Surprising reproduction strategies and fitness in the monogynous ant *Cardiocondyla elegans*

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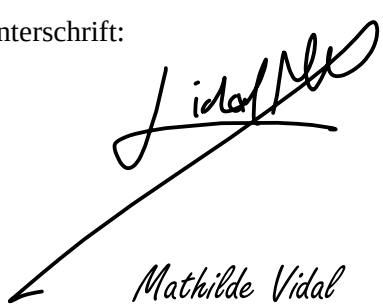

Two different angles picturing the same pair of *Cardiocondyla elegans* individuals. The ant worker is seen carrying a winged future queen on its back, holding it by the neck with its mandibles. This behaviour occurs during the reproduction season when multiple instances of workers carrying future queens between nests can be observed. *Photos by Mathilde Vidal.*

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Abstract

The influence of sexual selection constantly shapes individuals' reproductive strategies. Commonly, in the animal kingdom, reproduction is the concern of sexual organisms with compatible sexual apparatus, males and females. The surprising reproduction strategy of the ant *Cardiocondyla elegans* shows a rare case of third-party involvement by the sterile worker, which, in the long-term, might have a decisive influence on the colony fitness. Such third-party involvement in reproduction has only been shown in humans. However, few cases in the literature have reported non-sexual individuals influencing reproduction, in the insect class. Using behavioural and genetic analysis, our study aims to investigate how such influence on reproduction affects the fitness of a monogynous and polyandrous ant species. Workers of *C. elegans* have, indeed, been observed carrying the winged future queens (gynes) on their back, from what we know now to be their natal nest to another foreign colony, where they can mate with unrelated males. Our study shows the benefits of such gyne dispersion via the calculated 30% of outbreeding found in the species both in 2007 and 2021. Behavioural analysis revealed that specialised workers select particular colonies over others as recipients for the carried gynes. However, to this day, we remain unable to reveal the reason nor mechanism of such nest selection. Genetical analysis showed the possibility that transported gynes (alien gynes) be selected by unrelated workers for additional carrying during the reproduction season. After which, alien gynes are shown in our study to hibernate in the receiving nest, during the winter, before dispersing by foot prior to the next reproduction season. As a polyandric species, future queens of *C. elegans* can mate with multiple males during the reproduction period. Our study confirms the estimated number of males mated with a queen (patrilines) found in 2007, in a different population, and the possibility for queens to produce workers after mating without the need for unrelated genetic material. Finally, we revealed the possible existence of an optimal number of patrilines over which the colony foundation of *Cardiocondyla elegans*' queens can be negatively impacted.

1. Introduction

1.1. Sexual selection and inclusive fitness

Sexual selection is a highly influential process in evolution. As expressed by Anderson in 1994, following Darwin's thoughts: "sexual selection arises from differences in reproductive success caused by competition for access to mates" (Andersson, 1994, p. 3). In other words, when multiple individuals of the same sex and from the same species compete for a mating partner and only one of them can be selected for reproduction, the traits of the chosen individual will prevail over those of the others. By reproducing, individuals with a particular phenotype (saying green eye colour) can pass on their genes to the next generation. The resulting offspring, which might have inherited the distinct phenotype, can, in turn, convey their genes to their descendants, and so forth. Whenever an individual carrying the mentioned phenotype is selected for reproduction, increases the chances for the green eye colour to be inherited and for the phenotype to keep existing in the long term. The quantitative estimation of abundance and frequency of a phenotype (or genotype) in a population, called *fitness*, can assess the reproductive success of a trait over multiple generations.

Generally, traits which evolved under the pressure of sexual selection and keep being selected in the long term (many generations) are beneficial for individual fitness (Cally et al. 2019). When the benefits (increased chances of reproduction success) outweigh the risks (increased chances of predation) the preferred inherited phenotype will be maintained in the long term. If the risk of predation prevails over the benefit in reproduction success, individuals carrying the perilous phenotype will have a high chance of dying from predation before being able to reproduce. In the long term, the trait will stop being inherited by having no more living individuals carrying the risky phenotype.

Such inherited traits are called secondary sexual characteristics, which contrary to primary sexual characteristics (e.g., reproductive organs) are not directly used for reproduction but increase the chances of mating success. Sexually selected traits can be observed, for instance, through physical appearances like size, colours, or ornaments (e.g., the abundant size and distinctive patterns of a peacock tale), and/or a specific behaviour (e.g., the elaborate birds-of-paradise mating dance) which enhance the reproductive success of an individual. Commonly shown by males, equivalent secondary sexual traits can be found in insects (e.g., the famous fireflies bioluminescence patterns) to attract a partner and/or improve the success of mating (e.g., the eastern dobsonfly's curved jaws used to attach to the female during mating (Darwin 1871)).

In eusocial insects, like bees and ants, sexual selection is often based on male's size and involves physical competition (Heinze and Hölldobler 1993; Heinze and Tsuji 1995; Boomsma et al. 2005; Couvillon et al. 2010). In the case of eusociality, where caste specification can lead to sterile individuals, the estimation of fitness for a given genotype is not as simple as previously mentioned.

Indeed, the *direct* fitness of an individual can be estimated through its reproductive success in passing its genes on to its direct offspring. However, in 1964, Hamilton defined the term of *inclusive* fitness (Hamilton 1964a, b) which provided an explanation for the evolution of altruism in the case of eusocial insects. In evolutionary biology, an individual is called an altruist when its behaviour is beneficial to other organisms' reproductive success while decreasing its own. Hamilton's definition of inclusive fitness stipulates that a trait that appears beneficial for the inheritance of an organism's genes will be favoured by natural selection, regardless of which organism directly produced offspring. In the case of altruism, an individual's gene pool can still win in inclusive fitness by helping closely related kin, which is sharing an important percentage of its own genes, to successfully reproduce (kin selection, (Darwin 1859)).

Hamilton proposed a mathematical rule, describing the conditions necessary for natural selection to favour altruistic traits: $c < rb$ (Hamilton 1964a, b). Where *c* represents the direct reproductive *costs* to the altruistic, *b* represents the direct reproductive *benefits* to the individual who received the help and *r* expresses the *coefficient of relatedness* between the two (the probability to have similar alleles at a random locus, via a common ancestor). In other words, Hamilton's rule stipulates that the existence of altruistic behaviour can be explained if the benefits given to the recipient's reproductive success, multiplied by the relatedness between the altruist and the recipient, are higher than the direct costs in the altruist's offspring production. Hence, to gain inclusive fitness, sterile workers in eusocial species show altruistic behaviour towards the more closely related kin.

1.2. Coefficient of relatedness and inbreeding depression

As previously mentioned, the measure of a coefficient of relatedness (or simply relatedness) between two individuals is an important indicator of the chances of sharing common genes, by inheritability. One approach to estimate the relatedness among individuals is to compare the length of selected DNA sections called microsatellites, or Short Tandem Repeat (STR). These DNA segments are composed of short motifs (between one to potentially more than six base pairs) repeating up to 50 times (figure 1.1. and (Richard et al. 2008; Gulcher 2012)). The high mutation rate and heritability of microsatellite regions make them a convenient estimator of relatedness. Indeed, as shown in figure 1.1, an individual carrying eight repetitions of two base pairs (dinucleotide microsatellite) at both alleles of a particular locus, will produce offspring carrying at least one allele at the same locus with eight repetitions. By isolating STRs regions and comparing their length as the number of base pairs, it is possible to estimate a value of relatedness between individuals. As such, the value of relatedness is given by the probability that individuals share similar inherited alleles, relative to a base population (or population control) where all individuals have different ancestors (Oliehoek et al. 2006). Multiple calculations of pairwise relatedness between two individuals can be given as a correlation coefficient (Wang 2017) or a regression coefficient (Queller and Goodnight 1989) offering values between 1 and minus 1. Hence, high positive values of relatedness indicate

that the two individuals are related to one another. In contrast, negative values of relatedness indicate a negative relationship between alleles of both individuals. In other words, the tested individuals would be shown to be less related than they would be related on average to a random individual in the population. Therefore, negative values of relatedness and values approximating 0 (no relationship between the data), indicate unrelated individuals.

A particular case of relatedness among individuals can be observed in ants, along with other Hymenoptera. Indeed, unlike mammals who use chromosomal sex determination, ants use arrhenotokous parthenogenesis (production of haploid males from unfertilized eggs) which leads to haplodiploidy as a sex determination mechanism. Haplodiploidy is defined by the production of haploid males from unfertilised eggs whereas diploid females come from fertilised eggs. Under such conditions, sibling ant workers of a monogynous (one single queen) and polyandrous (multiple males that can mate with the queen) species can have a relatedness coefficient of either 0.25 (half-sister) or 0.75 (full sister) (figure 1.2.). As such, if we compare the relatedness coefficient of a set of many ant workers from such species, we can expect the average value of relatedness to be between 0.25 and 0.75. In this case, the more males had the queen mated with, the closer the average of relatedness among workers would be to 0.25.

Driven by natural and sexual selection, most organisms tend to prefer circumstances that increase their fitness, therefore, avoiding the risks of excessively breeding with closely related individuals but see de Boer et al. 2021. The loss of genetic diversity in such conditions can lead to a decrease in fitness in the whole population, also called inbreeding depression. Indeed, a high level of inbreeding leads to the augmentation of partially recessive and possibly detrimental mutations, due to the increase in homozygosity (Darwin 1876; Charlesworth and Charlesworth 1987; Charlesworth and Willis 2009). Thus, as a defence mechanism, multiple organisms have developed different strategies to avoid sibling-mating (e.g., Pusey & Wolf, 1996). For haplodiploid organisms, high rates of inbreeding can have additional consequences (Zayed and Packer 2005; Harpur et al. 2012). Indeed, it has been shown in numerous studies on Hymenopteran that an increase in sib-mating often results in the production of diploid males. Such diploid males are commonly non-viable or sterile (Van Wilgenburg et al. 2006; Heimpel and de Boer 2008). A study by Doums in 2013 showed that diploid males of the species *Cataglyphis cursor*, if still fertile, produced sterile triploid workers, which, in a long term might be disadvantageous. In consequence, many social insects have evolved several techniques to avoid detrimental sib-mating. Some approaches, for example, involve producing males earlier, for them to disperse before the maturation of female sexuals. Other ant species had been shown to have specialised colonies focusing on the production of one sex over the other to avoid inbreeding (Helms and Rissing 1990). As a commonly known example, some ant species use large nuptial flights to increase the chances to mate with unrelated males, away from their original nest.

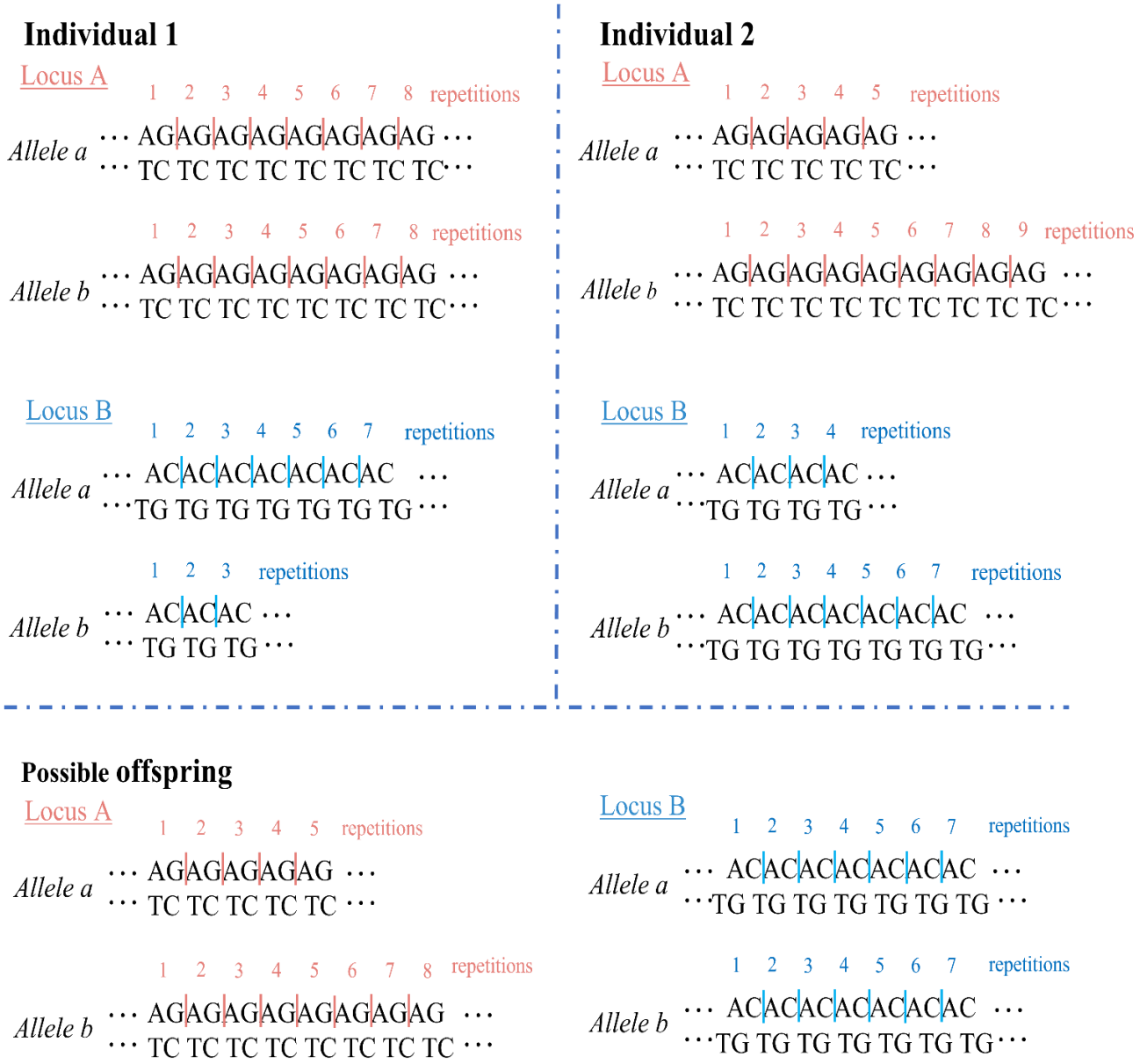

Figure 1.1. **Illustration of microsatellites heritability.** Simplified representation of the numbers of dinucleotide microsatellites repetitions for two DNA sections (locus A and Locus B). Each locus is represented by two alleles (allele a and allele b) for diploid organisms; individual 1, individual 2 and one case example of their possible offspring receiving half of its parents' genetic material.

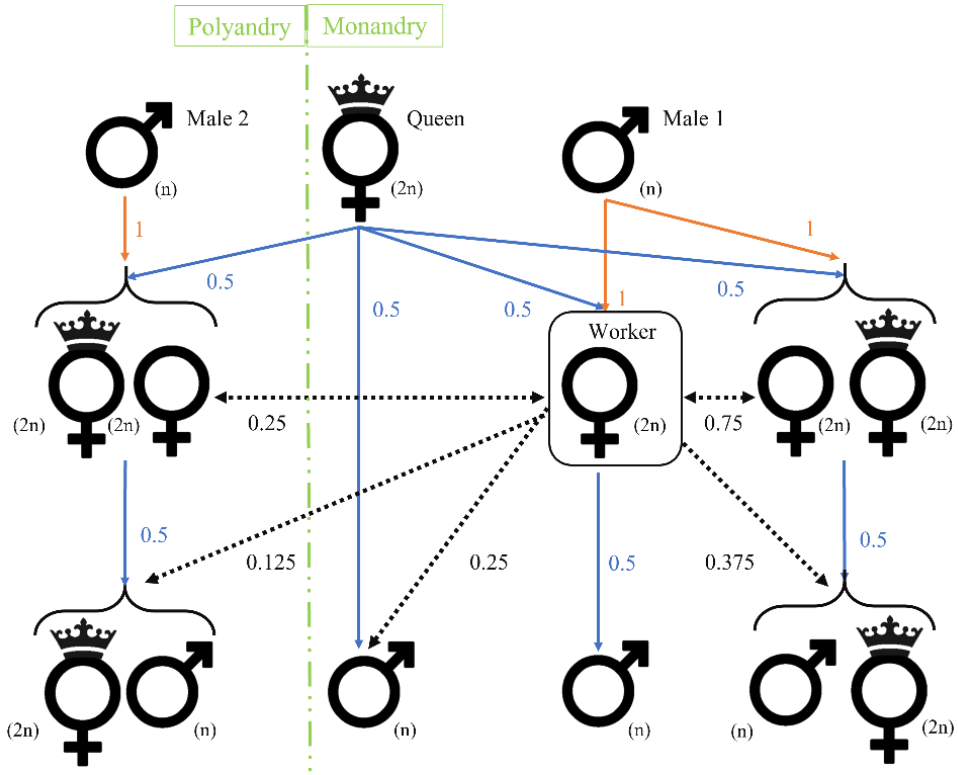

Figure 1.2. **Relatedness coefficient among cast members of a monogynic haplodiploid organism.** Numbers next to each arrow represent the pairwise relatedness between the two individuals, for a monandry species (right to the green line) and a polyandrous species (the whole drawing). Male symbols represent males (n; haploid), female symbols represent workers and female symbols with a crown represent the principal queen (in the first line) or produced future queens (both 2n; diploid). *Modified from* (Lenoir 2006).

1.3. *Cardiocondyla elegans* as a model organism

As mentioned above, many techniques used to avoid sib-mating involve ant dispersion. A high number of ant species famously disperse through nuptial flights. However, the production of sexual individuals that had lost the capability of flying makes outbreeding harder for some ant species. The ant genus *Cardiocondyla* is characterised by the presence of wingless (ergatoid) males in almost all its known species (Seifert 2003) except *C. zoserka* (Heinze 2019). Only a fraction of species (e.g., *C. emeryi*, and *C. obscurior*) conserved the presence of dispersible winged males. Moreover, a dimorphism in future queens (gyne)' mesosoma dimensions in multiple species (e.g., *C. batesii*, and *C. elegans*) suggests the presence of winged gynes yet unable to fly (Seifert 2003). Therefore, almost all matings in the genus *Cardiocondyla* happen inside the nests (intranidal mating).

Ergatoid males of the genus *Cardiocondyla* are equipped to stay inside the nest for their whole life cycle; they have a light orange-brown colouration (due to the lost necessity to invest in black cuticular pigmentation) and worker-like mandible, useful in case of male competition or to grab female during mating (Seifert 2003). Contrary to the common rule in Hymenoptera order, ergatoid males of *Cardiocondyla* have an unlimited sperm supply during their whole life. Hence,

opposed to winged males with limited sperm supply, ergatoid males can reproduce with multiple gynes until the end of their life cycle (Heinze and Hölldobler 1993; Heinze et al. 1998).

The high intranidal mating, thus increased sib-mating, in the genus *Cardiocondyla* makes it a likely candidate for inbreeding depression. In the case of polygynous species, where each nest contains multiple fertile queens, a necessary percentage of outbreeding can be reached by the acceptance of alien queens into established colonies, which will in terms produce alien sexuals (Creighton and Snelling 1974; Heinze et al. 2006). Winged males produced by some *Cardiocondyla* species equally increase outbreeding by dispersing prior to mating (Kinomura and Yamauchi 1987). Yet, the genus *Cardiocondyla* contains many Palearctic species which have evolved obliged monogyny, where each nest contains only one established fertile queen (Schrempf and Heinze 2007; Heinze and Foitzik 2009). A phenomenon called worker policing regularly eliminates any supplementary fertile queen in the nest (Schmidt et al. 2016a). Furthermore, Palearctic species of *Cardiocondyla* only contain ergatoid males which are very unlikely to disperse or for a very short distance (Oettler et al. 2010; Heinze 2017). The absence of mating dispersal or acceptance of alien queens led Palearctic species to be highly inbred (Schrempf et al. 2004; Lenoir et al. 2007; Schrempf 2014).

However, studies showed that monogynic species of the genus *Cardiocondyla* displaying a high inbreeding rate, have evolved yet unknown sex determination mechanisms in which neither sib-mating nor mother-son mating led to the production of disadvantageous diploid males (Schrempf et al. 2004; Schmidt et al. 2014). Nonetheless, the presence of a few unrelated matings in these highly inbred species has been documented (Schrempf et al. 2004), as well as the decline in colony fitness over multiple generations in closed laboratory colonies (shorter queen life spend and higher brood mortality) (Schrempf et al. 2006). This suggests that the reproduction history of *Cardiocondyla*, even as resistant to inbreeding as it appears to be, involves a small fraction of outbreeding events.

Observations made by Lenoir and Mercier in 2007, of *Cardiocondyla elegans* workers carrying valuable gynes on their back to distant colonies, seemed to suggest a unique behaviour that evolved in a Palearctic species to promote outbreeding. The work done by Lenoir during his doctoral thesis with *C. elegans* in Tours (Loire, Northwest region of France) provided valuable information about the species ecology (Lenoir 2006). Thanks to this previous work, nesting preferences, as well as colony organisation in *C. elegans*, were well documented (see paragraph 2.2). Their work provided the scientific community with five specific primers targeting microsatellite sections of *C. elegans*' DNA (Lenoir et al. 2005). Their studies on the genetic structure of the species indicated an expectedly high inbreeding coefficient and showed the implication of unrelated mating accounting for 30% of outbreeding (Lenoir et al. 2007). Hence, following the work done by Lenoir and Mercier in Tours, our study tends to broaden the existing knowledge surrounding the remarkable gyne carrying behaviour of *Cardiocondyla elegans*.

1.4. Aim of the thesis

The peculiar behaviour of *C. elegans*' workers towards future queens makes it a very interesting case for the study of colony fitness in a highly inbred species. As of today, in the animal kingdom, only a few cases of non-sexual individuals influencing reproduction have been reported in the literature (Cronin et al. 2011; Sunamura et al. 2011; Walker et al. 2011; Helft et al. 2016; Hölldobler 2016). The anecdotal behaviour of *C. elegans*' workers carrying winged female sexuals between nests during the mating season suggests a potential key-role in the species reproduction.

Using behavioural observations in the field and microsatellite genotyping, our study aimed to deepen our knowledge in the biology of the monogynous and polyandrous *Cardiocondyla elegans*, as a promising studied species. Doing so, we investigated the relationship between carrier and gyne, compared their relatedness and hypothesized that the dispersion of future queens selected nests promoted outbreeding. By defining donor and receiver colonies, our research answered the question asked by Lenoir in 2007 concerning the direction to which gynes were transported. We revealed that worker select gynes to carry in their joint natal nest, before walking up to several meters to non-randomly selected colonies. Workers can then drop their sister gyne in a foreign nest entrance where it can mate with non-brother males. However, given the occasional occurrence of unrelated pairs of workers and gynes, we hypothesised that gynes might be carried more than once between nests.

The reported presence of alien gynes into strictly monogynous colonies in 2007 lead us to wonder how long such intruders would be welcomed in foreign colonies. A study led by Schrempf in spring 2016 investigated the fate of gynes after hibernation and revealed the presence of alien gynes in foreign nests which will then disperse by foot on their own, before the new reproduction season, to found new colonies. To determine the necessary reproductive conditions for a gyne to be founding its colony we investigated their worker production. By making artificial nests in the laboratory, we monitored the colony founding of isolated queens and recorded the number of workers produced. Finally, using genotyping analysis, we hypothesised that an increase in the number of males mated with a queen will result in enhanced colony fitness. Our study revealed an estimated optimal number of patrilines above which queens tend to have a decreased colony founding efficiency.

2. Materials and methods

2.1. Previous unpublished studies

The research done during this doctoral thesis was based on previous studies of *Cardiocondyla elegans* that have been realised in the University of Regensburg between 2014 and 2017 by Dr Alexandra Schrempf, Dr Julia Giehr, MSc Florian Königseder and BSc Larissa Kalb, under Prof Dr Jürgen Heinze's supervision. These preceding studies were unpublished prior to the beginning of the doctoral thesis in 2017. Between 2017 and 2021, the previous data have been analysed by me, completed when necessary and included in the paper by Vidal et al., 2021. For a better understanding of the study, past experiments will be referred to and described in this monograph.

2.2. *Cardiocondyla elegans* as a study species

Cardiocondyla elegans Emery (Emery 1869a, b), is a small ant of 2-3 mm which belongs to the Formicidae family and Myrmicinae subfamily. The species can be found principally in the Mediterranean region, where the climate is characterised by warm dry summers and mild wet winters (Bernard 1968). These ants can commonly be found near rivers in sandy malleable soil, where they dig their nests vertically, looking for fresher temperatures during the hot summer season. The nests can thus go as deep as one meter and consist of multiple pea-sized narrow cavities called "chambers" which can be found along the way (Seifert 2003; Lenoir 2006; Lenoir et al. 2007).

As a polyandrous and strictly monogynous species, *C. elegans*' colonies are composed of a single queen that had mated with multiple males during its mating season (mean number of patrilines ± SD = 4.52 ± 1.6, (Lenoir et al. 2007)). For most of the year, each nest contains an established queen in charge of reproduction, about one millimetre bigger than each of the multiple workers in charge of nursing brood and foraging for food outside the nest. When the temperature approaches the dry summer season, the queen produces sexual individuals. Males, of the same size as the workers and wingless (ergatoid), differ from other individuals of their species by their orange colour (figure 2.1). In contrast to other *Cardiocondyla* species, being strictly ergatoid, the males do not disperse and are surprisingly mutually tolerant (Heinze 2017). Shortly after male production, the queen produces new virgin queens called gynes (figure 2.1). Gynes being the same sized as established queens are yet recognizable by the presence of wings attached to their thorax. Once established a queen sheds its wings. It is possible to observe the traces of shed wings (two black dots) in the established queen's thorax.

The mating season occurs during the Mediterranean summer. Unlike many other ant species, the sexual individuals of *Cardiocondyla elegans* do not practice nuptial flights. Instead, they have

intranidal matings. Special chambers are used, during the reproduction season, to localise sexual individuals near the nest entrance, few centimetres below the surface (Lenoir 2006). Mating occurs between males and gynes inside these mating chambers.

By the end of the summer, a unique behaviour, exclusive to this species can be observed. Few workers per colony can be seen carrying gynes, one by one, from one nest to another. Gynes are placed at the entrance of a colony where they can reach the mating chamber and mate with males. Thanks to their unlimited sperm production, males of *C. elegans* are capable of inseminating multiple gynes by the end of their life cycle. After the summer, all males will die. During the cool winter, the queen, her workers, a few non-carried gynes and multiple "alien gynes" (transported from another colony) hibernate in the same nest (see paragraph *3.1.3 Fate of carried gynes*, and Lenoir et al., 2007). As neither the queen nor her workers tolerate egg-laying by additional queens (Heinze 2017), the inseminated gynes will have to disperse and establish new colonies on their own when the temperature arises in spring.

Figure 2.1. **Picture of gyne, worker and male of the ant *Cardiocondyla elegans*.** The picture shows a winged gyne (vertically oriented), a worker (in the left), and a male (naturally of orange colouration in the bottom right) of *Cardiocondyla elegans*.

(Photo by M. Vidal)

2.3. Studied sites

Between 2014 and 2020, eight expeditions were conducted to the South of France (Gard, Languedoc-Roussillon) at a minimum of one per year. Seven different sites (between Beaucaire and Remoulins) were used for all behavioural observations, population structure and ant collection: (**SM**: N 43° 51′ 10.5″, E 4° 37′ 2.2″; **RFRK**: N 43° 55′ 43.9″, E 4° 34′ 5.1″; **P**: N 43° 56′ 31.0″, E 4° 33′ 34.5″; **H**: N 43° 55′ 2.7″, E 4° 35′ 4.2″; **FK**: N 43° 55′ 39.8″, E 4° 34′ 18.1″; **CP**: N 43° 51′ 9.9″, E 4° 37′ 2.4″; **BN**: N 43° 50′ 38.1″, E 4° 36′ 59.5″). All sites were sparsely vegetated with soil consisting mostly of sand and scattered small stones (figure 2.2). Except for the sites P and SM, all were positioned over a few meters from the rivers Gardon or Rhône which grants the easiest access to cooling temperatures in the deeper level of the soil during the dry summer. The site P was an unpaved, sandy parking lot in the centre of Remoulins and the site SM was located between two roads.

Consequently, the presence of *C. elegans* in the sites P and SM was only reported until 2017 when a critically dry summer is thought to be partially responsible for their disappearance.

In 2014 the expedition was conducted by Dr Giehr who mapped colonies, observed, and collected ants. Dr Schrempf, along with Larissa Kalb in 2015 and Florian Königseder in 2016, conducted a total of four expeditions where they mapped colonies, observed, and collected ants. From 2017 to 2020, I conducted one expedition per year where I either mapped colonies, observed, or collected ants.

On average in Languedoc-Roussillon, temperatures over the day can vary between 3-15°C during the winter (December to March), 8-21°C in spring (March to June) and autumn (September to December) and 19-35°C in summer (June to September). In summer 2017 the maximum temperatures reached a record of 45°C. Precipitations principally occur in autumn and early winter.

Figure 2.2. **Field expedition picture.** The picture was taken in August 2019 on the site RFRK. Each wooden stick represents a nest entrance of the ant *Cardiocondyla elegans* (Photo by M. Vidal).

2.4. Expeditions to the South of France

2.4.1. Observation

All behavioural observations were made by eye, sitting a few centimetres away from the ants. *C. elegans* being the smaller ant in the studied area, was easy to differentiate from other ant species. Gyne carrying behaviour could be spotted by the light reflection of the sun into the gyne's wings (figures 2.1 and 3.1). Nest entrances were sometimes distinguishable by the presence of a small pile of sand surrounding the entrance, composed of sand grains removed by ants to dig their nest. Other times, nest entrances were found by following forager individuals carrying food back to their colony. To differentiate nests, each was tagged with an individual numbered flag attached to a wooden stick (figure 2.2).

Above the ground, ants were collected using an aspirator tube (length x diameter: 6.3 x 2.3 centimetres). To ensure the collection of nestmate workers from a chosen colony, ants were taken when observed leaving or entering a particular nest entrance.

To identify the source and recipient colony of a gyne carrying event, we observed nest entrances, preferentially ones showing signs of worker activities (e.g., foraging, bringing sand grains out the nest, and guarding). Such nest entrances were examined until witnessing the departure of a worker carrying a gyne. The pair was then followed, from a respectable distance, until the gyne was dropped in a recipient nest. Once the gyne was delivered, the workers would go back to their original colony. It was therefore possible to assess source and recipient colonies by observing a random pair of worker and gyne at any time during their transport. Once the recipient colony reached, following the worker on its way back would determine the source nest.

2.4.2. Collection and mapping

For later conservation in the laboratory and genetic analysis pairs of carriers and gynes were collected. More than 300 pairs were collected without information on their origin nor destination, to compare the relatedness between carrier and gyne, as well as to investigate the worker production (see paragraphs 2.6 and 2.8). 51 pairs were collected with known source and intended recipient colonies to compare the relatedness to both carrier and gyne between source, recipient and passed colonies. In this case, the origin and destination were assessed as described above, however, the pair of carrier and gyne was collected a few millimetres from the recipient colony entrance before the gyne could be released. Non-targeted colonies were determined by selecting the three to five detected nests at a similar or lower distance from the source colony than was the recipient one. To compare the relatedness to both carrier and gyne between source, recipient and passed colonies, an average of 25 workers were collected at each nest entrance.

Tagged colonies were mapped in all sites, using a string to objectively delimit a rectangular perimeter around the observation area. The vertical and horizontal distance to the string was recorded for each marked colony in the area. A representation was constructed for each mapped area and arrows were added to represent the observed gyne carrying behaviour between nests. In 2014 Giehr mapped and observed colonies in the sites BN, CP, P, RFRK and SM. In 2015 Schrempf and Kalb mapped and observed colonies in all seven sites. Because of the presence of trees to provide shading while observing and the abundance of *C. elegans* colonies, I chose to map and observe colonies in the site RFRK exclusively, in 2018 and 2020.

2.4.3. Excavation

To study the number of individuals from different castes which compose a nest, colonies were excavated at the end of each observation period. Along the excavation process, by hand or with a spoon, ants were collected with the aspirator. All excavated colonies were left in the aspirator tubes, fed with cookie scrambles, and given a 1 x 1 centimetre piece of wet cut kitchen paper for humidity.

The ants could survive in these conditions for a couple of weeks. Some excavated colonies were kept in aspirator tubes until counting.

2.4.4. Transport

Individuals were then either stored in alcohol 100% for later genetic analysis or transferred in vertical nests for the creation of laboratory colonies. The 51 pairs of carrier and gyne collected with known source and intended recipient colonies, were directly stored in alcohol 100% in 1.5 mL Eppendorf tubes for later genetic analyses. All pairs of carrier and gyne which needed to be kept alive for later experiments or to create laboratory colonies were initially collected with the aspirator before being transferred in modified 15 mL Eppendorf tubes for transport until the University of Regensburg. A quarter of each transport tubes was filled with distilled water and plugged with cotton. Each pair of ants was kept between the humified cotton and a second cotton piece blocking the entrance without preventing airflow. Cookies crumbs and one small local leaf was provided between the two pieces of cotton as a food source and to provide shelter. The carrier and gyne could survive up to three weeks in these conditions while being transported to Germany and before their transfer into artificial nests.

Overall, the ants were collected with the approval of the Access and Benefit-Sharing Clearing-House (ABSCH). We obtained a certificate of compliance allowing the collection of *Cardiocondyla elegans* in the Gard (Languedoc-Roussillon, France)(ABSCH 2019).

2.5. Maintenance in the laboratory

Once transported to Regensburg, the ants which were not stored in 100% alcohol were placed in artificial plaster nests. Prior to my arrival at the University of Regensburg, Schrempf and Königseder designed a new type of nest to enhance the survival of *C. elegans*' laboratory colonies. Contrary to other ant species, like *Cardiocondyla obscurior*, which can survive in small horizontal plaster nests of 10.5 x 10.5 x 2 centimetres, *C. elegans* prefer to be kept in vertical artificial nests to better mimic their natural habitat (supplementary figure 2.1). The newly designed nests were made of dry plaster carved with tunnels and a dozen chambers. Two glass plates of 14 x 24 centimetres and three plastic bar frames of eight millimetres width were holding the nest vertically. For increased stability, each nest was maintained by three metal clips and a three centimetres deep base of dried plaster, in an open plastic box. The top of the nest was concealed by a fourth plastic bar made of two 4.3 x 0.5 centimetres holes covered by a net, to provide air supply. To allow colony observation, the carved tunnels and chambers were made on one side of the plaster and visible through the glass plate. However, to ensure more natural conditions for the colonies, the glass plate was covered with a carton or an opaque plastic, removable for punctual colony assessment. These spacious vertical nests successfully maintained large laboratory colonies of hundred individuals for multiple years.

In 2019, however, a new experiment required the long-term survival of isolated gynes. To save space for hundreds of replicates and ensure the survival of isolated ants, small vertical nests were designed (supplementary figure 2.2). Round, two parted Petri dishes of 94 x 16 millimetres with diametral separator were used. To provide a nest entrance, the plastic line dividing the box in two was melted using a soldering iron over five millimetres in the middle. Plaster was put only in half of the petri dish and carved with a tunnel and two to three chambers. Similarly, to the large vertical nests, a cut microscopic slide of 37 x 25 millimetres was used to cover the carved part of the plaster that mimics the nest. As these small colonies of isolated individuals needed to be assessed daily, a red, transparent plastic, of equal dimensions, was attached to the cut microscopic slide. Petri dishes were enclosed by a perforated lid whose hole had been covered by a net to provide air supply. Nests were kept vertically by a two millimetres plaster base.

Both laboratory and experimental colonies were maintained in incubators (RUMED – Rubarth Apparate GmbH – Types 3001 – 3601, version D/30-36/02-2001 and D/30-35/12-95) set with 12/12 hours of light/darkness to represent days and nights. The temperatures were set following the natural conditions in Remoulins and Beaucaire. Accordingly, decreasing or increasing the temperature up to four degrees, once per week, until reaching respectively; hibernation or summer condition (table 2.1).

Diet preferences of *Cardiocondyla elegans*, in natural conditions, classify the species as opportunistic feeders. Foragers can be observed carrying diverse food sources to their colonies, such as dead insects, seeds, cookie crumbs and plants. They have been observed in contact with flower pistils and drinking out of honey drops deposited on the floor by humans. Thus, the ants were fed twice a week, with an alternation of thawed fruit flies (*Drosophila melanogaster*) and cockroaches' pieces (*Nauphoeta cinerea*). The number of fruit flies or the size of cockroaches' pieces given per nest was adjusted according to the nest's size and the number of individuals. In addition, each nest was given 1 x 1 centimetre of kitchen paper soaked with distilled water and a second 1 x 2 centimetres of folded kitchen paper containing a drop of honey. To prevent the accumulation of fungi in the nest, leftovers from the prior feeding were always removed and replaced with fresh food.

Localised near rivers, *C. elegans*' nests in their natural habitat have high humidity. Lenoir, in his doctoral thesis, reported that the studied population of *C. elegans* in Tours had "0.12 m^3 of water per m^3 of sediments" (Lenoir 2006). According to his research, nests had a higher humidity a few centimetres under the surface and lower the deeper to the ground, for most of the year. During the summer season, this tendency reversed to show a dryer layer right under the surface. Accordingly, both laboratory and experimental nests were humidified once per week by gently pouring distilled water from the top of the nests, through the entrance. The inevitable growth of fungi in the nests helped keep the humidity, thus no distilled water was introduced during the summer season.

	Hibernation temperatures	Summer temperatures
Hours	Degrees	
06 am	5°C	17°C
08 am	7°C	23°C
10 am	10°C	29°C
12 pm	13°C	34°C
06 pm	11°C	28°C
08 pm	9°C	24°C
10 pm	6°C	20°C

Table 2.1. **Artificial winter and summer temperatures.** Daily temperature variation set in the incubators where the ants of *C. elegans* were stored. The temperatures were set following the habitat conditions of the species, in Beaucaire and Remoulins (Gard, Languedoc-Roussillon, France). The ants were kept for a few months at hibernation temperatures during the winter (December to March) and summer temperatures during the summer (June to September). Temperatures were changed by zero to four degrees each week between these two seasons.

2.6. Microsatellite analysis

2.6.1 Protocols

As mentioned in paragraph 1.2, the relatedness between individuals was calculated by estimating their alleles differences over multiple microsatellites located in numerous parts of their genome. To assess and compare the number of paired base repetitions in ants' genomes, the first step was to extract the DeoxyriboNucleic Acid (DNA) of each individual. The DNA was extracted, from each whole ant's body, using the Cetyl TrimethylAmmonium Bromide (CTAB) method (modified from Sambrook & Russel, 2001, for details see supplementary method 2.1) and diluted in 30 µl of TE buffer (Tris- Ethylenediamine tetraacetic acid). To confirm successful DNA extractions, 5 µl of the sample were mixed with 1 µl of loading dye, migrated by electrophoresis on a 1% agarose gel and visualised under UV lights.

Once the DNA was isolated, we needed to target specific regions of the genome where microsatellites were previously recorded. This second step required the use of designed primers to amplify such focus regions of the DNA. Polymerase Chain Reactions (PCRs) were performed in a 20 µl reaction volume using 1 µl of DNA with 19 µl of master-mix (7 µl H2O, 10 µl GoTaq, 1 µl of each forward and reverse primers). Samples were amplified following Lenoir et al. (2005) with an initial denaturation step at 94 °C for 3 min, 40 cycles at 94 °C for 45 s, an annealing temperature set according to the primer for 45 s (supplementary table 2.1), followed by a step at 72 °C for 45 s, and a final extension step at 72 °C for 7 min. Similar to the method used to confirm DNA extraction,

successful PCR was visualised by migrating 1 µl of PCR product in a 1% agarose gel. The visualisation under UV lights of the migrated component informed us, not only on the presence or absence of PCR products but on their quantity as well. The brighter the gel band, the more PCR products were contained in the sample tube.

Ants from 2015 were analysed at a maximum of five microsatellite loci, specifically designed for *Cardiocondyla elegans* in 2005 (CE2-3A, CE2-4A, CE2-4E, CE2-5D and CE2-12D) (Lenoir et al. 2005). In 2018, we aimed to increase the number of microsatellite loci and tested seven existing primers from other *Cardiocondyla* species (Schrempf et al. 2004; Schmidt et al. 2016b) and five universal primers (Butler et al. 2014) (supplementary table 2.2). A primer would be added to our total if the targeted region would show a sufficient allele diversity between individuals of *C. elegans* from different colonies. DNA samples of six ants were compared at each new loci. All the five universal primers showed insufficient diversity. One primer from *C. obscurior* (Cobs 13) (Schmidt et al. 2016b), as well as one primer from *C. bateseii* (Card 8) (Schrempf 2014), were considered relevant to the comparison of relatedness between individuals of *C. elegans* and added to the sum. Starting from 2018, all samples were analysed with seven microsatellite loci (CE2-3A, CE2-4A, CE2-4E, CE2-5D, CE2-12D, Card 8 and Cobs 13) (supplementary table 2.1.).

As a final step, amplified microsatellites regions were sent through a sequencer to determine allele sizes for each locus. 0.1 to 0.3 µl of PCR product (following its detected band thickness in the agarose gel) was mixed with 25.2 µl of ABI master-mix (25 µl formamide and 0.2 µl standard size T486). Allele size was determined using allele migration speed with the sequencer GeneScan® 500 TAMRA dye size standard. Each sample contained 0.1 to 0.3 µl of amplified DNA from a specific microsatellite locus, attached to a (blue, green or black) fluorescent primer and 0.2 µl of red standard size T486. Differently sized DNA fragments, and red standard size, migrated along a small pipe in the formamide. Throughout their migration, a laser coupled with a light detector sensed the passing of each fragment and could detect their fluorescent colour. The given size of each red standard fragment recognised by the sequencer at a given time provided a size calibration. Primers of different fluorescence could be combined in the ABI-master mix and differentiated by the sequencer. A total of three primer combinations were used: CE2-3A, CE2-4E, CE2-12D; CE2-4A, CE2-5D and Card 8, Cobs 13.

GeneScan® 3.1 software (Applied Biosystems) was used to visualise, for each colour, the number of fragments that passed through the laser (supplementary figure 2.3). Due to slight paired based loss of the size standard, allele size of the highest peak approximated the number of paired bases constituting each microsatellite loci. The final number was similarly rounded for all individuals.

2.6.2. Relatedness and inbreeding calculation

Average or pairwise relatedness between nestmates and between gynes and carriers was always calculated following the equation by Queller & Goodnight (Queller and Goodnight 1989). Using mainly the *related* package (Frasier 2018) on R software-4.0.3 and occasionally the software Relatedness v4.2 (Queller and Goodnight 1994). Part of the genetic data from 2015 to 2016 was analysed using the software package GDA (Lewis and Zaykin 2002).

The coefficient of inbreeding (F) for any individual, can be defined by the probability that two alleles at a given locus are identical (homozygous) because of a proportion of related parents in its ancestry lineage. The fixation index or fixation coefficient (F_{ST}) is given by the average expected coefficient of inbreeding in a population. Values of F and F_{ST} were estimated using the software GenAlEx v6.51b2 (Peakall and Smouse 2006). The frequency of matings that occurred with siblings (sib-mating), noted α, was calculated using the equation from (Pamilo 1985): $F_{ST} = \alpha/(4-3\alpha)$.

2.6.3. Multiple carrying

To investigate the possibility that gynes might be carried more than once, the relatedness between gynes and their carrier was compared for early collected pairs and pairs collected at the end of the excursion in 2019. Indeed, at the beginning of the reproduction season gynes and carriers are produced by the same queen, hence have a minimum of 0.25 relatedness coefficient. However, the numerous alien gynes arriving in the nest along the days would increase the chances for a worker to select an unrelated gyne to carry. No pairs were observed in the site RFRK between the 8th and 14th of August. Six first pairs of gyne and carrier were collected between the 14th and 16th of August, at the beginning of the identified reproduction season. 6, 15, 9, 14 and 23 pairs were collected on the 19th, 20th, 21st. 26th and 27th, respectively. Finally, 14 pairs were collected on the 30th of August which was the last day of the excursion to the South of France.

2.8. Worker production

2.8.1. Workers estimate and queen survival

To study the colony founding potential of carried gynes, 155 pairs of gyne and carrier were collected in the RFRK site during the summer of 2019. Gradually, upon their arrival to the laboratory, all pairs were removed from their transport Eppendorf tubes. 112 of the gynes were placed alone in a small vertical plaster nest and 43 of the collected gynes were placed with one or multiple males produced in summer 2019 by five different laboratory colonies from two different excursion years (RFRK9-2016, RFRK132-2016, RFRK300-2016, RFRKC2-2018, RFRK42-2018). All carriers were stored in 100% alcohol for later multiple carrying studies. 16, 13 and 14 gynes were individually placed in temporary vertical nests with either one, two or three males, respectively, for one day. The time necessary for mating was estimated by witnessing males in mating positions after a few hours

grouped with a gyne. Moreover, it has been shown that wing shed in ants is often correlated with mating (Heinze and Tsuji 1995), and gynes of *C. elegans* grouped with at least one male for one day always resulted in wing shedding. In contrast, gynes grouped with their carrier in Eppendorf tubes never shed their wings after one day. After 25 days of transport in Eppendorf tubes, 45% of gynes were dealate. After spending one day with one or multiple males, the gynes were transferred into individual new vertical nests and isolated for the rest of the experiment without the addition of workers nor brood.

The collection of all 155 pairs of gynes and carriers was distributed over 16 days (between the 11th and 30th August 2019). As all pairs were not collected the same day, the ones gathered earlier in the field were transferred to small vertical nests before later collected pairs. Such procedure provided that each pair spent an equal time of 25 days in the transport Eppendorf tubes. Because of possible stress due to handling from tubes to artificial nests, all ants which died between day 1 and day 5 of the experiment were removed from survival curves (21 gynes removed: 14 gynes grouped with a minimum of one male in the laboratory and 7 gynes directly isolated from carrying). Similarly, gynes that died before the first counting of workers were removed from the survival curves (19 gynes removed: 2 gynes grouped with a minimum of one male in the laboratory and 16 gynes directly isolated from carrying).

The survival of the left 134 isolated gynes was monitored daily. To allow ants to experience a reproduction cycle as natural as possible, passing through a hibernation phase (December to March), the experiment extended from September 2019 until November 2020. After hibernation, the apparition of the first workers produced by isolated queens was recorded in May 2019 during the first counting. Since then, worker production was estimated weekly, for 38 consecutive weeks, by counting the number of workers present in each small nest, under a binocular (LEICA – S8AP0: magnifying from x 3.6 to x 30). Once the number of workers reached 30 in such small nests, the amount counted was approximated to ± 5 workers (e.g., 35, 40, 45, …, 105, 110).

2.8.2. Genotyping

After November 2020, at the end of the previously described worker producing experiment, all surviving queens that had produced workers were frozen at -20°C. To determine the number of males that had mated with a queen (patrilines) we manually compared worker and queen genotypes. Conforming to the haplodiploid reproduction system used in ants, a queen will share 50% of her alleles with its daughters, for each microsatellite loci (see purple highlighted alleles in supplementary tables 2.3). The daughters, in this case workers, will be given the other 50% of their alleles by one of the male's genetic data stored in the queen 'spermatheca (see all other colour highlighted alleles in supplementary tables 2.3). Males, being haploid, give 100% of their genetic data to their descendants. Therefore, the number of different colours used for each table gives us the minimum estimated number of males that had mated with the queen. Which male's genotype is used by the

queen to produce workers is not always random, for there exist cases of sperm competition, prior or inside the spermatheca (Parker 1970; Simmons 2002; Aron et al. 2016).

To estimate the number of patrilines, we consider that two males cannot have identical genotypes, which in the case of double mating could lead to a non-detection error. However, according to Lenoir's studies in 2007 in *C. elegans*, based on Chapuisat 1998, the probability that two random males had mated with the same gyne and shared the same genotype, for five polymorphic microsatellite markers was 0.0004 (Chapuisat 1998; Lenoir et al. 2007). Our study increased the microsatellite loci to seven and therefore lowered the already negligible non-detection probability. A total of 18 queens were genotyped, using the genetic data of 8 to 12 produced workers. 11 of these queens had been coupled with one to three males in the laboratory, whose genotypes were also analysed (supplementary tables 2.3).

2.8.3. Exclusive sibling mating

As shown by Lenoir in 2007, gynes can be mated with both brothers and foreign males (Lenoir et al. 2007). To evaluate the reproductive potential of queens mated with siblings only, newly produced gynes and males from the same sealed laboratory colony were grouped in a small vertical nest for eight weeks. Contrary to the previously mentioned experiment, gynes were not individually grouped with one or multiple males. In the exclusive sibling mating experiment, between 10 to 20 gynes per laboratory colony were collected at the same time and gathered in an empty small vertical plaster nest with up to 10 brothers. Therefore, a longer grouping time (October to November 2020) before isolating the gynes helped increase the chances of mating for each future queen. After weeks of grouping with their siblings, a total of 47 gynes from four different laboratory colonies, were transported and isolated in new small vertical nests. All surviving males were killed and discarded. The experiment lasted for 250 days, between November 2020 and August 2021. Survival was monitored similarly to previously described. However, the number of workers produced by surviving gynes was recorded only once in August 2021, at the end of the experiment.

2.8.4. Reproductive status

To investigate a gyne mating status, the spermatheca and ovaries can be isolated from the rest of the ant's body and observed under a microscope. Dissections were realised with the help of fine forceps of 0.05 x 0.02 millimetres tip dimensions, under a binocular microscope (LEICA – MEB126: magnifying from x 10 to x 80). The spermatheca could be recognised by its oval to round shape, attached to the two ovarian tubes which are each composed of three ovaries (figure 2.3). Spermatheca and ovaries were isolated on a microscopic slide from other tissues in one drop of distilled water. The slide was then topped with a slipcover and observed under a microscope (ZEISS – Primo Star + Moticam: magnifying from x 40 to x 600). To detect sperm movement inside the spermatheca, the queen needed to be sacrificed a few seconds prior to dissection by decapitation.

Frozen samples showed the presence of sperm by its brown colour under the microscope (figure 2.3) but as the sperm was no longer viable, no motion could be identified.

In April 2016, 20 gynes were collected by Schrempf from five different collecting sites (BN, CP, H, RFRK, and SM) and dissected to investigate the reproductive status of alien gynes, after hibernation and before their dispersal. From 2019 to 2020, to analyse further the mating success of experimentally isolated gynes, all dead ants were kept in a 1.5 mL Eppendorf tube at -20°C, for later spermatheca analysis.

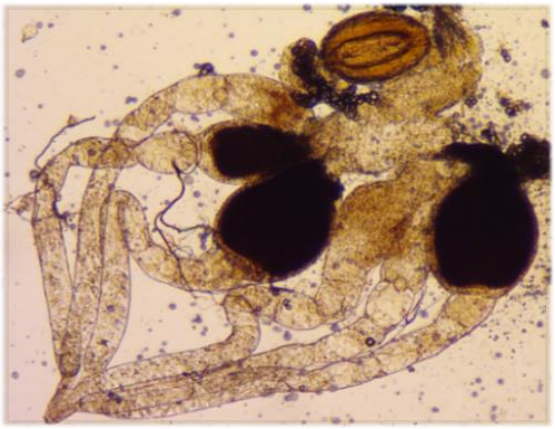

***Figure 2.3. Cardiocondyla elegans* queen reproductive system.** Picture of a spermatheca (brown-orange, oval, in the top) connected to the base of the two ovarian tubes, each constituted of three ovaries. The spermatheca is considered full due to the orange colour given by the presence of sperm. Three of the six ovaries contain, each, an egg in development (black shapes). The picture was taken at x 40 magnification.

2.9. Statistical analyses

The standard deviation of the mean (SD) was calculated using the STEDV.S formula in Microsoft Excel 2021-2103(16.0.13901.20400). The formula calculates the SD as the square root of the variance S^2, estimated from a sample drawn from a population: $S^2 = \frac{\sum_{i=1}^{n}(x_i-\bar{x})^2}{n-1}$. Where x_i is the ith observation from a sample of the population, $\bar{x}$ is the sample mean and n - 1 are the degrees of freedom. The standard error of the mean (SEM or SE) was calculated by dividing the SD by the square root of the sample size. For data from 2015 and 2016, standard errors of means were obtained by jack-knifing by groups. The Mantel test was made using the *vegan* and *geosphere* packages (Hijmans et al. 2019; Oksanen et al. 2020) in R software-4.0.3. Graphics were made using Microsoft Excel 2021-2103 (16.0.13901.20400) (figures 3.2, 3.4 and 3.6) and the *ggplot2* package (Wickham et al. 2021) using R software-4.0.3 (figures 3.3, 3.5, 3.7, 3.8 and 3.10). The survival curves were created using *survival* and *survminer* packages (Alboukadel et al. 2021; Therneau et al. 2021) for R software-4.0.3 (figure 3.9). One and two samples *t*-test, One-way ANOVA and all post hoc tests were done using R software-4.0.3. All R codes are available online (Vidal et al. 2021b).

In 2015, colonies, gynes, and carriers were collected in four sites (BN, CP, RFRK, SM) with an F_{ST} value of 0.042 ± 0.051 (jackknifed over sites). A single sample *t*-test showed that the F_{ST} value was not significantly different from 0 ($t = 0.823$, $p > 0.1$). Therefore, we considered that the four sites belonged to a single population that extends along the rivers. From the years 2018 to 2020 we focused exclusively on the more populated site, RFRK.

3. Results

A recently published research paper (Vidal et al. 2021a) was based on a consequent part of the following results, thus, several graphs and tables presented here will be taken from the publication and properly cited. All raw data are publicly accessible through figshare.com with an Attribution 4.0 International (CC BY 4.0) license (Vidal et al. 2021b).

3.1. Field observations

3.1.1. Colony composition

Laboratory colonies of *C. elegans* showed that the resident queen and brood were kept in the lower chambers of the large vertical nests. During excursions to the South of France, as the soil, composed of sand and stones, was very mobile, the probability of not finding individuals while excavating increased with depth. Schrempf reported a very efficient excavation technique used to dig up to one meter until finding a chamber containing brood and the resident queen. Despite the same technique being used, no resident queen has been found in any excavated nests after 2016.

The reported numbers of individuals per castes and year represent the chance of finding ants during the excavation process (table 3.1). The absence of reported individuals cannot exclude the possibility of having missed them among the sand (non-detection error). Moreover, anecdotal observations made in the laboratory showed that workers situated in the highest region of artificial vertical nests proved to be efficiently moving with increased speed to deeper levels of the nest when they perceived human disturbances.

Nonetheless, the excavation of 171 colonies of *C. elegans* helped confirm the previous observation of the ant in a region farther north in France (Loire) by Lenoir between 2005 and 2007. Regardless of the period nor the year, colonies were composed principally of workers and gynes (table 3.1). During the excavation process, many workers were collected below the nest entrance. Additionally, they were found in tunnels and chambers deeper along the way. Nests were dug up to one meter and composed of multiple chambers distributed vertically. When recorded, the resident queen was found with brood and workers in one of the deepest chambers.

Mating chambers could often be found under a larger stone close to the surface, which presumably helps protect the ants from insolation. These chambers could contain a hundred gynes, numerous workers and a few males (table 3.1). All males were situated in the mating chamber, while gynes could sometimes be collected in deeper chambers in the nest.

In the excursion of 2017 to the South of France, temperatures reached record values of 45°C. Workers were observed foraging above the ground and entering nests. However, the totality of excavation attempts was unsuccessful. Looking for humidity, ants had built their chambers deeper in the ground where it became hard to dig in a mobile sandy soil.

Nest excavations in spring 2016 reported a non-presence of males. The sand being more humid, therefore more compact in April, decreased the probability of non-detection errors. Laboratory colonies confirm the absence of males during spring-like artificial temperatures.

Field collections	# Colonies	Workers	Gynes	Males
September 2014	23	33 ± 33 (2-139)	42 ± 57 (0-253)	1 ± 2 (0-9)
August 2015	26	103 ± 92 (2-318)	160 ± 125 (0-413)	4 ± 4 (0-19)
April 2016	59	56 ± 39 (3-160)	26 ± 30 (0-130)	0
June 2016	45	9 ± 12 (0-50)	3 ± 4 (1-13)	0 ± 0.05 (0-1)
July 2016	54	33 ± 31 (0-150)	13 ± 16 (0-60)	0.1 ± 0.5 (0-2)
August 2018	15	39 ± 22 (15-96)	23 ± 31 (0-123)	2 ± 3 (0-10)
August 2019	10	45 ± 18 (17-65)	26 ± 21 (0-65)	3 ± 3 (0-8)
August 2020	26	66 ± 19 (22-112)	43 ± 34 (0-146)	1 ± 1 (0-5)

Table 3.1. **Composition of colonies of the ant *Cardiocondyla elegans***: Number of colonies of the ant *Cardiocondyla elegans* excavated, number of workers, gynes, and males found in the nest; mean ± SD (range) in different collection years. Periods from July to September are considered as summer and mating seasons. The collection period of April 2016, highlighted in purple, is considered as spring (post hibernation). *Modified from Vidal et. al. CommsBio, 2021.*

3.1.2. Behavioural description

Among the eight excursions, the foraging activity of *C. elegans*' workers was always observed. During the summer season, the behaviour of the foragers depended on the ambient temperature. Dry summer days in the South of France could vary between 20°C at night and the highest 35°C in the early afternoon. Temperatures started decreasing again in the late afternoon. In the early morning (between 7 and 9 a.m., about 24°C) most workers were still in their nests. The few foragers observed outside with cooler temperatures were notably slower than those observed between 10 a.m. and 12 p.m. when the temperatures were about 28-30°C. Similarly, at the same time of the day, in the same site, a slight difference of activity could be recognized between cooler shaded and warmer sunny areas. Anecdotal observations revealed that between 12 and 2 p.m. workers' moving speed increased considerably until the temperatures would reach their maximum. No ants could be observed between 2 and 5 p.m., especially in the sunny areas. Due to their protection from the sweltering heat, shaded areas could still show some limited activity in the middle of the afternoon. Between 6 and 9 p.m. workers were observed outside their nests. During windy days, temperatures in the ground were decreased and workers were observed longer outside their nests. However, highly robust wind or heavy rain would interfere with their normal activities and no ants were recorded outside their nests.

Tandem running between two workers had been observed on numerous occasions. The leading forager would guide the following individual for an extended period in the foraging area. The following ant was seen touching the abdomen of the leading worker with its antennae. If the contact between the two ants were to be lost, the leading ant would wait for the following individual to reinstall it. Few occasions of tandem running led to a food source (e.g., honey drops or flower nectar) but possible other uses of this behaviour by the species, such as teaching newly produced workers to recognise its surroundings, remain undiscovered.

Gyne carrying behaviour was regularly observed throughout most excursions (table 3.2). Workers would carry gynes for several minutes up to a maximum distance of 14.8 meters (n = 182, the mean distance between source and recipient colonies = 3.1 ± SD 0.2 m, range = 0.3–14.8 m) to a recipient colony (figure 3.2). Similar to the technique used by other genera of the subfamily *Myrmicinae* to transport nestmates during nest moving (Möglich and Hölldobler 1974), a worker of *C. elegans* would carry a gyne on its back, holding it by the neck with its mandibles (figure 3.1). Workers seemed to not drop gynes randomly, e.g., into the nearest nest entrance, but instead passed several nests on their way to the selected recipient colony (mean = 2.3 ± SD 0.3 passed nests, n = 57 pairs in five collecting sites; figure 3.2).

A carrier's behaviour towards a gyne was notably different from a forager' attitude. If disturbed, by human tools or other insects, a forager would usually drop the food source it was carrying. Foragers from different colonies have been observed aggressively competing over food but for only a few seconds, the vanquished individual was always observed leaving the area immediately. Cases of carriers dropping a gyne were rare. More commonly, due to intense wind, intertwined branches or being repeatedly physically disturbed with human tools. When such a phenomenon happened, the lost gyne was not trying to come back to its original colony nor to find a way to keep moving. Instead, the dropped gyne could be observed turning in a circle, staying close to the initial dropping zone. The carrier would seem agitated and looking for the gyne, once found, it would immediately carry it back and keep its way to the recipient colony. The gyne's behaviour when dropped unintentionally was similar to the one she would have when delivered to a receiver nest entrance. Aggression from the gyne towards the carrier prior to or post transport had never been recorded.

Cardiocondyla foragers typically search for food on tortuous paths (Creighton and Snelling 1974) Indeed, *C. elegans* foragers have been observed walking in the field seemingly randomly until finding a food source. In contrast to specialised workers that were carrying the gynes in a straighter line to the recipient colony. These were deviating from their targeted direction only when forced by environmental obstacles (e.g., stones or dense and intertwined branches). Anecdotal observations revealed that carriers rarely entered the nest of the recipient colony. After releasing the gynes to the targeted colonies, they always returned to their initial nest with similar speed and direction.

Occasionally, two of three different workers carrying a different gyne have been observed simultaneously outside, leaving or coming back from or to the same nest. All carriers from the same nest commonly targeted the same set of recipient colonies. More commonly, one worker per nest, at a time, was observed carrying a gyne. Shortly after the carrier came back to its original colony, another gyne transport to the same recipient colony could be observed (see supplementary video of Vidal et al. 2021a). At the right temperatures, a source colony could be observed transporting gyne up to 11 times, one pair at a time. As explained in paragraph 2.4.2, pairs of gynes and carriers were collected in the field for later studies in the laboratory. The experimental removal of the currently travelling pair, by aspiration, away from the nest entrance of a recently observed trading colony, always resulted in transport interruption. The source colony would not resume gyne transport until the next day. The fact that carriers were not immediately replaced by other workers suggested that only a few specialised individuals were able to transport gynes at a given time.

Similar to foraging behaviour, carrying instances were recorded at a specific time of the day, depending on the temperatures. High chances of observing a pair of gyne and carrier were recorded almost exclusively between 9 a.m. and 1 p.m. Anecdotal observations revealed that no matter the temperature when transporting gynes, workers were always walking faster than observed foraging speed (with or without food). In summer 2016 no pairs of gyne and carrier were observed (table 3.2), probably due to inadequate temperatures. Only two pairs were observed in September 2014 (table 3.2). Gyne carrying behaviour was generally observed close to the warmest months of the year. Surprisingly, carrying behaviour was abundantly reported in July 2015, whereas, starting from 2017, no pairs could be observed before mid-August (table 3.2). The colonies seemed to have shifted their preferred carrying month, according to the temperature changes in their environment.

A total of 515 pairs of carriers and gynes were observed between 2014 and 2020 in the South of France (table 3.2). Among these pairs, 177 were observed from their source to their recipient colonies. When studies were not conducted on the collected individuals, they were used to create laboratory colonies. Overall, a cumulative number of 210 colonies were mapped (table 3.2). In August 2015, colonies were mapped for all seven sites. From 2018 to 2020 we focused, particularly, on the site RFRK.

All mapped areas were scaled and represented in two dimensions (figure 3.2, Kalb 2016). The multiple arrows representing gyne transfer in this figure show that carriers passed neighbouring colonies on the way to the recipient one. Such gyne distribution appears to be non-random.

In 2015, the total of transfers observed between colonies was lower than for 2018 and 2020. This phenomenon can be explained by researchers' objectives during collection. For the genetic analysis in 2015, the number of nests and sites involved were maximised, over the number of pairs observed per site. From 2018 to 2020 all observations were made in the site RFRK for the entire duration of the expedition.

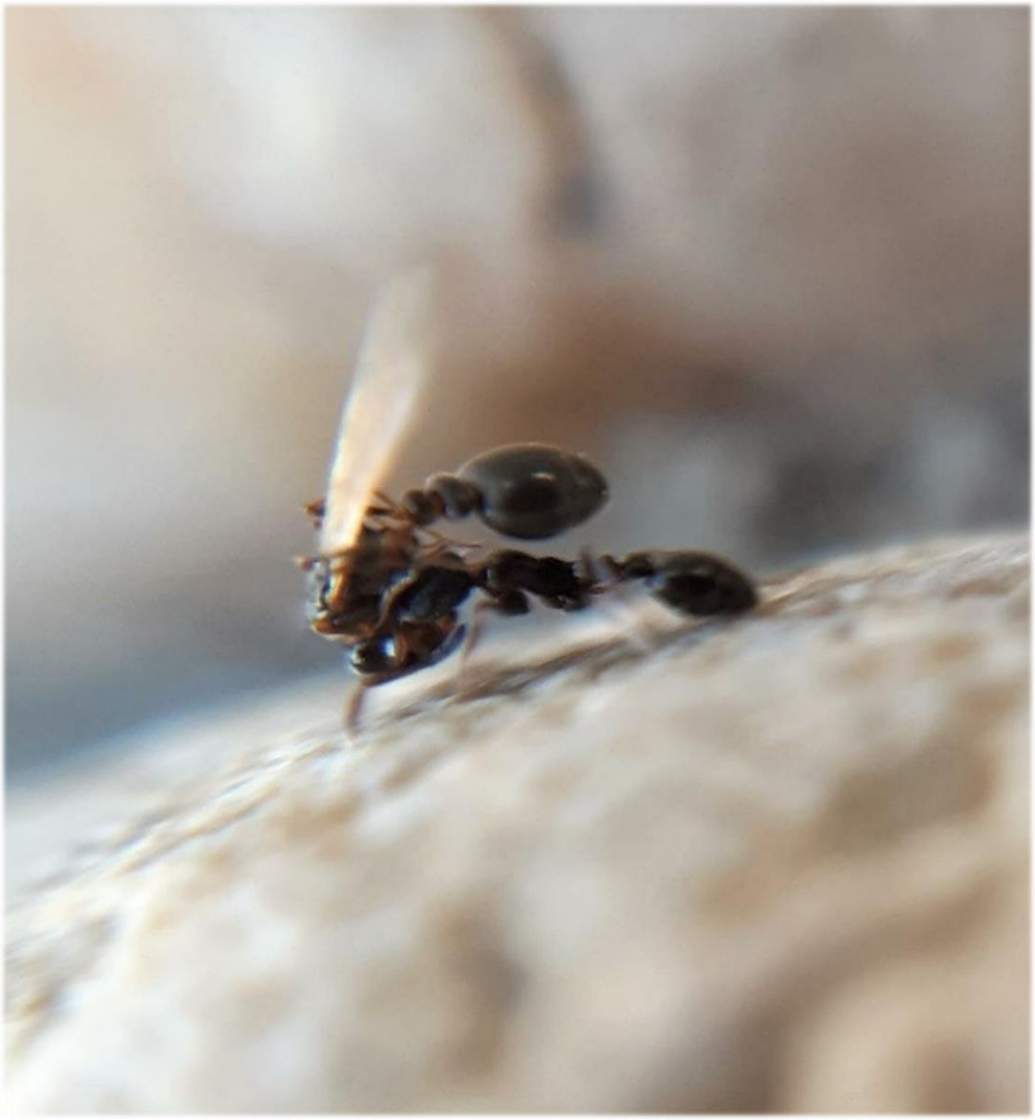

Figure 3.1. **Gyne carrying in the ant *Cardiocondyla elegans*.** The picture shows a worker of the ant *Cardiocondyla elegans* carrying a winged female sexual (gyne) to the nest entrance of another colony to allow outbreeding (photo by M. Vidal). *Modified from Vidal et. al. CommsBio, 2021*.

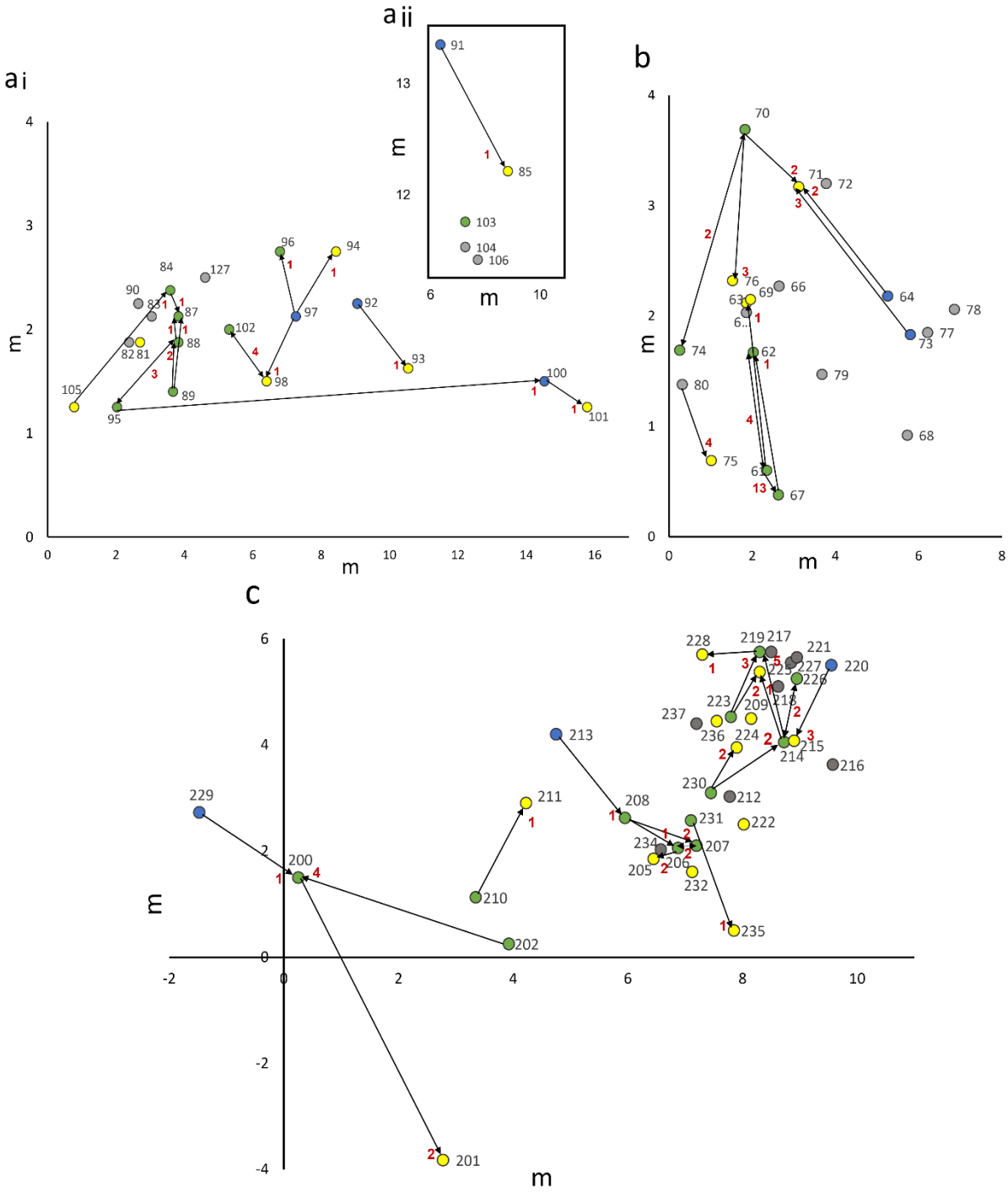

Figure *3.2.* Direction of gyne transport in the ant *Cardiocondyla elegans*. Location of colonies of the ant *Cardiocondyla elegans* in the collecting site RFRK (N 43°55'43.9", E 4°34'5.1") in 2015 (ai and aii), in 2018 (b) and in 2020 (c) in which the transport of female sexuals (gynes) by workers was observed. Shown are the exact localisations (in meters) of colonies used in the microsatellite analysis. Colonies marked by a yellow dot only received carried gynes, colonies marked with blue only donated gynes, and colonies marked in green were both sources and recipients. Colonies not involved in gyne carrying are indicated in grey. Simple arrows indicate the direction of gyne transport. Double arrows indicate transport in both directions. Coloured circles without arrows represent colonies for which the origin or destination of carried gynes could not be determined. The red numbers near the arrows indicate the total number of transfers observed between nests. *Modified from Vidal et. al. CommsBio, 2021*.

Month - Year	September 2014					August 2015						
Population	P	BN	CP	RFRK	SM	P	H	FK	BN	CP	RFRK	SM
Colonies mapped	9	8	8	10	5	18	11	15	21	12	25	7
Pairs of GC observed	0	0	1	0	1	16	4	0	24	16	44	17
Pairs of GC observed with known destination	0	0	0	0	0	16	2	0	19	13	28	5
Pairs of GC collected, with known destination	0	0	0	0	0	0	0	0	25	13	7	4

Month - Year	April 2016				July – August 2016			
Population	BN	CP	RFRK	SM	BN	CP	RFRK	SM
Colonies mapped	0	0	0	0	0	0	0	0
Pairs of GC observed	0	0	1	0	0	0	0	0
Pairs of GC observed with known destination	0	0	0	0	0	0	0	0
Pairs of GC collected, with known destination	0	0	0	0	0	0	0	0

Month - Year	August 2017				August 2018	August 2019	August 2020
Population	BN	CP	RFRK	SM	RFRK	RFRK	RFRF
Colonies mapped	0	0	0	0	20	6	35
Pairs of GC observed	20	10	50	0	82	167	62
Pairs of GC observed with known destination	0	0	0	0	55	3	36
Pairs of GC collected, with known destination	0	0	0	0	0	3	8

Table 3.2. **Colonies and pairs of carriers and gynes sampled in a population of the ant *Cardiocondyla elegans*.** Colonies of the ant *Cardiocondyla elegans* mapped in the collection sites in France and the number of pairs of gyne (G) and carriers (C) that were observed in the field. In 2014 and 2015 several collecting sites were studied, which later were not investigated again. Due to changes in environmental conditions and to the short time range for collection, we focused proprietary on populations BN, CP, SM and RFRK. *Modified from Vidal et. al. CommsBio, 2021*.

3.1.3. Fate of carried gynes

At the end of the mating season, many gynes had been transported to alien colonies. As the temperatures decreased, ants were preparing their nests for hibernation. No gynes have been observed leaving the nest on their own between summer and winter. Schrempf organised an excursion in spring 2016 to analyse nest compositions after hibernation (table 3.1; "April 2016" purple highlight). 59 colonies were excavated and revealed the presence of up to 130 winged or dealate gynes (mean = 25 ± SD 30), but no males. This confirmed that alien gynes hibernated in transported colonies. Occasional observations of solitary winged or dealate gynes dispersing on foot indicated that future queens were leaving alien colonies before the reproduction season. No dispersing gyne was observed flying but rare instances of gynes moving their wings have been recorded. Only one early case of gyne carrying was observed.

Among the excavated gynes, 20 (nine winged and 11 dealate) were dissected. All spermathecae showed the presence of sperm, meaning that all gynes were mated. The dissections even showed developed eggs in the ovaries of 15 of them.

3.2. Genetic analysis

3.2.1. Population structure and estimation of relatedness

Genetic analysis of *C. elegans* from the South of France over the years revealed a relatedness among nestmates similar to the values obtained by Lenoir for the same species in a region farther north of France in 2007 (table 3.3). Single sample *t*-tests rejected the following hypothesis for all four collection years: the mean nestmate relatedness is equal to the value of relatedness among full-sisters (0.75) (2015: $t = -8.5$, $p < 0.0001$, 2018: $t = -7.4$, $p < 0.0001$, 2019: $t = -3.3$, $p = 0.01$, 2020: $t = -8.5$, $p < 0.0001$) and the mean value of fixation coefficient for all loci equal to 0 (2015: $t = 6.8$, $p = 0.003$, 2018: $t = 8.9$, $p = 0.0001$, 2019: $t = 4.1$, $p = 0.007$, 2020: $t = 10.2$, $p < 0.0001$). The mean nestmates relatedness being significantly lower than 0.75 matches with the assumption of monogyny and polyandry. The estimation of sib-mating, using the value of F_{ST}, significantly different from 0, indicates that an average of 69% of the males, that a gyne had mated with, were its brothers (matching 70,4% sib-mating reported by Lenoir in 2007).

As observed during field excursions, distinct nests entrances could sometimes be found a few centimetres from each other (figure 3.2). Despite this potential proximity, there was no evidence for neighbouring nests to be part of the same colony. It appeared that genetic distances between colonies were not significantly correlated with spatial distances (Mantel test; $r = -0.012$, $p = 0.64$, figure 3.3).

	Number of individuals / colonies	Mean nestmate relatedness (± SE)	F_{ST} (± SE)	Sib-mating frequency
2015 (5 loci)	246 / 42	0.49 ± 0.03	0.46 ± 0.07	77%
2018 (7 loci)	119 / 17	0.33 ± 0.06	0.30 ± 0.03	63%
2019 (7 loci)	42 / 6	0.49 ± 0.08	0.43 ± 0.10	75%
2020 (7 loci)	113 / 16	0.39 ± 0.04	0.28 ± 0.03	61%

Table 3.3. **Genetic composition of colonies of the ant *Cardiocondyla elegans*.** Mean nestmate relatedness, fixation coefficient (F_{ST}) and sib-mating frequency in populations of the ant *Cardiocondyla elegans* among four collecting years. *Modified from Vidal et. al. CommsBio, 2021.*

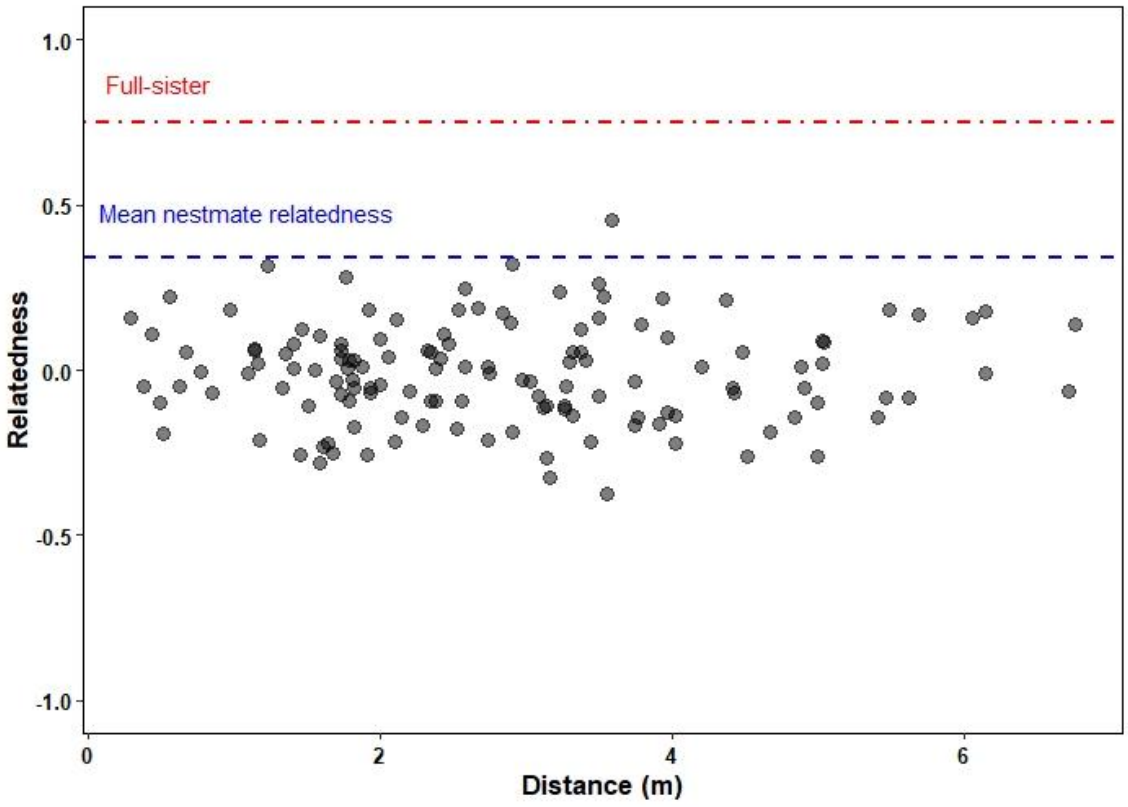

Figure 3.3. Absence of correlation **between genetic relatedness and spatial distance among nests in a population of the ant *Cardiocondyla elegans*.** Association between genetic relatedness and spatial distance among 16 colonies (n = 7 workers per colony) of the ant *C. elegans* in collecting site RFRK in 2018. The red dashed and dotted line represents the value of relatedness expected for full sisters (0.75), the blue dashed line represents the empirically determined mean relatedness among nestmates (2018: 0.33 ± 0.06, see table 3.3). *Taken from Vidal et. al. CommsBio, 2021.*

3.2.2. Original nest and targeted recipient colonies

The mean relatedness between gyne and carrier ± SE (2015: 0.34 ± 0.08, 2019: 0.39 ± 0.03, 2020: 0.23 ± 0.08) was calculated for 139 collected pairs between 2015 and 2020, over the sites CP, BN, SM and RFRK (table 3.4 and figure 3.4). Statistics (single sample *t*-tests) revealed that, for each year, the mean relatedness between gyne and carrier was significantly lower than the value of relatedness between full-sisters (0.75) but not lower than the value of relatedness between half-sisters (0.25) (table 3.5). This suggested that, on average, the relatedness between gyne and carrier was in the ranged of relatedness between nestmates of a monogynous and polyandrous ant species (figure 1.2). We concluded that, on average, workers carry related gynes. However, as shown in figure 3.4, the distribution of relatedness between carrier and gyne can vary from -0.8 to 1. The fact that some pairs of worker and gyne are under the half-sister threshold in figure 3.4 and the low values of relatedness between gynes and donor colonies in 2020 (table 3.4), indicates the possibility for gynes to be carried more than once.

51 pairs of moving gyne and carrier were collected with known source and recipient colonies (2015: n = 40, 2019: n = 3, 2020: n = 8, table 3.4 and figure 3.4). Similar to the mean relatedness between gyne and carrier, the mean relatedness between gyne or carrier to workers of the source colony ranged, for all years, between relatedness values of half and full sisters (table 3.5). In contrast, for all three years, the mean relatedness of both carrier and gyne to workers from recipient colonies were not significantly different from 0 (table 3.5). Moreover, genetic relationship representation in figure 3.4 helped visualise that in both a and b, values of relatedness between individual and source colony (blue dots) were principally above the half-sister threshold. This suggested that workers commonly carry their sisters away from their initial colony to an averagely non-related neighbouring colony.

Comparably as for the relatedness between gyne and carrier to workers of their recipient colony, the mean relatedness of both gyne and carrier to workers from non-targeted colonies did not differ significantly from 0 for all three years (table 3.5). Therefore, gynes are not significantly more related to non-targeted colonies than they are to the recipient ones. In other words, the selection of a targeted colony by a carrier does not seem to be done following the gyne' relatedness to it.

Mean relatedness ± SE	Gyne or carrier	Source colony	Recipient colony	Non-targeted colonies
	2015			
Gyne to	0.34 ± 0.08	0.33 ± 0.05	-0.07 ± 0.05	-0.06 ± 0.04
n =	40	40	40	15
Carrier to	0.34 ± 0.08	0.29 ± 0.06	-0.04 ± 0.04	NA
n =	40	40	40	0
	2019			
Gyne to	0.39 ± 0.03	0.29 ± 0.14	0.03 ± 0.21	-0.03 ± 0.03
n =	91	3	3	3
Carrier to	0.39 ± 0.03	0.43 ± 0.04	-0.20 ± 0.14	-0.09 ± 0.04
n =	91	3	3	3
	2020			
Gyne to	0.23 ± 0.08	0.21 ± 0.06	-0.04 ± 0.07	-0.06 ± 0.02
n =	8	8	8	8
Carrier to	0.23 ± 0.08	0.29 ± 0.04	-0.14 ± 0.08	-0.12 ± 0.02
n =	8	8	8	8

Table 3.4. **Mean relatedness from the pair of gyne and carrier to source, recipient, and non-targeted colonies.** Mean pairwise relatedness per year between gyne or carrier and workers from source, recipient, and non-targeted colonies. n = represents for each relatedness value the number of gynes or carriers analysed against 5 – 7 workers per source, recipient, and non-targeted colonies. We considered an average of 3 (range: 1-5) non-targeted colonies per transport.

(A) Relatedness between	Gyne and carrier	Gyne to source	Carrier to source
Single sample *t*-test		**2015**	
H_o: μ = 0.75 **H_1: μ < 0.75**	t = -5.3 p < 0.001	t = -8.3 p < 0.001	t = -7.1 p < 0.001
H_o: μ = 0.25 **H_1: μ < 0.25**	t = 1.2 p = 0.89	t = 1.6 p = 0.94	t = 0.68 p = 0.75
		2019	
H_o: μ = 0.75 **H_1: μ < 0.75**	t = 11.6 p < 0.001	t = -3.3 p = 0.04	t = -7.3 p = 0.009
H_o: μ = 0.25 **H_1: μ < 0.25**	t = 4.6 p = 1	t = 0.16 p = 0.56	t = 4.04 p = 0.97
		2020	
H_o: μ = 0.75 **H_1: μ < 0.75**	t = -6.6 p < 0.001	t = -9.2 p < 0.001	t = -11.8 p < 0.001
H_o: μ = 0.25 **H_1: μ < 0.25**	t = -0.22 p = 0.41	t = -0.68 p = 0.26	t = -1.1 p = 0.85

(B) Relatedness between	Gyne to recipient	Carrier to recipient	Gyne to non-targeted	Carrier to non-targeted
Single sample *t*-test		**2015**		
H_o: μ = 0	t = -1.4 p = 0.17	t = -0.9 p = 0.36	t = -0.69 p = 0.50	NA
		2019		
H_o: μ = 0	t = 0.13 p = 0.92	t = -1.5 p = 0.27	t = -1.7 p = 0.23	t = -0.86 p = 0.48
		2020		
H_o: μ = 0	t = -0.6 p = 0.59	t = -1.8 p = 0.12	t = -1.3 p = 0.22	t = -1.8 p = 0.11

Table 3.5. **Statistical calculations.** Tables A and B show results of single sample *t*-test examining whether the mean relatedness per year between gyne or carrier and workers from source (A), recipient, and non-targeted colonies (B), is statistically lower than the value of full-sister relatedness (0.75), higher than the value of half-sister relatedness (0.25) (A) or different from 0 (B). μ represent the mean relatedness per group. H_o and H_1 represent, respectively, the null and alternative hypotheses. We reject the null hypothesis and accept the eventual alternative hypothesis when the *p*-value is inferior or equal to the significance level of 0.05. Values of *t* are the calculated differences between groups, represented in units of standard error.

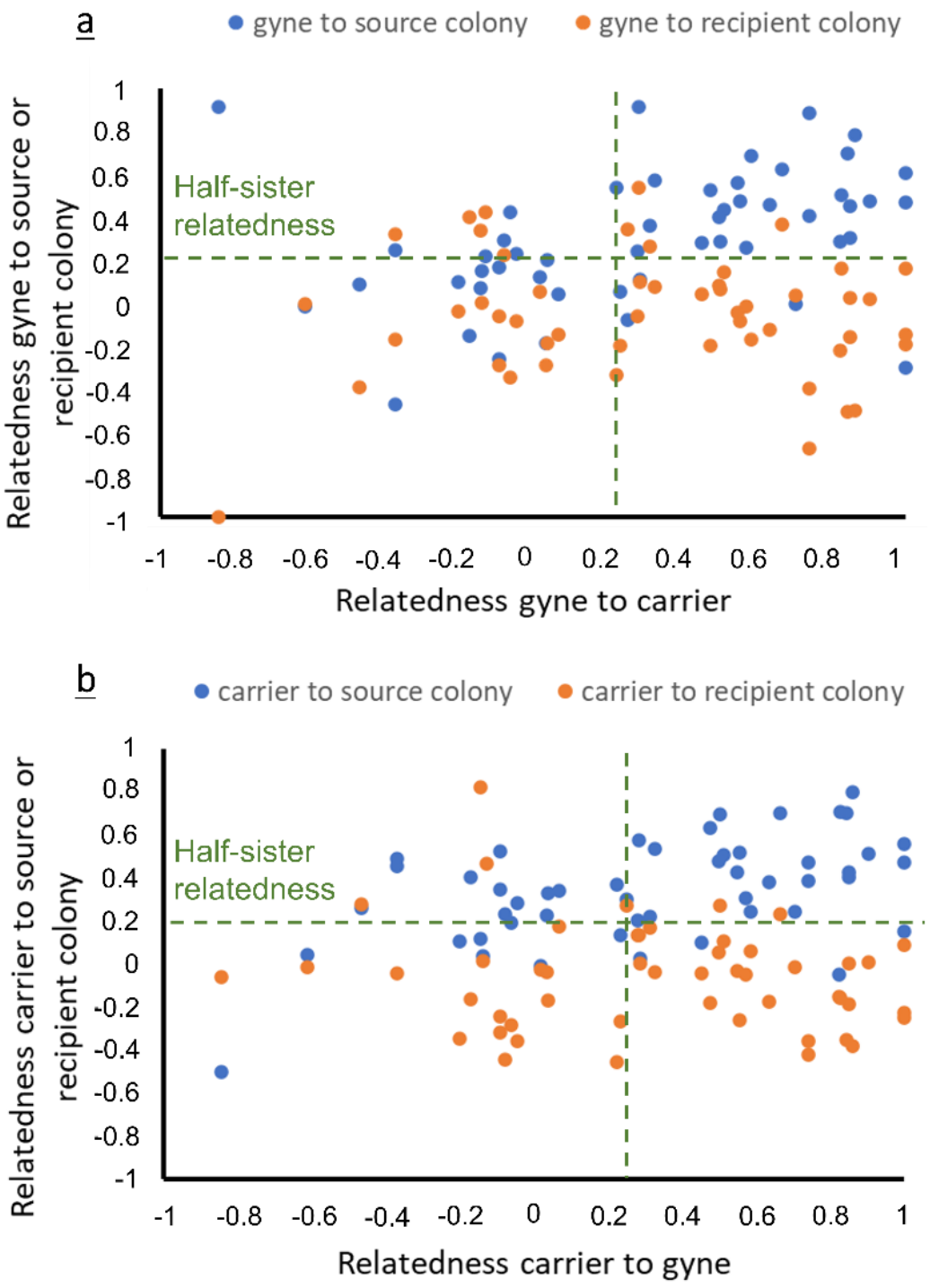

Figure 3.4. **Genetic relationship of gynes and carriers to source and recipient colonies**. Association of the relatedness of gynes (a) and of carriers (b), which were collected during gyne carrying, to the source colony (blue) and the recipient colony (brown) with the relatedness between gyne and carrier (n = 51 pairs of gyne and carrier collected in 2015, 2019 and 2020). Green dashed lines represent the value of relatedness between half-sisters (0.25). *Modified from Vidal et. al. CommsBio, 2021.*

3.2.3. Multiple carrying

With a maximum observation of 167 pairs of gyne and carrier in August 2019 (table 3.2), by the end of the reproduction period, numerous nests contain alien gynes in their mating chambers. The genetic analysis of two *C. elegans* nests in the Loire in 2007 showed that respectively 40% and 100% of all gynes were alien females transported from another colony (Lenoir et al. 2007). To investigate the possibility that workers might also carry alien gynes to targeted recipient colonies, the mean relatedness between carried gynes and their carrier was compared for seven different collection dates (figure 3.5). When comparing the two extreme collection dates: the 15th and the 30th of August, the mean relatedness between individuals of a pair is significantly different (two samples *t*-test; *t*-value = 3.03, df = 25, *p*-value = 0.005). However, the mean relatedness between gynes and carriers stays high until the 27th of August.

Because of haplodiploidy in ants and the polyandry in *C. elegans*, the mean relatedness between gyne and carrier ± SE (2015: 0.34 ± 0.08, 2019: 0.39 ± 0.03, 2020: 0.23 ± 0.08; table 3.4) can be ranged between the value of relatedness of half and full-sisters (0.25 to 0.75, figure 1.2, (Lenoir 2006)). As such, gynes and carriers can be considered related (e.g., produced by the same queen) as soon as the value of relatedness between the two is equal or higher than 0.25. However, the first representation in figure 3.5 does not show the number of pairs related to each other but the mean relatedness of all pairs collected the same day. To better visualise the multiple carrying hypothesis, we calculated the probabilities of encountering a non-related pair (with a value of relatedness under 0.25) over all collected couples, for each collection date (figure 3.6). Such representation suggests that the probability of collecting a non-related pair of gyne and carrier, during the reproduction season, increases with time.

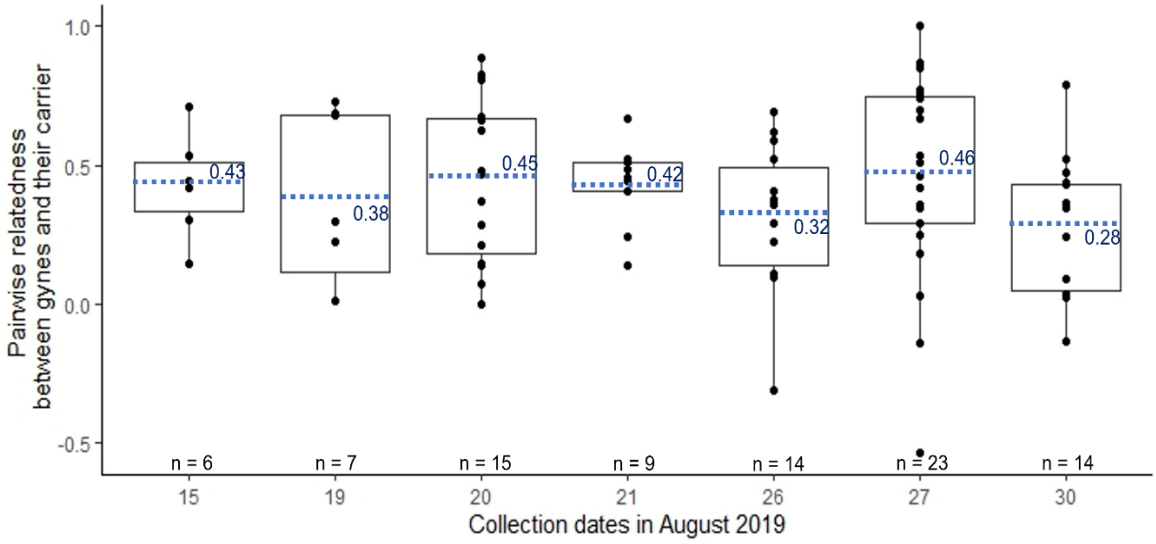

Figure 3.5. **Fluctuations of relatedness between carriers and gynes.** Values of pairwise relatedness between gynes and their carrier for seven different collection dates in August 2019, at the site RFRK (n = number of gyne and carrier pairs collected). Each boxplot contains between 25% and 75% of the data. The blue dashed line and the blue number, represent the mean values of pairwise relatedness for each date.

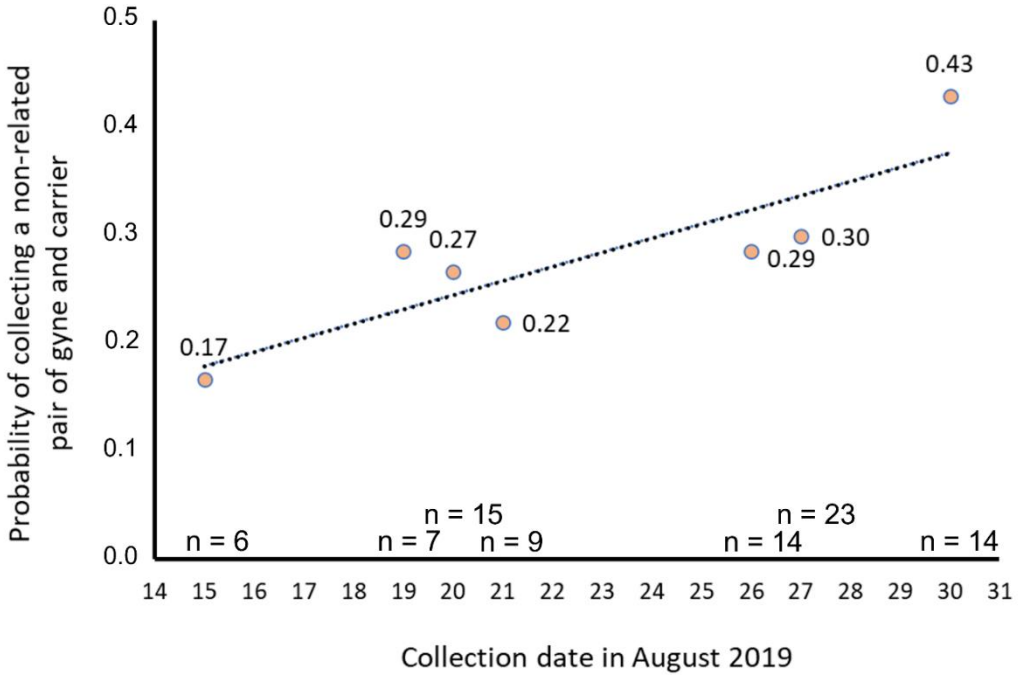

Figure 3.6. **Time variation of the probability to collect non-related pairs of carrier and gyne.** Calculated probabilities to collect a pair of gyne and carrier with a relatedness lower than half-sister (0.25) for each collection date in August 2019, in the RFRK site. n represents the number of pairs used to calculate the probability. The coefficient of determination R^2=0.7303 is represented by a linear trendline. Pearson correlation test gave a *p*-value = 0.01, at the 5% significance level, we rejected the null hypothesis of no correlation. We, therefore, conclude that there is a significant linear relationship between the probability to collect non-related pairs and the time of collection.

3.3. Worker production

A total of 155 gynes were collected while being carried by a worker, 43 were grouped in the laboratory with up to three unrelated males. 112 gynes were isolated without extra-induced mating. Worker production of isolated gynes was recorded from May on, after artificial hibernation, every week for a total of 38 weeks. Among the gynes that survived until the first worker counting, 88% of laboratory mated queens produced workers, against 16% for gynes isolated immediately from being carried (table 3.6). Interestingly, 10 of the 13 gynes isolated without induced-mating, which produced workers, had already shed their wings in the Eppendorf tube during the France-Germany travel.

Spermatheca observations of all 43 laboratory-mated gynes revealed the absence of sperm in only four cases. Two of them died before the first counting while the second half did not produce any workers. Out of the 43, the spermathecae of 9 gynes, dead before counting, could not be found. Similarly, 54 spermathecae out of 112 gynes isolated immediately after being carried were not found during dissections. Out of the 58 remaining gynes, 21 had sperm in their spermatheca (with 13 of them having produced workers) and 37 had empty spermathecae. In August 2015, the genital apparatus of eight carried and four solitarily moving gynes were dissected. Sperm was present in six carried and all four solitary queens.

	Number of gynes	Percentage of gynes surviving until first counting	Percentage of gynes producing workers
Mated in the laboratory	43	60%	88%
Non-mated in the laboratory	112	74%	16%

Table 3.6. **Worker production of carried gynes with or without induced mating**. Number of gynes isolated (for a maximum of 38 weeks) from each of the two categories: mated or non-mated in the laboratory. All gynes were previously collected while being carried by a worker, in the site RFRK in August 2019. The first worker counting was done in May 2019, four weeks after hibernation. Gynes that had produced a minimum of one worker before dying were included in the percentage of gynes producing workers.

3.3.1. Genotyping results

For each queen (gyne which had produced workers), the number of patrilines was calculated by the quantity of different male genotypes found by analysing worker genetic material (supplementary 2.3). The calculated mean number of patrilines for gynes mated or non-mated in the laboratory ± SD = 3.3 ± 2.3 n = 12 and 4.3 ± 1.6 n = 6, respectively. Gynes coupled with unrelated males in the laboratory did not significantly mate with more males than gynes being carried in the field (two-sample *t*-test, $t = 1.2$, p-value = 0.3). The genotyping of gynes to which we induced mating in captivity showed that, almost always, regardless of the number of males presented to them, after one day of grouping only one male had mated with the gyne. Only two cases of laboratory-induced mating showed that two males had mated with the gynes after one day of grouping (supplementary tables 2.3).

3.3.2. Number of patrilines' influence on worker production

Given the queen genotyping results, we compared the number of workers produced by 18 queens, according to their estimated number of males mated (patrilines). Isolated gynes were given time to hibernate (December to March) before the first counting of the number of workers produced (May). Statistical analysis revealed that the number of patrilines significantly influences the number of workers produced (linear regression model, p-value < 0.001). 38 weeks after the first counting, queens that were estimated to have mated with only one male had produced more workers than the one with an estimated seven patrilines (figure 3.7). Compared to single-mating, multiple-mating in *C.elegans* queens significantly decreased the number of workers produced after 38 weeks (figure 3.8, linear regression model, F-statistic = 12.61, p-value < 0.001). An increased number of matings appeared to have a negative effect on the first steps of colony foundation.

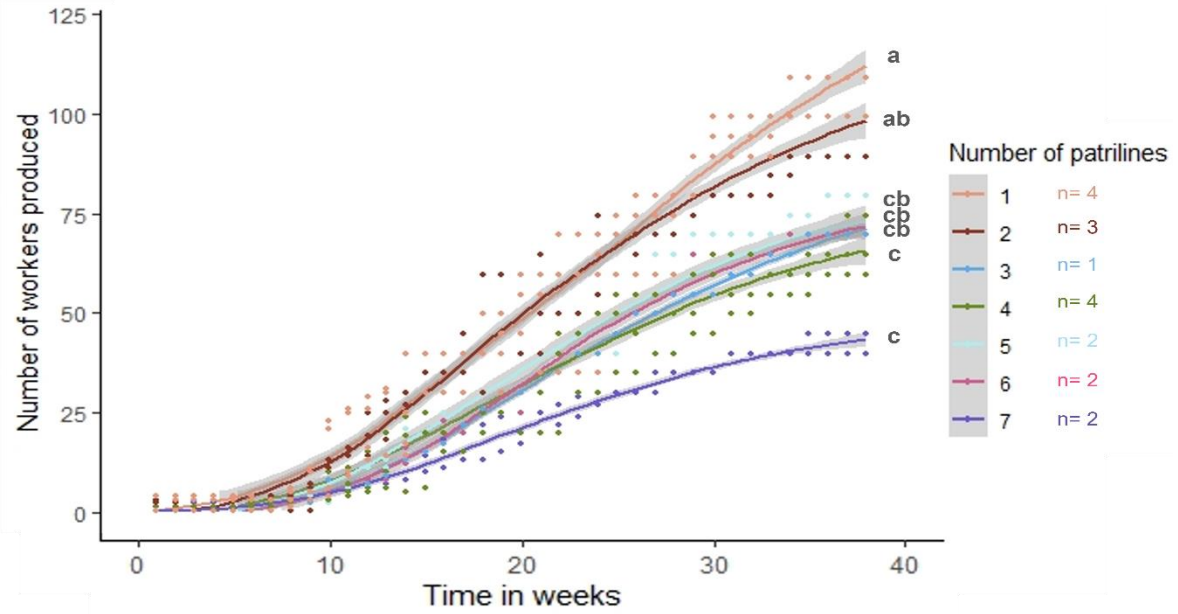

***Figure 3.7*. Influence of the number of patrilines on the worker production.** Number of workers produced per week, for 18 isolated queens, for 38 weeks, two months after hibernation. Each queen was taken from the site RKRF in 2019 while being carried by a worker. Queens were grouped according to the number of patrilines (from 1 to 7) estimated via genotyping (see supplementary tables 2.3). n represents the number of queens in each group. Letters a, b and c represent significant differences between the means, with a 95% family-wise confidence level (multiple comparisons of means: Tukey Contrasts, supplementary figure 3.1).

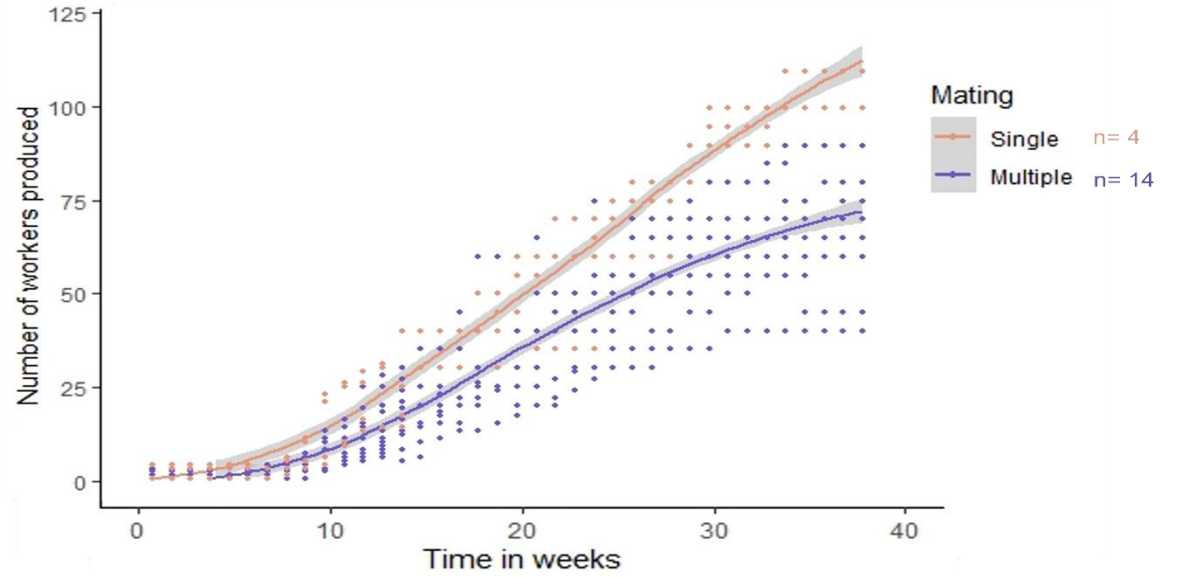

***Figure 3.8*. Influence of the mating condition on worker production.** Number of workers produced per week, for 18 isolated queens, during 38 weeks, two months after hibernation. Each queen was taken from the site RKRF in 2019 while being carried by a worker. Queens were grouped according to being singly or multiply mated, estimated via genotyping (see supplementary tables 2.3). n represents the number of queens in each group.

3.3.3. Survival probability

Using the results of worker production by isolated gynes, two survival curves were represented (figure 3.9). The probability of survival of all 80 gynes which did not produce workers was compared to the probability of survival of all 36 queens which had produced at least one worker. Only four out of the 80 isolated gynes which did not produce workers, had been grouped with unrelated males in the laboratory. Therefore, 95% of the gynes present in the "no worker produced" group had been isolated immediately after being carried. The "worker produced" group contains the remaining gynes to which mating was laboratory-induced and 13 gynes isolated directly after being carried. Interestingly, 10 out of these 13 worker-producing gynes had already shed their wing prior to being isolated without lab-induced mating. The curves and *p*-value both attest to a significant survival probability difference between the two groups. As it is often depicted in eusocial insects (Keller and Genoud 1997) reproductive individuals live longer than non-reproductive ones.

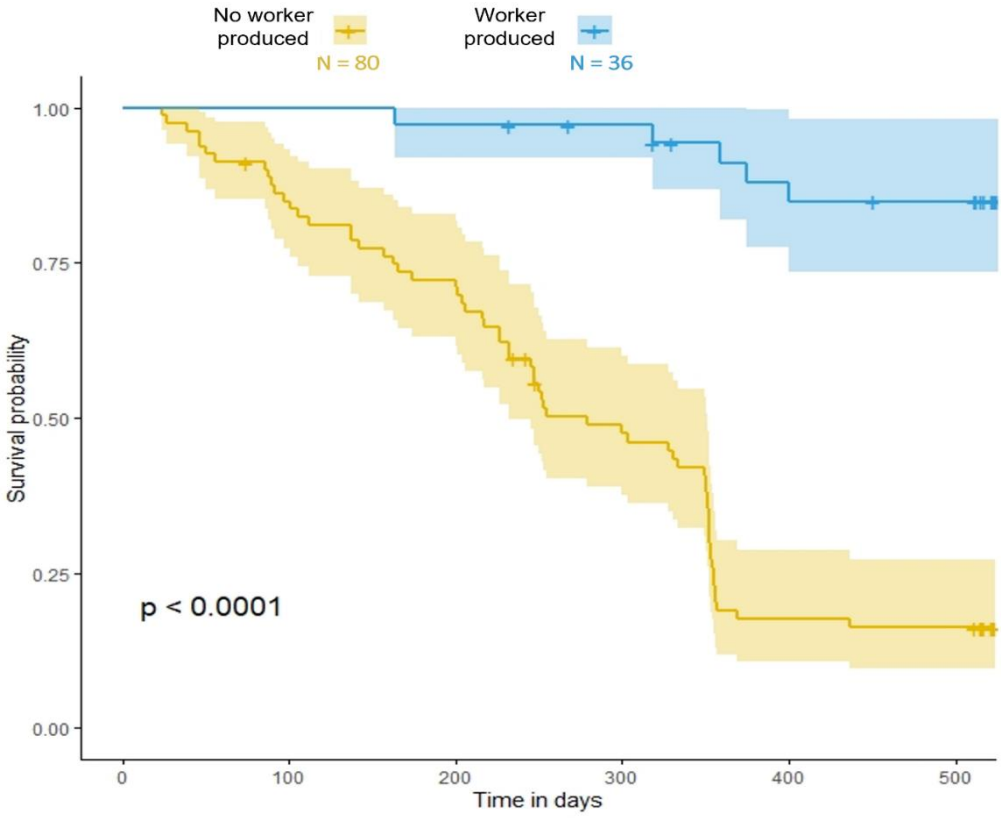

Figure 3.9. **Survival curves *Cardiocondyla elegans*' queens, according to their reproductive status.** Survival probability of isolated gynes, according to their worker production. The worker production was assessed at the end of the experiment (530 days). A queen (gyne that had produced workers) was counted in the worker-produced group if it had produced a minimum of one worker. The *p*-value < 0.0001 represents the log-rank *p*-value from the score test for the entire curve.

3.3.4. Exclusive sibling mating

Siblings' sexual individuals were grouped to investigate the capability for *Cardiocondyla elegans*' queen to produce workers while only mated with brothers. The experiment ended after 250 days of gyne isolation. Among the 47 sibling-mated gynes isolated, 22 were alive after hibernation. At the end of the experiment, all 22 gynes had produced from 5 to 32 workers. This result revealed that queens of *Cardiocondyla elegans* can produce worker offspring without requiring sperm from males of a different genetic lineage.

The number of workers produced by queens mated exclusively with brothers was only recorded once, in August 2020, two months after hibernation. To compare the number of workers produced by exclusive sibling mating with multiply and singly mated queens, we used the values of worker production of August 2019 (similarly, two months after hibernation) for 18 queens with a known estimated number of patrilines (see figure 3.7 and 3.8). Such comparison revealed that, at a given time, queens multiply mated had produced significantly fewer workers than singly mated queens (as seen in figure 3.8) but also significantly fewer workers than queens mated exclusively with brothers (figure 3.10).

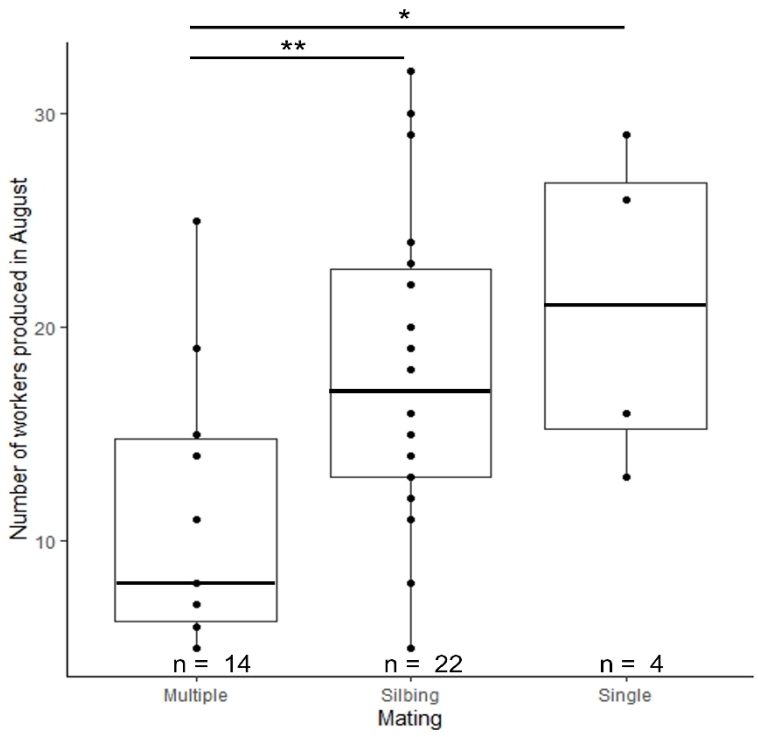

Figure 3.10. **Influence of the type of mating on the worker production of queens.** Comparison of the number of workers produced by multiply mated queens ("multiple mating": mated with a minimum of two males and can include both related and unrelated males), by inbred queens mated exclusively with brothers ("sibling mating": unknown number of matings) and by singly mated queens ("single mating": mated with only one unrelated male). Each boxplot contains between 25% and 75% of the data and n represents the number of queens in each group. One-way ANOVA revealed that at least one group was significantly different from the others (*p*-value = 0.01). Significance between groups is given by Tukey post hoc tests (*p*-value signification code : * 0.1 ** 0.05).

4. Discussion

4.1. Influence of gyne transports on colonies' fitness

To avoid inbreeding depression, organisms have evolved different strategies which prevent or decrease the potentiality to mate with siblings. From sex-biased dispersal and delayed maturity to kin recognition. In the case of *Cardiocondyla elegans*, which only contains intranidal mating between ergatoid (wingless) individuals, reaching enough outbreeding is achieved by workers' action to carry young queens (gynes) from their joint natal nest to another.

The high coefficient of inbreeding found in the Southern and North-western France population (respectively 69% and 70% of all matings involve siblings, see paragraph 3.2.1, table 3.3 and Lenoir et al. 2007), as well as the confirmed polyandry (average number of males mated with a queen ± SD = 3.6 ± 2.1 (our study) and 4.52 ± 1.6 (Lenoir et al. 2007)), indicated that young gynes have a high chance of mating with siblings in their natal nest. Behavioural observations and the comparison of mean relatedness between gynes, carrier, source, and recipient colony, revealed that specialised workers of *C. elegans* carry their sexual sisters for several meters, in the most direct path possible, to selected and seemingly unrelated recipient colonies. There, workers drop the carried gyne into the chosen nest entrance, where the newly arrived young sexual can go to a mating chamber, under the surface, to mate with non-sibling males. This gyne carrying behaviour is thought to be responsible for the left 30-31% of outbreeding.

Moreover, according to Lenoir's study of the *Cardiocondyla elegans*' habitat in 2006, the impact of floods during autumn and cold temperatures during the winter influenced individual survival from one year to the other. Lenoir's study reported that 40% of nests disappeared over the winter. Due to this environmental challenge, for ants of this species to nest, the soil must be composed of a specific grain size such as alluvial deposits, possibly to create air cavities in case of floods. Environmental studies of *C. elegans*' habitat in the South of France are yet to be conducted to confirm similar flooding recurrence. A summary of hydraulic data made public by the French Ministry of Ecological Transition showed an average interannual flow of the Rhône (river close to locations C and SM) near Beaucaire almost twice as high in February (maximum) as it is in August (minimum). Similarly, hydraulic data revealed an average interannual flow of the Gardon (river bordering all seven sites), almost 16 times higher in January (maximum) than in July (minimum). If such information needs to be confirmed by accessing the full data in the ministry archives, the existence of flow variations in these rivers suggests the possibility of floods during the winter. Some sites like P and SM might originally have been located very close to either the Rhône or the Gardon before floods relocated them further away from the rivers. Eventually, intense droughts might have been responsible for their disappearance. The remaining sites that are located near a river might still be subject to flooding. Hence, more than for outbreeding purposes alone, it is easy to imagine the

additional benefit of gyne dispersion. Facing a possible loss over the winter, the transport of gynes in different nests could increase the chances that some of them survive the floods, thus, enhance the inclusive fitness of the individuals in the source colony.

For some ants species with "social hybridogenesis", inbred matings only lead to the production of future queens (by thelytokous parthenogenesis, which is the production of female individuals from unfertilised eggs) and cannot produce worker offspring without the genetic contribution from a different lineage (Volny and Gordon 2002; Julian et al. 2002; Fournier et al. 2005; Ohkawara et al. 2006; Kuhn et al. 2020). In contrast, our study showed that queens of *C. elegans* are able to produce worker offspring after mating with brothers only. The long-term fitness of the inbred individuals produced, however, stays unknown. The fact that such a costly carrying behaviour was maintained over the generations, nonetheless, suggests that the benefits given by the increased genetic diversity as well as spatial dispersion are valuable.

The excursion made by Schrempf in spring 2016 confirmed that the alien gynes, previously transported to a recipient nest, hibernate in the receiving colony. As we mentioned earlier, workers of *Cardiocondyla elegans* do not tolerate additional egg laying than the one from their established queen. As such, mated alien gynes might spend the winter in the receiving colony but will be forced to disperse by the time that their ovaries start sending signals of maturation (Heinze 2017) e.g. (Monnin et al. 1998; Heinze et al. 2002; Hartmann et al. 2005).

Whether future queens use their wings to disperse after the winter is still unclear. As Seifert described in 2003, it is possible to observe a discrete gyne dimorphism in wing length. One group of gyne having weaker flight muscles and shorter wings than the second group. The first one (microsomatic) would be expected to disperse by foot, due to their incapability to flight, and the second (macrosomatic) via the air for a farther dispersal (Seifert 2003). Seifert considered the wings length and muscles of macrosomatic gynes of *C. elegans* viable for small flight dispersion after hibernation. To this day, neither gyne dimorphism in wing length nor flight dispersion had been observed in our studied populations. However, in March 2006, Lenoir and Mercier reported the observation of solitary flying gynes which would shed their wings and found a new colony once they reached the ground (Lenoir 2006). If the possibility of dispersion flight exists for gynes of *C. elegans*, the field collection of April 2016 might have been already too late to observe such behaviour and only gynes incapable of flying were left to disperse by foot. In this case, wings would not only be a vestigial feature, costing a lot of energy to produce but also a tool for enhanced dispersion. To add to the possible increase in gyne dispersal, wings are also very advantageous for the gyne's protection against the sun during their transport by workers.

4.2. Third-party involvement in reproduction

Over the years, multiple studies focused on the influence of sexual selection in sexual behaviour but mainly directed their attention on male's developed traits to mate with the female (Darwin 1871; Bateman 1948). It has recently become clear that females might also develop particular phenotypes to reach access to males (Bonduriansky 2001; Edward and Chapman 2011). In the case of *Cardiocondyla elegans*' reproduction strategy, males are mostly stationary and females are the ones that need to travel in order to reach suitable mating partners. The risks or possible costs of such behaviour affect the survival of gynes rather than males.

The increased visibility and intrinsic danger for a gyne to be carried outside its nest must have a positive impact on the long-term fitness of the individuals. From a natural selection point of view, for such a behaviour to evolve and sustain, its benefits for the individuals must outweigh the risks of predations (Darwin 1871). Our study revealed important positive outcomes for the species survival such as an increase in outbreeding, which enhances genetic diversity, and the dispersal of sexual individuals, to promote the survival of related gynes in case of floods.

However, the case of *Cardiocondyla elegans* is more complex than any female secondary sexual trait, for it involves the participation of a third-party individual: the worker. While such a third party involvement in sexual selection would be natural for zoophilic plants, which need the participation of a pollinator to achieve reproduction (Willson 1990), it is a rare case in the animal kingdom. It can only be found in humans, through matchmaking and arranged marriages leading to cultural exogamy and intermarriage (Walker et al. 2011). The topic, however, was mentioned by researchers working on eusocial insects, like ants. As of today, third-party involvement in the reproduction of sexual individuals had been suspected in few ant species when matings occur inside the nest. In this condition, workers can have an influence on the selection of a mating partner for the young queens by aggressively refusing or granting access to chosen males. This phenomenon was observed in Argentine ants (*Linepithema humile*), where workers would specifically attack, therefore forbid the entrance to males coming from a different supercolony (Sunamura et al. 2011). Likewise, third-party influence on mating partner selection was described in *Cataglyphis cursor*, where workers directed stronger attacks on large males, therefore favouring the reproduction of smaller males (Cronin et al. 2011; Helft et al. 2015, 2016). Similarly, studies on Army ants (*Eciton*) and Legionary ants (*Onychomyrmex* and *Leptanilla*) revealed the strong influence of workers on deciding which gyne will become the future queen and which males will have access to mating, all according to potential fitness (Hölldobler 2016).

All these examples and the recent case of *C. elegans*' workers' influence on specific nest choosing, therefore male selection, suggests that third-party influence on reproduction might be more common than expected in eusocial individuals. Inclusive fitness described by Hamilton's rule and the haplodiploidy of ants would likely justify such a phenomenon. Indeed, in the point of view of the

altruistic individual (the sterile worker) enhancing mating success and offspring survival of their close related kin will increase its own inclusive fitness. Therefore, the possible influence of workers in selecting a mating partner is justifiable from a natural selection point of view.

4.3. Multiple carrying hypothesis

The distribution of relatedness between carrier and gyne in figure 3.4, ranging from 0.8 to 1, and the presence of pairs under the half-sister threshold, suggests that gynes might be carried more than once. This hypothesis correlated with the increasing probability to collect non-related pairs along the reproduction season, as shown in figure 3.6.

According to the life circle of individuals in *C. elegans*, the existence of multiple carrying is relevant. Indeed, as males and gynes start to be produced in late spring and early summer, all sexuals are directed to the mating chambers. As we know, a high inbreeding value suggests important sib-mating. As such, males do not seem to be able to differentiate sisters from alien individuals and mate with the first encountered gyne. In summer, the carrying behaviour increases from July to September. When coming back from a previous trip, carriers have been observed and suspected to take only a few seconds to leave again with a new individual to transport. This would suggest that workers only need to enter the mating chamber, right under the surface to select their next gyne to carry. If workers, like males, were not capable to differentiate a sister from an alien gyne inside the mating chamber, the chance of picking a non-related individual to carry would increase with the number of alien gynes present in the mating chamber.

This hypothesis could be supported by the species' intrinsic tolerance for alien gynes. Several studies showed that nestmate recognition and acceptance in ants is principally based on cuticular hydrocarbon (CHC)'s composition in the surface of their body (Dahbi et al. 1996; van Zweden and d'Ettorre 2010). Therefore, producing gynes with an overall low variation in cuticular profile for all populations would increase their acceptance, thus dispersal. Unpublished results from Schrempf, Lucas and Heinze in 2016 showed no significant difference in CHC profiles of gynes from two different populations.

Behavioural studies of Lenoir in 2007, however, revealed that the introduction of gynes in laboratory colonies of *C. elegans* resulted in worker aggression towards the young queens. When alien males were free to enter the artificial nest into the established laboratory colony, gynes were aggressively rejected. Yet, the result of their study does not reject our hypothesis, since laboratory receiving colonies used did not contain any males, nor were the introduced gynes dissected to assess their ovarian development. Therefore, workers might have been rejecting the coming gynes because they were already ready to found a new colony, ergo replace their established queen, or because their nest was in inadequate conditions to mimic sexual season (presence of males). In both cases, since

gynes do not move in or out of the nest on their own before spring dispersal, workers' aggression could have been directed towards the incoming of a solitary gyne rather than its cuticular profile.

Moreover, our study showed that exclusive sibling mating was enough for queens to produce workers after 250 days of isolation. Consequently, the fact that 84% of isolated gynes collected during a carrying, were not able to produce workers during the 500 days of the experiment, adding to the fact that 33% of them had empty spermatheca suggest that workers might carry virgin individuals. However, we could easily imagine that to optimize the increase of their fitness, carriers would gain more by carrying away gynes that had already mated with their brothers. Hence, the carrying of virgin gynes, probably in the middle of the reproduction season, when the mating chambers are full, might result from the carrier incapability to distinguish between mated or non-mated gynes.

The distribution of carriers' relatedness to source colonies in figure 3.4 (b) equally supports the multiple carrying hypothesis. Indeed, by comparison with the gynes' relatedness to source colonies which are sparser, the relatedness of carriers to source colonies tends to be better distributed over the half-sister threshold. In reverse, relatedness values of carriers to recipient colonies tend more evenly towards lower values than it can be observed for gynes. This suggests that the decision to target a recipient colony over another depends on its own relatedness to it, which in term increases the outbreeding of sisters gynes and randomly distributes alien gynes after additional carryings.

From a worker's point of view, carrying an alien gyne that had probably mated with one or more of its brothers would enhance the survival of the male's genetic material; hence increase the worker's inclusive fitness. Moreover, the fact that all colonies might additionally carry alien gynes, increases the chances for its own sister gyne to mate with other unrelated males.

4.4. Influence on gyne direct fitness by the number of mating partners

According to evolutionary theory, male insects, which are generally short-lived and have a limited set of abilities, would be expected to have an increased in fitness by increasing the number of mates. The more females they mate with, the more copies of their genes they succeed to spread, the higher the chances that viable offspring pass on their genes to the next generation. Female insects, however, are expected to select one or sometimes few males to reach the necessary reproduction success. Mated females even tend to live longer than non-reproductive ones (Keller and Genoud 1997). Our results showed that queens of *C. elegans* which can reproduce live significantly longer than gynes which did not produce any workers (figure 3.9). However, mating in insects can be associated with risks like infection or predation (Wing 1988; Daly 2015). Therefore, a male would gain in mating with as many partners as possible before dying, opposite to a female which would not produce more offspring by multiply mating and would lose all chances to pass on its genes if it were to die before laying any eggs.

Nonetheless, studies have shown that polyandry in insects is more common than first expected (Yasui 1998; Arnqvist and Nilsson 2000; Fjerdingstad et al. 2003; Denny et al. 2004). These studies showed that mating does not only procure the sperm necessary to create offspring but can help increase female lifespan, fertility, and egg production rate. Still, in 2000, Arnqvist and Nilsson proposed the existence of "an intermediate optimal female mating rate, beyond which a further elevated mating rate is deleterious". Indeed, each mating increases the risks mentioned above. If the benefits gained by the female in terms of lifespan, fertility, and egg production outcome the risks, multiple mating is advantageous. However, as soon as the number of males involved increases the risks associated with mating, higher than the benefits, polyandry becomes deleterious (Watson et al. 1998; Arnqvist and Nilsson 2000; Schrempf et al. 2015). Our study showed that multiply mated queens had a lower worker production than singly mated ones (figure 3.10).

In the case of *Cardiocondyla elegans*, an increase in the number of male partners enhance the chances of outbreeding, especially after transport to another colony. Although multiply mated isolated queens have a significantly lower worker production than inbred queens (figure 3.10). Moreover, as gyne carrying implies bringing the future queen outside the nest, the risks of overheating, being displaced by the wind or predation increases with each transport. Our study suggested that an estimated one to two patrilines stimulated the rapid production of workers (figure 3.7). In contrast, the estimated seven patrilines seemed to slow down the production of workers. If this study does not reflect the total worker production (until 38 weeks only), nor the offspring viability, it could point out a possible deleterious effect of seven mating partners on a queen's early establishment. As previously shown, the average number of males mated with a queen of *C. elegans* ± SD is equal to 3.6 ± 2.1 (our study) and 4.52 ± 1.6 (Lenoir et al. 2007). Furthermore, the two queens that had an estimated seven patrilines had an original estimate of four to five patrilines before being introduced with males in the laboratory (supplementary tables 2.3). In other words, none of the 18 studied queens did mate with seven males in natural conditions. Hence, we could theorise that mating with three to five males is the optimal female mating rate for our studied species. Yet, it is necessary to remember that our dataset is too small to generalise our results to the whole species (n = 2 – 4 queens per group). Therefore, additional studies and genotyping of *C. elegans*' queens would be necessary to confirm or reject our theory.

Finally, if multiple mating with foreign males might be beneficial in a long term, our data show no short-term benefits in terms of worker production by multiply mated queens. As theorised by Trontti in 2007 with the ant *Plagiolepis pygmaea*: "multiple mating may also evolve as a pure male strategy without benefits to queens or colony functions" (Trontti et al. 2007). In such phenomenon called convenience polyandry (Alcock et al. 1978; Trontti et al. 2007) males, with an unlimited sperm supply, try to maximise their number of copulation and female accept the extra copulations because the costs linked with refusing the mating would be higher than the eventual costs linked with surrendering to the mating. In our case, for gynes to refuse a supplementary mating while

being in a natal or foreign mating chamber, they would need to leave the nest. In the severe heat of the summer and the cold of the following winter, a solitary gyne without a nest to find shelter in would be unlikely to survive until colony founding in spring. Convenience polyandry could explain the high number of patrilines found in queens despite the possible costs in the number of workers produced.

4.5. Hypothesis on the nest selection process by workers

Our study showed that chosen recipient colonies are on average unrelated to the gynes and their carriers. However, as seen in figure 3.3, the distribution of colonies relatedness does not correlate with their distance to one another. In other words, it would not be enough for a worker to drop a gyne as far away from its original nest as possible, to increase its chance to encounter an unrelated receiver colony. Moreover, mapped colonies and recorded transports between colonies (e.g., figure 3.2) showed that the distance for each carrying and the number of passed colonies were highly variable. Another point of our study showed that the relatedness of gynes to skipped colonies is not significantly different from 0. This would suggest that the colonies non-selected are not excluded because of their relatedness to the gynes but for yet unknown reasons.

Two essential interrogations remain: *why* and *how* do carriers select particular colonies over others as recipients?

4.5.1. Why?

According to the multiple mating hypothesis, alien gynes might be randomly selected by workers in the mating chamber and transported additionally, thus enhancing their possible number of patrilines. Nonetheless, like all evolutionary traits, the risks of such carrying behaviour should not overcome the benefits. It is easy to imagine that each transport puts the gyne at risk from predation, desiccation, loss (due to wind or changing terrain) etc. As mentioned before, a lost gyne is unlikely to survive summer and winter conditions due to a lack of shelter. As such, selecting a recipient nest without sexual individuals would increase the risks of losing a gyne, since it could result in rejection of the incoming sexual individual by the receiving workers. Moreover, as seen previously, a possible negative impact on the colony founding for a new queen might affect gynes that have mated with a high number of males (figure 3.7). Hence, the additional carrying of a queen should remain occasional. In which case, recipient colonies best have a high number of gynes in their mating chamber, to decrease the chances for recipient carriers to select all the alien gynes for additional carrying. Likewise, a colony rich in gynes in its mating chamber would likely have produced multiple males (with an average of 5.27 ± 4.31 males per nest found in 2007 by Lenoir, and a maximum of 19 males collected in a nest in 2015, table 3.1). Hence, such a sexual rich colony enhances the chances for transported gynes to encounter and mate with a foreign male without having to be re-carried.

Isolation of gynes in the laboratory showed that inseminated queens do not always produce sexual individuals in the first year of their reign (personal observations). Sexuals seemed to be produced only for nests with a high enough number of workers (approximately 50 to 70 workers in a small vertical nest minimum). Moreover, figure 3.7 showed that a high number of patrilines might increase the time needed for a queen to produce enough workers for the colony to be considered: "mature". Hence, in the field, newly established colonies might not be an evolutionary interesting choice for the workers to drop their sister gynes in. Therefore, a possible characteristic that would influence worker's colony selection could be the number of sexual individuals present in the receiving colony.

A second hypothesis that we cannot yet refute is the possibility that workers simply randomly come across colonies while solitarily foraging and tag, in their spatial memory, the first encountered nest of the day, possibly of the season. At last, we could imagine that workers of *C. elegans* evolved towards behaviour which combines both hypotheses. Indeed, our field observations of foraging activities showed that workers do not seem to have predefined patterns in their foraging paths. Instead, foragers appeared to be randomly moving and changing direction according to environmental obstacles or their encounters with workers from different nests. Following this idea, a future carrier would start as a forager which appeared to have randomly come across a suitable receiving colony and tagged it as such.

4.5.2. How?

Studies on ant navigation showed that they preferentially used a view-based strategy to orient themselves in their environment (Collett 1996; Harris et al. 2007). In 2009, it has been proposed that ants used a "global viewing approach" where they considered the globality of their environment and navigated using multiple clues until reaching their nest where a "specific viewing approach" was used, as particular individual landmarks could be recognised (Collett 1996; Bisch-Knaden and Wehner 2003; Wystrach and Beugnon 2009). In the sandy soil where *C. elegans* nests, small scale fluctuations of the environment happen regularly (e.g., wind, animal steps…etc. (Lenoir 2006)). Therefore, an adaptive strategy requiring the use of their global view of the landscape to navigate, instead of focusing exclusively on individual landmarks, would be justifiable to have appeared in *Cardiocondyla elegans*' workers. Overall, it had been suggested that some ants can navigate through their environment using global views of their surroundings, scan and map their environment before storing this information in the long-term memory (Collett and Collett 2000; Wystrach and Beugnon 2009; Collett et al. 2013).

If it were to be the case for *C. elegans* workers, it would explain how carriers manage to remember and recognise the way to a targeted colony as well as the way back home. Following carriers in the field, we observed that if the path between the source and targeted receiver colony, taken by a carrier was very similar for each of its transport, it was not exclusively identical. The

carrier was seen to adapt to any change in the path to always find the targeted colony as well as its way back home. This observation suggests the possibility for *C. elegans*' workers to use a global viewing on their environment rather than the use of only particular clues to navigate. This theory could be tested in later studies adopting the setup described by Wystrach and Beugnon's in 2009. In this case, workers of *C. elegans* could have randomly encountered a suitable recipient nest during a previous exploration of the environment and memorised the location to either teach another worker the way (by tandem running) or transport a gyne by themselves.

The possible strategies used by *C. elegans*' workers to assess suitable receiving colonies remain unknown. A first possible explanation would be that workers can detect scent clues in the nest entrance of encountered colonies. Indeed, individuals of *C. elegans*' have been observed continuously bringing sand from their nest to the nest entrance. This behaviour is thought to be due to soil movements that force workers to regularly reconstruct their chambers by bringing sand outside the nest. In the reproduction season, because sexual individuals are stored in the mating chamber under the surface, workers presumably need to enlarge this chamber during the summer, hence, bringing sand imprint with gyne and/or male odours outside their nest. If a worker were to detect such a specific scent in the nest entrance of a neighbouring colony, it could attest to the presence of males and gynes. In this case, the stronger the scent the more likely it would be that the colony contains sexual individuals.

It had already been shown that ergatoid males from the genus *Cardiocondyla obscurior* were capable of differentiating winged males from queens via scent (Cremer et al. 2002). This study revealed differences in the hydrocarbon profiles of workers, ergatoid males, winged males, and virgin queens. Unpublished studies from Schrempf, Lucas and Heinze in 2016 showed no significant difference in CHC profiles between carriers and their carried gynes but did not involve males. These results could be due to a carrier's ability to mimic a gynes' scent while carrying it, or to a non-detection to highly specific differences. The fact that Cremer's study in 2002 found differences in hydrocarbon profiles of small size individuals of *C. obscurior* (1 mm), could be an indicator that by adapting this highly sensitive protocol to assess again the CHC profiles of cast members of *C. elegans* ants (2-3 mm) we could confirm previous results or witness possible differences. Supposing it were possible to isolate the specific scent signature of sexual individuals (males and gynes) from workers of *C. elegans*, they could be compared to sand collected in the entrance of the receiving nests in the field. Thus, assessing the high or low presence of sexual individuals in recipient and passed colonies.

Similarly, a possible indicator of the number of individuals inside a nest could be reflected by their territorial marking (Jaffe and Sanchez 1984) or simply their dejections (Giehr et al. 2019). In both cases, the amount of any existing "alarm pheromone" as well as the number of dejections in the nest entrance could signal to a passing worker on the status of the colony.

A second theory involved the capacity of workers to deduce the presence of gynes by the level of aggression encountered at the entrance of a visited nest. Indeed, anecdotal observations made during our excursions to the South of France suggest that a couple of minutes before the departure of a carrier with a gyne from the source colony, the nest entrance would be aggressively "guarded" by one (possibly the carrier) or a succession of workers. What we will call the guarding carrier seemed to survey the nest entrance for few seconds, walking extremely fast and reacting to any stimuli violently (e.g., other workers) before going back inside the nest. The same worker (or another one) was seen to repeat this behaviour after a few seconds before going back inside…etc. This aggressive behaviour repeated multiple times before one worker could be seen leaving the nest with a gyne. By doing so, the worker(s) potentially assessed any immediate danger for the gyne to be carried, as well as meteorological conditions. Moreover, we observed that *C. elegans*' workers from different colonies, if reasonably complaisant when encountered while walking in their shared territory (site), were aggressive towards each other to defend a food source or their respective nest entrances. However, the aggression level of a worker defending a nest entrance, if compared, would probably be lower than the one of a worker preparing the carrying of a gyne. Therefore, if a worker passing by a randomly discovered neighbouring colony were more aggressively received at the nest entrance than usual, it could be an indicator for a probability to contain a high number of gynes, ergo males.

5. Conclusion

Our research project focused on a population of *Cardiocondyla elegans* from the South of France, as such, we confirmed the relatedness among nestmates and the 70% coefficient of inbreeding found in the population from a region of France farther north. Behavioural observations showed that workers did not drop the gyne randomly in neighbouring nests, instead had a short selection of colonies targeted as recipients. A deepened investigation on the relatedness between carrier worker and carried gyne revealed that workers select a young queen inside their own natal nest before carrying it. Furthermore, it appears that gynes can be transported to multiple colonies during the reproduction season. A project led by Schrempf and Heinze in 2016, showed that at the end of the reproduction period, migrated gynes hibernate in the unrelated nest they have last been transported to, before dispersing by foot in spring. By analysing ant genotypes, our study confirmed the polyandrous behaviour of *Cardiocondyla elegans*' queens. The estimated number of males that had mated with a single queen correlated with the values from Lenoir in 2007. Finally, our research expanded our understanding of reproductive fitness in *C. elegans* by showing a decreasing rate of worker production with an increased number of estimated patrilines (number of males mated with a queen). Overall, if this dissertation deepens our knowledge on *Cardiocondyla elegans*' reproductive strategies to a point that highlights the unusual third-party involvement in reproduction as well as the possible disadvantage of multiple mating, it also points at interesting questions remaining unanswered which would gain in being studied further.

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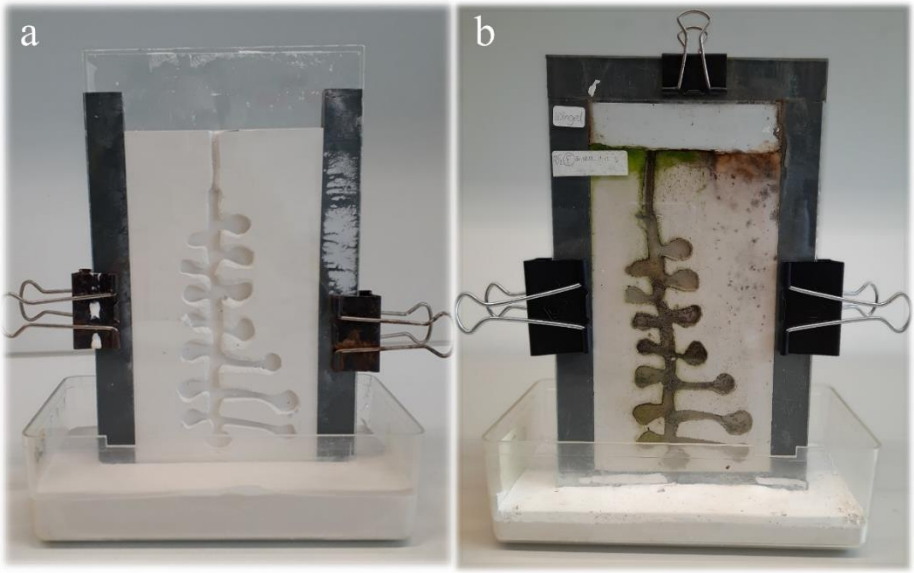
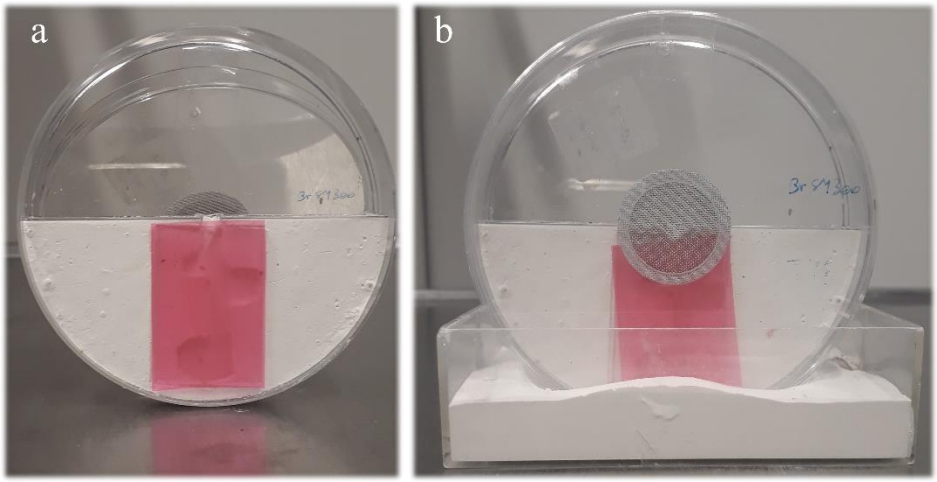
Supplementary

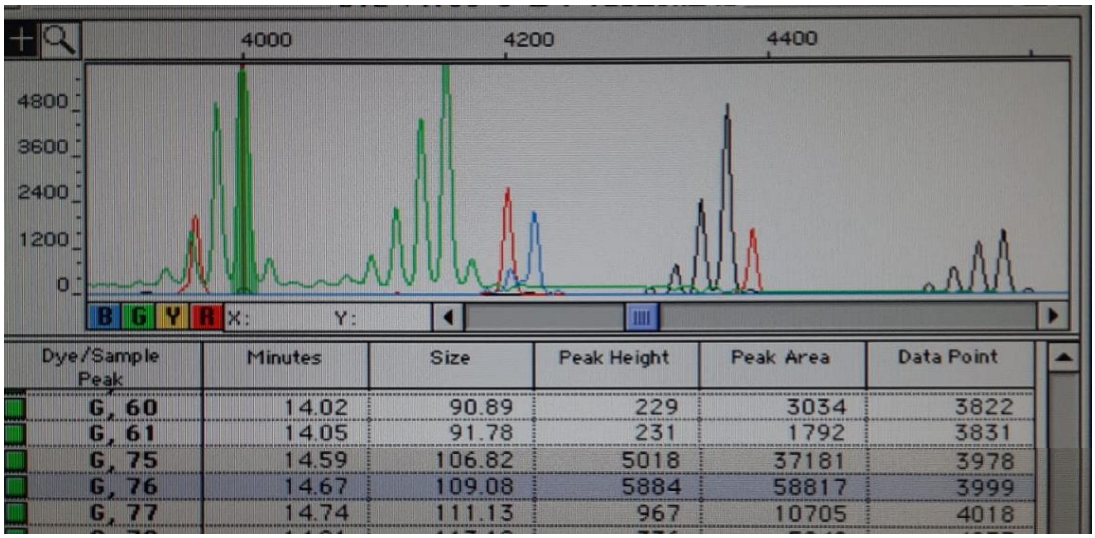


Supplementary figure 2.1. Large artificial vertical nest. Picture of artificial plaster nests conceived to enhance the survival of *Cardiocondyla elegans* in the laboratory. The left picture shows an open and newly created plaster nest (a) whereas in the right picture (b) ants have been surviving for more than one year in a closed nest.



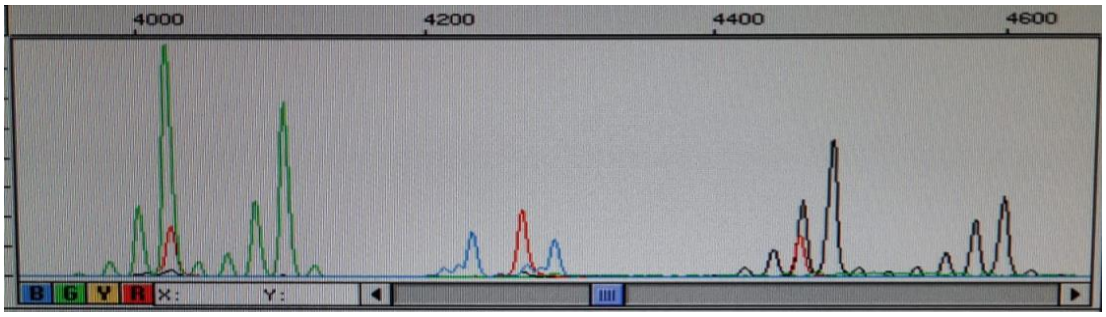
Supplementary figure 2.2. Small artificial vertical nest. Picture of artificial plaster nests conceived to enhance the survival of a reduced number of individuals of *Cardiocondyla elegans* in the laboratory. Both pictures show a newly created plaster nest, either open (a) or closed and with a plaster base (b).

A.

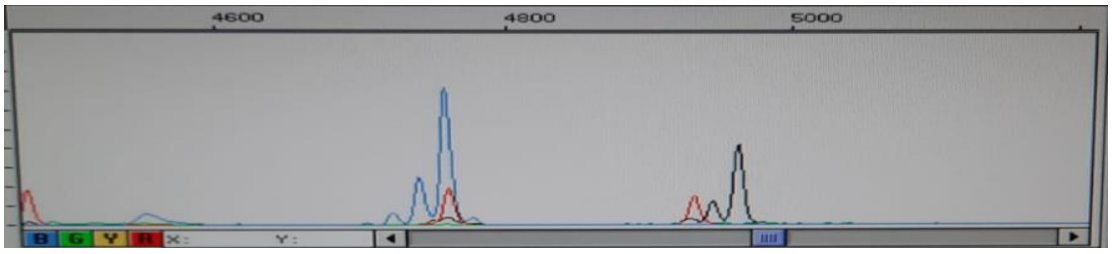


Dye/Sample Peak	Minutes	Size	Peak Height	Peak Area	Data Point
G, 60	14.02	90.89	229	3034	3822
G, 61	14.05	91.78	231	1792	3831
G, 75	14.59	106.82	5018	37181	3978
G, 76	14.67	109.08	5884	58817	3999
G, 77	14.74	111.13	967	10705	4018

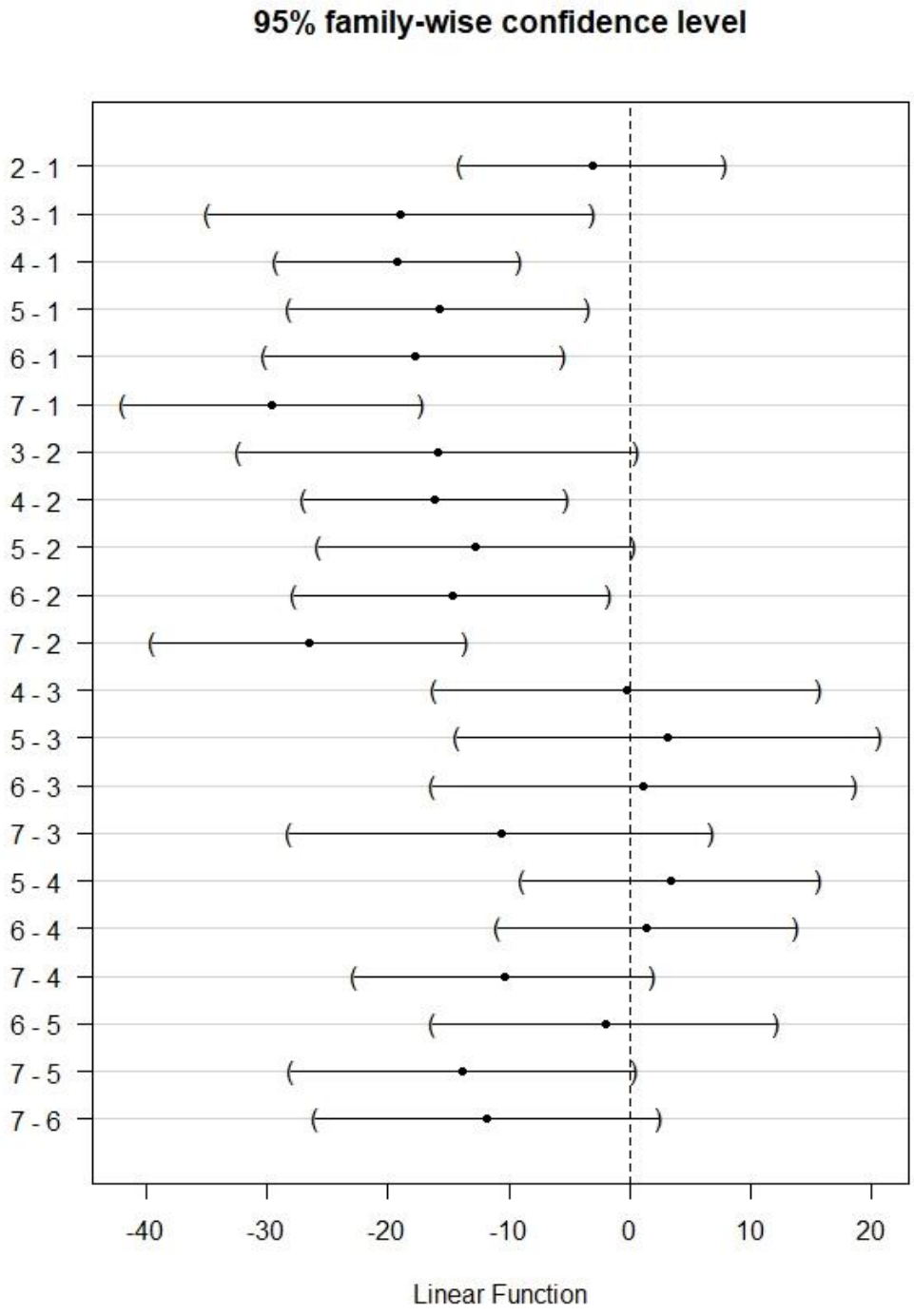
B



C



Supplementary figure 2.3. Visualisation of allele's sizes. Computer screens of allele size by the GeneScan® 3.1 software (Applied Biosystems). (A) Each highest peak represents the allele attached to the primer's fluorescence. Small peaks are broken and amplified DNA that had lost one, two or three pair bases. For each combination of primers, green, blue, and black peaks represent different primers. Red peaks represent the size standard. The number of pair bases at a given allele is given by the column "size" for each peak. The amount of DNA with the same "size" passing through the laser is given by the column "Peak Height". A locus was considered heterogeneous when two high peaks could be found, as shown in picture B (the individual is heterogenous for every three loci). A locus was considered homogeneous when only one high peak could be found as it is visible in picture C (the individual is homogenous for both loci). In picture A, the individual is heterogeneous at both loci green and black but homogenous at the blue locus.



Supplementary figure 3.1. Estimations of the Tukey Contrasts post hoc test. Estimations of the 95% family-wise confidence level of the Tukey Contrasts post hoc test made after the linear regression model comparing the number of workers produced according to the number of males mated with a queen (patriline). Each number on the left represents a quantity of patriline. Paired numbers are conditions being compared to one another.

Supplementary method 2.1. DNA extraction with CTAB method (modified from Sambrook and Russel 2001).

Material list:

- Thermomixer
- Pipettes for transferring volumes of 2µl, 40 µl, 150µl, 300 µl, 350 µl, and 500 µl
- Enough space in a freezer (-20 °C) to store one Eppendorf cup per sample, one Eppendorf cup rack, and a few Falcon tubes
- Labelling stickers that fit on Eppendorf cup lids
- Crushed ice in a container large enough to store all samples in Eppendorf cups
- TE buffer (pH8)
- 70% EtOH (p.a.)
- 100% EtOH (p.a.)
- Isopropanol
- Chloroform / Isoamyl alcohol 24 : 1
- Natrium acetate (1/10 Vol., 3 M, pH = 4.8)
- CTAB solution: 0.75 M NaCl, 50 mM Tris/HCl (pH = 8.0), 10 mM EDTA, 1% Hexadecyltrimethylammoniumbromid (add after autoclave); you need 500 µl per sample
- Water bath if thermomixer is not used for warming up
- Clean pestles for crushing the material
- Fluid nitrogen
- Centrifuge for 4 °C and room temperature (14000 rounds per minute)
- PCR water (Sigma Aldrich Chemie GmbH) for DNA storage or TE (Tris/EDTA) buffer for long-term storage of DNA (40 µl each for ants, thoraces or larvae, 25 µl each for eggs or legs)

Part I

Preparations:

- Prepare 1% CTAB solution (500 µl per sample) and warm it to 65 °C (either in the water bath or in the Thermomixer, for the latter use large Eppendorf cups).
- Prepare crushed ice for cooling samples in Eppendorf cups.
- Fluid nitrogen
- Proteinase K (MBI Fermentas) (2-10 µl per sample)
- Chloroform / Isoamyl alcohol (24 : 1)

Procedure:

- ✓ Ants that have been stored in alcohol: dry over kitchen towel paper.
- ✓ Place each individual in a **1.5 ml Eppendorf cup** (without fluid), put a label (colony number, individual ID) on the cup, put cups on ice.
- ✓ Put **fluid nitrogen** in each Eppendorf cup, quickly close the lid for a short time (for preventing the sample to "jump out"), and **immediately crush** animals with clean pestles; then add **500 µl** of warmed **(65 °C) 1%** (or 2%) **CTAB** solution.
- ✓ Incubate the mixture for **15 min (short protocol) to 1 h (long protocol) at 65 °C** in the Thermomixer (650 rpm *for the DNA to not stay down the tube*).
- ✓ Let the mixture cool down to 55 °C (and set the Thermomixer to 55 °C), then add **3 µl Proteinase K** and incubate in the Thermomixer for ~**2h at 55 °C** *(or longer)*.

Part II

Preparations:

- Prepare Chloroform / Isoamyl alcohol 24 : 1 (500 µl per sample).
- Prepare NaAc (1/10 Vol., 3 M, ph 4.8) (40 µl for each sample).
- Prepare one fresh 1.5 ml Eppendorf cup for each sample (with an equal label).
- Put Isopropanol (350 µl for each sample) in the freezer (-20 °C).

Procedure:

- ✓ Add **500 µl Chloroform / Isoamyl alcohol**, vortex (or shake by hand), and centrifuge for **5 min at 14000 rpm** (rounds per minute) (RT).
- ✓ After centrifugation put the **upper phase of each sample in a fresh cup** (take the 200µL pipette).
- ✓ Add **40 µl NaAc** (1/10 Vol., 3 M, ph 4.8)mix and **350 µl Isopropanol (-20 °C)**, shortly shake the cup, then incubate **40 min at -20 °C** *(or longer)*.

Part III

Preparations:

- Put 70% and 100% EtOH (p.a.) in the freezer (-20 °C) (300 µl of each per sample).
- Prepare 70% EtOH (p.a.) (150 µl per sample, **room temperature (=RT)**).
- Prepare PCR water (Sigma Aldrich Chemie GmbH) or TE buffer (pH8) for resolving the DNA pellet.
- Prepare a cooled metal cup rack (by putting it in the freezer at -20 °C) or crushed ice for handling the samples while cooled.
- Cool down the centrifuge.

Procedure:

- ✓ Centrifuge for **15 min at 4-6 °C and 14000 rpm**; discard supernatant carefully with pipet (*1000µL, no need to change the cone) – let a few among of liquid in the tube.
- ✓ Place the cups on ice or in a cooling rack; wash each pellet with **300 µl 100% EtOH (p.a.) (-20 °C)**, then centrifuge **7 min at 4-6 °C and 14000 rpm**; discard supernatant carefully with pipet– let a few among of liquid in the tube.
- ✓ Place the cups on ice or in a cooling rack; wash pellet with **300 µl 70% EtOH (p.a.) (-20 °C)**, then centrifuge **5 min at 4-6 °C and 14000 rpm**; discard supernatant carefully with pipet– let a few among of liquid in the tube.
- ✓ Wash pellet with **150 µl 70% EtOH (p.a.) (RT)**, then centrifuge **4-5 min at RT and 14000 rpm**; discard supernatant carefully with pipet (200µL) – do not let liquid on the tube.
- ✓ **Dry** DNA pellet in open Eppendorf cup for **about 5-10 min** at **RT until there is no liquid anymore** (in the flue (Abzug)!).
- ✓ Resolve the pellet in PCR water or **TE buffer (pH8)**: use **30 µl** (with filter tips). Leave enough time for dissolving (e.g., **1 h in the fridge**) *(or longer)*.

Locus name	Primer sequences (5′to 3′)	Repeat type	TA (°C)	Size range (bp)	No. of alleles	Label of F-primer
CE2–3A	F: CCGTCTTTTCCACTCAC R: GGAATCGTCGAGAGAGA	(AG)	60	97–135	18	TET
CE2–4A	F: TGCGAGTGGATGTATGA R: CCCACCTTACAGCAATATC	(AG)	60	175–193	9	FAM
CE2–5D	F: AGACGTAAGGTTTGAAGAGA R: ACAACTATGCCAAATTAAGTAT	(AC)	60	202-206	3	HEX
CE2–12D	F: TCCGCTAAATTATCATGG R: TCGAGTGCATAAAGGAATA	(AG)	60	127–143	8	FAM
CE2–4E	F: ATACAAAAGAATATGAAGTAATACA R: GTGTGCTTATGTATCTGGTAT	(AC)	50	135–179	22	HEX
Card 8	F: TCGCCGTCTATTCTGTCGTTA R: CTATTATCGGCAATGTGC	(AC)	54	118–132	5	FAM
Cobs 13	F: TATCTTTTCAACCCTCTCGC R: TATTCCGCGATAGCTTAAAT	(CT)	60	74-86	5	TET

Supplementary table 2.1. **Primer microsatellites used for the ant *Cardiocondyla elegans.*** Showing: primer sequences, repeat motif, primer pair-specific annealing temperature (TA), size range in base pair, the observed number of alleles, and fluorescent primer labels.

Locus name	Primer sequences (5′to 3′)	Locus name	Primer sequences (5′to 3′)
Ant11893	F: CAGGCTCGGRACGTTAATGC R: GGTGCCGACGTCTAGCTAGC	**Cobs 8**	F: TTATCGTGAGGATTTTGAGGC R: TTTCGACAATGACAAACCGAGC
Ant2936	F: GGGGGATCCGGTAATCCTCT R: TCGCCCTGCAGTTAATGTGT	**Cobs 9**	F: ACTCAGTGCCAATTCG AATAAACAGC R: TGAACCGGGTAGAATCAATTA
Ant3993	F: TGATCCGCTCTTAAAATTTAGATGGA R: ACTTTCCGCRGCATTAAACATTTTCTT	**Cobs P3**	F: ACTCTCACAATCGCTACGC R: GACGTACGGCCAGATGTCA
Ant575	F: TCAGGTTCGACACATGTGCC R: TCAAGATCGTTTGTCAGGCTGA	**Cobs 13.2**	F: AATCGCGCCTGCGACGGCG R: AGTTTCTCACTTTTGCTCG
Ant1343	F: TCGGTCCCGTGCCTTCGATT R: GRGGGCGCGTCAAATTTGCT	**Card 21**	F: GAATCGTGACGAAGCATAC R: GTAATGGCCAACGCCTCGC

Supplementary table 2.2. **Rejected primers for genetic studies of the ant *Cardiocondyla elegans.*** Showing: primer sequences, repeat motif, primer pair-specific annealing temperature (TA), size range in base pair, the observed number of alleles, and fluorescent primer labels. All primers in this table showed insufficient allele diversity. Five universal primers named Ant11893, Ant2936, Ant3993, Ant575 and Ant1343 (Butler et al. 2014); four primers from *Cardiocondyla obscurior* named Cobs 8, Cobs 9, Cobs P3 and Cobs 13.2 (Schmidt et al. 2016b); and one primer from *Cardiocondyla batesii* named Card 21 (Schrempf et al. 2004) were rejected, due to the absence of allele diversity, to study relatedness in *Cardiocondyla elegans*.

Supplementary tables 2.3. Patrilines deduction. Deduction of the minimum number of males genotypes contained in each queen spermatheca, according to produced workers' genotypes. Use of seven different microsatellites loci for a minimum of eight workers per queen. If a queen had been introduced to males in the laboratory (lab male), their genotypes for each locus were analysed. Purple highlighted colours are alleles inherited by workers from the queen. Each other colour represents a different male genotype. The total of male genotypes per queen is its estimated number of patrilines.

- Queens without induced mating in the laboratory:

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen A	127	131	111	111	141	149	204	204	181	181	122	122	82	84
Worker 1	131	135	101	111	141	155	204	206	181	181	122	122	80	84
Worker 2	131	135	101	111	141	145	204	206	181	187	122	126	80	84
Worker 3	131	135	101	111	149	155	204	206	181	181	122	122	80	84
Worker 4	131	135	101	111	145	149	204	206	181	187	122	126	80	84
Worker 5	127	135	101	111	149	155	204	206	181	181	122	122	80	82
Worker 6	127	135	101	111	141	145	204	206	181	187	122	126	80	82
Worker 7	127	135	101	111	149	155	204	206	181	181	122	122	80	82
Worker 8	131	135	101	111	149	155	204	206	181	181	122	122	80	84
Worker 9	127	135	101	111	141	145	204	206	181	187	122	126	80	82
Worker 10	131	135	101	111	149	155	204	206	181	181	122	122	80	84
Males' genotypes														
Male 1	135		101		155		206		181		122		80	
Male 2	135		101		145		206		187		126		80	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen B	133	135	113	133	151	165	204	204	175	179	122	122	82	82
Worker 1	135	139	111	113	151	159	204	204	179	181	122	122	80	82
Worker 2	133	139	111	113	159	165	204	204	175	181	122	122	80	82
Worker 3	135	139	111	113	159	165	204	204	175	181	122	122	80	82
Worker 4	133	139	117	133	151	159	204	204	175	181	122	122	80	82
Worker 5	135	139	113	133	151	159	204	204	175	181	122	122	80	82
Worker 6	133	139	117	133	159	165	204	204	175	181	122	122	80	82
Worker 7	135	139	113	117	159	165	204	204	179	181	122	122	80	82
Worker 8	133	139	113	133	159	165	204	204	175	181	122	122	80	82
Worker 9	133	139	117	133	151	159	204	204	179	181	122	122	80	82
Worker 10	135	139	113	113	151	159	204	204	175	181	122	122	80	82
Males' genotypes														
Male 1	139		111		159		204		181		122		80	
Male 2	139		113		159		204		181		122		80	
Male 3	139		117		159		204		181		122		80	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen C	127	135	111	119	155	165	204	204	181	183	124	126	80	82
Worker 1	127	127	111	119	155	165	204	204	181	183	124	124	82	82
Worker 2	133	135	111	111	155	165	204	204	181	183	124	124	80	82
Worker 3	127	133	111	111	165	173	204	204	183	183	124	124	80	80
Worker 4	127	127	111	111	165	165	204	204	183	183	124	124	80	82
Worker 5	127	135	111	119	155	165	204	204	181	183	124	124	82	82
Worker 6	127	127	111	119	165	165	204	204	181	183	122	126	80	82
Worker 7	127	135	111	111	155	165	204	204	181	183	124	126	82	82
Worker 8	127	127	111	119	155	165	204	204	183	183	124	126	80	82
Worker 9	127	127	111	111	155	165	204	204	181	183	122	124	80	82
Worker 10	127	135	111	119	155	165	204	204	183	183	124	126	82	82
Males' genotypes														
Male 1	133		111		155/	165	204		181/	183	124		80/	82
Male 2	133		111		173		204		183		124		80	
Male 3	127		111		165		204		183		124		82	
Male 4	127		111		165		204		181/	183	122		80/	82

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **D**	133	135	111	125	149	175	204	204	181	183	124	124	80	82
Worker 1	133	133	111	111	151	175	204	204	181	183	122	124	80	82
Worker 2	133	135	111	125	149	165	204	204	181	183	122	124	80	82
Worker 3	133	135	111	125	149	175	204	204	183	183	124	124	80	82
Worker 4	133	133	111	111	149	165	204	204	183	183	124	124	80	82
Worker 5	133	135	111	125	151	175	204	204	183	183	124	124	80	82
Worker 6	133	135	111	111	149	175	204	204	181	183	122	124	80	82
Worker 7	133	133	111	111	149	175	204	204	183	183	124	124	80	82
Worker 8	133	135	111	111	151	175	204	204	181	183	122	124	80	82
Worker 9	133	135	111	125	149	165	204	204	183	183	122	124	80	82
Worker 10	133	135	111	111	151	175	204	204	181	183	124	124	80	82
Males' genotypes														
Male 1	133		111		151		204		181/	183	122		80/	82
Male 2	133/	135	111/	125	165		204		183		122		80/	82
Male 3	133		111		149/	175	204		183		124		80/	82
Male 4	133		111		165		204		183		124		80/	82
Male 5	133/	135	111/	125	151		204		183		124		80/	82
Male 6	133/	135	111		149/	175	204		181/	183	122		80/	82

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **E**	127	133	109	109	153	165	204	204	183	187	122	122	82	82
Worker 1	133	135	109	109	153	165	204	204	183	187	122	122	82	82
Worker 2	127	133	105	109	153	165	204	204	183	187	122	122	75	82
Worker 3	133	135	109	109	153	165	204	204	183	187	122	122	75	82
Worker 4	133	135	105	109	153	165	204	204	183	187	122	122	82	82
Worker 5	133	135	105	109	155	165	204	204	183	187	122	122	82	82
Worker 6	133	135	105	109	155	165	204	204	183	187	122	122	82	82
Worker 7	133	135	105	109	153	165	204	204	183	187	122	122	82	82
Worker 8	133	135	109	109	155	165	204	204	183	187	122	122	82	82
Males' genotypes														
Male 1	135		109		153/	165	204		183/	187	122		82	
Male 2	135		109		153/	165	204		183/	187	122		75	
Male 3	127/	133	105		153/	165	204		183/	187	122		75	
Male 4	135		105		153/	165	204		183/	187	122		82	
Male 5	135		105		155		204		183/	187	122		82	
Male 6	135		109		155		204		183/	187	122		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **F**	133	133	103	109	145	151	204	204	181	181	122	124	80	84
Worker 1	133	133	103	111	145	145	204	204	181	181	122	124	82	84
Worker 2	133	133	103	111	151	151	204	204	181	185	122	122	80	82
Worker 3	133	135	103	111	151	151	204	204	181	185	122	124	80	84
Worker 4	133	135	109	111	145	151	204	204	181	185	122	122	82	84
Worker 5	133	135	103	111	151	151	204	204	181	185	122	124	82	84
Worker 6	133	135	103	111	NA	NA	204	204	181	185	122	124	80	82
Worker 7	133	135	109	111	145	145	204	204	181	185	122	122	80	82
Worker 8	133	133	109	111	145	151	204	204	181	181	122	124	80	82
Worker 9	133	133	103	111	145	151	204	204	181	181	122	122	80	82
Males' genotypes														
Male 1	133		111		145		204		181		122		82	
Male 2	133		111		151		204		185		122		82	
Male 3	135		111		151		204		185		122/	124	80/	84
Male 4	135		111		151		204		185		122		82	
Male 5	135		111		145		204		185		122		82	

- Queens with induced mating in the laboratory:

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **G**	135	143	109	119	145	149	204	204	179	183	122	122	82	84
Worker 1	131	135	107	109	145	157	204	204	181	183	120	122	82	82
Worker 2	131	143	107	119	145	157	204	204	181	183	120	122	82	82
Worker 3	131	135	107	109	145	157	204	204	179	181	120	122	82	82
Worker 4	131	135	107	119	149	157	204	204	181	183	120	122	82	84
Worker 5	131	143	107	109	145	157	204	204	181	183	120	122	82	82
Worker 6	131	135	107	119	145	157	204	204	179	181	120	122	82	84
Worker 7	131	135	107	119	145	157	204	204	181	183	120	122	82	84
Worker 8	131	143	107	109	149	157	204	204	181	183	120	122	82	82
Worker 9	131	135	107	119	149	157	204	204	181	183	120	122	82	84
Worker 10	131	143	107	109	149	157	204	204	179	181	120	122	82	82
Males' genotypes														
Male 1	131		107		157		204		181		120		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **H**	127	135	129	129	155?	155?	204	204	175	181	122	122	74	80
Lab male 1	133		125		171		204		181		122		82	
Worker 1	133	135	125	129	155	171	204	204	181	181	122	122	74	82
Worker 2	127	133	125	129	155	171	204	204	181	181	122	122	74	82
Worker 3	133	135	125	129	155	171	204	204	181	181	122	122	74	82
Worker 4	127	133	125	129	155	171	204	204	181	181	122	122	74	82
Worker 5	133	135	125	129	155	171	204	204	175	181	122	122	74	82
Worker 6	127	133	125	129	155	171	204	204	181	181	122	122	74	82
Worker 7	127	133	125	129	155	171	204	204	181	181	122	124	74	82
Worker 8	133	135	125	129	155	171	204	204	181	181	122	124	74	82
Worker 9	133	135	125	129	155	171	204	204	175	181	122	122	74	82
Worker 10	133	135	125	129	155	171	204	204	181	181	122	124	74	82
Males' genotypes														
Male 1	133		125		171		204		181		122		82	
Male 2	133		125		171		204		181		124		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **I**	133	135	111	131	149	155	204	204	181	183	122	124	82	82
Lab male 1	133		125		171		204		181		122		82	
Worker 1	133	135	111	125	149	171	204	204	181	181	124	122	82	82
Worker 2	131	133	111	125	149	171	204	204	181	183	122	122	80	82
Worker 3	133	135	111	131	149	155	204	204	183	185	124	124	80	82
Worker 4	133	135	111	125	149	171	204	204	181	181	124	122	82	82
Worker 5	131	133	111	125	149	171	204	204	181	181	122	122	80	82
Worker 6	133	135	111	131	149	155	204	204	181	185	124	124	80	82
Worker 7	133	135	111	131	149	155	204	204	181	185	122	124	82	82
Worker 8	133	133	111	125	149	171	204	204	181	183	122	124	82	82
Worker 9	133	135	111	131	149	155	204	204	183	185	122	124	82	82
Worker 10	133	133	111	125	149	171	204	204	181	183	122	124	82	82
Males' genotypes														
Male 1	133		125		171		204		181		122		82	
Male 2	131		125		171		204		181		122		80	
Male 3	133		131		155		204		185		124		80	
Male 4	133/	135	111/	131	149/	155	204		185		122/	124	82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **J**	133	135	109	109	153	163	204	204	181	183	122	122	82	82
Lab male 1	137		103		163		204		181		122		82	
Lab male 2	133		125		171		204		181		122		82	
Worker 1	135	137	103	109	153	163	204	204	181	183	122	122	82	82
Worker 2	135	137	103	109	163	163	204	204	181	183	122	122	82	82
Worker 3	135	137	103	109	153	163	204	204	181	183	122	122	82	82
Worker 4	135	137	103	109	153	163	204	204	181	181	122	122	82	82
Worker 5	135	137	103	109	153	163	204	204	181	183	122	122	82	82
Worker 6	135	137	103	109	163	163	204	204	181	183	122	122	82	82
Worker 7	135	137	103	109	153	163	204	204	181	183	122	122	82	82
Worker 8	135	137	103	109	163	163	204	204	181	181	122	122	82	82
Worker 9	135	137	103	109	153	163	204	204	181	181	122	122	82	82
Worker 10	135	137	103	109	153	163	204	204	181	181	122	122	82	82
Males' genotypes														
Male 1	137		103		163		204		181		122		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **K**	133	135	117	125	149	155	204	204	175	181	122	122	80	82
Lab male 1	137		103		163		204		181		122		82	
Lab male 2	133		125		171		204		181		122		82	
Worker 1	135	137	103	125	155	163	204	204	181	181	122	122	80	82
Worker 2	135	137	103	125	149	163	204	204	175	181	122	122	80	82
Worker 3	133	137	103	125	155	163	204	204	181	181	122	122	80	82
Worker 4	135	137	103	125	155	163	204	204	181	181	122	122	80	82
Worker 5	135	137	103	117	155	163	204	204	175	181	122	122	80	82
Worker 6	131	133	109	117	149	167	204	204	181	181	122	122	82	82
Worker 7	133	137	109	125	155	163	204	204	175	181	122	122	82	82
Worker 8	133	137	103	125	149	163	204	204	175	181	122	124	82	82
Worker 9	133	137	103	125	149	163	204	204	181	181	122	124	82	82
Worker 10	135	137	109	117	149	163	204	204	175	181	122	122	80	82
Males' genotypes														
Male 1	137		103		163		204		181		122		82	
Male 2	137		109		163		204		175/	181	122		80/	82
Male 3	131		109		167		204		181		122		82	
Male 4	137		103		163		204		181		124		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **L**	133	135	111	113	149	149	204	204	181	185	122	122	82	82
Lab male 1	137		103		163		204		181		122		82	
Lab male 2	133		125		171		204		181		122		82	
Worker 1	135	137	103	111	149	163	204	204	181	185	122	122	82	82
Worker 2	133	135	113	125	149	171	204	204	181	181	122	122	82	82
Worker 3	133	135	113	125	149	171	204	204	181	181	122	122	82	82
Worker 4	133	135	113	125	149	171	204	204	181	181	122	122	82	82
Worker 5	133	135	111	125	149	171	204	204	181	185	122	122	82	82
Worker 6	135	137	103	111	149	163	204	204	181	185	122	122	82	82
Worker 7	135	137	103	113	149	163	204	204	181	181	122	122	82	82
Worker 8	135	137	103	113	149	163	204	204	181	185	122	122	82	82
Worker 9	133	135	111	125	149	171	204	204	181	181	122	124	82	82
Worker 10	135	137	103	111	149	163	204	204	181	181	122	124	82	82
Males' genotypes														
Male 1	137		103		163		204		181		122		82	
Male 2	133		125		171		204		181		122		82	
Male 3	133/	135	125		171		204		181		124		82	
Male 4	137		103		163		204		181		124		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **M**	133	135	113	115	141	153	204	204	181	183	122	122	82	84
Lab male 1	137		103		163		204		181		122		82	
Lab male 2	133		125		171		204		181		122		82	
Worker 1	135	137	103	115	141	163	204	204	181	181	122	122	82	82
Worker 2	133	137	103	115	141	163	204	204	181	181	122	122	82	84
Worker 3	133	133	115	125	153	171	204	204	181	183	122	122	82	84
Worker 4	135	137	103	115	141	163	204	204	181	181	122	122	82	82
Worker 5	133	135	115	125	153	171	204	204	181	181	122	122	82	82
Worker 6	133	135	115	125	153	171	204	204	181	183	122	122	82	82
Worker 7	133	137	103	115	141	163	204	204	181	183	122	122	82	84
Worker 8	133	133	115	125	153	171	204	204	181	181	122	122	82	84
Worker 9	133	135	115	125	141	171	204	204	181	183	122	122	82	82
Worker 10	133	133	115	125	141	171	204	204	181	183	122	122	82	84
Males' genotypes														
Male 1	137		103		163		204		181		122		82	
Male 2	133		125		171		204		181		122		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **N**	137	139	113	113	153	159	204	204	181	181	122	122	82	84
Lab male 1	137		113		163		204		181		122		82	
Lab male 2	139		113		159		204		181		122		82	
Worker 1	129	139	113	113	153	159	204	204	181	181	122	122	82	82
Worker 2	129	139	113	113	159	165	204	204	181	181	122	122	82	82
Worker 3	129	137	107	113	153	165	204	204	181	181	122	122	82	84
Worker 4	137	139	113	113	159	165	204	204	181	181	122	122	82	84
Worker 5	129	139	113	113	153	159	204	204	181	181	122	122	82	82
Worker 6	137	139	113	113	153	159	204	204	181	181	122	122	82	84
Worker 7	129	139	111	113	159	165	204	204	181	181	122	122	82	82
Worker 8	129	139	113	113	153	159	204	204	181	181	122	122	82	84
Worker 9	137	139	113	113	159	163	204	204	181	181	122	122	82	84
Worker 10	129	139	113	113	159	165	204	204	181	181	122	122	82	82
Males' genotypes														
Male 1	137		113		163		204		181		122		82	
Male 2	139		113		159		204		181		122		82	
Male 3	129		113		165		204		181		122		82	
Male 4	129		107		165		204		181		122		82	
Male 5	129		113		153/	159	204		181		122		82	
Male 6	137	139	113		165		204		181		122		82	
Male 7	129		111		165		204		181		122		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **O**	127	137	111	119	141	145	204	204	181	181	122	126	82	82
Lab male 1	137		113		163		204		181		122		82	
Lab male 2	139		113		159		204		181		122		82	
Worker 1	127	139	111	113	141	159	204	204	181	181	122	126	82	82
Worker 2	127	139	113	119	141	159	204	204	181	181	122	126	82	82
Worker 3	137	139	113	119	145	159	204	204	181	181	122	122	82	82
Worker 4	137	139	111	113	141	159	204	204	181	181	122	122	82	82
Worker 5	137	139	111	113	141	159	204	204	181	181	122	126	82	82
Worker 6	127	139	113	119	141	159	204	204	181	181	122	122	82	82
Worker 7	127	139	111	113	141	159	204	204	181	181	122	126	82	82
Worker 8	137	139	111	113	141	159	204	204	181	181	122	122	82	82
Worker 9	127	139	113	119	141	159	204	204	181	181	122	122	82	82
Worker 10	137	139	111	113	145	159	204	204	181	181	122	122	82	82
Males' genotypes														
Male 1	139		113		159		204		181		122		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **P**	131	133	107	111	151	157	204	204	181	185	120	122	82	84
Lab male 1	131		107		157		204		181		120		82	
Lab male 2	135		125		141		204		181		122		82	
Lab male 3	137		113		159		204		181		122		82	
Worker 1	131	133	107	111	151	157	204	204	181	183	120	124	82	84
Worker 2	131	131	107	111	151	157	204	204	181	185	120	122	82	84
Worker 3	131	131	107	111	145	157	204	204	181	183	120	124	82	84
Worker 4	131	131	107	111	151	157	204	204	181	185	120	122	82	84
Worker 5	133	133	107	111	145	157	204	204	181	183	120	124	82	82
Worker 6	131	131	107	123	151	157	204	204	181	185	120	122	82	84
Worker 7	131	133	107	123	151	157	204	204	181	185	120	122	82	82
Worker 8	131	131	107	123	145	157	204	204	181	183	120	122	82	84
Worker 9	131	133	107	111	151	157	204	204	181	185	120	122	82	82
Worker 10	131	131	107	111	145	157	204	204	181	185	120	122	82	84
Males' genotypes														
Male 1	131		107		157		204		181		120		82	
Male 2	131/	133	107/	111	151/	157	204		183		124		82/	84
Male 3	131		107/	111	145		204		183		124		82/	84
Male 4	133		107/	111	145		204		183		124		82	
Male 5	131		123		151/	157	204		181/	185	120/	122	82	
Male 6	131		123		145		204		183		120/	122	82/	84
Male 7	131		107/	111	145		204		181/	185	120/	122	82/	84

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **Q**	133	135	113	125	147	147	204	204	181	183	122	122	82	82
Lab male 1	133		125		147		204		183		122		82	
Lab male 2	137		105		165		204		181		122		82	
Lab male 3	133		113		163		204		181		122		82	
Worker 1	133	135	111	125	147	147	204	204	181	183	122	122	82	82
Worker 2	133	135	113	125	147	153	204	204	181	183	122	122	82	82
Worker 3	133	135	113	125	147	147	204	204	181	183	122	122	82	82
Worker 4	133	135	111	125	147	147	204	204	181	183	122	122	82	82
Worker 5	133	133	113	125	147	153	204	204	183	187	122	122	82	82
Worker 6	133	133	111	125	147	153	204	204	181	183	122	122	82	82
Worker 7	133	135	113	125	147	147	204	204	181	183	122	122	82	82
Worker 8	133	135	113	125	147	153	204	204	183	187	122	122	82	82
Worker 9	133	133	113	125	147	153	204	204	183	187	122	122	82	82
Worker 10	133	133	111	125	147	147	204	204	181	183	122	122	82	82
Worker 11	133	133	111	125	147	147	204	204	183	183	122	122	82	82
Worker 12	133	135	113	125	147	147	204	204	181	183	122	122	82	82
Males' genotypes														
Male 1	133		125		147		204		183		122		82	
Male 2	133		111		147		204		183		122		82	
Male 3	133/	135	113/	125	153		204		183		122		82	
Male 4	133		113/	125	153		204		187		122		82	
Male 5	133		111		153		204		181/	183	122		82	

Primers	**Ce2- 12D**		**Ce2-3A**		Ce2-4E		**Ce2- 5D**		**Ce2-4A**		Card 8		**Cobs 13**	
Caste														
Queen **R**	135	135	109	111	151	169	204	204	181	181	122	122	82	84
Lab male 1	133		125		147		204		183		122		82	
Lab male 2	137		105		165		204		181		122		82	
Lab male 3	133		113		163		204		181		122		82	
Worker 1	133	135	109	125	147	169	204	204	181	183	122	122	82	82
Worker 2	133	135	111	125	147	151	204	204	181	183	122	122	82	82
Worker 3	133	135	111	125	147	169	204	204	181	183	122	122	82	84
Worker 4	133	135	109	125	147	169	204	204	181	183	122	122	82	84
Worker 5	133	135	109	125	147	151	204	204	181	183	122	122	82	84
Worker 6	133	135	111	125	147	169	204	204	181	183	122	122	82	84
Worker 7	133	135	109	125	147	151	204	204	181	183	122	122	82	84
Worker 8	133	135	109	125	147	151	204	204	181	183	122	122	82	82
Worker 9	133	135	109	125	147	151	204	204	181	183	122	122	82	84
Worker 10	133	135	111	125	147	151	204	204	181	183	122	122	82	82
Males' genotypes														
Male 1	133		125		147		204		183		122		82	

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