arboreality might have been a result of a selective pressure to counteract inquiline invasion. We might be facing the two elements of an arms race between termites hosts and their inquilines: as these latter find ways to profit from the ontogeny of the host colony, the hosts use nest arboreality to meet these invasions.

Of course, this reasoning depends on inquilines posing costs to the hosts and that is still a wide open research avenue. If they do not pose any cost at all, there will be not reason to avoid them. In the absence of more concrete data, however, we refrain from exploring it any further by now.

One thing is for sure: arboreal termitaria are not typically known for an expressive load of inquilines. This is true not only for the species here in focus (*C. cyphergaster*) but also for others such as *M. exiguus*, according to (Campbell et al., 2016). Opposite examples can be found too: termitaria known to house the longest list of inquilines are precisely the epigeous ones built by *C. cumulans*.

Besides reducing the chance of success of inquilines, the arboreality of the host nest may also confer other advantages to the host colony. As proposed by Boomsma et al. (2005), arboreality may be protective by also reducing the chance of success of other threats from the soil, such as predators, pathogens, and micro-parasites.

The next question which comes to mind would be: how can this be stable? While arboreality seems obviously advantageous for the hosts, can it be advantageous for the inquilines too? It seems that the answer is “yes”. The only inquilines which are found in *C. cyphergaster* nests, in normal situations, are *I. microcerus* and *I. fur*. The only record of other inquilines species in these nests reports it to happen in abnormal situations (Costa et al., 2019). Thus, if arboreality impairs the invasion by inquilines, but *Inquilinitermes* spp., these latter would profit from an environment free of potential competitors. Streching a little bit this argument would lead us to suspect that arboreality can also impair other types of invaders, in addition to inquiline termite species, such as the long array of predators already reported to cohabit termitaria (de Visser et al., 2008). If this is so, *Inquilinitermes* spp. will also profit from an *enemy-free space* in arboreal nests.

It remains to unveil the mechanism behind the impairement posed by arboreality on inquilines. Some hypotheses come to mind, but it is worth noting that these are mere speculations, as the biology of inquilines in general, and *Inquilinitermes* spp. in particular, is still largely unkown. A promissing research route points out that host nest arboreality would impair inquilinism by reducing the number of inquiline reproductives able to reach host nests at all. This would happen due to a series of factors among which one could think of the inherent difficulty to find the right tree with a suitable host nest. Additionally, as compared to other termites, inquiline dispersal reproductives would be longer exposed to predation during swarm (Lucena et al., 2022) due to their need to search in a two-dimensional space. Other termites' searching space is limited to one dimension (horizontal soil surface). Inquilines, on the other hand, must combine the horizontal search on the soil surface with the vertical search upon the tree. We warn that although inquiline termite dispersal biology might hold the answer to which mechanism is behind this pattern, this is a research avenue still to be explored. We urge researchers to focus on the cost of the dispersal of reproductive inquiline termite species, especially regarding energy limitation, predation risks, and eventual arms race with their host.

Finally, we also emphasize that arboreal termitaria position should be incorporated in cohabitation studies in termites along with nest size and colony ontogeny, which are already known to affect inquiline establishment (Cristaldo et al., 2012; DeSouza et al., 2016; Rodrigues et al., 2018, 2021). Accounting for nest vertical position, along with the other covariates here studied, would also allow for sampling optimization, guiding collection efforts towards nests that are likely to attend experimental needs. That is to say, one could combine these three variables to choose the right nest to be inspected, minimizing the inherent insuccess of a random sampling. This will speed up collecting efforts while sparing nests that are unfit for the research aims.

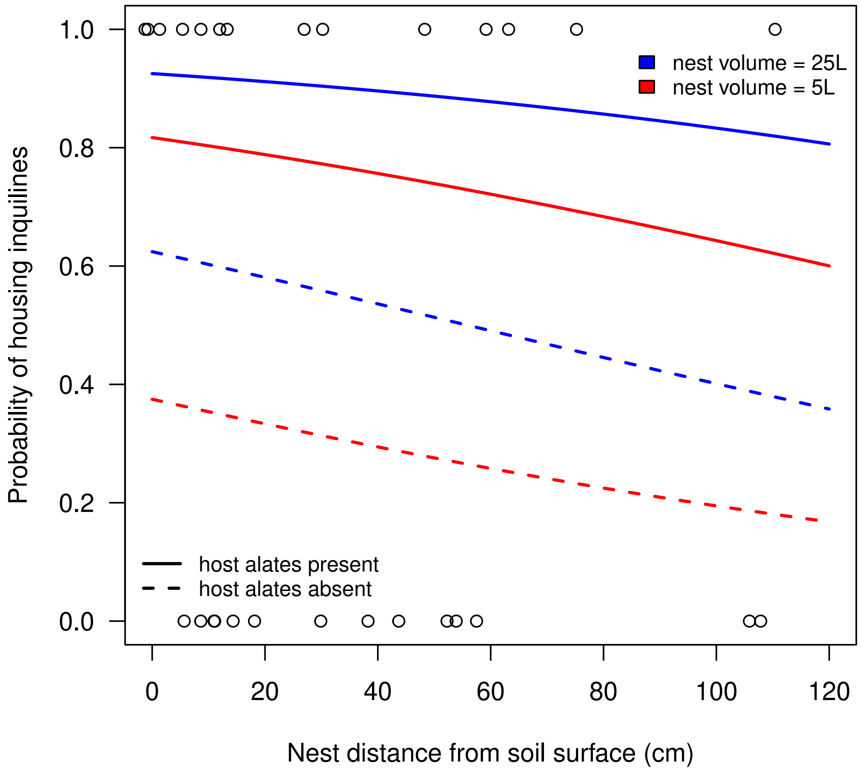

Figure 1: Nest-soil distance (i.e., distance between the bottom part of a net and the ground surface, x-axis) affects inquilinism occurrence (i.e., probability of housing inquilines, y-axis) across nests with different volumes (blue and red lines) in colonies where (pre)alates are present (continuous lines) and absent (dashed lines).

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7 Appendix

Table 2: Raw data table. Nest ID is the nest identification; Nest volume is in liters; Distance between nest and ground (i.e., degree of arboreality) is in centimeters; Inquilinism is the absence (0) or presence (1) of inquilines in the nest.

Nest ID	Nest volume	Nest-soil distance	Inquilinism
2021FN01	5.3328	0	1
2021FN02	10.7056	39	0
2021FN03	11.9931	55	0
2021FN04	3.9904	5	0
2021FN06	15.3869	9	1
2021FN05	20.7363	43	0
2021FN07	24.1966	52	0
2021FN08	18.8586	109	0
2021FN09	8.4485	15	0
2021FN10	16.9896	47	1
2021FN11	17.5413	75	1
2021FN12	10.1563	62	1
2021FN13	5.1684	18	0
2021FN14	7.0889	115	1
2021FN15	8.9948	105	0
2021FN16	2.4121	58	0
2021MN01	0.0992	8	0
2021MN02	16.9928	30	0
2021MN03	27.6992	6	1
2021MN04	2.1929	13	1
2021MN05	4.7901	10	0
2021MN06	2.5657	10	0
2021MN07	17.1799	0	1
2021MN08	13.0576	0	1
2021JN01	12.3254	58	1
2021JN02	16.0464	26	1
2021JN03	40.3249	31	1
2021JN04	18.6985	110	1
2021JN05	5.1830	11	1

Chapter 2

Hormonal triggering of termite inquilinism

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Contents

Abstract

Symbiosis, the long-term intimate relationship of unlike organisms, is ubiquitous. Understanding the proximate mechanisms enabling symbiosis can further the comprehension of evolutionary history and origins of biodiversity. The symbiotic system composed by the termite host *Constrictotermes* spp and the termite inquiline *Inquilinitermes* spp is a good working example to such studies, as several trends of this relationship are well known. In this system, the probability of cohabitation goes beyond 50% after host nests attain a threshold volume and, as a consequence of their growth, the colonies change in soldier-worker castes proportion, reproduction activity and defense behavior. Through gene expression, we investigated the hormonal mechanism that trigger such changes, mostly by shifting Juvenile Hormone (JH) titters, creating a window of opportunity for inquiline invasion. Our results show that juvenile termites from colonies with and without inquilines seems to produce similar levels of JH in their Corpora Allata as the Juvenile Hormone epoxidase expression was equivalent between them. Conversely, Krüppel Homolog 1 (Kr-h1) expression in these juveniles is significantly and positively correlated to the presence of inquilines, indicating that general titters of JH (the sum of individual and social physiology) differ in termite hosts from colonies with and without inquilines. This correlation between Kr-h1 high expression levels and the presence of inquilines could indicate that (i) the window of opportunity was used and that is why inquilines are present in bigger mature nests after a reproductive event and (ii) inquilines, perhaps, have some effect upon the host physiology, becoming an active gear in the social regulation of colony hormonal levels, especially JH.

Resumo

A simbiose, a relação íntima de longo prazo entre organismos diferentes, é onipresente. A compreensão dos mecanismos próximos que permitem a simbiose pode promover a compreensão da história evolutiva e das origens da biodiversidade. O sistema simbiótico composto pelo cupim hospedeiro *Constrictotermes* spp e pelo cupim inquilino *Inquilinitermes* spp é um bom modelo de trabalho para tais estudos, já que diversas tendências dessa relação são bem conhecidas. Neste sistema, a probabilidade de coabitação ultrapassa 50% depois que os ninhos hospedeiros atingem um volume limite e, como consequência do seu crescimento, as colônias mudam na proporção de castas soldados-trabalhadores, na atividade reprodutiva e no comportamento de defesa. Através da expressão gênica, investigamos o mecanismo hormonal que desencadeia tais alterações, principalmente pela alteração dos títulos do Hormônio Juvenil (HJ), criando uma janela de oportunidade para a invasão inquilina. Nossos resultados mostram que cupins juvenis de colônias com e sem inquilinos parecem produzir níveis semelhantes de JH em suas Corpora Allata, já que a expressão do hormônio juvenil epoxidase foi equivalente entre eles. Por outro lado, a expressão do Krüppel Homolog 1 (Kr-h1) nesses juvenis está significativa e positivamente correlacionada com a presença de inquilinos, indicando que os títulos gerais de JH (a soma da fisiologia individual e social) diferem em hospedeiros de cupins de colônias com e sem inquilinos . Esta correlação entre os altos níveis de expressão de Kr-h1 e a presença de inquilinos pode indicar que (i) a janela de oportunidade foi aproveitada e é por isso que os inquilinos estão presentes em ninhos maduros maiores após um evento reprodutivo e (ii) os inquilinos, talvez, tenham algum efeito sobre a fisiologia do hospedeiro, tornando-se uma engrenagem ativa na regulação social dos níveis hormonais das colônias, especialmente da JH.

1 Introduction

Symbiosis, the long-term intimate relationship of unlike organisms (Oulhen et al., 2016; Rodrigues et al., 2021), is ubiquitous (Margulis and Fester, 1991; Margulis, 2008). Understanding the proximate mechanisms enabling symbiosis can further enhance the comprehension of evolutionary history, origins of biodiversity, and hierarchical evolutionary transitions (Losey Jr, 1993; Higashi and Abe, 1997; West et al., 2015; Estrela et al., 2016). The symbiotic system composed by the termite host *Constrictotermes* spp and the termite inquiline *Inquilinitermes* spp is a good working example to study symbiosis initiators, as several determinants of their cohabitation in the host nest have been elucidated (Cunha et al., 2003; Cristaldo et al., 2012, 2014, 2016; Barbosa-Silva et al., 2016; DeSouza et al., 2016; Jirošová et al., 2016; Rodrigues et al., 2018, 2021; Lucena et al., 2019; Hugo et al., 2020; Cruz et al., 2023).

For example, cohabitation between these termites is more likely to occur in reproducing host colonies that hold nests bigger than 13 liters (Cristaldo et al., 2012). That was shown to be correlated with colonies becoming reproductively mature and, during the annual reproductive season, going through drastic changes in caste proportions. This change in the caste-proportion homeostasis seems to increase host tolerance or diminish host resistance to inquilines, easing their entrance into the nest. The mechanism behind this change was hypothesized to be a trade-off between defense and reproduction, which ultimately reduces the development of soldiers (i.e., colony defense) and workers (i.e., foraging activity) in favor of the development of dispersal reproductives (Rodrigues et al., 2018, 2021).

However, it is still unclear what mechanism triggers the trade-off itself, although a hormonal one was considered. This mechanism is likely to rely mostly on juvenile hormone (JH) synthesis and metabolism, as JH is the main physiological factor determining post embryonic development and developmental plasticity in termites (Watanabe et al., 2014; Korb, 2015; Korb et al., 2021). Once triggered, this hormonal mechanism would lead to a shift in the development of first instar termite juvenile (L1), as described below.

Right after the colony's foundation, the first instar juveniles (L1) produced by the royal couple experienced high hunger levels due the lack of foraging activities in the incipient colony. This state of hunger (Cymborowski et al., 1982; Corona et al., 2007), combined with JH transferred from the queen (Lanzrein et al., 1985; Maekawa et al., 2012), leads L1 to develop into soldiers. These soldiers produce a primer pheromone (i.e., soldier primer pheromone or SPP), which reduces JH titters in the new L1 (Lüscher, 1972; Lefeuve and Bordereau, 1984; Okot-Kotber, 2014). These, in turn, develop into workers. Soldiers and workers forage together, reducing colony hunger enough to sustain this alternating balance of soldier and worker production by sustaining high and medium JH titters. As the contingent of workers and soldiers attain enough numbers and the colony is well-fed, JH titters would drop enough for L1 to develop into dispersal reproductives, starting the reproductive season. Consequentially, the replacement of soldiers and workers becomes insufficient, leading to reduced foraging and defense activities.

At this moment, a window of opportunity for invasions is open due to such decrement in colony defense. Simultaneously, the lack of workers forces the colony into a starvation state, eventually resuming the cycle previously described of high-medium JH titters in juveniles' metabolism. This window of opportunity would remain open until the high-medium JH titters stabilizes the soldier and worker contingent, closing the reproductive season. After a period of homeostasis, the colony is again well-fed and the SPP effect is enhanced by a larger soldier contingent, then JH titters will drop sufficiently low to trigger a new reproductive event (Figure 5).

In summary, low JH titters direct juveniles to the nymphal lineage (NL), through which they become dispersal reproductives, so-called alates (A). Medium JH titters lead to the development of workers through the apterous lineage (AL). Higher JH titters direct L1, also through the AL, to develop into soldiers. These different JH titters are determined by both individual metabolism and social context in the nest (Figure 5) (Watanabe et al., 2014; Korb and Belles, 2017; Jongepier et al., 2018). It follows that JH titters define the abundance of workers, soldiers, and dispersal reproductives in a colony across time, whereas the intraspecific interactions between those castes influence JH titters available to the metabolism of caste-less individuals from L1 developmental stage. When L1 individuals are developing into alates during the reproductive season, the host colony is prone to inquiline invasion.

Hence, we hypothesise that fluctuations in JH titters can open a window of opportunity for inquiline invasion. A proxy of JH titters is the relative expression of JH pathway genes, which we predict to be expressed differently in colonies housing and not-housing inquilines. To test that hypothesis, we measured the expression of Krüppel-homolog 1 (Kr-h1) and JH epoxidase (JHepox) in first instar juveniles from host colonies with and without inquilines.

2 Materials and methods

2.1 Terms definition

We understand "symbiosis" in de Bary's definition, which is the intimate and long-term "living together of unlike organisms" (Oulhen et al., 2016), independently from positive or negative outcomes from their interactions. The terms "cohabitation", "coexistence", and "inquilinism" are used to describe the simultaneous presence of two different termite species in the same nest. The termite "host" (also known as the "builder") and the termite "inquiline" (also known as the "guest") share a nest. Obligatory inquilines are not capable of constructing their own nest. The physical structure built by the host termite is called a "nest" or "termitarium". A monospecific collection of termites closely related to each other is known as a "colony". Termites are monophyletic eusocial cockroaches (Blattodea) that live in colonies composed of individuals that differ in form and function, classified in "castes". The termite juvenile stage, which hatches from the eggs, has no caste determined yet and is referred to as "first instar juvenile" (L1). The sterile castes comprise workers (W) and soldiers (S), formed through the apterous development lineage (AL). The reproductive castes, royal couple (the "queen" (Q) and the "king" (K)), and their dispersing progeny (immature dispersals are known as "pre-alates"(PA) and mature dispersals are called "alates"(A)) develop through the so-called nymphal lineage (NL) (Figure 1).

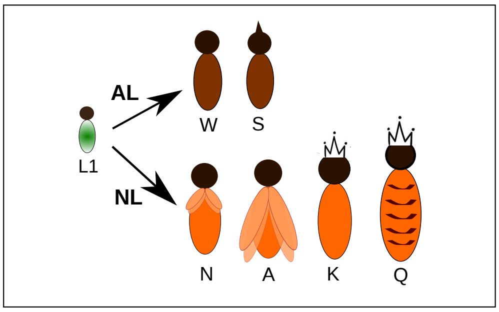

Figure 1: Schematic bifurcated developmental pathway in termites such as *Constrictotermes cyphergaster*. The fluctuation of hormone titters will direct the first instar larvae to one of the two developmental lineages of the developmental pathway. Sterile castes, workers (W) and soldiers (S), develop through the apterous lineage (AL). The reproductive castes, royal couple (the "queen" (Q) and the "king" (K)), and their dispersing progeny (immature dispersals are known as "pre-alates"(PA) and mature dispersals are called "alates"(A)) develop through the so-called nymphal lineage (NL). High and medium JH titters recruit L1 to the AL, whereas lower titters direct them to the NL.

2.2 Field sampling

Eight termite nests (see section 5) were collected in 2021 in the district of São José das Lajes, city of Cordisburgo (19° 07' 30" S 44° 19' 15"), Minas Gerais state, Brazil. The vegetation in the site is Cerrado *stricto sensu* (Brazilian savanna), composed of arboreal and shrub-herbaceous strata. Trees are small to medium heights with irregular branches and thick barks (Graeff, 2015). The soil is classified as dark-red latosol, presenting a rocky/sand appearance. The climate is tropical Aw according to Köppens classification, presenting a dry winter and rainy summer. The mean annual precipitation is 1200mm and the mean temperature is 21.5ºC. These nests varied between an oval and spherical form which can be visually split into hemispherical caps and cylinders to facilitate nest volume inference (Cristaldo et al., 2012).

Immediately after collection, nests were opened and inspected for inquilines and juveniles from the host species. Inquilines are commonly found in isolated peripheral chambers while hosts are concentrated at the center of the nest structure. The host first instar larvae are lethargic and can be found in groups, usually near egg masses at the very central part of the nest structure. The L1 was carefully collected using small painting brushes cleaned with alcohol to avoid damaging the individuals. The juveniles were directly put in 1.5mL Eppendorf vials containing the stabilization solution RNAlater (Sigma Aldrich), and left in a fridge (-6°C) available at the field for 48h. After that, they were transported to the lab in thermal boxes filled with ice and placed in an -80°C fridge.

2.3 Gene expression experiment

To infer JH titters, we performed qRT-PCR experiments on Kr-h1 and JHepox (P450) genes. The JHepox gene codes for enzymes that catalyse the final steps of JH synthesis, being a proxy of JH synthesis in the Corpora Allata of an individuals' body. The expression of the gene Krüppel-homolog 1 (Kr-h1), on the other hand, is a proxy of general titters of JH. That is, JH available to an individuals metabolism (Figure 2). Hence, by analyzing JHepox and Kr-h1 expressions, we target the JH titters before and after social effects, which is key to studying a hormonal mechanism that relies on both individual and social physiology (Korb, 2015; Jongepier et al., 2018).

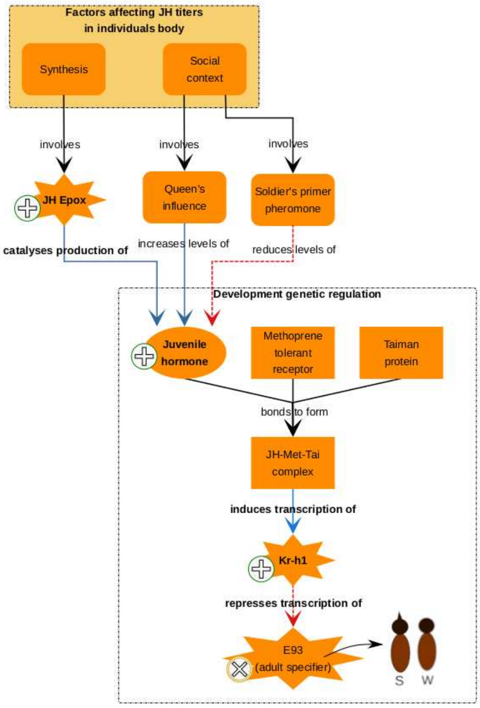

Figure 2: Fluxogram describing factors affecting JH titters in the body of juvenile termites (i.e., larvae) and their interactions with the genes JHepox and Kr-h1. JHepox expression levels are a proxy for JH synthesis in an individual CA, as it expresses enzymes that catalyze the final steps of JH synthesis in the CA. Meanwhile, Kr-h1 expression is a proxy for general levels of JH available for an individual's metabolism, encompassing both the individual synthesis and social-physiology effects. Mainly, the soldier caste and royal couple contribute to the regulation of JH titters in the juvenile's metabolism. The queen's CA is well developed and active presenting high JH titters in its body fluids. Through proctodeal trophallaxis, the queen's body fluids increase JH titters in the juvenile's body. Soldiers, on the other hand, produce a primer pheromone which has inhibitory effects upon JH availability to juvenile's metabolism. General high JH titters, for example, would hence correlate to high expression of Kr-h1, since the complex formed by JH, Met, and Taiman is responsible for inducing transcription of the Kr-h1 gene. High levels of Kr-h1 expression, in turn, would lead to the inhibition of the gene E93 (ecdysone pathway gene) preventing the development of adult features, such as reproductive organs. Therefore, the expression of both JHepox and Kr-h1 is positively correlated to JH titters.

The qRT-PCR followed Elsner et al. (2018). We extracted total RNA from whole L1 individuals as they were too small to be dissected. Each sample used for RNA extraction was composed of 10 L1 individuals from 8 different colonies, with and without inquilines. Extraction followed the peqGOLD TriFast protocol. Concentration and purity of RNA were measured using Nanodrop 2000c (peqLab), followed by dilution to 25 ng/µl with nuclease-free H2O. Reverse transcription and qRT-PCR were done in a LightCycler 96 (Roche), using QuantiTect SYBR Green RT-PCR Kit (Qiagen) with a reaction volume of 25 ng/µl. Reverse transcription lasted 1,800 s at 50°C, activation followed for 900 s at 95°C, and then 50 amplification cycles were run. Amplification cycles consisted of 15 s of denaturation at 95C, 30 s of annealing at 62°C, and 70 s of extension at 72°C. This was followed by a melting step with 10 s at 95°C, 60 s at 65°C, and 1 s at 97°C, followed by a cooling step for 30 s at 37°C. The amplification and melting curves were checked to ensure target specificity. To normalize expression, we used the gamma-tubulin (y-tubulin) gene, known to be expressed in a stable fashion (Dumontet et al., 1996). Three technical replicates per gene and sample were performed.

2.4 Statistical analysis

The software R Studio (version 2023.06.2+561) was used for analysis. A Wilcoxon rank-sum test (also called Mann-Whitney U) (Haynes et al., 2013) was applied to compare expression levels between two groups: (i) host colonies with and (ii) without inquilines. The non-parametric statistical Wilcoxon rank-sum test has the advantage of not depending on *a priori* assumptions about the data set distributions and can be applied to small sample sizes.

3 Results

We compared the expression of the genes Kr-h1 and JHepox by using a total of eighty L1 individuals from eight different colonies, four with and four without inquilines. Our results show that JHepox expression was not significantly different between colonies with and without inquilines. Whereas, Kr-h1 expression is significantly and positively correlated to the presence of inquilines.

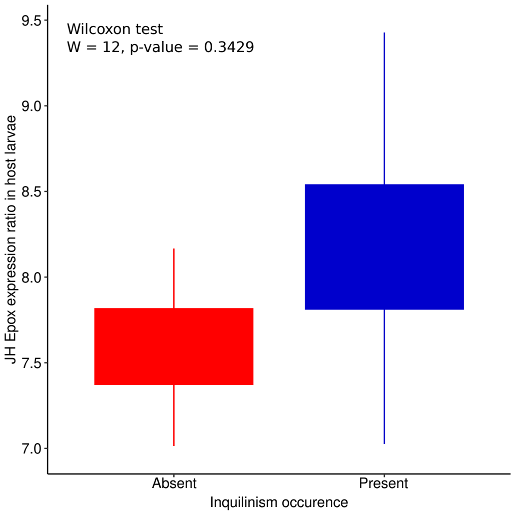

Figure 3: Comparison of JHepox expression levels (y-axis) in colonies not-housing (red) and housing inquilines (blue). There was no differential expression in colonies with and without inquilines (p-value = 0.34), pointing out that the JH titter promoted by JH synthesis in the CA is not correlated to inquiline presence.

Low expression levels of Kr-h1 in L1 (the red points in figure 4) indicate that the window of opportunity (i.e., reproduction event) is beginning to start and these L1 individuals are set to develop into alates in approximately five months (Moura et al., 2011; Lucena et al., 2019) through the NL. High expression levels of Kr-h1 (the blue points in Figure 4) indicate L1 is set to develop into soldiers and workers through the AL.

Regarding the window of opportunity for inquiline invasion (Rodrigues et al., 2021), which we hypothesize to be triggered by a JH-linked hormonal mechanism (Figure 5) the correlation between kr-h1 expression and inquilines suggests that (i) the starting point of the invasion opportunity seems linked to lower Kr-h1 expression levels, meaning that L1 set to become alates; the colony is entering reproduction; and (ii) the closing of the opportunity seems linked with higher Kr-h1 expression levels; L1 set to become workers and soldiers, the colony is ending reproduction and recovering its homeostasis, marked by workers and soldiers prevalence in caste proportions.

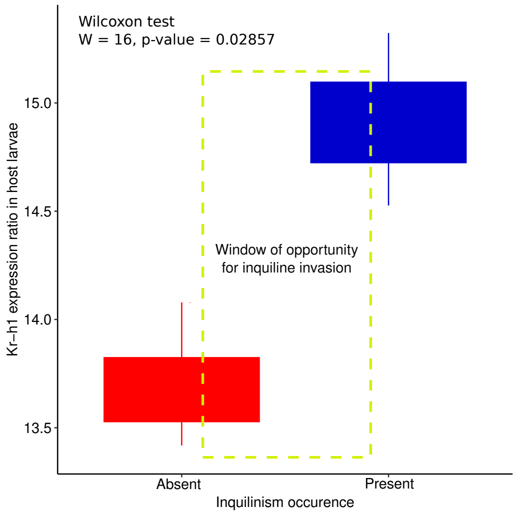

Figure 4: Comparison of Kr-h1 expression levels (y-axis) in colonies not-housing (red) and housing inquilines (blue).The differential expression in colonies with and without inquilines (p-value = 0.03) points to a correlation between inquilinism and JH titters in L1 individuals. The yellow dotted line represents the window of opportunity for inquiline invasion, which takes place from the start of the defense-reproduction trade-off, throughout the homeostasis recovery after the reproduction event. The start of the trade-off is signaled by the lower Kr-h1 expression, in red (lower JH titters), and its end is signaled by the higher Kr-h1 expression, in blue (higher JH titters).

Hence, this correlation between Kr-h1 high expression levels and the presence of inquilines could indicate that (i) the window of opportunity was used and that is why inquilines are present in larger nests which, after reproducing, are back to recruiting workers and soldiers; (ii) inquilines have some effect upon the host physiology, suggesting that once the inquilines are inside the nest they might be an active gear in the social regulation of colony hormonal levels. Below, we will discuss these two explanations of current results.

4 Discussion

Our results point to a correlation between inquiline presence and expression of the JH-pathway genes (Kr-h1 and JHepox) in hosts, implying a correlation between inquilinism and host physiology. This is in line with the evidence that inquilinism is linked to host ontogeny, namely, host nest volume, fluctuations in caste proportion, and defense activities (Cristaldo et al., 2012; DeSouza et al., 2016; Rodrigues et al., 2018, 2021).

We observed that host juveniles from colonies without inquilines presented lower expression of Kr-h1 indicating that they are in line to become alates. The opposite is true, juveniles in colonies with inquilines presented higher expression of Kr-h1, signaling that such colonies are drifting back to recruiting workers and soldiers (Figure 4).

In addition, JHepox expression in colonies without inquilines did not differ from those in colonies with inquilines (Figure 3). JHepox is known to be independent of social context, being uniquely related to the JH synthesis in the CA gland. On the other hand, kr-h1 expression is a consequence of the JH titters available to metabolism. Those titters are a combination of JHepox activity plus SPP and parental influence, hence, Kr-h1 expression depends on the social context (Figure 5).

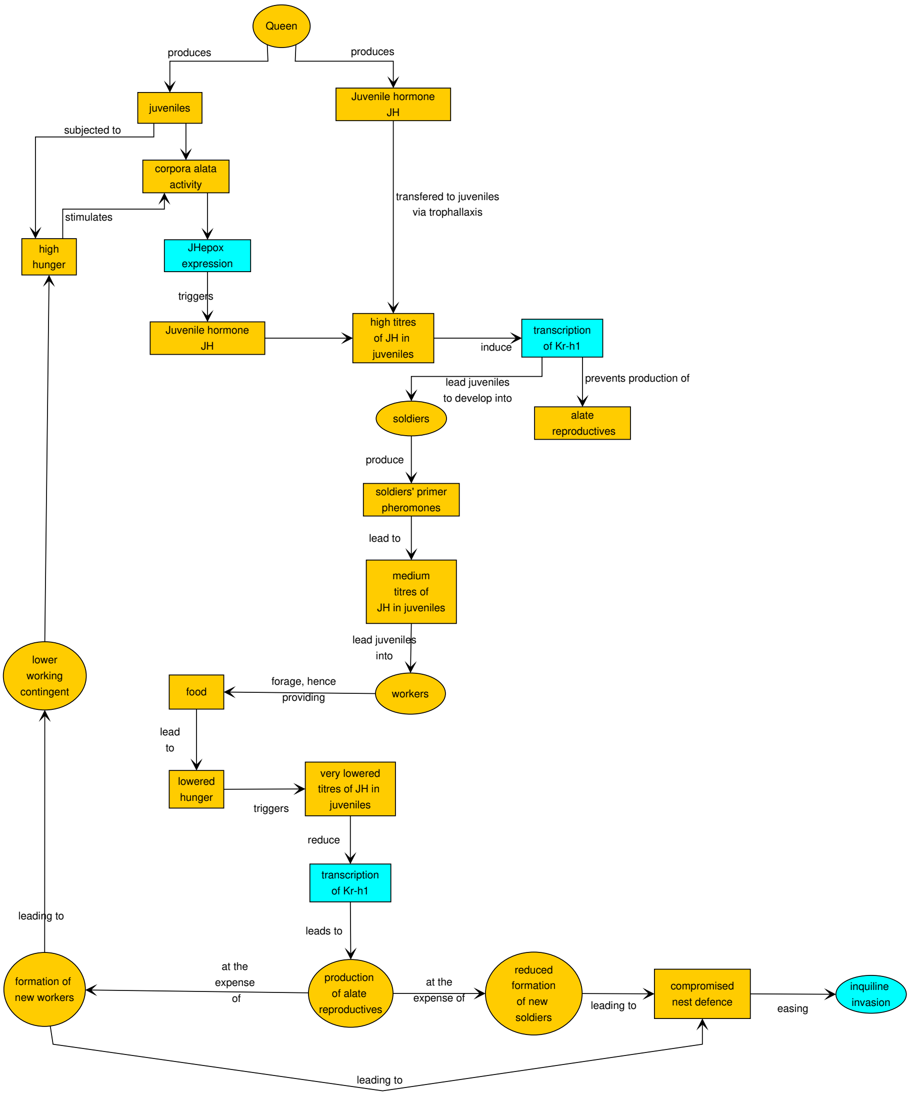

Figure 5: Fluxogram describing the overall rationale of the hormonal triggering in termite inquilinism.

The social context in colonies where inquilines were not present is expected to be the following: juveniles are in line to become alates (kr-h1 expression was lower, meaning lower JH titters), hence, soldier and workers population won't be replenished, being reduced as a cost of producing the alates that will be fully developed in approximately five months (Lucena et al., 2019). This shortage in soldiers is in line with the diminished defense activity shown by DeSouza et al. (2016) for nests with sizes close to the nest volume threshold for inquilinism.

Colonies with inquilines, on the other hand, had juveniles who were in line to become soldiers and workers (kr-h1 expression was higher, meaning higher JH titters), signaling that the social context was one of a colony that was set to reestablish its defensive and foraging capacities after the swarming of alates. This is in line with the hypothesis sired by Rodrigues et al. (2021) that the reproductive event, triggered by hormonal mechanisms, is a window of opportunity for inquiline to enter the nest.

This correlation between Kr-h1 high expression levels and the presence of inquilines could indicate that (i) the window of opportunity was used and that is why inquilines are present in larger nests which are back to recruiting workers and soldiers; (ii) inquilines have some effect upon the host physiology, suggesting that once the inquilines are inside the nest they might be an active gear in the social regulation of colony hormonal levels.

In the first scenario, the symbiosis between inquiline and host would be facilitated by the defense-reproduction trade-off triggered by a hormonal mechanism, which disrupts the host colony homeostasis, easing inquiline invasion. This is in line with the known correlation between symbiosis and different types of disturbance (Seckbach and Grube, 2010), such as homeostasis ruptures (Rodrigues et al., 2021), environment disturbance such as fire (Monteiro et al., 2017) and environmental harshness (Viana-Junior et al., 2021).

In the second scenario, the correlation between inquilinism and host physiology would be seen as an outcome of cohabitation. For this scenario to be considered plausible, perhaps two primary premises would be required, (i) capacity for interspecific recognition between hosts and inquilines and (ii) evidence of behavioral change when recognition occurs. Indeed, chemical mechanisms have been pointed to modulated cohabitation and interactions between these two species, ultimately affecting their behavior. For example, it has been shown that both inquilines and hosts are capable of recognizing each other's chemical cues, hence, being able to establish a spatial separation inside the nest and avoid conflict (Hugo et al., 2020; Jirošová et al., 2016).

Nonetheless, it seems like ecological benefits could come out of a possible interference of inquilinism in host physiology and reproduction. Symbionts, especially obligatory ones (i.e. organisms permanently living on the host's body or nest), such as *Inquilinitermes*, rely on host survival and reproduction to enable their survival (Mestre et al., 2020).

In this scenario, inquilines cohabiting in the host nest could benefit from the host colony's ability to recover homeostasis after the reproductive event (i.e., the window of opportunity for inquiline invasion). Such recovery allows host colonies to defend and expand the nest, as well as reestablish foraging activities. When those activities are fully happening in the nest there should be an increment of area and organic matter available inside the nest. That could favor inquilines as they seem to feed on such organic matter (Florencio et al., 2013; Barbosa-Silva et al., 2016).

Additionally, this recovery is critical for host colony survival and reproduction in the next season, which will open again the window of opportunity for invasions and create an opportunity for the dispersal of cohabiting inquilines. Hence, a hypothetical increase of JH caused by the presence of inquilines could be beneficial if it could catalyze the recovery of the work and soldier contingents in the host colony. This seems like a research road worth exploring.

Finally, these correlations between JH-pathways gene expression in hosts and inquiline presence reinforce that the mechanisms mediating this symbiosis are linked to ontogeny and social context, which are mediated by hormones and gene expression. One question emerges from these results, which is: are the inquilines cycle simply aligned with the host's physiology, or, as the symbiosis begins, inquilines become an active gear in the host colony's physiological regulation?

5 Appendix

5.1 Colonies features

Table 1: *Constrictotermes cyphergaster* colonies sampled in this study. Each row represents a host colony.

Nest ID	**Inquiline presence**	**Presence of (pre)alate**	**Nest volume**
N01	Absent	Absent	0.1 L
N02	Absent	Present	16.9 L
N03	Present	Present	16.0 L
N04	Present	Present	27.7 L
N05	Present	Present	18.7 L
N06	Absent	Present	4.8 L
N07	Absent	Absent	2.6 L
N08	Present	Present	17.5 L

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