

# **Seasonal changes in host ant aggressiveness towards social parasites of different types**

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## Highlights

- Social parasites are social insects that exploit other social insects
- Defence costs against parasites vary, driving the evolution of defensive strategies
- The aggressiveness of the hosts depends on the type of social parasite
- The aggressiveness of the hosts is influenced by neighbouring social parasites
- Seasonal changes in host aggressiveness align with the activity of social parasites

## Abstract

Social parasites are social insects that exploit the labour and resources of other social insects. In ants, several types of social parasites are known, including temporary parasites, in which host workers are present only during colony founding, and slave-maker ants, which continually supplement their workforce with host workers throughout the colony's lifetime. Because defence against parasites is costly, host species are expected to modulate their responses according to parasite-imposed risks. In this study, we investigated the seasonal patterns of aggressiveness in a common host *Formica fusca* (silky ant), towards its temporary social parasite (*Formica polyctena*, European wood ant) and a slave-maker (*Formica sanguinea*, blood-red ant). Behavioural assays were conducted in spring, summer, and autumn using host workers collected from colonies located (1) near *F. polyctena* colonies, (2) near *F. sanguinea* colonies, and (3) in parasite-free (control) areas. Hosts from areas dominated by *F. polyctena* were more aggressive towards *F. polyctena* in spring and summer than in autumn, showed peak aggression towards *F. sanguinea* in summer, and were more aggressive towards *F. sanguinea* than *F. polyctena* in summer and autumn. Hosts from areas with *F. sanguinea* displayed the highest aggression towards both parasite species in spring and summer, with similar responses to both parasites across all seasons. Control colonies showed an intermediate pattern, combining elements of both responses. Seasonal variation in host aggressiveness appears to track

differences in the timing and severity of threats imposed by the two parasites: colony founding by *F. polychaeta* in spring and early summer, and slave raids by *F. sanguinea* in summer. Such temporal adjustment of defensive behaviour may help minimize the costs of defence against social parasites by increasing aggression only when the threat is greatest.

**Keywords:** behavioural flexibility, defence costs, *Formica fusca*, *Formica polychaeta*, *Formica sanguinea*, slave-making, temporary social parasitism.

## Introduction

Parasites, due to their impact on host fitness, are a powerful driving force of evolution (Buckingham & Ashby 2022). They can shape the genetic diversity of host populations (Altermatt & Ebert 2008; Kaunisto et al. 2013) and influence their reproductive strategies, including the evolution of sexual reproduction (Hamilton 1980; Lively 1996; Mostoway et al. 2010; Ashby & King 2015). Parasites can even affect the social behaviour of their hosts (Bonds et al. 2005; Prado et al. 2009; Ashby & Farine 2022). Conversely, hosts also impose selective pressures on their parasites, driving co-evolutionary processes such as arms races. These arms races are fuelled by ongoing adaptations and counter-adaptations between hosts and their parasites (Futuyma & Kirkpatrick 2017; Buckingham & Ashby 2022).

Hosts typically defend themselves against parasites through a variety of mechanisms, ranging from molecular to behavioural defences, aimed at avoiding parasitism, or if that fails, mitigating its costs (Schmid-Hempel 2021). Defences can be constitutive, i.e. expressed constantly regardless of whether a parasite is present, or inducible, i.e. only expressed after an encounter with parasites (Harvell 1990; Westra et al. 2015). Constitutive defences entail constant costs, which can be disadvantageous when parasites are absent, although they provide immediate

protection if needed (Steppuhn & Baldwin 2008). Inducible defences, on the other hand, are expressed only after contacting the parasite, but this delay can lead to additional fitness costs. Social parasitism, which occurs in eusocial Hymenoptera, including ants, halictid and apid bees, and vespid wasps, is a form of parasitism in which the parasite exploits the social behaviours of its host, such as nest building, foraging, and brood care (Buschinger 1986, 2009; Hölldobler & Wilson 1990; De La Mora et al. 2020; Rabeling 2020). Social parasitism is generally classified into two categories: “compound nests”, in which host and parasite brood are kept separate, and “mixed colonies”, where host workers rear the parasite brood (Hölldobler & Wilson 1990; Buschinger 2009; De La Mora et al. 2020; Rabeling 2020). However, in most cases, only mixed colonies are considered “true” social parasitism. “Compound nests” include forms such as plesiobiosis, cleptobiosis, lestobiosis, parabiosis, and xenobiosis. In contrast, the focus of this study is on “mixed colonies”, which include inquilinism, temporary parasitism and slave-making (dulosis). In inquilinism, found in ants, bees, and wasps, parasitic queens infiltrate nests of the host species, where they typically cohabit with the host queen, often reducing her fertility (Rabeling & Bacci 2010; Cover & Rabeling 2024). Inquilines are often workerless, producing only sexuals (gynes and males) that are cared for by the host workers. However, in some bees and wasps, the parasite is not necessarily a queen, but drifted workers who lay their eggs in the nest of other (often queen-less) colonies (Birmingham et al. 2004; Beekman & Oldroyd 2008; Oliveira et al. 2016). In temporary social parasitism, known in ants and wasps, the parasite is dependent on its host only during the phase of colony founding (Buschinger 1986; Hölldobler & Wilson 1990; Buschinger 2009; De La Mora et al. 2020; Rabeling 2020). Temporary parasitic queens infiltrate queen-less host nests or eliminate (by killing or driving away) the host queen, and take over the colony. Brood care is carried out by the host workers, which eventually die out and are replaced by workers of the parasite species. In case of slave-making, that is only known in ants, the colony founding resembles that of temporary parasites.

97 However, in this case, host workers do not disappear from the colony. Instead, parasite workers  
98 raid nearby host colonies to steal offspring (primarily pupae, as they do not require special care).  
99 Once the adult host workers eclose within the parasite nest, they integrate into the social parasite  
100 colony, performing tasks for its benefit (d'Ettorre & Heinze 2001). Both temporary parasitism  
101 and slave-making can be facultative or obligatory. Facultative social parasites can survive  
102 without their hosts, while obligatory social parasites are completely dependent on them  
103 (Hölldobler & Wilson 1990). Hosts and parasites are often closely related phylogenetically, a  
104 phenomenon known as Emery's rule (Lopez-Orsio 2021). In its strict sense, this means that  
105 hosts and parasites are sister taxa (i.e. they are the closest relatives of one another); more  
106 broadly, they may simply be closely related within the same clade.

107 Defence against social parasites involves mechanisms that can work both before and after an  
108 'infestation' (Grüter et al. 2018). Pre-infestation defences include effective differentiation  
109 between nestmates and non-nestmates (d'Ettorre & Lenoir 2010), the ability to distinguish own  
110 brood and parasite brood (Achenbach et al. 2010; Chernenko et al. 2013) and aggression  
111 towards intruders (Pamminger et al. 2011; Jongepier et al. 2014). Other strategies include  
112 colony evacuation and its relocation in response to an attack (Jongepier et al. 2014), resistance  
113 to the pheromones used by parasites to manipulate hosts (Lenoir et al. 2001), and the  
114 maintenance of a distinctive colony odour through limited acceptance of additional conspecific  
115 queens (Keller & Passera 1989). Post-infestation defences include behaviours such as slave  
116 'rebellions', when host workers display aggression towards slave-makers or destroy parasite  
117 brood (Heinze et al. 1994; Achenbach & Foitzik 2009; for review see Czechowski & Godzińska  
118 2015). In special circumstances, even the full emancipation of slaves may occur, which in  
119 certain eventualities adopt their conspecific queens (Czechowski & Godzińska 2015).

120 However, as with all parasites, defence against social parasites is associated with costs (Sheldon  
121 & Verhulst 1996), especially when the parasite has a morphological advantage (e.g. larger body

size) over its host (De La Mora et al. 2020; Rabeling 2020). The optimal defensive strategy depends on the cost-benefit ratio between the risk of parasitic infestation and the expenditure on defence outlays (von Beeren et al. 2011). Since parasites are not always present or active, inducible defences (that are activated only after contact with the parasite) are often advantageous (Harvell 1990), especially in resource-limited environments (Westra et al. 2015). Temporary social parasites, for example, are most dangerous during their colony founding period, usually in spring or early summer, whereas slave-makers are most destructive during the summer raiding period (Czechowski et al. 2012; Seifert 2018). Accordingly, some hosts exhibit seasonal changes in the defensive behaviours, such as increased aggression that aligns with the seasonal activity of social parasites. These changes may serve as an induced defence even in the absence of direct parasite contact at the time (d'Ettorre et al. 2004; Pamminer et al. 2011).

The ant genus *Formica* is ideal for studying social parasitism, as it exhibits all major social parasite strategies (i.e. temporary parasitism, slave-making and inquilinism) and harbours the highest number of socially parasitic species among all ant genera (Borowiec et al. 2021). According to Romiguier et al. (2018) and Borowiec et al. (2021), social parasitism in *Formica* has a single evolutionary origin, stemming from facultative dependent colony founding via budding. Within this evolutionary framework temporary social parasitism arose first, followed by the loss of independent colony founding. Slave-making evolved once within the temporary parasitic lineage, whereas inquilinism arose independently from temporary parasitic ancestors. In this study, we investigated differences in the host defences of ants against two types of social parasites: *Formica polyctena* Först., a temporary parasite, and *Formica sanguinea* Latr., a facultative slave-maker. We assessed the aggressiveness of *Formica fusca* L., a common host species of both parasites, with particular emphasis on seasonal variation in the intensity of worker responses. Specifically, we investigated whether (1) host aggressive responses differs

between these two parasite types; (2) seasonal changes in aggressiveness occur in parasitized and parasite-free (control) host populations; and (3) seasonal patterns of host aggression differ in relation to each parasite species. We expected that (1) hosts would respond more strongly to slave-makers than to temporary parasites, given the higher fitness costs imposed by *F. sanguinea* during the raiding season; (2) seasonal changes in host aggression would occur only in populations exposed to parasites but not in parasite-free areas, as these hosts must allocate defensive effort to periods of greatest threat; (3) the seasonal pattern of aggressiveness would differ for each parasite. Temporary parasites pose the greatest threat to host colonies during the colony-founding period (typically in spring), when young queens establish new colonies; also a time of intense competitive pressure from *F. polyctena* on neighbouring ant colonies (see Materials and methods: Study species). In contrast, slave-makers exert the greatest threat during their raiding season in summer, when *F. sanguinea* colonies not only capture slaves but also prey on the workers and brood of other ant colonies. This is also the period when *F. sanguinea* functions as a temporary parasite, with young queens searching for colonies of host species to take over (see Materials and methods: Study species).

## Materials and methods

### Study species

In the experiment, we used *Formica fusca* (silky ant) as a model host species and its two social parasites (and at the same time competitors): *Formica polyctena* (European wood ant), a temporary social parasite, and *Formica sanguinea* (blood-red ant), a facultative slave-maker. *Formica (Serviformica) fusca* Linnaeus, 1758 is a common northern Palaearctic, fairly thermophilic species of diverse (open and forest) habitats (Czechowski et al. 2012; Seifert 2018). Nests are built in the ground, under stones, in decaying wood and litter, and in tussocks

of mosses and grasses. Colonies usually consist of several hundred workers (but can sometimes number a few thousand) and 1–15 queens. A typical opportunistic submissive ant species on the lowest level of the interspecific ant competition hierarchy; workers forage singly, preying on small invertebrates, and feeding on honeydew and extrafloral nectar. Of all *Serviformica* species, *F. fusca* is the most frequent host of temporary socially parasitic *Formica* species, as well as a target of slavery by *F. sanguinea* and *Polyergus rufescens* (European Amazon ant). Nuptial flights are in late July and in August, and colony-founding queens lay eggs either shortly afterward or in the following spring. Colonies hibernate without any brood, and egg laying begins again in early spring. Workers eclose in 90 days at 17 °C and in 34–38 days at 22 °C (Seifert 2018). Cephalic size/index of workers (as proxy for body size):  $1343 \pm 130$ .

*Formica polyclena* Förster, 1850 is a common northern Palearctic, moderately thermophilic wood ant species that inhabits mainly coniferous and mixed forests (Czechowski et al. 2012; Seifert 2018). It builds nests with large mounds, up to 2.5 m in height and often more than 3 m in diameter, of dry organic material. Individual colonies typically contain more than one million workers and up to several thousand queens, usually forming multi-nest (polydomous) systems that can span large extents of forest. Long, up to approx. 200 m foraging routes radiate from the nests towards aphid-bearing trees; although workers primarily collect honeydew, a significant proportion of their foraging involves non-selective predation and scavenging on various invertebrates. New colonies are initiated either through (facultative) temporary social parasitism in *Serviformica* nests or, more commonly, through colony splitting. *F. polyclena* is a very aggressive and territorial species; together with other territorial species, it constitutes the highest level in the competition hierarchy of multi-species ant assemblages. Nuptial flights take place in spring (May–June). Mated queens begin egg-laying either after their adoption into a conspecific colony or following the invasion of a heterospecific host colony. Workers develop

in approximately 35 days (Seifert 2018). Cephalic size/index of workers (as proxy for body size):  $1666 \pm 181$ .

*Formica (Raptiformica) sanguinea* Latreille, 1798 is a common southern Palearctic thermophilic species of dry and sunny habitats, occurring both in light woodlands and open areas (Czechowski et al. 2012; Seifert 2018). It nests in decaying tree stumps partially covered with dry plant material, and also in the ground, often under stones. Colonies number several thousand workers and usually have one reproductively active queen. Extremely aggressive and predaceous ants; they prey on various invertebrates, including the brood of other ant species, and also feed on honeydew. New colonies are founded through temporary social parasitism in *Serviformica* nests, through colony splitting, or through adoption of conspecific young queens by a group of workers. It is a facultative slave-maker that raids *Serviformica* species. The proportion of hosts in a mixed workforce is usually 10–20%. Its competitive pressure on other ant species and possible territoriality is debatable. For example, Czechowski (2000) describes it as a territorial species, which during its raiding period does not respect the boundaries of territories of other territorial species (including those of the subgenus *Formica* sensu stricto). Nuptial flights usually occur in August–September, coinciding with the raiding season. During raids, mated gynes often participate by digging into and entering the target nests. Colony founding occurs mainly during this period, either by usurpation by mated queens that join raiding parties or by solitary queens taking over initial or queen-less mature host colonies. Cephalic size/index of workers (as proxy for body size):  $1763 \pm 189$  (Seifert 2018).

### **Collection and housing of colonies**

Mature, similar sized colonies of *F. fusca* were collected (dug up with their nests during warm, rainless weather) in southern Hungary in three seasons of 2023: May, July, and late August (hereafter: spring, summer, and autumn, respectively; Supplementary Table S1). Although these

months may appear to be close in time, we adjusted our collection periods to the actual weather conditions of the year, which can alter ant activity and seasonal cycle. In 2023, spring was unusually cold in Hungary, resulting in low ant activity, and autumn arrived earlier than usually. Therefore, in our study these months correspond to the peak activity of *F. polychena* foundresses, the period of most intense activity and raiding behaviour of *F. sanguinea*, and the period during which *F. fusca* experiences relatively low pressure from both social parasites in the study area.

Host colonies were collected from areas of three types: (1) the territory of *F. polychena* (temporary parasite), (2) the vicinity of *F. sanguinea* (slave-maker) colonies, and (3) control areas, i.e. areas without social parasites. Host colonies collected near *F. sanguinea* were located 10–25 meters from parasite nests, while those near *F. polychena* were 20–50 meters from active nest mounds. Control colonies were collected from sites with no recorded parasite presence within a 300-meter radius. Sites with *F. polychena* and *F. sanguinea* were located in Scots pine (*Pinus sylvestris*) forests near the village of Ásotthalom, while control sites were in deciduous forests near the city of Szeged (Supplementary Table S1). ). It is worth noting that colonies in parasite-free areas might still be exposed to sporadic attacks by gynes of each of the parasites, as they can disperse over great distances to establish new colonies (Hakala et al. 2019). Four *F. fusca* colonies were collected from the areas of each type in each season, resulting in a total of 36 colonies (3 seasons  $\times$  4 colonies  $\times$  3 area types. Colonies of *F. fusca* were collected along with workers from the nearest social parasite colony. Separate colony pairs were used in each case. In social parasites, only sub-colonies (i.e. only the necessary number of workers to perform the tests described below) were collected, without destroying the nests. In all three seasons, tests were preceded by a one-week acclimatisation period in the laboratory. The collected *F. fusca* colonies and sub-colonies of social parasites were housed in plastic boxes (L 37 cm  $\times$  W 25 cm  $\times$  H 21 cm). Nesting material (soil, rotten wood, litter) was provided from

their natural habitats. Each nest box was connected to a foraging arena (L 60 cm × W 30 cm × H 15 cm) with a plastic tube (1 cm Ø, 10 cm long). The ants were kept under constant temperature ( $24 \pm 4^{\circ}\text{C}$ ) and humidity (42–43%) conditions, and 12:12 h light and dark cycle. The ants were fed every second day in the foraging arenas with food commonly used to maintain laboratory colonies of ants (Bhatkar & Whitcomb 1970), while water was provided ad libitum.

## **Behavioural observations**

Behavioural tests comparing the responses of *F. fusca* workers to the corpses of their social parasites (i.e. *F. polychtena* and *F. sanguinea*) and to nestmate corpses were carried out for two weeks in every season. We used corpses to standardise the tests and eliminate the variability arising from different behavioural responses of parasites. The corpses used for the tests were produced by freeze-killing ( $-20^{\circ}\text{C}$ ) of anaesthetised healthy, uninjured individuals. The corpses were thawed 5 min before the beginning of the tests, and each corpse was used only once. To avoid the appearance of chemical cues characteristic of corpses, the corpses were used within 45 min (5 min after the start of thawing), ensuring that they served as proxies for living ants (see Choe et al. 2009).

Each test began by placing a *F. fusca* worker in a circular plastic Petri dish (Ø 5.3 cm) covered with a lid. After a 60 s acclimatisation, a corpse was carefully placed into the Petri dish through a small hole drilled in the lid. We tested 60 *F. fusca* workers from each colony: 20 with the corpses of the temporary social parasite, 20 with the corpses of the slave-maker and 20 with nestmate corpses. A total of 2160 tests (12 colonies × 60 workers × 3 seasons) were carried out across the three seasons. In each test involving social parasites, the workers were confronted with a corpse from the social parasite colony nested near the corresponding *F. fusca* colony (for detailed information on the origin of parasite corpses used in each colony's assays see Supplementary Table S2). Workers from control colonies were also exposed to 20 corpses of

each parasite type. However, these latter corpses were assigned randomly with respect to their colony of origin (from the 4 colonies per parasite types per season).

The responses of *F. fusca* workers to the different corpse types were videotaped for 80 s, and their aggressiveness was measured as the number of seconds during which the worker exhibited aggressive behaviours (mandible opening, biting, and gaster flexing or squirting formic acid; Fig. 1) towards the corpse. In total, 48 hours of video footage was recorded. A given *F. fusca* individual was tested only once, and after testing, it was isolated from the rest of the colony to prevent accidental reuse. The Petri dishes were cleaned and replaced after each trial and reused only after every fourth trial. All tests involving a particular colony were completed within one day. The responses of ants to the corpses were determined by analysing the recorded videos. The analyses were carried out blindly with respect to the type of corpse used.

### Statistical analyses

We used generalized linear mixed models (GLMM) with a Bernoulli error distribution, a logit link, and maximum likelihood estimation to test the effect of corpse type, season, site type, and their interaction on the workers' probability of showing an aggressive response (aggressive: 1, not aggressive: 0;  $N = 2160$ ). A separate GLMM with a Gamma error distribution, a log link, and maximum likelihood estimation was used to test the effects of the same variables on the duration of aggressive responses of *F. fusca* host workers. Only aggressive interactions were included in the latter model ( $N = 1112$ ). In both model types, corpse type, season, site type, and their interaction were included as fixed factors, and colony ID was included as a random factor. The data analyses were performed using the R software (R Core Team 2022). The generalised linear mixed models were built using the *glmmTMB* function of the 'glmmTMB' package (Brooks et al. 2017). To compare the different factor levels of the linear models (post-hoc: False Discovery Rate), the *emmeans* function ('emmeans' package, Lenth 2023) was used. The

goodness of fit of the models was tested using various diagnostics from the ‘DHARMA’ package (Hartig 2022). To reveal the exact significance levels a table-wide sequential FDR correction was applied.

## **Ethical note**

This research was not reviewed by an institutional or governmental regulatory body, because no protected species were used in the study, and no approval was required for work on invertebrates. We minimised the stress during the collection and transport of the colonies as much as possible. In the laboratory, to maximise the welfare and survival of the colonies, we kept them under conditions close to natural. During the tests, we only observed the spontaneous behaviours of the ants, and any stress beyond the intended scope of the assay (i.e. exposure to corpses of nestmates and parasites) was minimised. After the end of the experiment, the colonies were transported back to places of their origin and released near suitable nesting places.

## **Results**

### **Responses of *F. fusca* from the vicinity of *F. polychtena***

Host workers differed in their aggressive responses to the two parasite species across seasons. More host workers exhibited aggressive behaviours towards *F. sanguinea* than towards *F. polychtena* in summer (GLMM  $Z = 2.73$ ,  $P = 0.017$ ) and autumn ( $Z = 3.16$ ,  $P = 0.005$ ), although no significant difference was detected in spring ( $Z = 0.53$ ,  $P = 0.857$ ) (Fig. 2a; Suppl. Table S3). The number of aggressive workers was greater in spring and summer towards both types of parasite corpses than towards nestmates ( $Z > 3.02$ ,  $P < 0.007$ ), and in autumn workers were more aggressive towards *F. sanguinea* than towards nestmate corpses ( $Z = 3.50$ ,  $P = 0.0013$ ). However, in autumn, workers did not show increased aggression towards *F. polychtena* compared with nestmate corpses ( $Z = 0.4$ ,  $P = 0.92$ ).

Seasonal patterns of aggression towards parasites also varied within parasite species. More host workers exhibited aggression towards *F. polychtena* corpses in spring (GLMM  $Z = 2.43$ ,  $P = 0.04$ ) and summer ( $Z = 3.55$ ,  $P = 0.001$ ) than in autumn, while numbers did not differ between spring and summer ( $Z = 1.21$ ,  $P = 0.446$ ) (Fig. 2a; Suppl. Table S4). Aggression towards *F. sanguinea* was exhibited by more workers in summer compared with spring ( $Z = 2.79$ ,  $P = 0.015$ ) and autumn ( $Z = 3.47$ ,  $P = 0.002$ ) but did differ between spring and autumn ( $Z = 0.85$ ,  $P = 0.675$ ). The number of aggressive workers showing aggression towards nestmate corpses did not vary across seasons ( $Z < 1.27$ ,  $P > 0.414$ ).

The duration of aggressive responses further reflected species-specific differences. Host workers exhibited longer aggressive responses towards *F. sanguinea* corpses than towards *F. polychtena* in all seasons (GLMM  $Z > 3.08$ ,  $P < 0.006$ ), and parasite corpses elicited longer aggressive responses than nestmates in all seasons ( $Z > 4.67$ ,  $P < 0.0001$ ) (Fig. 2b; Suppl. Table S5). However, the duration of aggressive behaviours did not vary seasonally towards either *F. polychtena* ( $Z < 0.31$ ,  $P > 0.95$ ) or *F. sanguinea* ( $Z < 0.96$ ,  $P > 0.61$ ) (Fig. 2b; Suppl. Table S6). In contrast, the duration of aggressive responses towards nestmate corpses was marginally longer in summer than in spring ( $Z = 2.19$ ,  $P = 0.073$ ) and autumn ( $Z = 2.18$ ,  $P = 0.075$ ) but did not differ between spring and autumn ( $Z = 0.16$ ,  $P = 0.986$ ).

### **Responses of *F. fusca* colonies from the vicinity of *F. sanguinea***

The number of aggressive responses was greater in spring and summer towards both parasite species than towards nestmate corpses (GLMM  $Z > 4.03$ ,  $P < 0.0002$ ), and in autumn workers were more aggressive towards *F. polychtena* corpses than towards nestmates ( $Z = 3.09$ ,  $P = 0.006$ ) (Fig. 3a; Suppl. Table S3). However, in autumn, we found only marginally more aggressive responses towards *F. sanguinea* than towards nestmate corpses ( $Z = 2.27$ ,  $P = 0.06$ ).

We did not find significant differences in the number of host workers exhibiting aggressive behaviours towards *F. sanguinea* compared to *F. polychtena* in any season ( $Z < 1.01$ ,  $P > 0.572$ ).

The number of host workers exhibiting aggressive behaviours towards *F. polychtena* corpses was greater in summer than in autumn (GLMM  $Z = 3.65$ ,  $P = 0.001$ ), marginally greater in spring than in autumn ( $Z = 2.22$ ,  $P = 0.069$ ), and did not differ between spring and summer ( $Z = 1.53$ ,  $P = 0.277$ ) (Fig. 3a; Suppl. Table S4). Aggression towards *F. sanguinea* was exhibited by more workers than in autumn ( $Z = 4.11$ ,  $P = 0.0001$ ), marginally more in spring than in autumn ( $Z = 2.21$ ,  $P = 0.07$ ), while no difference was detected between spring and summer ( $Z = 2.04$ ,  $P = 0.103$ ). The number of aggressive workers towards nestmate corpses did not differ between spring and summer ( $Z = 1.76$ ,  $P = 0.183$ ) or between spring and autumn ( $Z = 1.48$ ,  $P = 0.3$ ), although more host workers were aggressive in summer than in autumn ( $Z = 3.15$ ,  $P = 0.005$ ).

The duration of aggressive responses did not differ between seasons for either *F. sanguinea* or *F. polychtena* corpses (GLMM  $Z < 1.5$ ,  $P > 0.292$ ), but parasite corpses consistently elicited longer aggressive responses than nestmates in all seasons ( $Z > 4.79$ ,  $P < 0.0001$ ) (Fig. 3b; Suppl. Table S5). We found no significant seasonal differences in the duration of aggressive behaviours towards *F. polychtena* ( $Z < 0.42$ ,  $P > 0.909$ ), *F. sanguinea* ( $Z < 1.6$ ,  $P > 0.247$ ), or nestmate corpses ( $Z < 0.77$ ,  $P > 0.72$ ) (Fig. 3b; Suppl. Table S6).

#### **Responses of *F. fusca* colonies from the control area**

Workers showed more aggressive responses in spring and summer towards both parasite species compared to nestmates (GLMM  $Z > 4.85$ ,  $P < 0.0001$ ), and in autumn aggression towards *F. polychtena* was higher than towards nestmate corpses ( $Z = 2.56$ ,  $P = 0.028$ ) (Fig. 4a; Suppl. Table S3). In contrast, aggression towards *F. sanguinea* in autumn did not differ from responses towards nestmate corpses ( $Z = 1.73$ ,  $P = 0.194$ ). We did not find significant

differences in the number of host workers exhibiting aggressive behaviours towards *F. sanguinea* compared to *F. polychtena* in any season ( $Z < 0.88$ ,  $P > 0.652$ ).

Seasonal shifts in the pattern of aggression towards parasites occurred within parasite species. The number of host workers exhibiting aggressive behaviours towards *F. polychtena* corpses was higher in summer than in both spring (GLMM  $Z = 2.37$ ,  $P = 0.047$ ) and autumn ( $Z = 3.85$ ,  $P = 0.0003$ ), with no difference between spring and autumn ( $Z = -1.58$ ,  $P = 0.257$ ) (Fig. 4a; Suppl. Table S4). Aggression towards *F. sanguinea* was exhibited by more workers in summer than in autumn ( $Z = 4.15$ ,  $P = 0.0001$ ) and marginally higher number of aggressive host workers were detected in spring than in autumn ( $Z = 2.31$ ,  $P = 0.055$ ), while spring and summer did not differ in this respect ( $Z = -1.96$ ,  $P = 0.123$ ). Aggression towards nestmate corpses did not differ between spring and summer ( $Z = -1.98$ ,  $P = 0.118$ ) or between spring and autumn ( $Z = -0.18$ ,  $P = 0.983$ ), although marginally more host workers were aggressive in summer than in autumn ( $Z = 2.15$ ,  $P = 0.08$ ).

The duration of aggressive responses differed by parasite species. Responses towards *F. sanguinea* were longer than towards *F. polychtena* in autumn (GLMM  $Z = 2.35$ ,  $P = 0.049$ ), and marginally longer in spring ( $Z = 2.13$ ,  $P = 0.084$ ), while no difference was detected in summer ( $Z = 0.06$ ,  $P = 0.998$ ) (Fig. 4b; Suppl. Table S5). Both parasite species elicited longer aggressive responses than nestmate corpses in all seasons ( $Z > 5.05$ ,  $P < 0.0001$ ).

In contrast, the duration of aggressive behaviours did not vary seasonally towards either *F. polychtena* (GLMM  $Z < 1.98$ ,  $P > 0.119$ ) or *F. sanguinea* ( $Z < 1.4$ ,  $P > 0.343$ ) (Fig. 4b; Suppl. Table S6). The duration of aggression towards nestmate corpses was longer in summer than in spring ( $Z = 2.61$ ,  $P = 0.025$ ), marginally longer in autumn than in spring ( $Z = 2.21$ ,  $P = 0.069$ ), and did not differ between summer and autumn ( $Z = 0.01$ ,  $P = 0.999$ ).

## Discussion

Our results show that *F. fusca* host colonies adjusted both the intensity and duration of aggressive responses towards social parasites in a season- and species-specific manner. Colonies from areas dominated by *F. polychtena* expressed stronger aggression towards *F. sanguinea* than towards *F. polychtena* in summer and autumn. Seasonal patterns differed between parasite species: aggression towards *F. polychtena* was elevated in spring and summer, whereas aggression towards *F. sanguinea* peaked in summer. Host workers responded longer to *F. sanguinea* than to *F. polychtena* in all seasons, however, the duration of aggression did not vary seasonally towards either parasite. Colonies from *F. sanguinea* areas exhibited similar levels of aggression towards the two parasite species in all seasons. As in the *F. polychtena*-adjacent colonies, the duration of aggressive responses did not differ seasonally. Seasonal patterns of aggression differed towards parasites, with marginally more workers showing aggressive responses towards both parasites in spring and more in summer compared to autumn. Colonies from control areas showed an intermediate response, exhibiting elements of both response profiles. They responded similarly towards both parasites, while the seasonal patterns of aggression varied between parasites. Hosts were most aggressive towards *F. polychtena* in summer, and towards *F. sanguinea* in summer, with marginally more aggressive workers in spring, than in autumn. Together, these patterns suggest that host colonies modulate their level of aggressive behaviour according to both the local parasite community and seasonal context, while maintaining a consistent qualitative distinction between parasite and nestmate cues.

Alloway (1990) demonstrated that hosts can differentiate social parasites from other enemies, such as competitors, suggesting they may also distinguish between social parasites of different types. While stronger aggressive responses towards social parasites than competitors have been reported in multiple studies (e.g. Pamminer et al. 2011; Scharf et al. 2011; Maák et al. 2014), it remains unclear under what conditions hosts can distinguish between different types of social parasites. In our study, *F. fusca* colonies collected from the vicinity of *F. polychtena*

responded differently to the two parasites, showing lower levels of aggression towards *F. polychtēna* both in duration of aggressive responses and number of workers exhibiting aggressive responses. This is also supported by Włodarczyk (2023), who found that *F. fusca* workers exhibited higher levels of aggression towards slave-makers than towards temporary parasite workers in the field. The lower levels of aggression towards *F. polychtēna* may reflect alternative defensive strategies against temporary parasites, such as the ability to effectively discriminate between nestmate and parasite brood, thereby preventing the rearing of parasite brood to adulthood (Achenbach et al. 2010, Chernenko et al. 2011, 2013). The presence of such strategies is also supported by the fact that temporary parasite *Formica* queens rarely succeed in taking over queenright host colonies, as they are typically killed by the hosts (Chernenko et al. 2013). Another possible explanation for the lower levels of aggression observed is that *F. fusca* behaves submissively towards *F. polychtēna* (Savolainen & Vepsäläinen 1988, Czechowski et al. 2012; Seifert 2018), which poses a relatively minor threat as a parasite compared to other similar *Formica* species, such as *F. rufa* (southern wood ant) or *F. lugubris* (hairy wood ant). *F. polychtēna* generally establishes new colonies through colony splitting, and even when temporary parasitism occurs, it typically targets queen-less host colonies that do not defend themselves as efficiently (Pisarski & Czechowski 1994). On the other hand, colony takeover by *F. polychtēna* may also occur through interference competition during colony splitting, when daughter colonies separating from maternal colonies aggressively displace nearby *F. fusca* colonies from favourable nesting sites (I. Maák, unpubl.). Similar competitive interactions with subordinate species are also known in other territorial ants, such as *Formica exsecta* (narrow-headed ant) displacing *Lasius flavus* (yellow meadow ant) (Pisarski 1982).

Thus, one could argue that the response of *F. fusca* to *F. polychtēna* may primarily reflect competitive interaction rather than defence against social parasitism, as *F. polychtēna* is a strong competitor of *F. fusca* (Savolainen 1991), particularly in spring and early summer. As a

dominant territorial species, it exerts considerable competitive pressure on subordinate species nesting within its territory to secure food resources (see Mabelis 1984; Champer & Schlenoff 2024). The persistence of *F. fusca* within such territories is likely facilitated by various strategies that minimize conflicts and reduce the likelihood of escalated interactions with the dominant competitor (Savolainen & Vepsäläinen 1988, Savolainen et al. 1989). Because *F. fusca* workers frequently encounter *F. polychtena* within its territories, maintaining persistently high aggression levels may be energetically costly or maladaptive. At the same time, living within the territory of wood ants can be advantageous for *F. fusca*. Territorial species such as *F. polychtena* can indirectly protect *F. fusca* from *F. sanguinea* by preventing slave-raids within their territories (Czechowski 2000; Czechowski & Markó 2006; Väänänen et al. 2010). Consequently, colonies located within wood ant territories may not need to invest as heavily in aggressive defence as those living outside these areas, a pattern also found by Włodarczyk (2023). Thus, the relationship between *F. fusca* and *F. polychtena* is much more complicated than it would first seem, which is also reflected in our results. In colonies collected from the vicinity of *F. polychtena*, the same number of host workers showed aggressive responses in spring (the season associated with increased parasite pressure from *F. polychtena*) towards both parasites, but more host workers exhibited aggressive responses towards *F. sanguinea* in the other two seasons. This pattern may reflect both the protective-parasitic-competitive context induced by *F. polychtena* and the higher threat posed by *F. sanguinea*. It is also important to note that this pattern differs from that of the control colonies – in which competition-based aggression patterns are most expected – where host worker aggression was lower towards *F. polychtena* in spring than in summer.

Given the significantly greater threat posed by the slave-maker *F. sanguinea* (see d’Ettorre & Heinze 2001), we expected *F. fusca* workers to show stronger aggression towards *F. sanguinea* than towards the temporary parasite *F. polychtena*. Although the pattern was not

consistent and varied between colonies from different sites, it remained detectable in our results. Even though both slave-makers and temporary parasites destroy host colonies during colony founding, slave-makers additionally raid neighbouring host colonies, imposing further fitness costs on the host population. These raids deprive host colonies of most offspring, including sexual pupae, and may result in the kidnapping of callow workers. Inclusive fitness of host colonies may be further reduced when slave-makers raid genetically related host colonies (Achenbach & Foitzik 2009). Moreover, raids also serve as a form of foraging in *F. sanguinea* (Mori et al. 2000), during which many adult host workers are killed and transported back to the slave-maker nest as prey (W. Czechowski, several observations). Such activity can lead to the complete destruction of host colonies within the raiding range of *F. sanguinea* (Seifert 2018).

Host colonies, particularly those of *F. fusca*, targeted by *F. sanguinea* raids have limited defensive options due to the slave-maker's aggression, physical strength, and the violence of the attacks. The most effective strategies are immediate nest evacuation or eliminating scouts or the first attackers, both of which require sufficient levels of aggression (Włodarczyk 2023, W. Czechowski, numerous observations; see also Czechowski 1977). Additionally, the severity of aggression can result from prior contact with social parasites. In *Temnothorax longispinosus* (long-spined acorn ant), for instance, workers exhibited increased aggression towards non-nestmate conspecifics after encountering slave-makers, while their aggression towards the slave-maker itself remained consistently high (Pamminger et al. 2011). In our experiment, colonies from the vicinity of *F. sanguinea* reacted similarly to both parasites, which may indicate that under sustained high parasite pressure, hosts show a more generalised, constitutive response pattern that maintain an elevated level of aggression against most type of threats. Such a behavioural shift could explain why aggression levels did not differ between the slave-maker and a temporal parasite/territorial competitor.

Contrary to previous examples and our second prediction, control *F. fusca* colonies also showed seasonal variation in aggression, with increased aggression in summer towards *F. polyctena* and in spring and summer towards *F. sanguinea*. Moreover, we found marginally longer duration of their responses in autumn and in spring towards *F. sanguinea*. This suggests that the seasonally regulated levels of aggression may represent a cost-effective innate strategy against potential social parasites as well as competitors. Maintaining aggressive defence can be advantageous during periods of increased threat, such as when young social parasite queens searching for host colonies appear. Nonetheless, previous research indicates that under intense social parasitic pressure, hosts may shift from aggressive defence to evacuation strategies (Jongepier et al. 2014). This suggests that both behavioural responses – i.e. modulating aggression and relocating the colony – may serve as adaptive cost-effective defences against social parasites. The increased aggression observed in control colonies during summer may reflect a general seasonal pattern driven by multiple threats. In addition to parasitic pressure, these colonies face strong competitive pressure from *F. sanguinea*, which can act not only as a slave-maker but also as an aggressive competitor (Ślipiński et al. 2018). However, since aggressive encounters entail risks such as injury or death (Georgiev et al. 2013), especially when confronting stronger opponents, the costs of maintaining high levels of aggression may constrain the intensity or duration of such responses.

The workers of *F. fusca* exhibited only low levels of aggression towards the corpses of their nestmates in all seasons, irrespective of their origin (i.e. whether they originated from areas with or without social parasites). Nestmate recognition in ants is based on ‘chemical distance’, i.e. the degree of difference in the cuticular hydrocarbon (CHC) profile between two interacting individuals (van Zweden & d’Ettorre 2010; Martin et al. 2012). Ants are also able to adjust their ‘recognition threshold’ (i.e. the level of CHC profile difference tolerated between two interacting ants; Brandt et al. 2005). Thus, one might expect a slight increase in aggression

towards nestmates during periods of heightened aggression towards social parasites, especially since social parasites often mimic the CHC profile of their hosts (Lenoir et al. 2001; van Zweden & d'Ettorre 2010). This was reflected also by our results related to the differences in the duration of low-intensity aggressive reactions towards nestmate corpses, particularly those collected near *F. polyctena* nests and control areas. However, based on our results, nestmate recognition remains effective even under moderately high parasite pressure; by accurately distinguishing between nestmates and parasites, host workers avoid the fitness costs associated with mistakenly attacking their own nestmates. It is important to note, however, that the two parasites in our experiment are not known to mimic the CHC-s of their hosts. Successful take-overs by temporary parasitic *Formica* species usually happen in orphaned host colonies (Chernenko et al. 2013), so *F. polyctena* may not possess a specialized strategy for mimicking host CHC profiles. *Formica sanguinea*, on the other hand, labels its hosts with its own CHCs rather than mimicking host profiles (Włodarczyk and Szczepaniak 2017).

The cost of aggressive defence can be reduced if workers express heightened aggression towards social parasites only during periods of greatest threat. For *F. fusca*, these periods occur in spring and early summer, when most wood ant species (including *F. polyctena*) establish new colonies after their nuptial flights, and again in mid to late summer, when the facultative slave-maker *F. sanguinea* conducts its raids along with colony founding by its young queens. Previous studies have shown that hosts of social parasites can adjust the intensity of their aggression seasonally (d'Ettorre et al. 2004; Pamminer et al. 2011). For example, colonies of *Formica rufibarbis* (red barbed ant) from areas threatened by raids of the obligatory slave-maker *Polyergus rufescens* responded more aggressively towards the slave-maker during the summer raiding period than the preceding and subsequent periods, whereas host colonies from unthreatened areas showed no such seasonal modulation (d'Ettorre et al. 2004). Seasonal modulation of aggression is not unique to ants, as similar patterns occur also in other social

insects. Honeybee guards, for example, adjust their guarding behaviour and acceptance thresholds according to the seasonal probability of honey-robbing (cleptoparasitism) by other bees (Downs & Ratnieks 2000; Couvillon et al. 2008). Differences in colony-level aggression and variation within colonies may arise because (i) some colonies contain a greater proportion of aggressive workers, (ii) colonies differ in the distribution of individual aggression (e.g. a few highly aggressive workers can raise the average aggression of a colony), or (iii) both factors operate simultaneously (Pinter-Wollman, 2012). Our results suggest that in *F. fusca*, colony-level regulation of aggression occurs primarily by adjusting the number of aggressive workers rather than altering the worker's individual aggression levels. This is consistent with Pinter-Wollmans' (2012) first hypothesis, which proposes that group level behavioural differences can emerge from variation in the proportion of individual behavioural types ("personalities") within the colony.

In summary, our results demonstrate that *F. fusca*, the primary host species of both studied social parasites, may distinguish between parasite species and adjust its responses according to the level of threat they pose. Seasonal shifts in aggression, aligned with the phenology of each parasite, appear to represent an effective strategy for minimizing the costs of defence. However, these strategies are strongly modulated by the identity of neighbouring rival colonies, allowing hosts to avoid the costs of maintaining constitutive defences over extended periods, as well as the risks of delayed responses, particularly when parasite activity follows predictable seasonal patterns. Taken together, host-parasite interactions are highly complex, shaped not only by parasite strategies and host defences, but also by the broader ecological context, including interspecific competition and the presence of neighbouring colonies.

## **Data Availability**

The data associated with this study are available as supplementary material.

## **Declaration of Interest**

The authors declare that we have no competing interests.

## **Author Contributions**

**Fanni Pécsy:** Conceptualization, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **István Elek Maák:** Conceptualization, Data Curation, Formal analysis, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Gábor Lőrinczi:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Ádám Lőrincz:** Investigation, Writing – review & editing. **Kata Anna Bán:** Investigation, Writing – review & editing. **Bonita Ratkai:** Investigation, Writing – review & editing. **Wojciech Czechowski:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

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#### Figure legends:

**Figure 1.** Aggressive behaviours of *Formica fusca* workers towards social parasite corpses: mandible opening (a), biting (b), and gaster flexing (c).

811

812 **Figure 2.** Number (a) and duration (b) of aggressive responses of *Formica fusca* workers, collected near  
813 the temporary parasite (*Formica polychtena*), to the different corpse types (*F. polychtena*, *F. sanguinea*,  
814 nestmate) across seasons. Boxplots display the median (central line), the 25th and 75th percentiles  
815 (lower and upper box boundaries), and whiskers extending to 1.5×IQR, with overlaid circular markers  
816 indicating the mean. Different letter combinations indicate significant differences between seasons and  
817 corpse types ( $P < 0.05$ ). Plots sharing the same letter are not significantly different from each other.  
818 Letters in italics mark marginally significant differences ( $0.5 < P < 0.1$ ). Letters without parenthesis  
819 indicate significant differences between seasons within each treatment group. Letters in parentheses  
820 indicate significant differences between treatment groups when comparing within the same season.

821

822 **Figure 3.** Number (a) and duration (b) of aggressive responses of *Formica fusca* workers, collected near  
823 the slave-maker (*Formica sanguinea*), to the different corpse types (*F. polychtena*, *F. sanguinea*,  
824 nestmate) across seasons. Boxplots display the median (central line), the 25th and 75th percentiles  
825 (lower and upper box boundaries), and whiskers extending to 1.5×IQR, with overlaid circular markers  
826 indicating the mean. Different letter combinations indicate significant differences between seasons and  
827 corpse types ( $P < 0.05$ ). Plots sharing the same letter are not significantly different from each other.  
828 Letters in italics mark marginally significant differences ( $0.5 < P < 0.1$ ). Letters without parenthesis  
829 indicate significant differences between seasons within each treatment group. Letters in parentheses  
830 indicate significant differences between treatment groups when comparing within the same season.

831

832 **Figure 4.** Number (a) and duration (b) of aggressive responses of *Formica fusca* workers, collected from  
833 control areas, to the different corpse types (*F. polychtena*, *F. sanguinea*, nestmate) across seasons.  
834 Boxplots display the median (central line), the 25th and 75th percentiles (lower and upper box  
835 boundaries), and whiskers extending to 1.5×IQR, with overlaid circular markers indicating the mean.  
836 Different letter combinations indicate significant differences between seasons and corpse types ( $P <$   
837  $0.05$ ). Plots sharing the same letter are not significantly different from each other. Letters in italics mark  
838 marginally significant differences ( $0.5 < P < 0.1$ ). Letters without parenthesis indicate significant

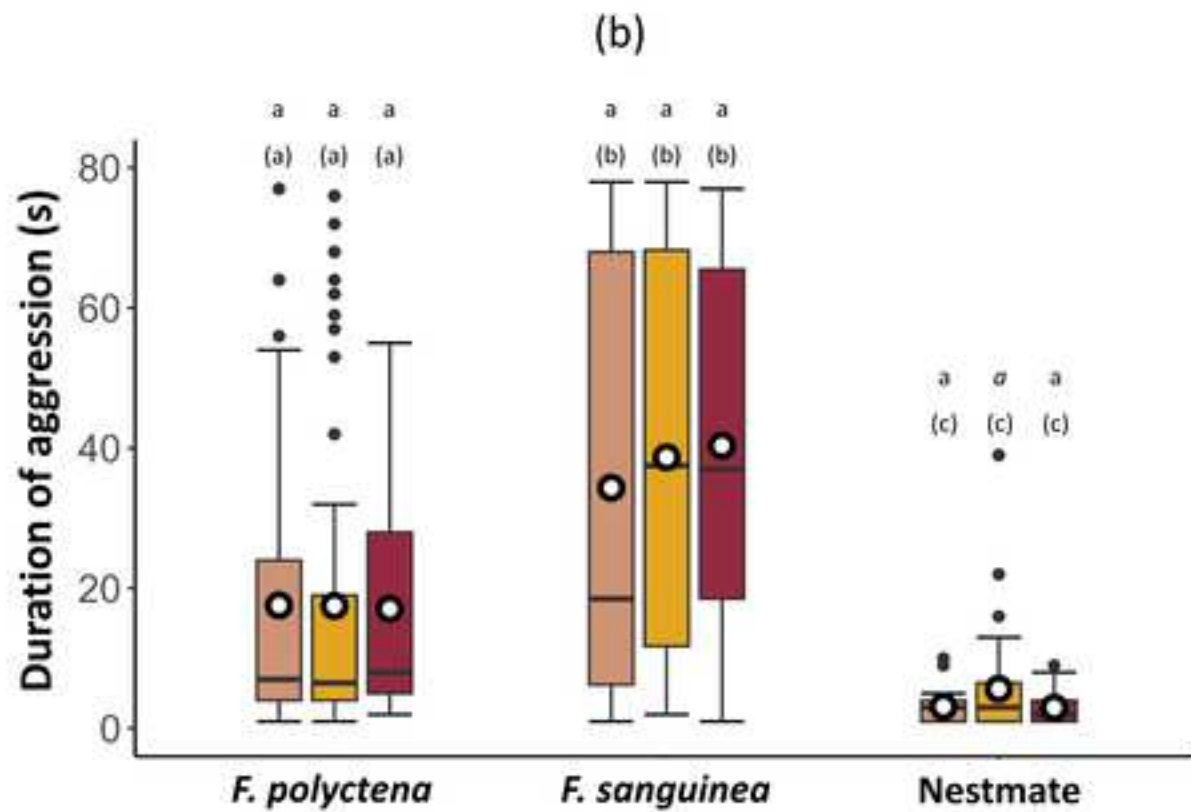
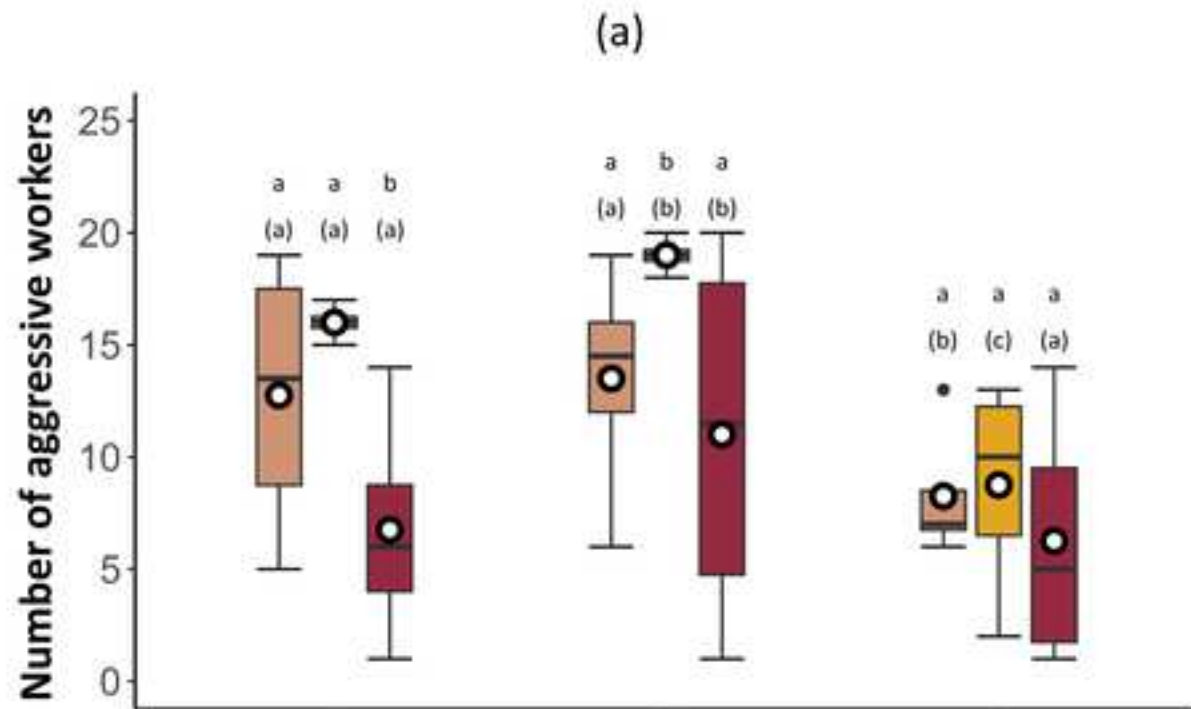
839 differences between seasons within each treatment group. Letters in parentheses indicate significant  
840 differences between treatment groups when comparing within the same season.

## Author Contributions

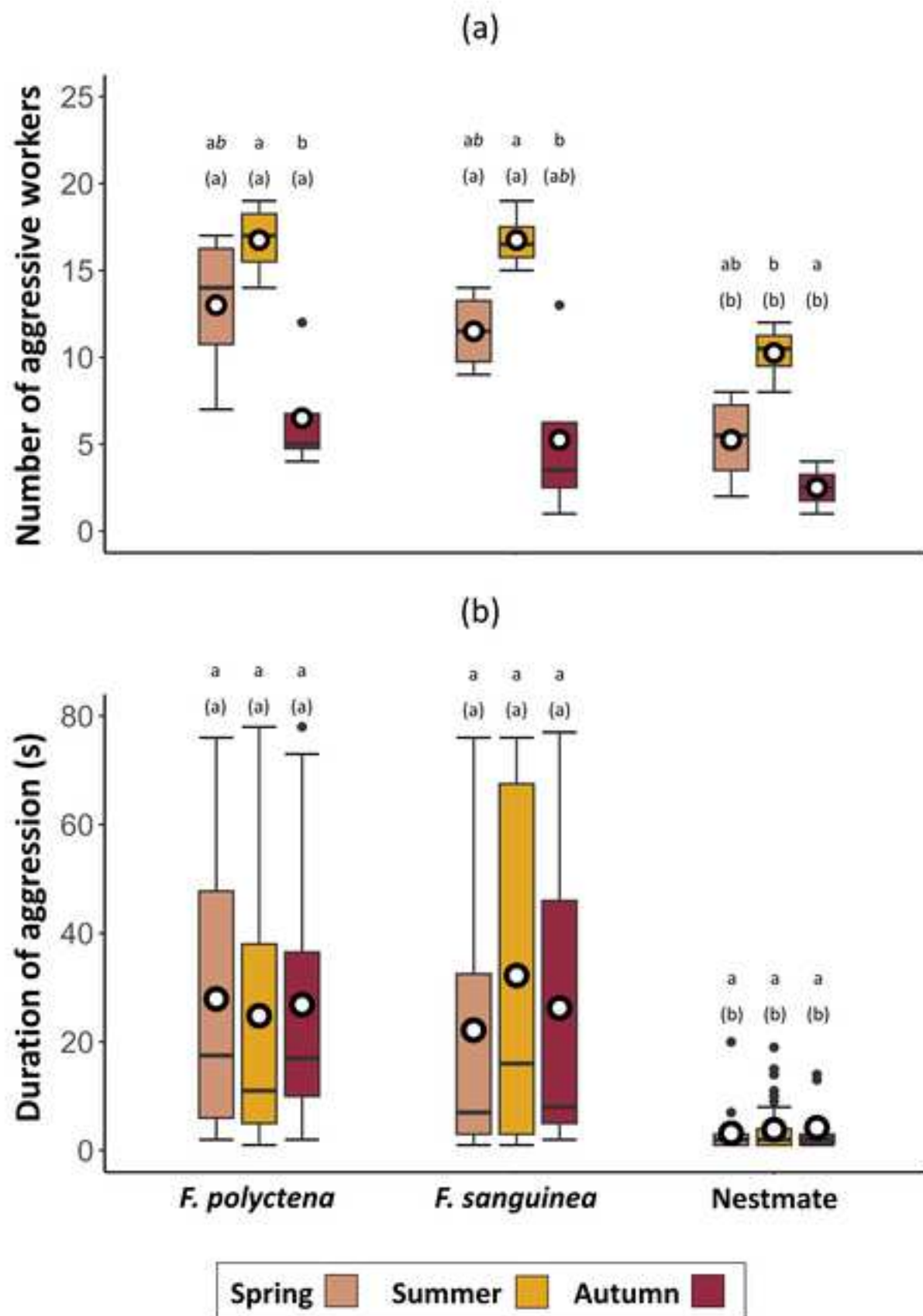
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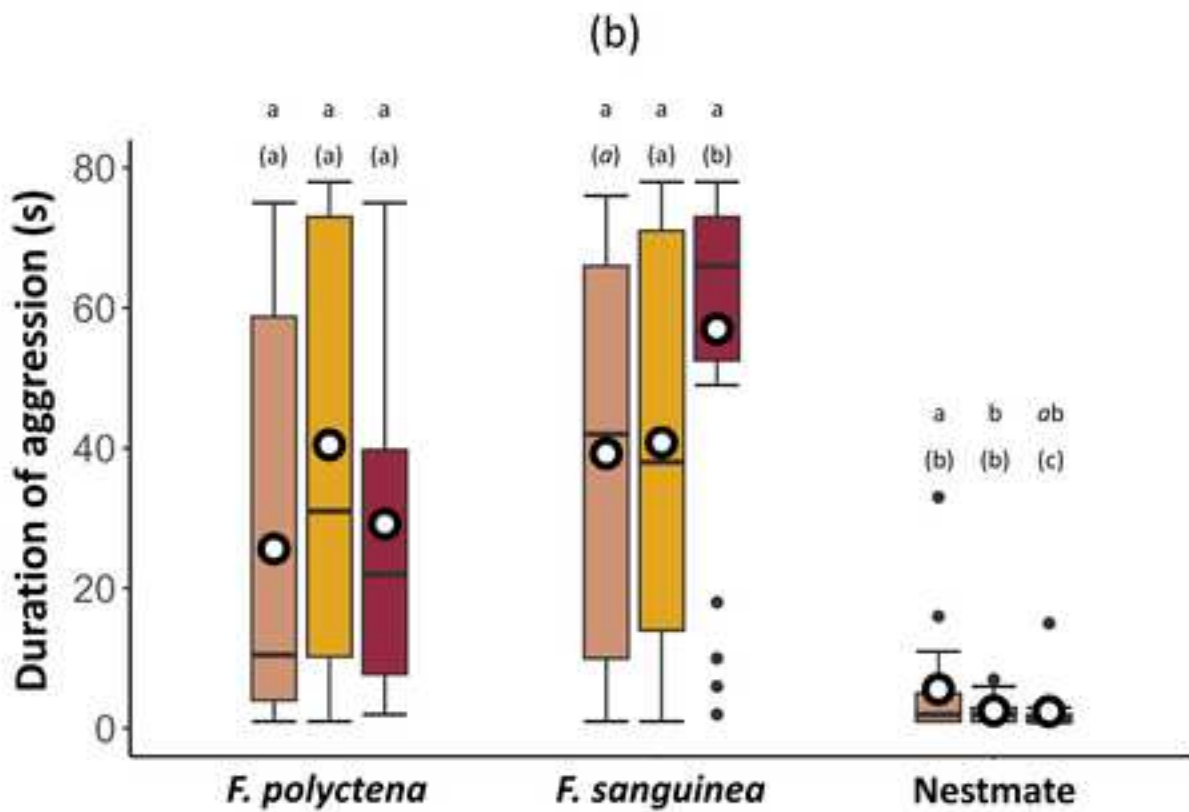
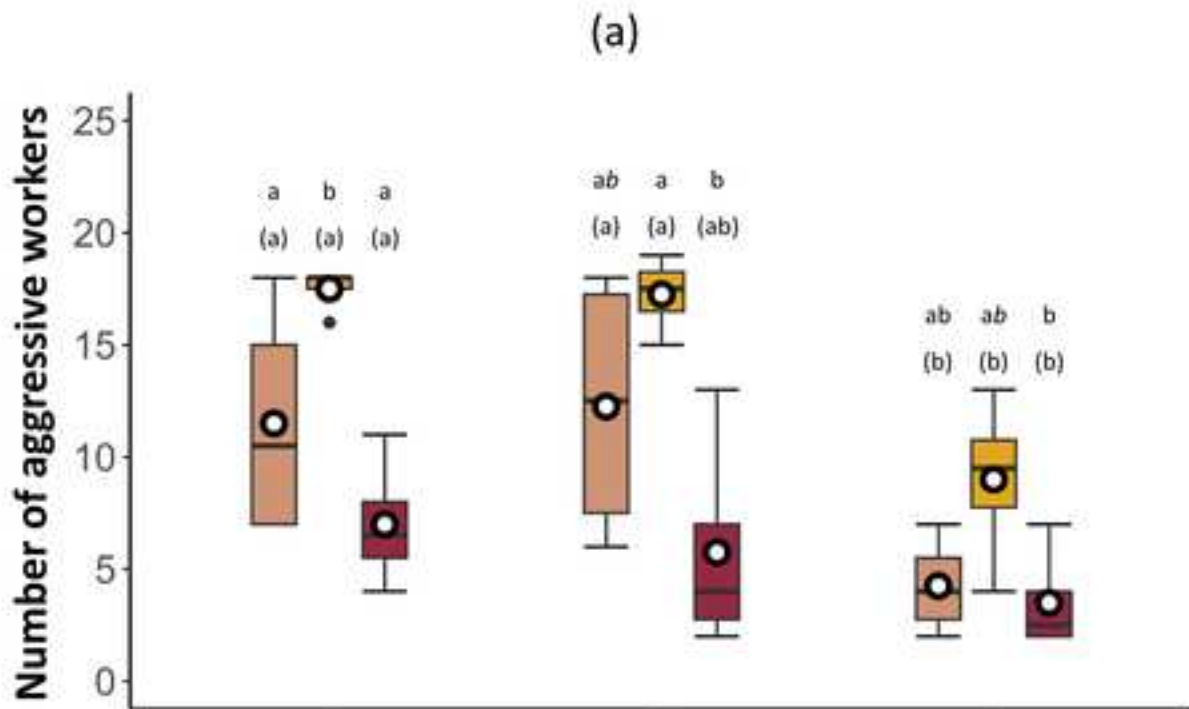
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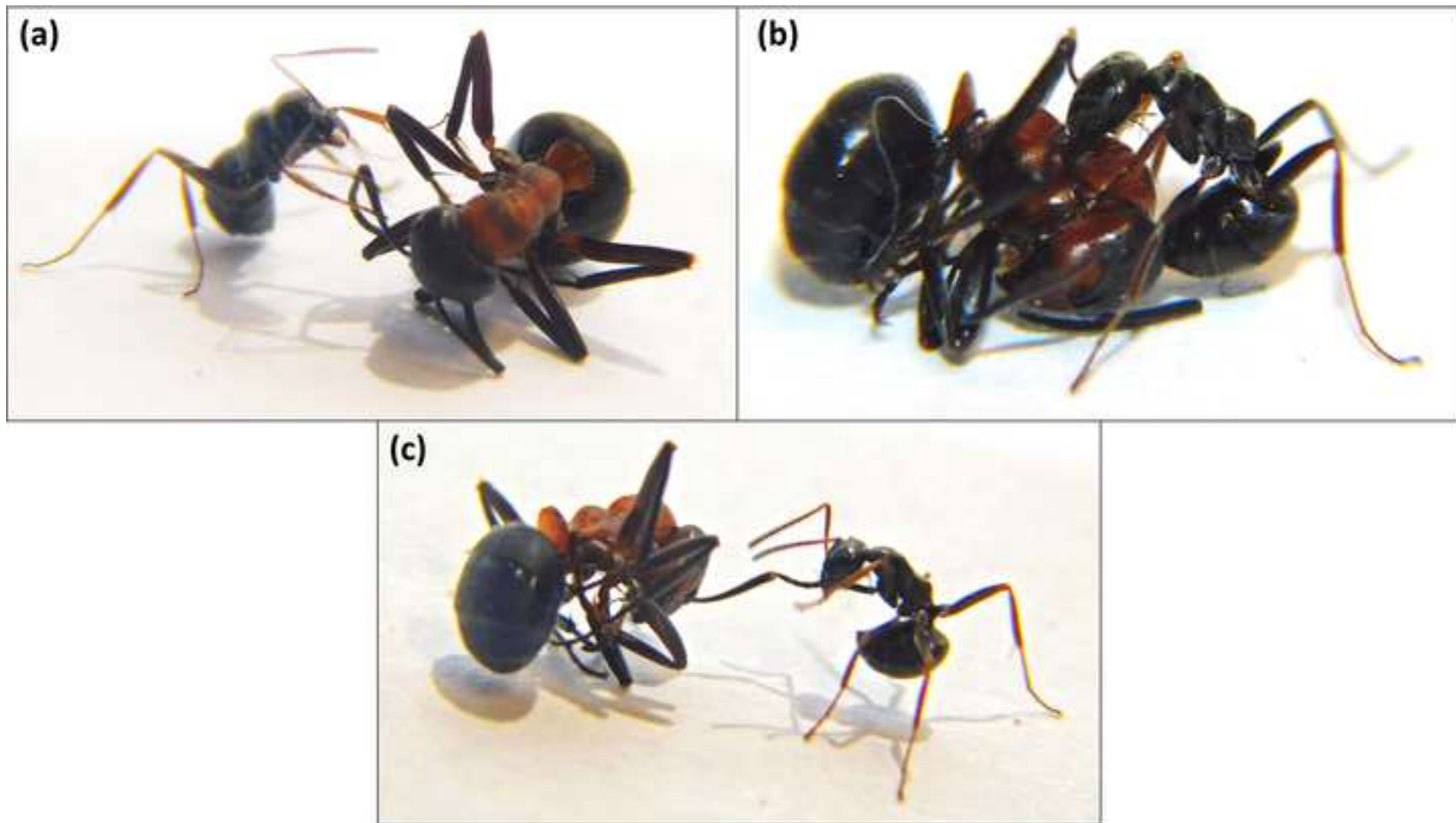


Spring Summer Autumn





Spring Summer Autumn

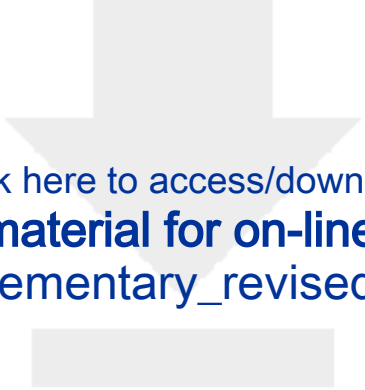




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