Demography and behavior of polygyne nests of the supercolonial *Cataglyphis niger*:

Does kinship matter?

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Abstract

The basic ant colony structure is presumed to have evolved trough kin selection. However, ants show a remarkable diversity in social organizations from a monogynous and monoandrous queen to the more derived states of polygyny with polyandrous queens. It is not clear whether kin selection can still explain these derived societies. The existence of polygyny is still an evolutionary enigma since kin selection theory predicts that queens should thrive for reproductive monopoly on the one hand, and workers are predicted to favor their own matriline in rearing gynes, on the other hand. Using a Quick Response (QR) - barcoding system that allows tracking individual queens and workers interactions, along with polymorphic DNA microsatellite markers indicating the matriline and patriline of each worker in the nest, we demonstrate the complex social interactions in polygyne nests of *Cataglyphis niger*. *C. niger* is not only polygyne but constitute a supercolony in the study site. Our findings that both the residing queens and the workers in the nest are not necessarily related to each other, are first for this species, support the supercolony structure of the population. Also in line with such a social structure, we demonstrate that they contribute equally to the nest production and rearing of the queens. Unlike invasive supercolonial species, *C. niger* is native in Israel, raising questions about the driving forces that stabilize this society, apart from kin selection.

Keywords: nepotism, social structure, supercolony, QR tracking, kin selection, polygyne

Introduction

In the middle ages, the imposed sterility of catholic popes has driven an inclusive fitness behavior of appointing their nephews as cardinals who will later on succeed them as popes, this is known as nepotism. Similarly, monogymous ant colonies, the postulated basic colony structure, presumably evolved via kin selection (Hamilton, 1964; Hölldobler and Wilson, 1990; Hughes *et al.*, 2008; Boomsma, 2009). Notwithstanding, more derived states where the colony is composed of multiple queens (polygyny) that may also be multiply inseminated, are very common and pose a conflict for inclusive fitness theory. Polygyny poses two problems: from the queens' perspective, queens are predicted to compete for monopoly in reproduction; from the workers' perspective, workers are predicted to favor their own matriline in rearing gynes (young unmated queens; (Keller, 1993). The social consequences of polygyny in ant colonies are variable, ranging from equal reproductive share among queens to functional monogyny (i.e., only one queen reproduces while all other queens behave as workers), and from complete openness to incoming queens to limited acceptance of only related queens (Hölldobler and Wilson, 1977). Social polymorphism is mostly believed to be the result of multiple selective pressures such as ecological constraints on nesting success, predation, increased genetic diversity, and is usually associated with profound changes in life history strategies and dispersal behavior (Bourke and Franks, 1995; Keller, 1995; Pamilo and Crozier, 1996; Ross, 2001; Chapuisat, Bocherens and Rosset, 2004). Inclusive fitness theory also assumes favorable discrimination of closely related individuals (e.g., nepotism), unless between-matriline competition hampers total colony reproductive output. This discrimination is likely mediated through kin recognition cues, similar to nestmate recognition cues. Although, recognition cues exist in ants (Soroker *et al.*, 1994; Lahav *et al.*, 1998) they seem to play a role in within-colony kin discrimination only in rare cases (El-Showk *et al.*, 2010). Additionally, in most cases relatedness in these polygyne nests is low thus questioning the benefits of inclusive fitness theory at all in these cases. In fact, so far nepotism in eusocial insects, specifically in ants has been mostly rejected ((DeHeer and Ross, 1997; Zinck, Châline and Jaisson, 2009; Friend and Bourke, 2012), except

for *Formica fusca* (Hannonen and Sundström, 2003) which was criticized for not having an actual behavioral assay and rather looked into the outcomes of behaviors (Holzer *et al.*, 2006; e.g. kin composition of brood), and *Leptothorax acervorum* ants (Gill and Hammond, 2011). Therefore, the extent of nepotism in eusocial insects is still unclear, and the need for actual behavioral assays exists. Boomsma and d'Ettorre (2013) suggested that within-colony kin discrimination might be maintained in a secondary polygyny where there is a high queen turnover and fluctuating relatedness in the colony.

The ant genus of *Cataglyphis* comprises over one hundred described species that are distributed over the palaerctic realm in arid environments (Agosti, 1990). *Cataglyphis* exhibits high social polymorphism from strictl monogyny (*C. sabulosa*, *C.bicolor* , C. emmae and *C. hispanica*) through facultative polygyny (*C. velox* and *C. livida*) to polygyny (*C. niger* and *C. mauritanica*; (Lenoir *et al.*, 2009; Boulay *et al.*, 2017). The genus also shows a diversity of reproduction modes (sexual and asexual), which allows us to investigate the evolution of sociality. Thus, the importance of understanding the mode of social organization in each species is high. Specifically, it is important to understand interactions within a polygyny nest where there are multiple possibilities of nest foundation and relatedness asymmetries (Keller and Reeve, 1995).

Here, we focus on a population of *C. niger* that has polygyne nests that are organized as a supercolony (Leniaud *et al.*, 2011). In this population structure, brood and workers are often translocated to other nests in the population according to the changing needs of the widespread colony. This complex social system encompasses multiple thoughts about the mechanisms leading to this evolutionary stable status. Nepotism has been investigated in the imported fire ant which has a similar population and social structure in its invasive range, refuting the existence of nepotism (DeHeer and Ross, 1997). However, *C. niger* is a native species, and therefore could behave differently and possibly show within-colony kin discrimination more than an invasive species.

Using a barcode system for tracking individual movement, we monitored worker-worker and workers-queen interactions in polygyne nests. We also genotyped the workers and queens in order to assess the within-nest relatedness. For the first time, to the best of our knowledge, we obtained extensive data of both individual interactions and relatedness between these individuals, showing the complex relationships present in polygyne nests, and dismissing the possibility of nepotism.

Methods

Ant collection and maintenance

Three complete polygyne nests (nests 677, 868 and 869 having 3, 3 and 4 queens, respectively) of *C. niger* (niger haplotype, as described in Eyer 2017) were excavated from the Tel-Baruch population in the semi-stabilized sand dunes of Tel Aviv, Israel. Prior to any manipulation, nests were kept in the lab for about a month under constant temperature and humidity and were given water and a diet according to (Bhatkar and Whitcomb, 1970), supplemented with tuna fish. For observations, a subset of randomly selected workers (n=200 for each of the 3 nests) and the queens were housed in artificial nests (20cm x 30cm) closed with an IR filtered roof and given water and food *ad libitum*. Workers and queens were each marked with individual 2mm QR barcodes using skin adhesive (SAUER-HAUTKLEBER, Manfred sauer GMBH). Using a Canon T3i we took snap shots of the nest every 2 seconds, for 10,000 continuous frames (approximately 5.5 hours). The following observation protocol was done for each of the 3 nests. Three days after tagging the first session began, constituting free roaming queens and workers, and lasted for 3 days. In the following session the queens were tethered at the petiole with fine nylon string (2-3 cm long), restricting each to a limited space in different corners of the nest, while allowing free movement of the workers throughout the nest. At the end of this session the queens were freed for three days, after which a second session of tethered queens began, in which the queens were placed at corners different from previous.

127 Statistical analysis

128 Behavioral analyses were performed using BugTag, a barcode analyzing system
129 (developed by *robiotec*). The tags allow for constant monitoring of the location of
130 each individual ant. The direction of the tags was corrected to match the position of
131 the head of the ants, indicative of the direction at which the ant faced. Workers with
132 low detection rates (their identity was certain in less than 30% of the 10000 frames)
133 were excluded from the analyses. In cases where missed detections occurred in
134 segments that were either fewer than five frames (less than 10 second apart), or if
135 the change in the individual's position before and after the detection gap was lower
136 than 2cm in distance, the position was interpolated using linear regression. Thus, the
137 numbers of workers in each analysis were 137 and 122 for nest 677, 149 and 161 for
138 nest 868, and 161 and 159 for nest 869.

139 Using MATLAB, we defined a circular virtual arena (2cm radius) around each queen,
140 hence termed "retinue space". Workers were considered to engaged in retinue
141 around the queen if they were positioned in the "retinue space" while facing the
142 queen for over 10% of the observation time. Ants that only occasionally interacted
143 with a queen or sporadically shared her roaming space were not considered as
144 involved in retinue behavior. The constancy of these groups over time, in terms of
145 ant identity, and the fidelity of each worker to a specific queen or task were also
146 measured. We compared our results with random permutations of worker-queen
147 sets, allowing random positioning of workers next to the queens.

148 Genetic analysis

149 At the end of the experiment, all the queens and workers were sacrificed for genetic
150 analyses. For nest 868 we analyzed the 3 queens and all the 165 workers in the
151 experimental nest. For the remaining 2 nests (677and 869) we analyzed the queens
152 and a sub set of workers (18 and 28 respectively), which included the top three
153 "retinue" workers for each queen (the minimal number of retinue workers for all of
154 the queens in all of the sessions) along with randomly selected nestmates. Queens'
155 ovaries were dissected for assessing their degree of activation. Under binocular we
156 compared the number of reproductively active ovarioles as well as the number and

157 size of oocytes in them, creating an activation index as follows: **0** - non-activated
158 ovarioles; **1** - 2 to 4 activated ovarioles with developed oocytes; **2** - 4 activated
159 ovarioles with small oocytes; **3** - all ovarioles with large, ready to lay eggs). We also
160 kept the spermathecal content of each queen for genetic analyses in ringer's buffer
161 at -80°c until DNA was extracted. DNA was extracted with 5% CHELEX (*BIO-RAD*) and
162 amplified with 7 microsatellite markers that had been designed previously for *C.*
163 *hispanica* (Ch23 ;Darras, Kuhn and Aron, 2014), *C. cursor* (Cc51,Cc89, and
164 Cc99;Pearcy *et al.*, 2004), and *C. niger* (Cn02, Cn04, and Cn08 ;Saar *et al.*, 2014)
165 using Type-it PCR mix (QIAGEN). PCR products were sequenced by ABI3500 genetic
166 analyzer and genotype analyses were performed with GeneMarker. Relatedness was
167 evaluated with Coancectry (Wang, 2011) and ML Relate (Kalinowski, Wagner and
168 Taper, 2006). We checked for possible correlation of relatedness to the mean
169 distances between all the individuals in the nest (workers and queens) using
170 SOCPROG 2.8. Network analysis was done using UCI6 and NetDraw.

171 **Results**

172 Nests compositions and within nest relatedness

173 The experimental nests had 3-4 queens (667- 3; 868- 3; 869-4), all of which were
174 multiply inseminated by 3-7 males. All the queens in the experiment (n=10) had well
175 developed ovaries with ready to lay eggs (level 3), except for one queen in nest 868
176 (Q3- tag # 552) and one queen in nest 869 (Q4- tag # 1189) who had medium size
177 oocytes (level 2). Table 1 presents the level of relatedness among the queens in the
178 experimental nests. In nest 869 three of the four queens were highly related (x>0.5;
179 full sisters or daughters), whereas the 4th queen was completely unrelated (x<0; not
180 different from relatedness=0). In nest 677 two of the queens were slightly related
181 (0.2181; half sisters or cousins), while the 3rd was not related to any of the other two
182 (x<0.1). All three queens in nest 868 were completely unrelated (x<0.1).

183 To assess nest demography, we genotyped all the workers and queens from nest 868
184 that were engaged in the tagging experiment (three queens and 165 workers). Since
185 these workers were randomly selected out of the approximately 1,000 workers that
186 populated the nest, we assume that they reflect a good assessment of the genotype

187 distribution within the entire nest. The genotype analysis revealed that 9.6% of the
188 workers were highly related (x>=0.5), 14.8% were moderately related (0.25<=x<0.5),
189 19.3% had low relatedness (0.125<=x<0.25) and 56.3% were unrelated (x<0.125).
190 Figure 1 shows a network analysis of relatedness values with ties indicating
191 relatedness greater than 0.25 and nodes are sized according to centrality measures.
192 Relatedness distribution of the workers to the different queens was as follows:
193 Highly related workers (x >=0.5, possible daughters or full sisters) Q1=17, Q2=15 (of
194 which 1 worker was similarly related to both Q1 and Q2), and Q3= 10. Moderately
195 related workers (0.25<=x<0.5, possible sisters) to the different queens was Q1=24,
196 Q2=26 (of which 4 workers were similarly related to both Q1 and Q2), and Q3= 4.
197 The remaining 63 workers were not related to any of the three queens, (X< 0.25).
198 Overall, nest relatedness for nest 868 was 0.058, and average relatedness of the
199 workers to each of the queens was similar (0.089±0.004). These results are slightly
200 lower than the within nest relatedness of the whole population of 0.112±0.009
201 (based on 129 workers from 6 nests; data not presented).

202 For nests 677 and 869 we genotyped a subset of workers as described in table 2. In
203 nest 677, five of 18 workers were highly related to one of the queens (relatedness
204 >=0.5): Q1=1, Q2= 4, and Q3=0. Seven workers were moderately related
205 (0.25<=x<0.5, possible sisters): Q1=5, Q2=1 (that was equally related to Q1), and Q3=
206 2. The remaining seven workers were not related to any of the queens (x<0.25). In
207 nest 869, eight of the workers were highly related to one of the queens (relatedness
208 >=0.5): Q1=2, Q2=1 (similarly related to Q1 and Q4), Q3=2 (one worker was similarly
209 related to Q4), Q4=5. Moderaltly related workers to queens were as follows: Q1= 4
210 (one similar to Q4), Q2= 6 (one similar to Q3 and one to Q4), Q3= 2, Q4=5. The
211 remaining 12 workers were not related to any of the queens.

212

213 Identity and genotype of "retinue workers"

214 The first set of experiments was done in an "open-range" arena where the queens
215 and workers could roam freely. Figure 2A depicts network analyses of the mean
216 distances between the individuals in the experimental nest 868. The network

presents only the ties between individuals that were in high proximity to each other over time (3 days; x<mean+2SD). The three queens as well as a small number of workers were highly central in the network, that is, they had close interactions with many individuals. These workers can be considered as ‘retinue workers’. Queens also tended to stay very close to each other. It is also evident that, over time, many of the workers did not retain high proximity to each other (had few or no ties). Figure 2B shows a network of relatedness of the ‘retinue’ workers only and the queens, depicting only ties higher than 0.25 (moderately to highly related). Queens were not very central with respect to relatedness values, having had only a few daughters among the retinue workers. Furthermore, the central retinue workers were not very centrally related to others in the nest. Aligning relatedness results and mean proximity locations of all the workers and queens showed no clear correlation (i.e., workers or queens do not interact more with highly related individuals; Mantel Z test= -0.005, p=0.75).

In the “open-range” arena queens tended to be near each other, which made it difficult to precisely determine which worker tended which queen. Therefore, to assess whether workers do prefer specific queens we spaced out the queens within the nest by confining each to a specific location in the nest while allowing workers free movement, and thus giving them the opportunity to selectively retinue specific queens. Figure 3 shows the number of workers (averaged over 500 frames) that occupied the retinue space around each queen. Queens' retinue consisted of 3.86±0.15 (±SE) workers (range of averages 2.8-4.9 of workers attending a queen through the whole session). In none of the nests there was apparent preference to specific queens. Workers varied in the time they spent interacting with the different queens (Fig. 4a+b), but most did not have prolonged interactions. Approximately a quarter of the workers spent more than 10% of their time in one of the queens' retinue space. The maximal proportion of time that workers spent next to a specific queen was 30%. The relatedness of the top three “retinue” workers (which spent more than 10% of their time in retinue) to the attended queens ranged between -0.29 to 0.54 (Fig. 5), demonstrating that the queens’ attendants were not necessarily related to the attended queen.

Workers were rarely loyal to the queens they initially attended to, but directed their retinue behavior also towards the other queens. Worker switches between queens occurred both during the same session as well as between the two replicate sessions (supplementary material 1). Finally, worker preference in retinue of specific queen was not different than random permutations of the workers' preference for the different queens.

Discussion

Interactions between nestmates in an ant colony are the main focus of many recent studies, all attempting to underline the mechanisms of 'self- organization' a hallmark in the evolution of social insects (Pinter-Wollman *et al.*, 2011; Mersch, Crespi and Keller, 2013; Heyman *et al.*, 2017). Here, for the first time, we combined individual tracking with genetic data, in order to elucidate the possible effects of relatedness and kin selection to worker in-nest behavior.

Polygyny is still an evolutionary enigma with respect to its adaptive value. Several explanations have been proposed for the mutual benefits of this social dynamic, from high relatedness of the co-inhabiting queens (the inclusive fitness explanation; Giraud *et al.*, 2001) to robustness of the colony (higher genetic diversity, and colony size explanations; Keller, 1995; Hughes, Ratnieks and Oldroyd, 2008; Heinze and Rueppell, 2014). In such complexed societies where relatedness varies highly between individuals, often reaching that of non-eusocial non-viscous population, we expect to see altruistic/nepotistic interactions, like those commonly found in many vertebrate societies (Eberhard, 1975; Clutton-Brock, 2002; Hatchwell, 2010). Theoretically nepotistic interactions should be favored unless they negatively affect the society's total fitness.

Additionally, in the present study, we assessed whether relatedness influences the dynamics of the nest (in terms of worker behavior) in this species case where polygyne nests also comprise a supercolony. As expected from a unicolonial system, we found that in *C. niger* inter- as well as intra-colony relatedness is very low; only about a third of nestmate workers are related to the level of sisters or half sisters. It was argued by Helanterä *et al.*(2009), that in order to maintain such a unicolonial

structures it is necessary to have nepotism. Otherwise there is a chance for the degradation of the social structure over evolutionary time scale. We could not detect in our system any nepotism.

Relatedness between the queens was also rather variable, ranging from sisters to un-related co-nesting queens. This suggests that this species exercises dependent colony foundation, conforming to the usual case in polygyne nests (Cronin *et al.*, 2013). Some of the queens return to their natal nests after mating or are transferred by workers to a new nest (budding; in the case of queens who are highly related) while other queens (with low relatedness) disperse and join existing nests (this could also be explained by colony fission). From our observations at the collection site, we observed only a few newly mated queens that attempted independent colony foundation, and very few nests with a single queen (unpublished data), thus dependent colony foundation seems to be a more preferred strategy in this population.

Our results also verify for the first time that this is a functional polygynous nest. Assessment of the ovarian status as well as the relatedness between the queens and workers indicate that all the queens contribute similarly to the nest population, thus is a true functional polygyny. Further contributing to the low relatedness is our finding that the queens are multiply inseminated. Finally, we observed frequent movement between nests of workers and brood. While we cannot exclude that this is simply nest relocation, we interpret this as free movement of workers brood and queens between the nests of the supercolony, according to changes in available resources (Robinson, 2014).

Queens in our experimental nests, were attended by the same number of workers on average, respectively they also showed similar oocyte development. These retinue workers, comprised only a small proportion of all the workers in the nest suggesting there is a retinue caste, as seen in honey bees (Seely, 1979), and in *C. fellah* as nurses' caste (Mersch, Crespi and Keller, 2013). These retinue workers were not closely related to the queens. In fact, it seems as the retinue workers attended the different queens at random, rather than being loyal to a specific queen. Indeed,

worker locations were not significantly difference in comparisons to random permutations. The same is true when we examine the relatedness between attendants and attendees. Nestmates do not associate more with highly related individuals, irrespective of whether they are workers or queens. All this emphasizes a stochastic intra-colony interactions, regarding both worker-queen and between workers interactions (i.e. the interactions are not different than expected at random) and thus, refutes any possibility of nepotistic behavior.

But is nepotism such a parsimony theory in this case? Under nepotism, we presume an unequal distribution of resources in favor of specific matrilines, which perturbs normal colony development, rendering polygyny evolutionary unstable. On the other hand, if nepotism is maladaptive, what is the driving force for workers to keep tending non-kin, and what are the mechanisms (if any) that prevent the downgrade of non-kin-biased worker behavior. This evolutionary conflict may explain why polygynous supercolonies are so rare, the benefits are mostly short lived and die out through the evolutionary timescale. For this kind of social structure to be stable, there should be other 'hard wired' mechanisms involved. An example is the 'social chromosome', a non combining chromosome that exists only in polygynous populations (Wang *et al.*, 2013; Purcell *et al.*, 2014). Future work should attempt to elucidate possible mechanisms that keep these populations stable, such as worker fixed sterility.

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Authors' contributions

TRB, OF and AH conceived the ideas and designed the methodology; TRB collected the data; TRB analyzed the genetic data; TRB and GB analyzed the tracking data; TRB and AH led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data Accessibility

Microsatellite genotypes, CSV files of ants' tracking and MATLAB analysis scripts will be available at: Dryad doi: to be announced after acceptance.

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Table 1: Relatedness values between queens

Nest - Queen	**869 Q2**	**869 Q3**	**869 Q4**
869 Q1	0.5304	-0.1901	0.5362
869 Q2	--	0.0626	0.6122
869 Q3	--	--	-0.0165
	667 Q2	**667 Q3**	--
667 Q1	0.2181	0.047	--
667 Q2	--	0.0794	--
	868 Q2	**868 Q3**	--
868 Q1	-0.1573	0.0873	--
868 Q2	--	-0.3619	--

	RELATEDNESS LEVEL	
NEST_QUEEN	**0.25**	**0.5**
677_Q1	6^{12}	1
677_Q2	5^{2}	4
677_Q3	2^{1}	0
869_Q1	$6^{12*°}$	2*
869_Q2	$7^{1*°\hat{}wy}$	1*
869_Q3	$4^{1\hat{}w}$	2^{1}
869_Q4	10^{12wy}	5^{1}

Table 2: Number of workers who have relatedness values of 0.25/0.5 to any of the queens. N= 18 for nest 677 and 28 for nest 869. $^{12*°\hat{}wy}$ indicate individuals that are related to both queens.

Figure legends:

Figure 1: Network analysis of relatedness values of the ants in nest 868 (with a threshold of 0.25). Queens colored in red. The size of the circle indicates its centrality (how many relationships he holds). The thickness of the line indicates the strength of the tie = relatedness value between individuals.

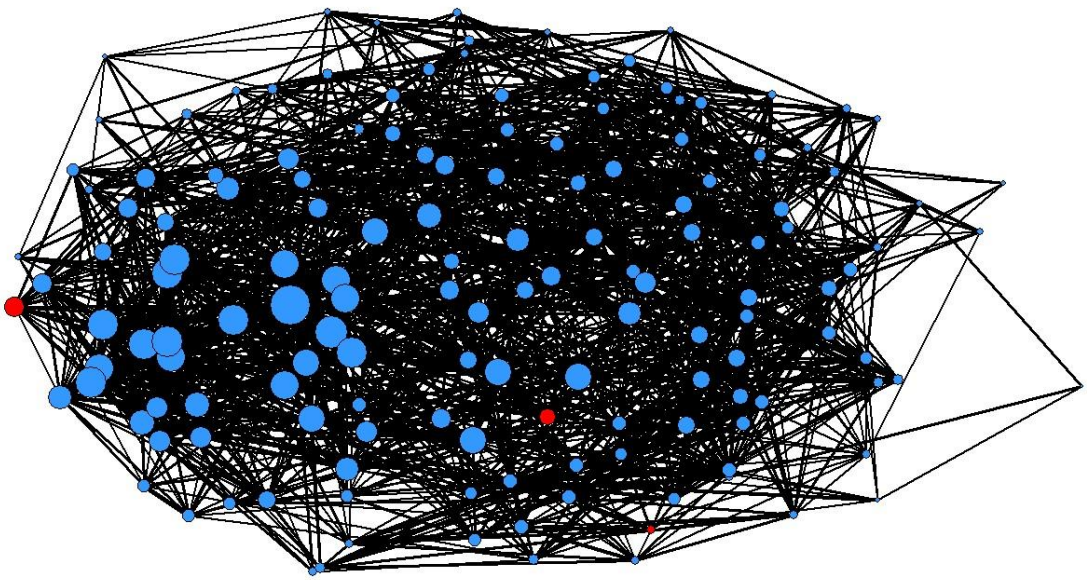


Figure 2: A) Network analysis of the averaged minimal distance (proximity) between the ants in nest 868 (threshold of x< 2*SD). Queens colored in red. The size of the square indicates its centrality (how many relationships he holds). The thickness of the lines indicate the strength of the tie= average proximity between individuals. B) Network analysis of relatedness values of the retinue ants in nest 868 showing ties larger than 0.25). Queens colored in red. The size of the circle indicates its centrality (how many relationships he holds). The thickness of the line indicates the strength of the tie = relatedness value between individuals.

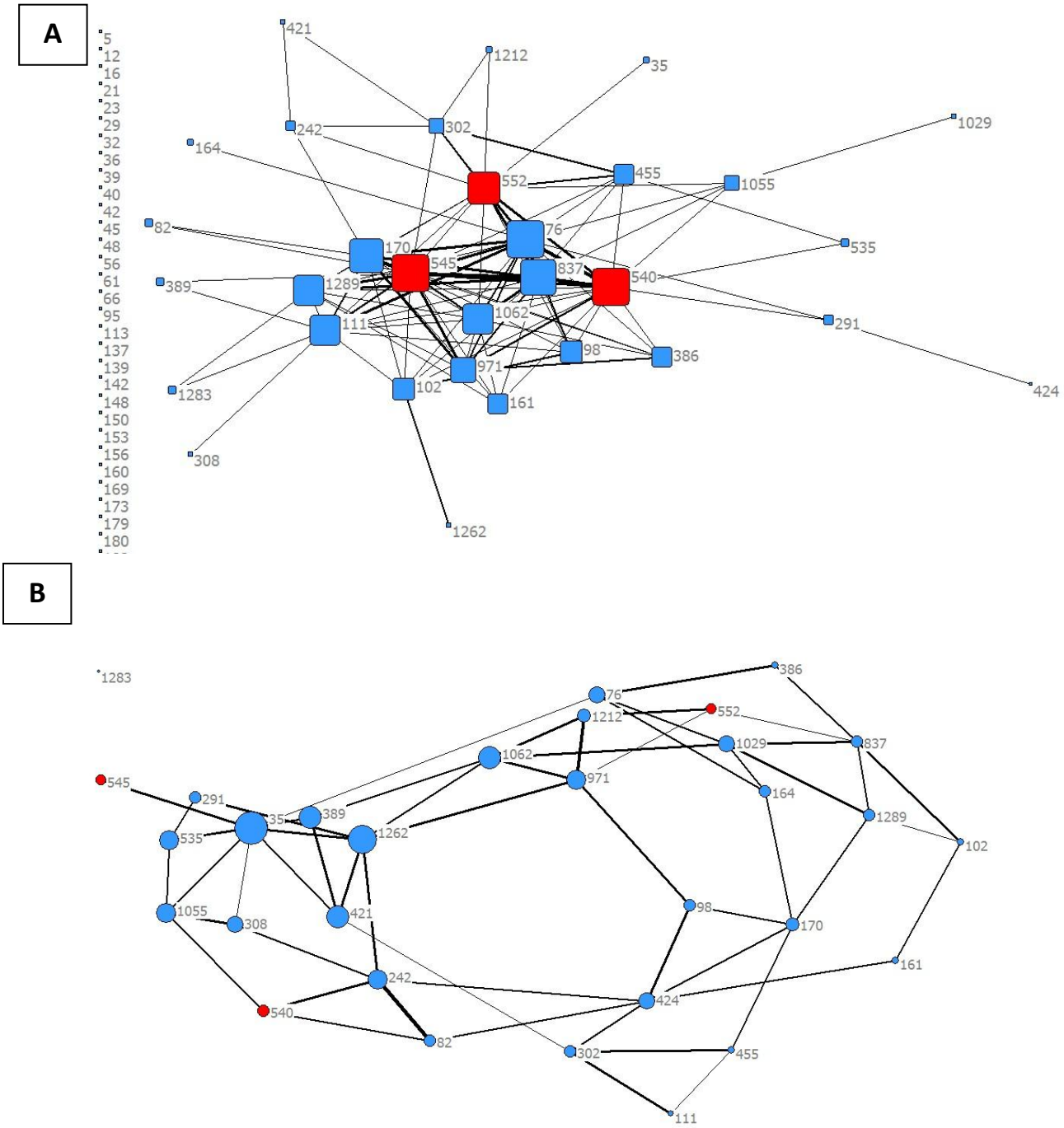


Figure 3: Assessment of queens' retinue, number of workers around each queen averaged for 500 frames. Average retinue was 3.86 (SE=0.15; range:2.8-4.9) workers for each queen. (a)nest 868; queen 552 in blue had smaller oocytes (b) nest 677 (c) nest 869; queen 1189 in purple had smaller oocytes.

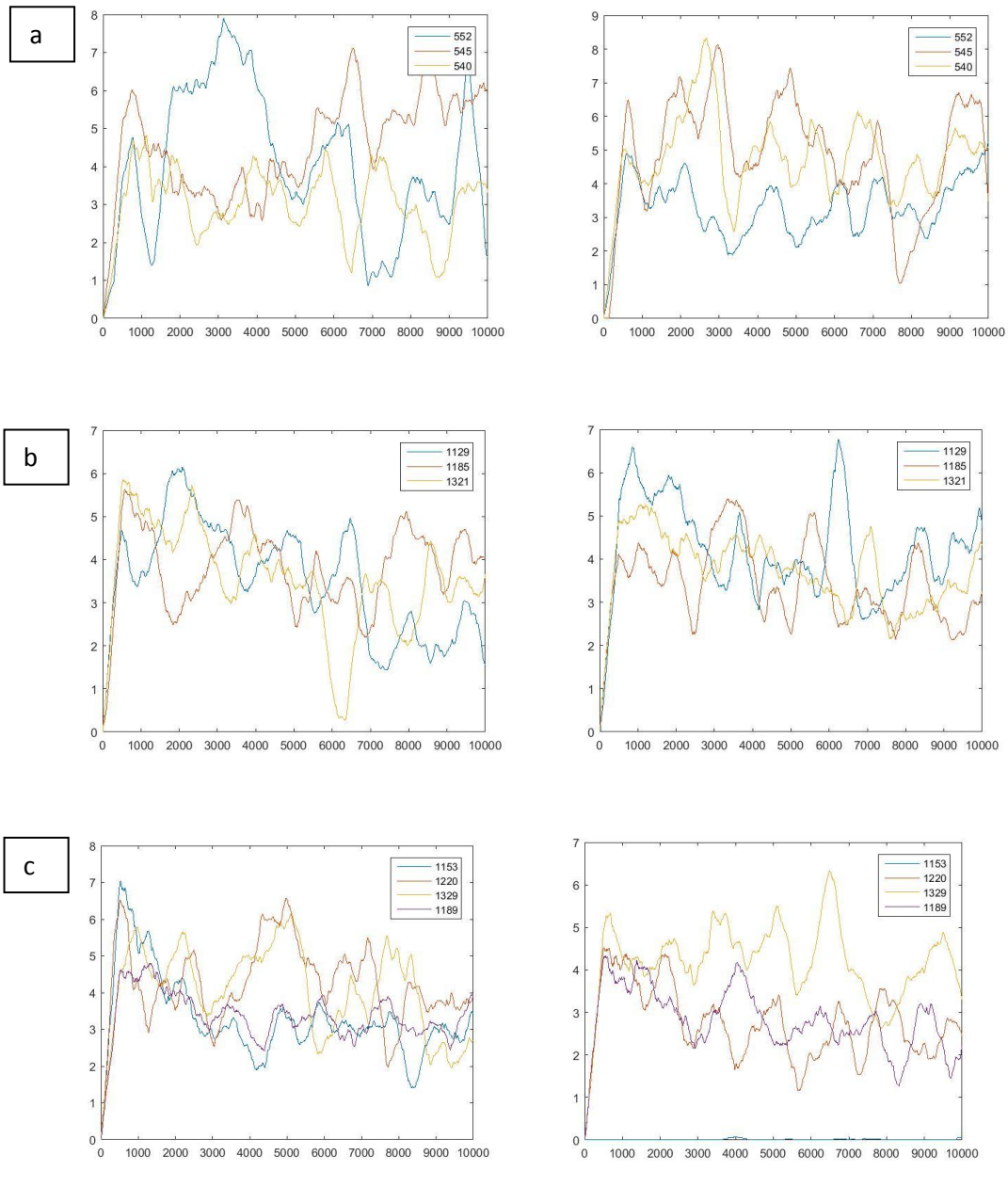
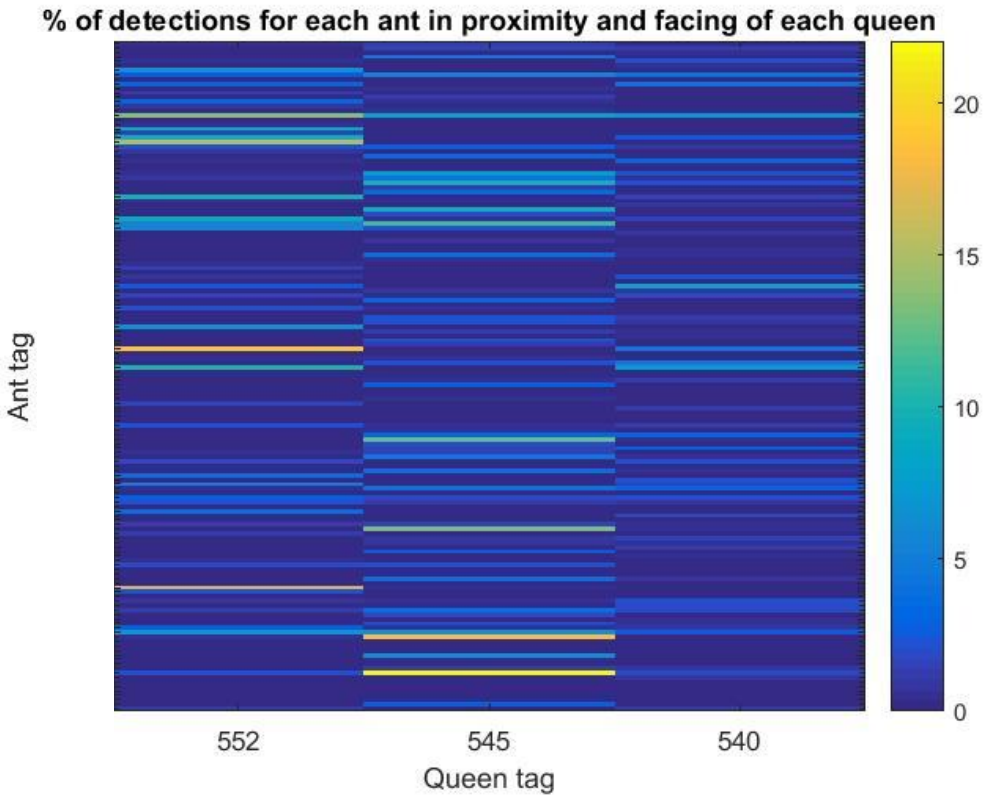


Figure 4: The proportion of time a worker interacted with a specific queen, normalized to each of the workers detection success in nest 868. a) First session where queens were tied. b) The second session of tied queens, after an intermediate time where queens were free.

a



b

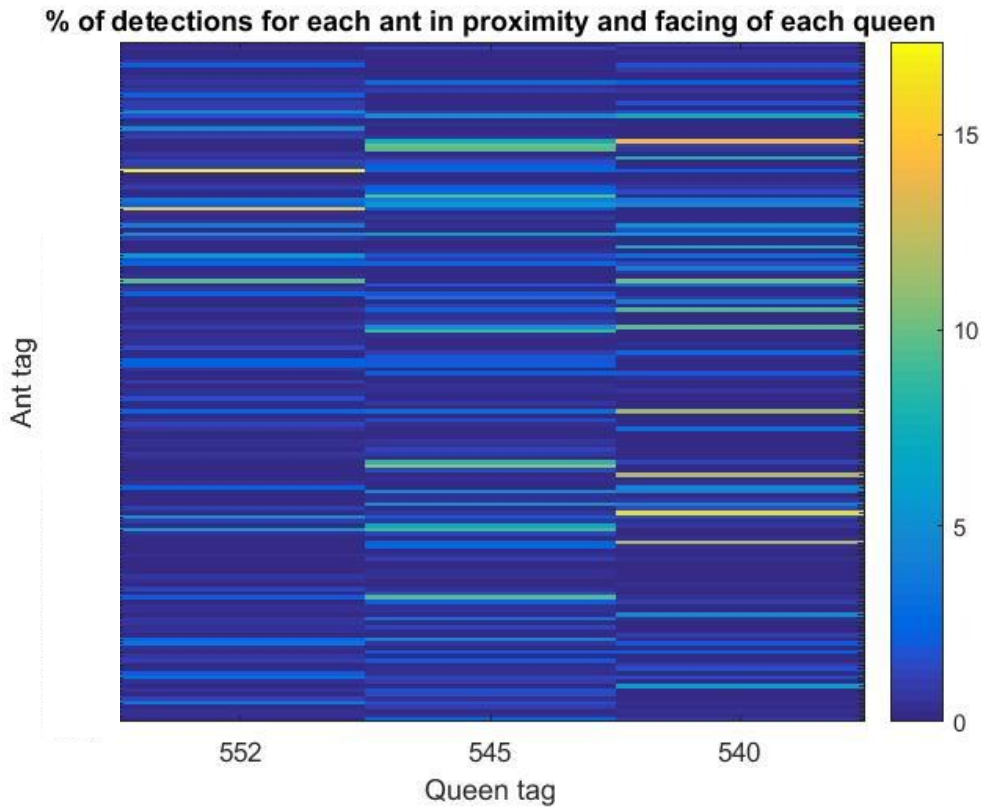


Figure 5: The level of Relatedness between individual ants, line thickness and length represents the level of relatedness, color and shape represent the different retinue groups of the queens (W=worker; Q= queen). Queens and their workers attendants (for each queen three of the workers who spent the most time next to the queen) in nest 677,868 (relatedness under zero was emitted from the network). A) nest 677 b) nest 868 c) nest 869

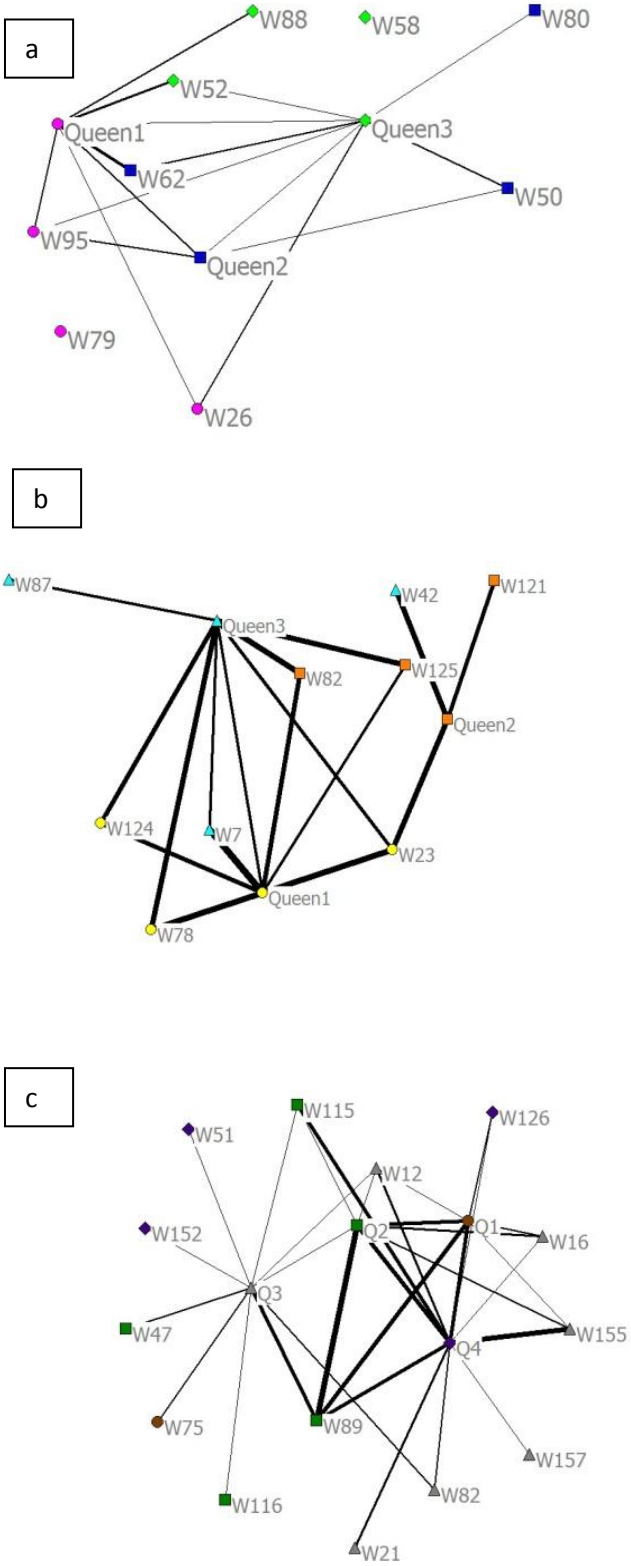
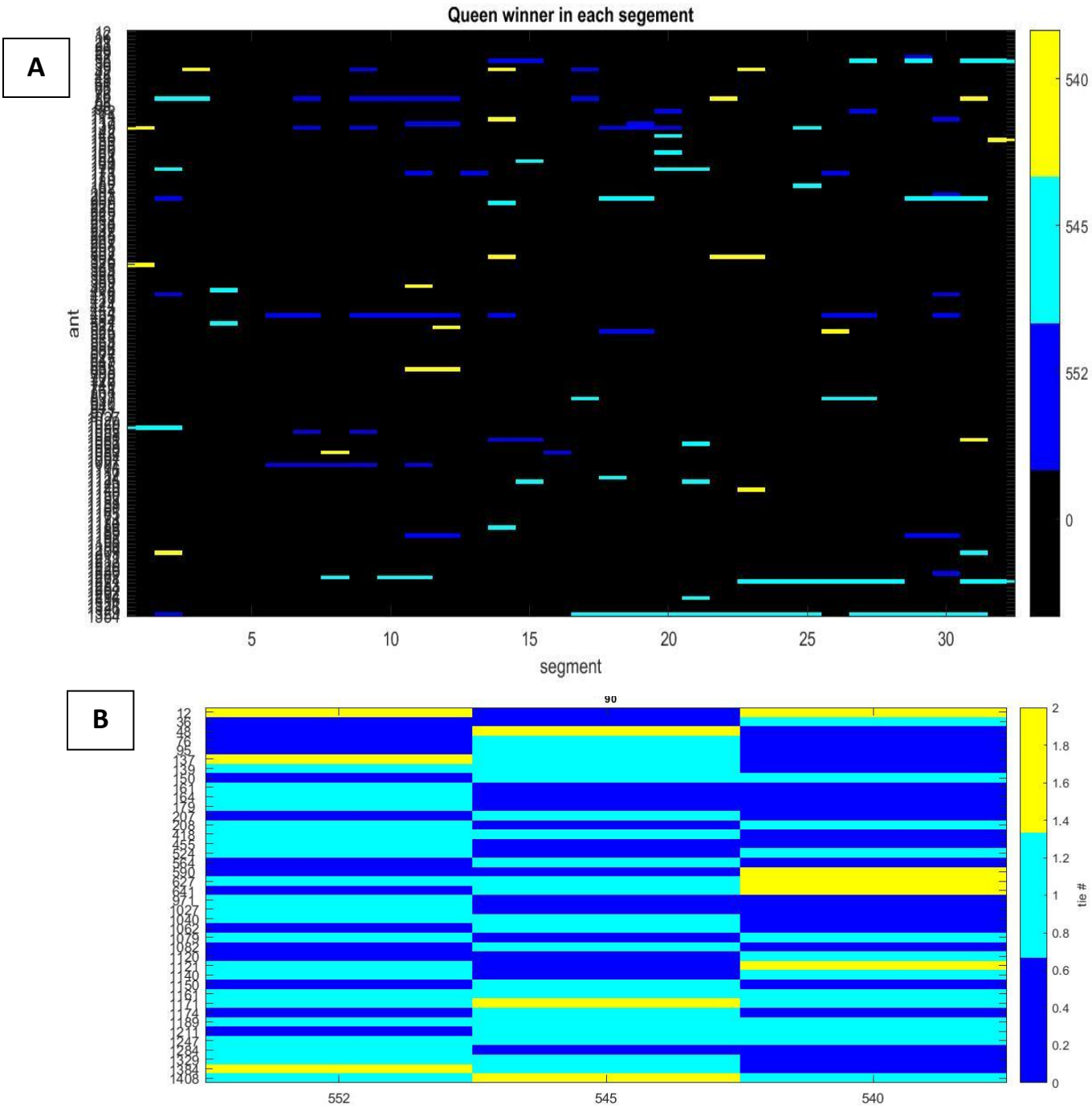



Figure Supplementary 1: a) Individual workers who spent more than X% out of 20 minute segments in a specific queen's retinue space. Each queen's retinue space is defined by a different color, other locations are black. b) Workers who attended the queens for more than 10% of the time. In dark blue the workers did not attend the specific queen, in light blue they attended the queen for only one repeat of the experiment, in yellow they attended to the queen in both repeats.