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Behavioural response of workers to repeated intergroup encounters in the harvester ant Messor barbarus

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Behavioural response of workers to repeated intergroup encounters in the harvester ant *Messor barbarus*

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Abstract

The evolution of cooperation in animal societies is often associated with the evolution of hostility towards members of other groups. It is usually predicted that groups under attack from outsiders should respond by becoming more cohesive or cooperative. However, the responses of individuals to real or simulated intergroup encounters vary widely, for reasons that are poorly understood. We tested how groups of workers of the harvester ant, *Messor barbarus*, responded to exposure to members of a different colony versus members of their own colony, and how previous exposure to an intruder affected the intensity of the within-group response. We found that workers increased in activity and had more contact with one another immediately following exposure to an ant from a different colony, but also showed a similar behavioural response to presentations involving an ant from their own colony. However, exposure to an intruder from a different colony resulted in much stronger behavioural responses to a second intruder, encountered shortly afterwards. Our results are consistent with studies of social vertebrates which suggest that exposure to intruders results in increased social cohesion. Our results also show that exposure to an intruder primes group members to respond more strongly to future intrusions. Our findings highlight a disconnect between the assumptions of theoretical models which study the effect of intergroup conflict on social evolution over many generations, and the short-term behavioural responses that are the usual focus of studies of intergroup conflict in insects and vertebrates.

Keywords: intergroup conflict, social evolution, cohesion, affiliation, priming

Introduction

Intergroup conflict is recognised as a major force influencing selection on social traits in organisms ranging from insects to humans (Darwin 1871; Reeve and Holldobler 2007; Bowles and Gintis 2011; Radford et al. 2016). Theoretical population genetic models have shown how intergroup conflict can favour the spread of altruistic alleles that increase between-group variation in fitness (and hence the strength of between-group selection) relative to within-group variation in fitness (Choi and Bowles 2007; Lehmann and Feldman 2008; Lehmann 2011). In addition, it is widely expected (largely on the basis of empirical studies) that groups under attack from other groups should pull together and become more cooperative (e.g. Radford 2008; Burton-Chellew et al. 2010). Thus conflict between groups is predicted to influence selection for altruism and cooperation within groups on an evolutionary timescale (i.e. over many generations), but is also predicted to affect the immediate, short-term behavioural responses of individuals to each other and to outsiders.

Empirical tests of the prediction that groups under attack from competitors should become more cohesive and cooperative have yielded conflicting results (Radford et al. 2016). For example, several primate species show an increase in grooming following group conflict (Cords 2002; Schino 2007; Majolo et al. 2016), and similar patterns have been observed in green woodhoopoes, *Phoeniculus purpureus* (Radford 2011) and laboratory groups of cooperative cichlids, *Neolamprologus pulcher* (Bruintjes et al. 2015). By contrast, in other species such as tufted capuchins, *Cebus apella* (Polizzi di Sorrentino et al. 2012), vervet monkeys, *Chlorocebus aethiops pygerythrus* (Arseneau et al. 2015), and bonnet macaques, *Macaca radiata* (Cooper et al. 2004), intergroup conflict has been shown to lead to an increase in within-group aggression.

The wide variation in observed behavioural responses to intergroup conflict may be linked to the inherent heterogeneity of groups in nature (Thompson and Cant 2018). Groups vary in their composition, and group members vary in status and incentive, which can alter decisions of when to

fight and the level of response to group conflict (Wilson et al. 2001; Cassidy et al. 2015; Arseneau-Robar et al. 2016). Individuals and groups can also vary in their past experience of conflict, which may differentially change individual responses to threats from competitors (Hsu et al. 2006; Esponda and Gordon 2015; Christensen and Radford 2018).

The ubiquity and intensity of between-group competition in ants makes them well suited to testing the effect of past experience on behavioural responses to intergroup conflict (Hölldobler and Wilson 1990; Bourke and Franks 1995; Adams 2016). Previous experimental studies in these systems have focussed on aggressive responses to conflict, with contrasting results. In some species, exposure to other groups resulted in a 'priming' response, where individuals in groups reacted more strongly to future encounters, or underwent developmental changes which increased their ability to fight or defend resources in future. For example, in Argentine ants, *Linepithema humile* (Van Wilgenburg et al. 2010), workers that were exposed to a non-colony member displayed increased aggression in subsequent encounters, and in interactions with ants from a colony that they had not previously encountered. In the ant *Pheidole pallidula,* exposure to intergroup conflict led to an increased production of soldiers (Passera et al. 1996). Theoretical and empirical work has shown that past experience can shape individual ants' criteria for non-nestmate recognition which collectively produces an aggressive colony-level response (Newey et al. 2010; Esponda and Gordon 2015). By contrast, some ant species have shown evidence of acclimatisation to the presence of neighbouring competitors, and a reduced aggressive response (*Pheidole* ants, Langen et al. 2000; *Streblognathus peetersi* and *Plectroctena mandibularis*, Tanner and Keller 2012). The causes of these varying responses to recent exposure are unclear. Moreover, in ants, little is known about the effect of exposure to intergroup conflict on social cohesion and within-group affiliation, the types of behaviour on which studies of primates and other vertebrates typically focus.

Here we test how simulated intergroup conflict affects affiliative behaviour and social cohesion in the harvester ant, *Messor barbarus*, and whether past experience affects the intensity of conflict responses. Specifically, we test the 'primed response' hypothesis which suggests that recent encounters with intruders reflect a high probability of subsequent, potentially costly, encounters. This hypothesis predicts that individuals that are exposed to intruders will exhibit elevated sensitivity and responsiveness to future intrusions, and higher levels of affiliation and social cohesion. We tested these predictions through an experiment in which we repeatedly exposed a group of workers to an individual from their own or an unfamiliar colony.

Methods

Colony maintenance

Twenty *M. barbarus* colonies were sourced from commercial suppliers in Spain and the Netherlands (AntHouse, Ants Kalytta, and Ant's Kingdom). Colonies were founded by multiple independent nuptial flights, meaning that relatedness between colonies was unlikely to be high. Colonies were kept in separate darkened nests made of 20 x 20 x 3 cm moulds of plaster of Paris, connected by a tube to a separate clear-plastic foraging area, in incubators kept at 25°C and with a day-night light regime. Colonies were checked 3 times a week, and water and food were added when necessary. Colonies contained a queen and individuals belonging to a major caste (the larger size class in the colony) and a minor caste (the smaller size class in the colony). Of the 20 colonies, 16 were used as experimental colonies (average colony size ± SE = 20.4 individuals ± 1.1; average ratio of minor to major caste individuals ± SE = 14.6 ± 0.7). Ants from the remaining 4 colonies (non-experimental colonies) were used as intruder ants in staged encounters with experimental colonies.

Staged experimental encounters

The behaviour of the 16 experimental colonies was analysed in response to experimental encounters with an intruder individual. Eight ants (1 major and 7 minors) were randomly selected from the

experimental colony and placed into a petri dish. Ants were recorded from above using a Canon DSLR camera and a Panasonic HC-VX980EB-K camcorder in a dark room under red light. Ants were left for 2 minutes to acclimatise before the start of the exposure experiment protocol.

After the 2 minute acclimatisation period, the ants were recorded for 10 minutes to generate baseline measurements of behaviour (the 'before' exposure phase). Ants were then given one of two exposure treatments: exposure to an 'intruder' ant (a randomly selected minor caste ant from one of the non-experimental colonies), or exposure to a 'home' ant (a randomly selected minor caste ant from their own colony). Ants were video recorded for a 10 minute period (the 'during' exposure phase). Exposure to a 'home' ant acted as a matched control to enable us to rule out the possibility that any changes in observed behaviour were the result of an increase in the number of ants in the petri dish, rather than the identity of the presented ant. We did not simultaneously expose experimental colonies to multiple presented individuals due to logistical constraints on the size of non-experimental colonies. The presented ant was marked with a small dot of white enamel paint applied to its head for identification during video analysis.

After 10 minutes of exposure, the presented ant was removed from the petri dish and ants were video recorded for a further 10 minute period (the 'after' exposure phase). After a 20 minute rest interval, the experiment was repeated to allow us to measure behavioural responses to a second, subsequent intruder. Similarly, the presented ant was either a 'home' ant or an 'intruder' ant. Again, there was a 10 minute period of video recording before, during and after the second exposure. Each experimental colony was exposed to four treatments in total: Intruder-Intruder (II), Intruder-Home (IH), Home-Home (HH), and Home-Intruder (HI). Each experimental colony received the four treatments on separate days and in a randomised order.

Video analysis

Video footage of behaviour was analysed using PotPlayer version 1.7.13622. We recorded three separate behaviours among ants in the experimental colony: time to first contact, activity, and social contacts. Time to first contact between the presented ant and an ant from the experimental colony (in seconds) was recorded in the 'during' exposure phase as a measure of the strength of response to intruders. We recorded the caste (major or minor) of the ant to make the first contact with the intruder. Activity was measured as the proportion of ants observed moving in the first 20 seconds of each minute of the 'before', 'during' and 'after' exposure phases. The number of social contacts was measured as the number of times two ants from the experimental colony touched heads or body parts and was recorded during each minute of the 'before', 'during' and 'after' exposure phases. Cases of grappling (when two ants bite and hold each other with the mandibles) between ants in the experimental colony, and with presented ants were also recorded. However, since grappling was very rare (21 observations in 8 out of 16 colonies), it was left out of formal statistical analysis. Observations were not blind to treatment or phase.

Statistical analyses

Statistical analyses were performed in R version 3.5.1 (R Development Core Team 2016) using linear mixed effects models (LMMs) and generalized linear mixed effects models (GLMMs), with binomial and Poisson error structures and logit and log link functions respectively, using the 'lme4' package (Bates et al. 2015). The residuals of LMMs were checked to confirm they were normally distributed with constant variance. For analyses in which the response variable was overdispersed, we used GLMMs fitted with an observational level random effect (Harrison 2014, 2015). In each analysis, we fitted the maximal model including all fixed effects and biologically relevant interactions, along with a random effect of colony ID to account for repeated measures of behaviours from the same experimental colonies. We used likelihood ratio tests to assess the significance of each fixed effect (Bates et al. 2015) and present parameter estimates and standard errors from the maximal model.

We did not perform stepwise model reduction by removing non-significant fixed effects from the model due to problems associated with such techniques (Whittingham et al. 2006; Mundry and Nunn 2009; Forstmeier and Schielzeth 2011). However, we did remove non-significant interactions to allow the significance of main effects to be tested (Engqvist 2005). Separate analyses were conducted on behavioural responses to first and second exposures to test for differences in response to intruders compared to home ants (on first exposure), and then for differences in response dependent on the first exposure. Post hoc Tukey's all-pairwise comparisons of means were conducted using the 'glht' function in the 'multcomp' package (Hothorn et al. 2008, 2016) to test for differences between levels of significant main effects of phase ('before', 'during' and 'after'), and treatment in the second exposure (II, IH, HH, HI).

Time to first contact

In 7 out of 64 cases in the first exposure, and 6 out of 64 cases in the second exposure, contact between an ant from the experimental colony and the presented ant did not occur during the 10 minute exposure period. We therefore removed these trials from our analysis and fitted the log-transformed time to first contact as the response variable in two LMMs (one analysing data from the first exposure, and one analysing data from the second exposure). Time to first contact was log-transformed to meet the assumption of normal distribution of residuals. We included treatment (I or H in the first exposure; II, IH, HH or HI in the second exposure), the caste of the ant that made the first contact (minor or major), and the interaction between these variables as fixed effects. One trial in the first exposure resulted in contact between a major and minor ant and the presented ant at exactly the same time, and for two trials in the second exposure we were unable to observe the moment of first contact accurately. These trials were subsequently removed from their respective analyses. We fitted these models to data on 56 and 58 trials (for the first and second exposure respectively) in 16 colonies.

Activity

We fitted the proportion of ants from the experimental colony that were active in the first 20 seconds after each minute of recording as the response variable in two GLMMs (one analysing data from the first exposure, and one analysing data from the second exposure). We fitted the models using a binomial error structure with an observational level random effect to correct for overdispersion of our response variable (Harrison 2015). We included treatment (I or H in the first exposure; II, IH, HH or HI in the second exposure), phase (before, during or after), and the interaction between treatment and phase as fixed effects. To test whether activity changed at a different rate between different treatments, we also included time (the minute of recording) and the interaction between time and treatment as additional fixed effects. We fitted these models to data on 1920 minutes of video recordings in 64 trials in 16 colonies (for both the first and second exposure).

Number of social contacts

We fitted the number of social contacts occurring between ants in the experimental colony during each minute of recording as the response variable in two GLMMs (one analysing data from the first exposure, and one analysing data from the second exposure). We fitted the models using a Poisson error structure with an observational level random effect to correct for overdispersion of our response variable (Harrison 2014). We included treatment (I or H in the first exposure; II, IH, HH or HI in the second exposure), phase (before, during or after), time (the minute of recording), and the interaction between treatment and phase, and treatment and time as fixed effects. We fitted these models to data on 1920 minutes of video recordings in 64 trials in 16 colonies (for both the first and second exposure).

Results

Time to first contact

We found no evidence that ants responded more quickly to intruder ants than home ants. On first exposure, there was no difference in the time that ants from the experimental colony took to make contact with an intruder ant (mean ± SE = 36.9 ± 19.4 seconds) compared to a home ant (mean ± SE = 62.8 ± 42.4 seconds). Nor was there any difference in the reaction of minor ants (mean ± SE = 39.7 ± 20.0 seconds) and major ants (mean ± SE = 74.6 ± 63.4 seconds) to the presented ant (GLMM, treatment x caste: $\chi^2_1 = 0.25$, P = 0.62; treatment: $\chi^2_1 = 0.63$, P = 0.43; caste: $\chi^2_1 = 0.43$, P = 0.51; model intercept ± SE = 3.35 ± 0.38). We also found no evidence that ants reacted differently to a second intruder ant depending on their previous experience. During the second exposure, there was no difference in time to first contact with the presented ant for castes in different treatments (GLMM, treatment x caste: β ± SE = -1.92 ± 1.45, $\chi^2_1 = 1.71$, P = 0.19). Ants did not react differently depending on whether they had previously been exposed to an intruder or a home ant (mean ± SE seconds for treatments: II = 25.7 ± 17.9; HI = 23.5 ± 8.3; IH = 51.4 ± 36.6; HH = 95.5 ± 81.0; GLMM, treatment: $\chi^2_1 = 7.06$, P = 0.07). There was also no difference in response time of minor ants (mean ± SE = 51.4 ± 25.9 seconds) and major ants (mean ± SE = 29.3 ± 28.5 seconds; GLMM, caste: β ± SE = 0.60 ± 0.66, $\chi^2_1 = 0.81$, P = 0.37).

Activity

In the first exposure, ants from the experimental colony were significantly more active when presented with an intruder ant compared to a home ant (GLMM, β ± SE = 0.26 ± 0.04, $\chi^2_1 = 37.24$, P < 0.001; Figure 1). Phase of exposure also had a significant effect on ant activity ($\chi^2_2 = 178.74$, P < 0.001; Figure 1) but this effect was independent of treatment (treatment x phase: $\chi^2_2 = 4.68$, P = 0.097; Figure 1). Ants were significantly more active during exposure to the presented ant than in the 'before' or 'after' phases (post hoc Tukey's test, 'before' vs 'during': 0.27 ± 0.052, z = 5.14, P < 0.001; 'during' vs 'after': -0.70 ± 0.052, z = -13.39, P < 0.001; 'before' vs 'after': -0.43 ± 0.051, z = -

8.36, P < 0.001; Figure 1). Ant activity decreased significantly during each trial (β ± SE = -0.071 ± 0.007, χ^2_1 = 91.97, P < 0.001), but this effect was independent of treatment (treatment x time: χ^2_1 = 0.027, P = 0.87).

In the second exposure, we found evidence of a primed response in the activity of experimental ants (GLMM, treatment: χ^2_3 = 46.11, P < 0.001). Ants that had previously been exposed to an intruder ant were significantly more active than ants that had been previously exposed to a home ant, although the comparison between IH and HI treatments was not significant (post hoc Tukey's test, HI vs II: β ± SE = 0.31 ± 0.061, z = 5.086, P < 0.001; HH vs II: β ± SE = 0.39 ± 0.061, z = 6.41, P < 0.001; HH vs IH: β ± SE = 0.20 ± 0.061, z = 3.29, P = 0.0054; HI vs IH: β ± SE = 0.12 ± 0.061, z = 1.95, P = 0.21; Figure 2). Ants that had been previously exposed to an intruder ant were also significantly more active when subsequently presented with an intruder compared to a home ant (IH vs II: 0.19 ± 0.06, z = 3.17, P = 0.0087; Figure 2), but not when they were previously exposed to a home ant (HH vs HI: 0.081 ± 0.061, z = 1.33, P = 0.54; Figure 2). Consistent with the first exposure, ants were also significantly more active in the 'during' phase than in the 'before' or 'after' phase (χ^2_2 = 337.38, P < 0.001; post hoc Tukey's test, 'before' vs 'during': β ± SE = 0.99 ± 0.053, z = 18.72, P < 0.001; 'during' vs 'after': β ± SE = -0.58 ± 0.052, z = -11.16, P < 0.001; 'before' vs 'after': β ± SE = 0.41 ± 0.053, z = 7.76, P < 0.001; Figure 3). However, the effect of treatment and phase were independent of one another (treatment x phase: χ^2_6 = 11.22, P = 0.082). Similar to the first exposure, ant activity decreased significantly during each trial of the second exposure (β ± SE = -0.065 ± 0.007, χ^2_1 = 73.94, P < 0.001), and this effect was independent of treatment (treatment x time: χ^2_3 = 2.97, P = 0.40).

Number of social contacts

In the first exposure, we observed significantly more social contacts between ants from the experimental colony when they were exposed to an intruder ant compared to a home ant (GLMM, β ± SE = 0.18 ± 0.02, χ^2_1 = 59.26, P < 0.001; Figure 4), but there was no significant difference between

264 treatments across phases (treatment x phase: $\chi^2_2 = 1.52$, P = 0.47; Figure 4). Independent of
265 treatment, phase had a significant effect on the number of social contacts ($\chi^2_2 = 20.49$, P < 0.001)
266 with significantly fewer contacts after exposure compared to before and during exposure (post hoc
267 Tukey's test, 'before' vs 'during': 0.047 ± 0.028, z = 1.71, P = 0.20; 'during' vs 'after': -0.13 ± 0.028, z =
268 -4.53, P < 0.001; 'before' vs 'after': -0.079 ± 0.028, z = -2.82, P = 0.013; Figure 4). Ants made
269 significantly fewer social contacts through the course of each trial, but this effect was not dependent
270 on treatment (treatment x time: $\chi^2_1 = 1.70$, P = 0.19; time: β ± SE = -0.063 ± 0.004, $\chi^2_1 = 236.44$, P <
271 0.0001).
272
273 Consistent with our findings of a primed response in the activity of experimental ants, we also found
274 that the number of social contacts between ants was significantly different depending on whether
275 they had previously been exposed to an intruder or a home ant (GLMM, treatment: $\chi^2_3 = 72.31$, P <
276 0.0001). Ants that had previously been presented with an intruder ant made significantly more social
277 contacts than ants that had been exposed to a home ant (post hoc Tukey's test, HI vs II: β ± SE = 0.24
278 ± 0.04, z = 6.03, P < 0.001; HH vs II: β ± SE = 0.33 ± 0.041, z = 8.21, P < 0.001; HH vs IH: β ± SE = 0.20 ±
279 0.041, z = 4.81, P < 0.001; HI vs IH: β ± SE = 0.11 ± 0.041, z = 2.60, P = 0.046; Figure 5). Ants that had
280 been exposed to an intruder ant in the first exposure made significantly more social contacts when
281 they were subsequently presented with an intruder compared to a home ant (IH vs II: 0.14 ± 0.039, z
282 = 3.43, P = 0.0034; Figure 5), but this difference was not observed when the first exposure was to a
283 home ant (HH vs HI: 0.093 ± 0.042, z = 2.21, P = 0.12; Figure 5). Phase had a significant effect on the
284 number of social contacts ($\chi^2_2 = 195.31$, P < 0.001), but this was not dependent on treatment
285 (treatment x phase: $\chi^2_6 = 3.98$, P = 0.68). Ants made significantly more contacts during exposure to
286 the presented ant compared to before, and this effect lasted throughout the 'after' exposure phase
287 (post hoc Tukey's test, 'before' vs 'during': β ± SE = 0.51 ± 0.036, z = 14.35, P < 0.001; 'during' vs
288 'after': β ± SE = -0.19 ± 0.034, z = -5.67, P < 0.001; 'before' vs 'after': β ± SE = 0.32 ± 0.037, z = 8.78, P
289 < 0.001; Figure 6). Ants made significantly fewer social contacts through each trial of the second

exposure (β ± SE = -0.083 ± 0.005, χ^2_1 = 247.67, P <0.001), and this effect was independent of treatment (treatment x time: χ^2_3 = 0.39, P = 0.94).

Discussion

Our study shows that workers of the harvester ant *M. barbarus* respond to the presence of an intruder by increasing their activity patterns overall, and in particular by increasing the rate at which they make contact with other colony members. This is consistent with the hypothesis that individual workers act to increase coordination or cohesiveness among members of their own group when confronted by members of a different group, which may serve as an indicator of invasion or attack by another colony. However, the behavioural response was statistically similar regardless of whether the presented ant was from their own colony or from a different colony, suggesting that when the ants first encountered an unfamiliar individual, they made no obvious distinction between members of their own or other colonies. Nevertheless, exposure to an unfamiliar intruder did have a large influence on the response of ants to a second intruder encountered shortly afterwards. Specifically, an encounter with an intruder from a different colony primed the ants to respond more strongly to a second intruder, particularly when the second intruder was also from a different colony. Thus our results suggest that recent previous experience of potential intergroup conflict increases the within-group response to a simulated intrusion, in line with the 'primed response' hypothesis.

These findings in a eusocial insect offer a complement to research on the behavioural responses of some social vertebrates to simulated intrusions. For example, in cooperative cichlids, *N. pulcher*, laboratory groups that are exposed to intruders subsequently engage in elevated rates of affiliative behaviour (e.g. soft touching and following; Bruintjes et al. 2015). Similar increases in affiliative behaviour following exposure to experimental intruders have been shown in green woodhoopoes (Radford 2008). In both cases the increase in affiliative behaviour is interpreted as an adaptive response which increases group cohesion, protecting the group from future attacks. These systems

316 differ from our ants in that group members each have the potential to reproduce, either currently or
317 in the future, whereas the ant workers are selected to behave in a way that maximises their indirect
318 component of fitness, realised via the assistance they can provide to the colony production of
319 reproductives. The fact that similar behavioural responses to intruders are seen in such different
320 systems is consistent with the hypothesis that increased contact or affiliation among group members
321 serves to prepare or strengthen the group against future attacks, and is therefore favoured by both
322 selection acting at the level of the individual and the level of the group (Okasha 2006; Robinson and
323 Barker 2017). Unlike the findings on cooperative vertebrates, however, our study suggests that
324 harvester ants respond to their first encounter with an unexpected individual in a similar way,
325 without making distinctions based on colony-of-origin.
326
327 We found evidence that the response to intruders in *M. barbarus,* in the form of social contacts and
328 activity, was enhanced by previous experience of an encounter with a non-colony intruder. This
329 result suggests that *M. barbarus* workers utilise experience of past enemy encounters to calibrate
330 future behavioural responses, in line with previous evidence in a variety of taxa (Rose and Brenowitz
331 1997; Monclús et al. 2014), including other ant species (Thomas et al. 2007; Newey et al. 2010; Van
332 Wilgenburg et al. 2010; Adams 2016). This result also provides support for predictions of recent
333 theory which shows that individual past experience results in quicker and more accurate recognition
334 of non-nestmates at the colony (Esponda and Gordon 2015). Ants could collectively gain experience
335 of enemy cuticular hydrocarbons (CHCs) used in nestmate discrimination during first exposure which
336 primes them to respond more intensely to non-nestmates on subsequent exposure (Guerrieri et al.
337 2009; Newey et al. 2010). Initial exposure to an intruder ant could affect patterns of movement (e.g.
338 by eliciting a patrolling response) and the structure of social networks, such that subsequent
339 encounters between ants in the colony are more likely. For example, an intruder ant could represent
340 a pathogenic threat to the colony that stimulates a change in within-colony social dynamics
341 (Stroeymeyt et al. 2018). Encounters with an intruder, even a single intruder as in our experiment,

342 may result in a step-change in the perceived level of risk of intergroup competition or attack. In
343 addition, the response of individuals to simulated intrusion may depend on the number of intruders
344 or the perceived strength or composition of a group of intruders (Roulston et al. 2003). Similarly,
345 intruders could represent a larger threat if they are more likely to be of a more aggressive
346 phenotype (Sturgis and Gordon 2013). The question of how groups assess each other's relative
347 strength and motivation has been little explored theoretically or empirically. In contrast to dyadic
348 animal contest theory, signals of resource holding potential (RHP) of groups depend on the
349 individual and combined signalling effort of individual group members, which may vary in complex
350 (non-linear) ways with group size and composition. Experimental approaches in ant systems such as
351 ours could be a powerful tool to elucidate some of the principles of intergroup signalling and
352 conflict, and how group size and individual RHP combine to determine group RHP (e.g. Batchelor et
353 al. 2012).
354
355 There are some limitations to our study which should be considered when interpreting our results.
356 Firstly, we observed fewer social contacts and a lower proportion of active ants in the 'before' phase
357 of the second exposure compared to the 'before' phase of the first exposure. This unexpected
358 difference in behaviour could reflect a lack of acclimatisation to the assay arena (itself an artificial
359 environment) before the first exposure, or fatigue from responding to the previously presented ant
360 before the second exposure. Fatigue in response to stimuli makes direct comparisons between initial
361 and subsequent exposures more difficult to interpret, but does not detract from our observed effect
362 of a primed response to an intruder. In fact, we might have observed an even stronger primed
363 response in the second exposure had ants not been fatigued. Secondly, colony sizes used in our
364 experiments were small. This may have affected colony response behaviour to intruders and could
365 go some way to explaining the lack of observed aggression to presented ants, particularly in light of
366 evidence that colony-level responses to non-nestmates are highly dependent on combined
367 individual-level experience and behaviour (Guerrieri et al. 2009; Newey et al. 2010; Esponda and

Gordon 2015). Similarly, we cannot rule out that the presentation of a single intruder may not elicit a behavioural response that accurately reflects the response of the colony as a whole (Roulston et al. 2003). Finally, we did not conduct behavioural assays blind to treatment. Non-blind studies are exposed to potentially inflated effect sizes (van Wilgenburg and Elgar 2013) and, as such, although our results provide evidence in support of a primed response to intruders, we should exercise some care when determining the certainty of our results.

Studies that show increased affiliative behaviour in response to simulated or real intergroup encounters are often taken as consistent with the predictions of theoretical models of the role of intergroup conflict as a promoter of cooperation and altruism within groups. However, there is currently a disconnect between theory and data on this point. Most formal population genetic and game theoretical models of intergroup conflict examine changes in the frequency, over many generations, of fixed genetic traits that influence behaviour toward members of a different group versus members of the own group (e.g. 'parochialism' and 'altruism', Choi and Bowles 2007; or 'belligerence' and 'bravery', Lehmann and Feldman 2008). These models do not analyse how individuals should respond plastically to changes in their social environment. The expectation that groups under attack should pull together and behave more cohesively is derived largely from verbal reasoning (starting with Darwin 1871), or empirical observations in humans and non-human animals (Radford et al. 2016; Kavaliers and Choleris 2017). Our findings are consistent with this expectation, but also draw further attention to this current disconnect between the assumptions of current theory and the types of behavioural responses that are measured in empirical studies. In particular, our behavioural results highlight the need for models which specify precisely what fitness benefit individuals and groups derive from behaviour that is determined to be affiliative (such as grooming or allopreening), and how this fitness benefit is realised. For example, does increased grooming among members of a primate group increase group cohesion through a process of reciprocity? Does increased social contact among worker ants communicate information on group strength, or

motivation and readiness to fight? New theoretical models which make explicit assumptions about the nature of intergroup competition and the function of within-group affiliation may help to explain why, in some circumstances, intergroup conflict (whether real or simulated) leads to increased social cohesion (this study; Cords 2002; Bruintjes et al. 2015), whereas in others it appears to exacerbate internal tensions (Cooper et al. 2004; Polizzi di Sorrentino et al. 2012; Arseneau et al. 2015).

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531

532 **Figure legends**

533 **Figure 1.** The effect of treatment on the proportion of ants active during the first 20 seconds of each
534 minute in each phase of the first exposure (N = 1920 minutes in 64 trials in 16 colonies). Points show
535 predicted means from a GLMM ± SE. In the first exposure, ants significantly increased in activity
536 when presented with an intruder ant (I), but they also showed a similar behavioural response when
537 presented with a home ant (H). Asterisks refer to post hoc Tukey's all-pairwise comparisons of
538 means. *** $P < 0.001$.
539

540 **Figure 2.** The effect of treatment on the proportion of ants active during the first 20 seconds of each
541 minute in each phase of the second exposure (N = 1920 minutes in 64 trials in 16 colonies). Points
542 show predicted means from a GLMM ± SE. In the second exposure, ants showed a primed response
543 if they had been exposed to an intruder ant in the first exposure (IH, II) compared to a home ant (HI,
544 HH). Asterisks refer to post hoc Tukey's all-pairwise comparisons of means. *** $P < 0.001$, ** $P <$
545 0.01. Non-significant pairwise comparisons are not labelled.
546

547 **Figure 3.** The effect of phase on the proportion of ants active during the first 20 seconds of each
548 minute (N = 1920 minutes in 64 trials in 16 colonies for both the first and second exposure). Points
549 show predicted means from a GLMM ± SE. Activity peaked during exposure to the presented ant in

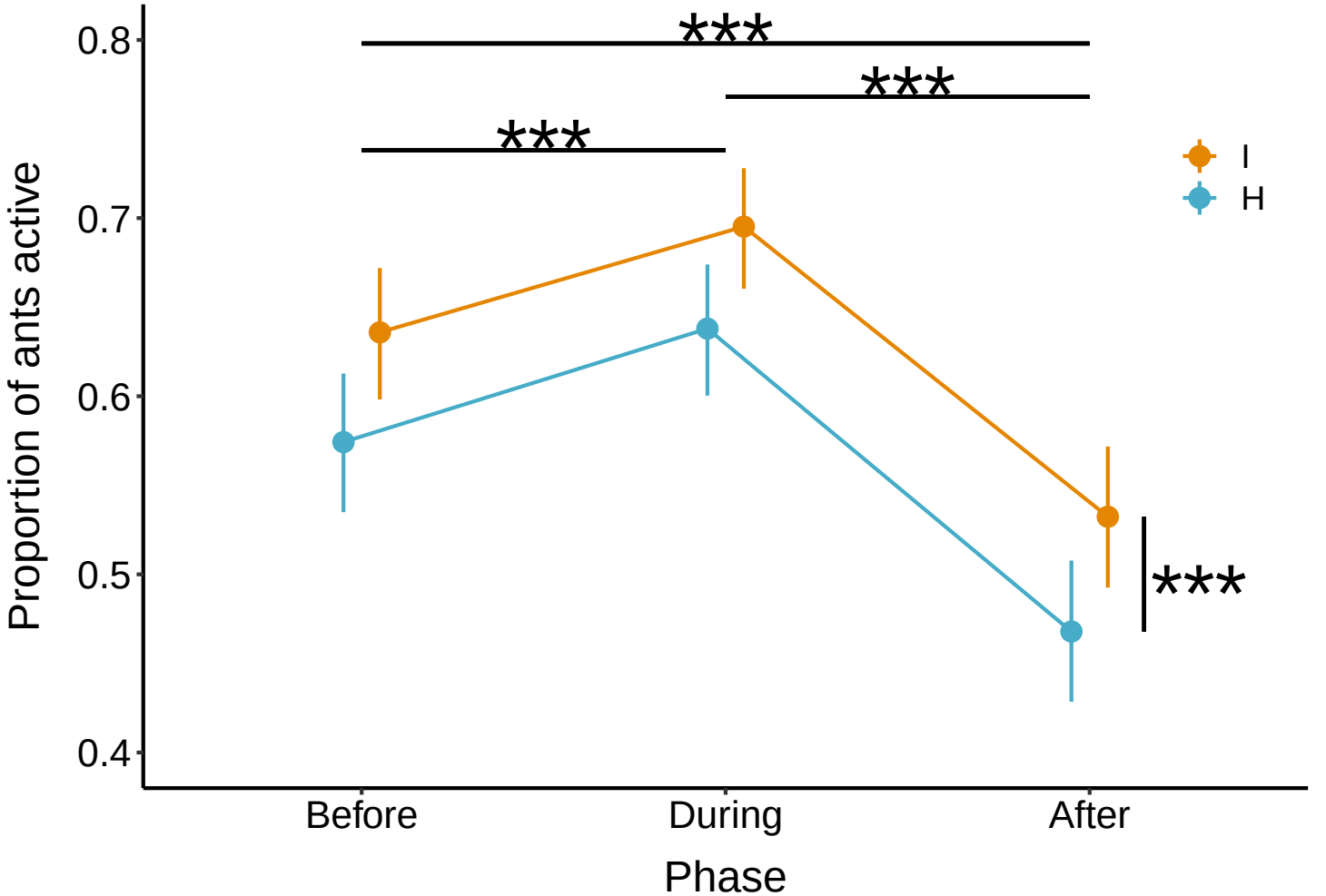
both the first and second exposure. Asterisks refer to post hoc Tukey's all-pairwise comparisons of means. *** $P < 0.001$.

Figure 4. The effect of treatment on the number of social contacts between ants in the experimental colony during each minute of each phase of the first exposure (N = 1920 minutes in 64 trials in 16 colonies). Points show predicted means from a GLMM ± SE. In the first exposure, ants performed significantly more social contacts when presented with an intruder ant (I), but they also showed a similar behavioural response when presented with a home ant (H). Asterisks refer to post hoc Tukey's all-pairwise comparisons of means. *** $P < 0.001$, * $P < 0.05$. Non-significant pairwise comparisons are not labelled.

Figure 5. The effect of treatment on the number of social contacts between ants in the experimental colony during each minute of each phase of the second exposure (N = 1920 minutes in 64 trials in 16 colonies). Points show predicted means from a GLMM ± SE. In the second exposure, ants showed a primed response if they had been exposed to an intruder ant in the first exposure (IH, II) compared to a home ant (HI, HH). Asterisks refer to post hoc Tukey's all-pairwise comparisons of means. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Non-significant pairwise comparisons are not labelled.

Figure 6. The effect of phase on the number of social contacts between ants during each minute (N = 1920 minutes in 64 trials in 16 colonies for both the first and second exposure). Points show predicted means from a GLMM ± SE. The number of social contacts was not different during exposure to the presented ant compared to before exposure in the first exposure, but peaked during exposure to the presented ant in the second exposure. Asterisks refer to post hoc Tukey's all-pairwise comparisons of means. *** $P < 0.001$, * $P < 0.05$. Non-significant pairwise comparisons are not labelled.

Figure 1


0.8
0.7
0.6
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Proportion of ants active
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I
H
Before
During
After
Phase

Figure 2

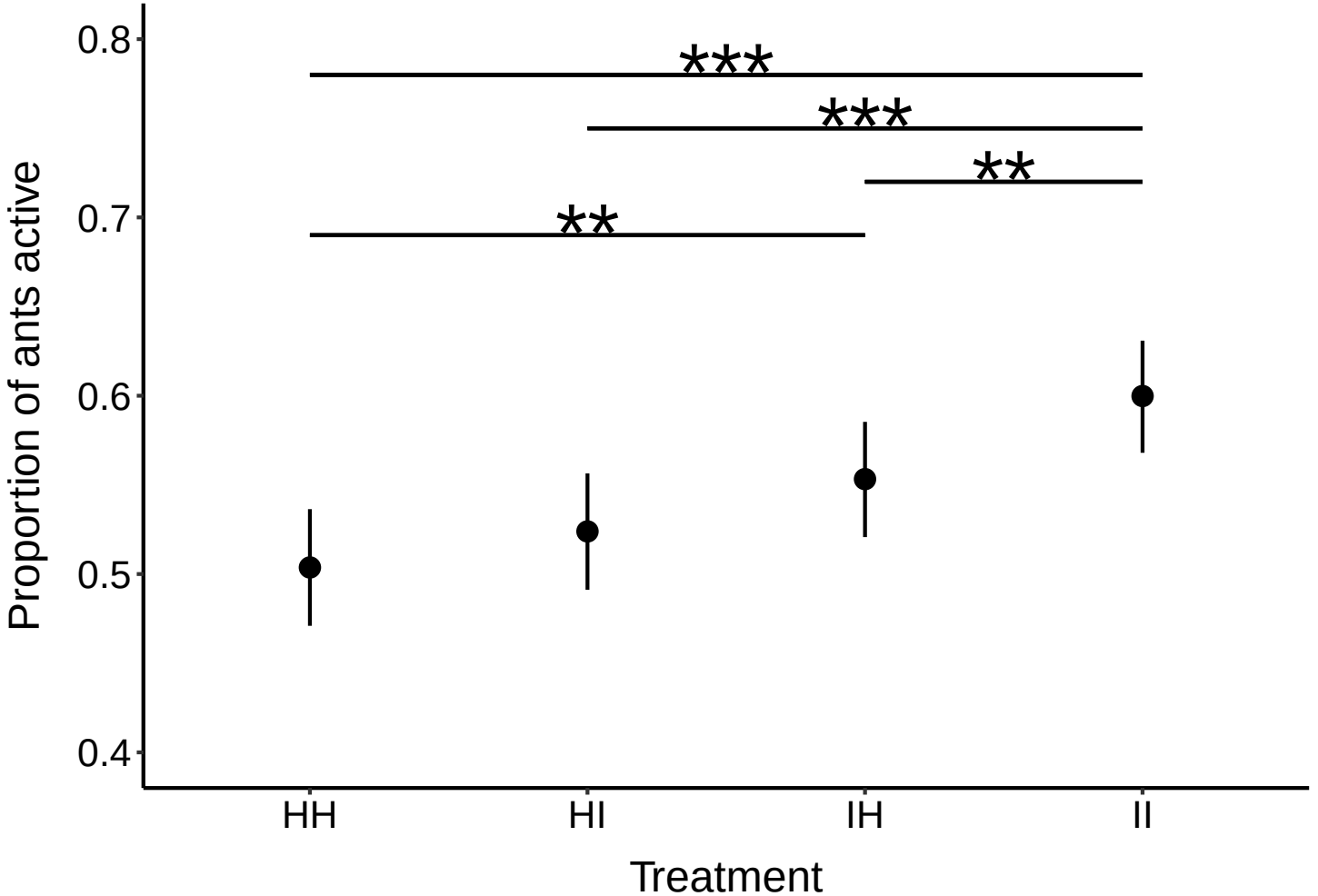
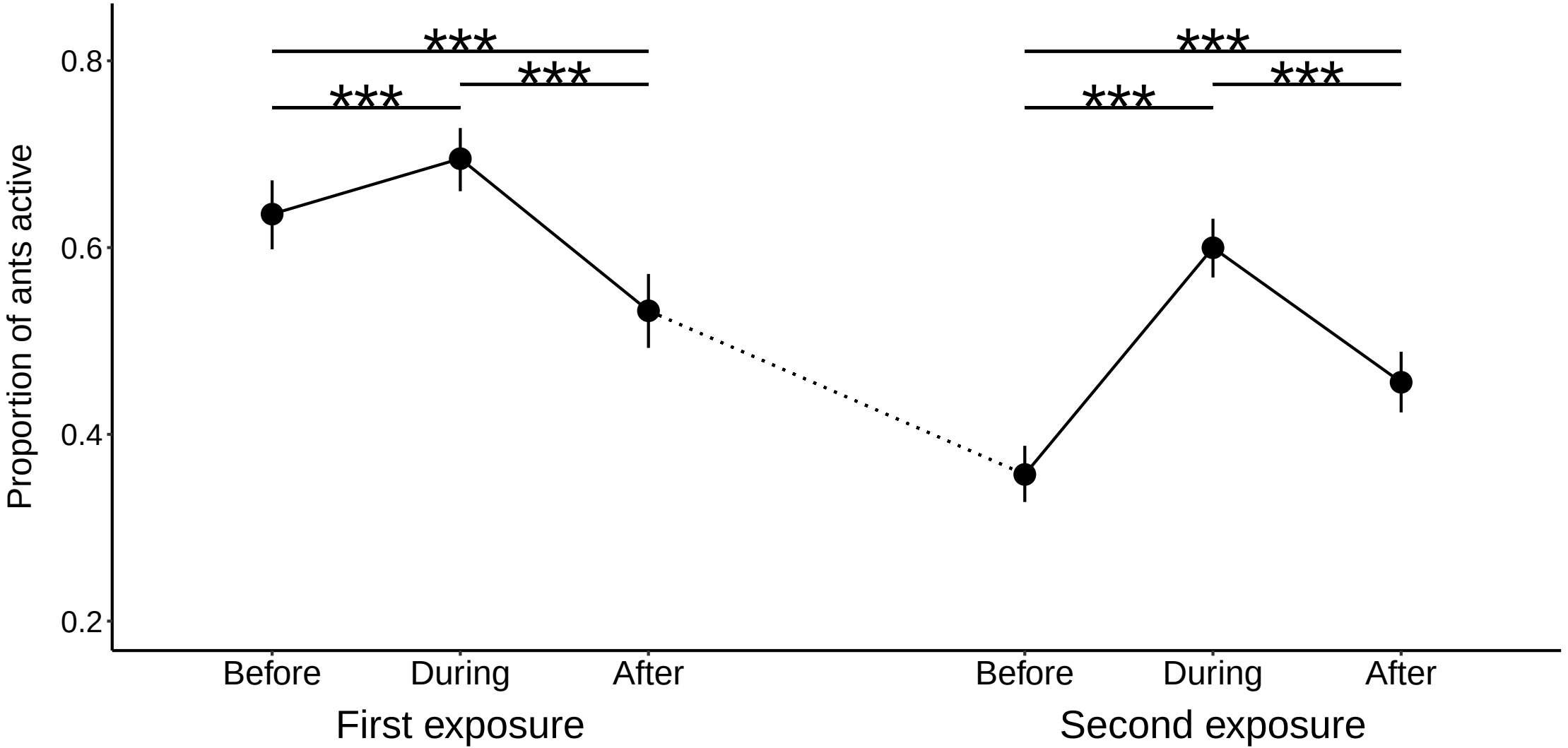


Figure 3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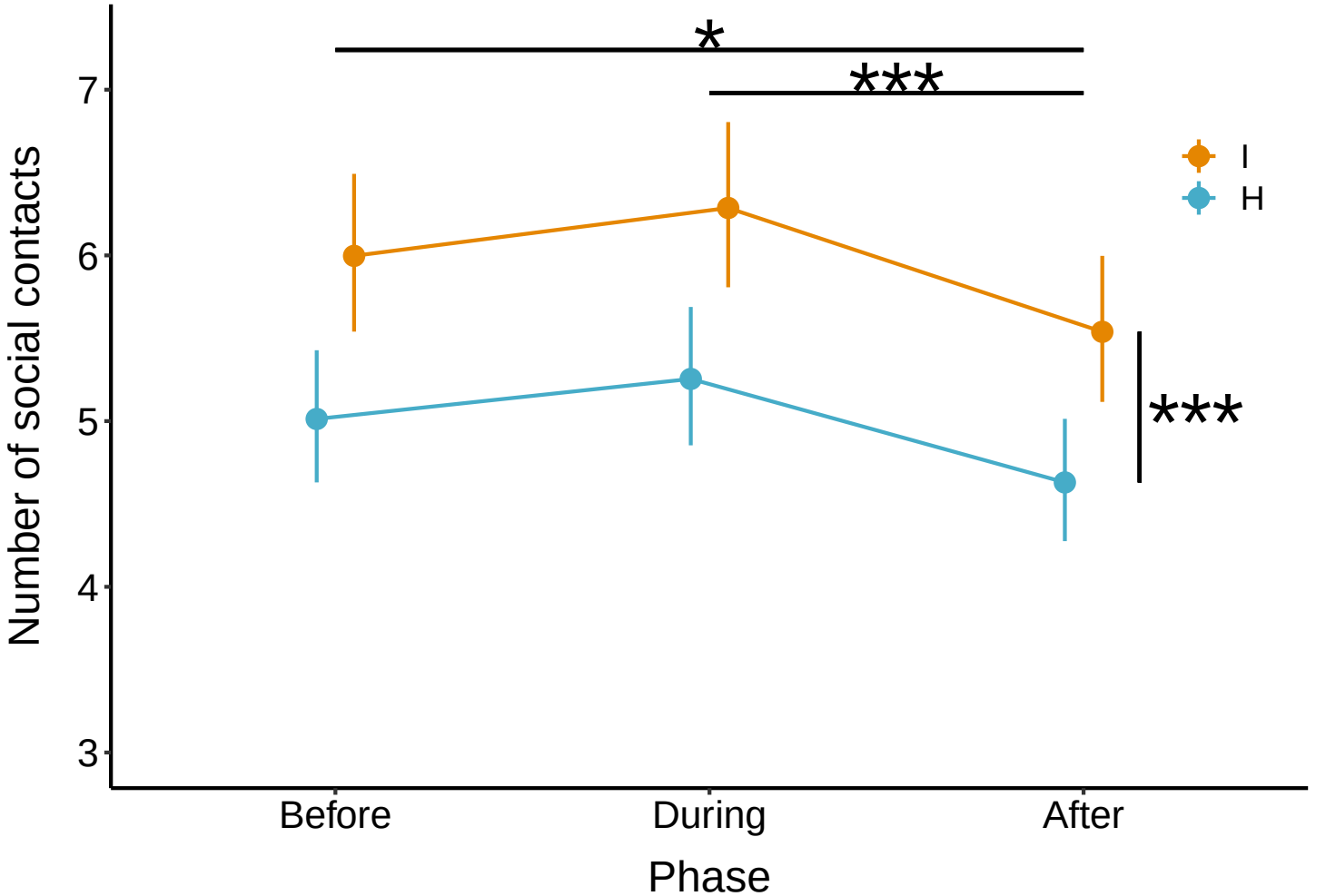
0.8
0.6
0.4
0.2
Proportion of ants active
Before
During
After
Before
During
After
First exposure
Second exposure

Figure 4


*
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***
I
H
Number of social contacts
7
6
5
4
3
Before
During
After
Phase

Figure 5

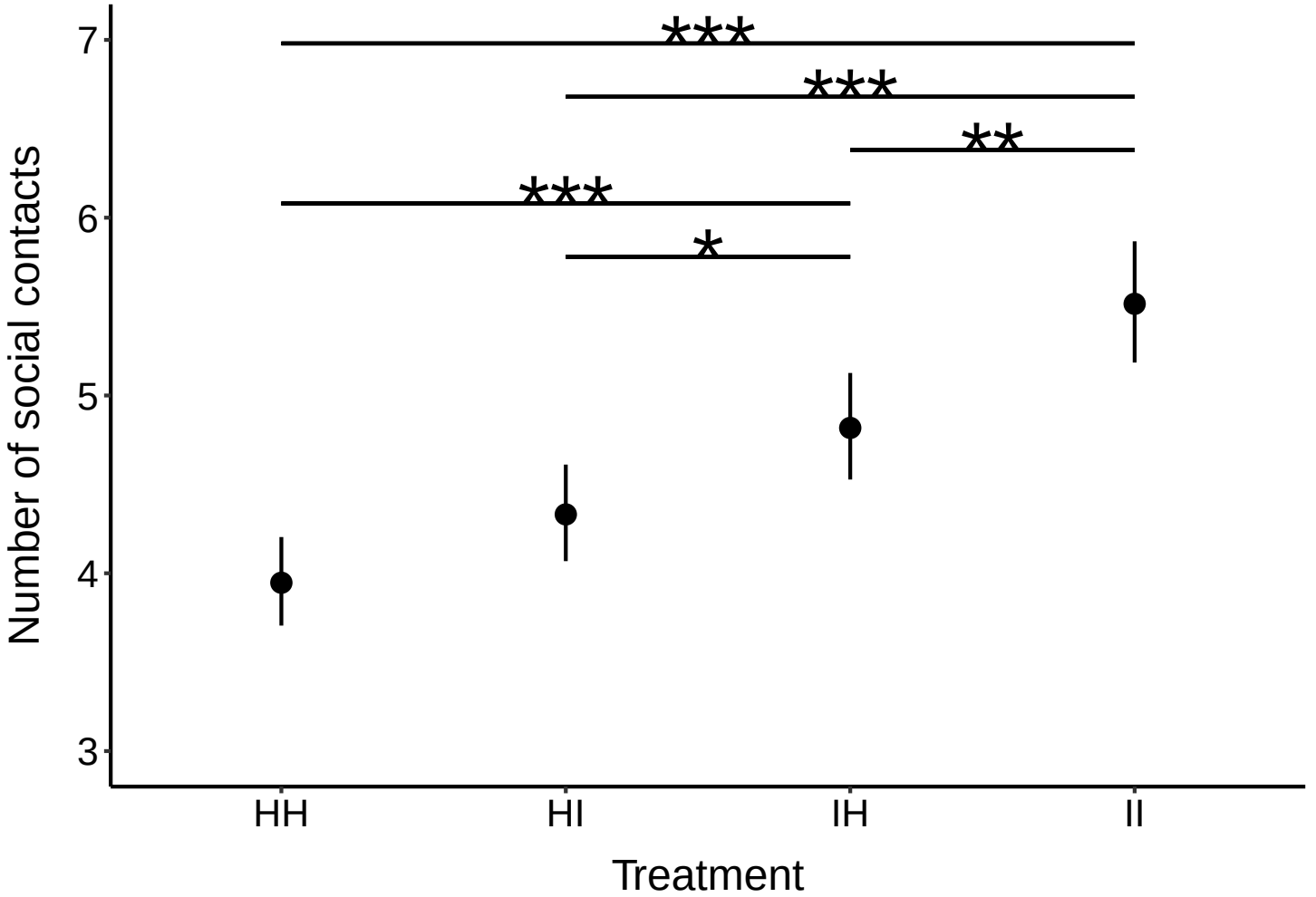
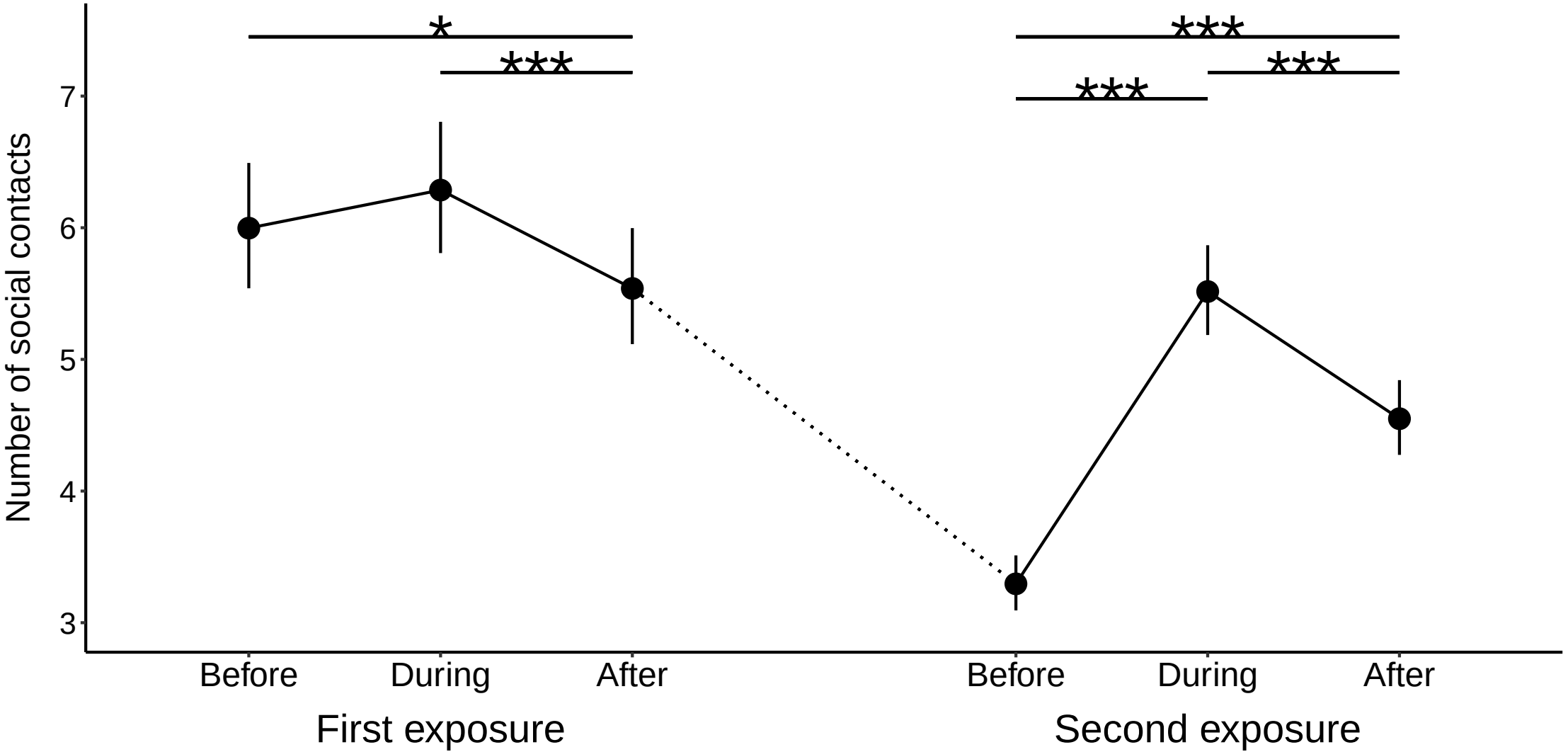


Figure 6


*
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7
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4
3
Number of social contacts
Before
During
After
Before
During
After
First exposure
Second exposure