

1 **Title:** Left Out in the Cold: Thermal Variation in an Ant-Plant Defense

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6

7 **Abstract**

8 Despite the vast research on the thermal ecology of ants, few studies have examined the
9 role that temperature plays in mediating protective ant-plant interactions. These mutualisms are
10 conditional interactions that depend on the ants' ability to repel herbivory and improve the
11 fitness of their host. To assess for temperature dependence, we observed baseline activity and the
12 aggressive response against a simulated herbivore in four symbiotic ant species on their
13 myrmecophyte host (*Acacia drepanolobium*) across a thermal gradient. Furthermore, we
14 experimentally removed the most aggressive ant species (*C. mimosae*) from trees to determine if
15 thermal variation in activity led to a functional response in a model herbivore (goats). Aggressive
16 responses to the simulated herbivore increased with surface temperature for each species, but the
17 two most aggressive species had a stronger response to temperature. As surface temperature
18 increased, goats took fewer bites and spent less time feeding from *A. drepanolobium* as a direct
19 result of increased *C. mimosae* aggression. However, we did not detect any thermal dependence
20 in baseline activity among the ant species. Overall, our study suggests that thermal variation
21 plays a role in mediating species interactions in a mutualism network.

22 **Keywords:** plant defense, ant-plant mutualism, thermal ecology, herbivory, abiotic

23

24 **Introduction**

25 Plants have evolved a wide variety of defenses in response to herbivory, including
26 chemical metabolites, physical armature, and protein-based defenses (reviewed in Freeman and
27 Beattie 2008). One unique defensive adaptation involves a mutualistic relationship with
28 protective symbiotic ants. Such ant-plant protection mutualisms have repeatedly evolved
29 throughout the tropics, involving over 100 species of flowering plants and 40 genera of ants
30 (Davidson and McKey 1993). Ants provide a number of services for their host plants, such as
31 defense against herbivory, pathogens, and encroaching plants, receiving nesting space and food
32 (i.e. nectar and/or food bodies) in return (reviewed in Heil and McKey 2003).

33 The ant defenders are considered an inducible defense, as they can vary the intensity of
34 their response depending on several biotic factors (i.e. plant disturbance, leaf damage, extrafloral
35 nectar secretion; reviewed in Agrawal and Rutter 1998). Comparatively, fewer studies have
36 considered abiotic factors that effect this plant defense (but see Kersch and Fonseca 2008;
37 Fitzpatrick et al. 2013, 2014). Because ants are poikilotherms, their activity levels tend to
38 increase with temperature (Hulbert et al. 2008), with peak activity occurring in each species'
39 optimal thermal range after which activity decreases due to risk of overheating or desiccation
40 (Cerdá et al. 1998, Bucy and Breed 2006, Azacarte et al. 2007). Since the efficacy of ants in
41 defending against herbivory is tied to their activity levels (e.g., Palmer and Brody 2007),
42 differences in thermal tolerance among ant species may therefore influence the defensive
43 efficacy of different ant species. For example, in the Sonoran desert, barrel cacti (*Ferocactus*
44 *wislizeni*) are defended by four ant species, the most effective of which has a narrower thermal
45 tolerance than the least effective mutualist (Fitzpatrick et al. (2014). As a consequence, as
46 temperatures peak during the hottest part of the day, the most effective defenders are replaced by

47 less effective defenders, suggesting that temperature can strongly influence the quality of
48 protection offered by ant mutualists. However, despite the potential for temperature-dependence
49 of ant defense of plants, to our knowledge there are no experimental studies evaluating how
50 temperature influences the ability of ants to defend plants from herbivore attack.

51 In this study, we examined interactions among the East African ant-plant *Acacia*
52 (*Vachellia drepanolobium*) and its ant symbionts across the natural temperature gradient that
53 occurs from sunrise to sunset. *Acacia drepanolobium* is a myrmecophytic tree that is broadly
54 distributed and locally abundant on clay-rich “black cotton” savannas throughout East Africa
55 (Herlocker 1974, Stapley 1998), frequently comprising >90% of total woody plant cover (Young
56 et al. 1997, 1998). Across this range, trees host single colonies (at any given time) of between
57 one and four sympatric symbiotic ant species, each of which imposes different costs and confers
58 different benefits to its host plant (Palmer and Brody 2007, Palmer et al. 2010). At our study site,
59 ants are exposed to average daily temperature cycles that vary by 16°C over the course of the
60 day, with even higher variation in surface temperatures, which allowed us to address three main
61 questions: (1) How does temperature affect ant activity on their host tree throughout the day? (2)
62 How does temperature affect the strength of a defensive response against simulated herbivory
63 throughout the day? (3) Does the efficacy of ant defense against a browsing herbivore (goats)
64 depend on temperature?

65

66 **Methods**

67 *Study System*

68 We conducted our study at Mpala Research Centre (0°17'N, 36°52'E, 1700-2000m
69 elevation) in Laikipia, Kenya. The lowest average monthly minimum and highest average

70 monthly maximum ambient temperatures are 11.0°C and 29.5°C, respectively. The study site is
71 characterized by a heavy “black cotton” vertisol (Ahn and Geiger 1987) dominated by several
72 grasses and a single tree species, *A. drepanolobium* (Young et al. 1997, 1998). In an ecosystem
73 with a broad range of large browsing herbivores, *A. drepanolobium* is well defended by a
74 combination of spines and symbiotic ants (Hocking 1970). While all spines function as
75 mechanical defenses, a subset is swollen to provide a nesting cavity for the protective ants.
76 *Acacia drepanolobium* also provide a food source for the ants by secreting a carbohydrate-rich
77 nectar from extrafloral nectaries located on the rachis of each leaf. Almost every tree is occupied
78 by a single colony of one of four symbiotic ant species: *Crematogaster sjostedti*, *C. mimosae*, *C.*
79 *nigriceps* and *T. penzigi*. While each tree only hosts one colony, a single colony can occupy
80 several neighboring trees. The defensive capabilities among ant species differ; *C. mimosae* has
81 been shown to defend their hosts the most aggressively, followed by *C. nigriceps*, *T. penzigi* and
82 *C. sjostedti* (Palmer et al. 2010). Removal of *C. mimosae* dramatically increased elephant
83 damage compared to controls, and removal of both aggressive ants (*C. mimosae* and *C.*
84 *nigriceps*) increased the proportion of browsed branch apices (Stanton and Palmer 2011).

85 *Ant Defensive Behavioral Observations*

86 To observe the relationship between ant activity and temperature, we surveyed 60 trees
87 within a 0.1 km² study area. We identified 15 trees occupied by each ant species that were 1.5-2
88 meters tall and not in contact with the canopies of surrounding trees. Selected trees occupied by
89 conspecifics were separated by at least 15 meters to ensure each replicate was an independent
90 colony. We separated the 60 trees into 12 spatial blocks and randomly sampled the five trees
91 within each spatial block using a random number generator. Every spatial block contained at

92 least one tree occupied by each species (except for one lacking a tree hosting *C. mimosae*) to
93 minimize a block effect.

94 For every tree, we measured ant behaviors from before sunrise to after sundown,
95 spanning a range of surface temperatures from 9°C (lowest recorded) to 49°C (highest recorded).
96 Each tree was observed at least once within the following time windows: dawn (before 6:31am),
97 dusk (after 6:30pm), and at two-hour intervals in between. Before behavioral observations were
98 taken, we recorded the time of day and surface temperature of an outer branch. The average
99 temperature was recorded by holding the Fluke 62 Max IR Thermometer (Fluke Inc., Everett,
100 Washington, USA) about 15 cm above a branch on the sunned side of the tree for five seconds.
101 We chose to measure surface temperature rather than ambient temperature because ants exist in
102 superheated boundary layers that can be considerable hotter than the air a few more millimeters
103 above the surface (Kaspari et al. 2015).

104 Two behaviors were observed: (1) baseline activity along undisturbed branches and (2)
105 swarming an artificial herbivore. First, we measured baseline activity by haphazardly selecting
106 healthy branches on each tree and counting the number of ants that passed into a 5 cm segment
107 of the branch over 30 seconds. The focal segment was the closest 5 cm section to an apical bud
108 free of any swollen thorns. We performed this procedure on two branches simultaneously to
109 produce a sum baseline activity level. Second, we simulated herbivory by rapidly sliding a
110 gloved hand 15cm down the branch towards the tip three times and then grasping the branch
111 within the 15 cm region for 30s, careful not to directly grab a swollen thorn (Palmer and Brody
112 2013). After the 30s period, we released the glove counted the number of ants that had crawled
113 onto the glove. The sum of the ants on both gloves was used as an index for the intensity of anti-

114 herbivory defense. Given that the ant colony sizes are in the thousands to tens of thousands, we
115 assumed that each glove test did not influence successive observations (Palmer 2004).

116 *Ant Defense against a Model Herbivore*

117 In our second experiment, we sought to determine whether temperature-dependent
118 variation in ant defensive level influenced herbivore behavior. We used goats (which widely
119 occur throughout our study region) as model browsers and examined their feeding behavior at
120 three distinct times on host plants with ants, and on host plants from which ants had been
121 removed. The goats were all female, approximately the same size, and hunger-motivated, as food
122 was withheld beginning the night before each experiment. First, we selected fourteen 1 meter tall
123 trees with similar canopy volumes that were occupied by *C. mimosae*. We then paired them
124 spatially and randomly selected a control (ant-occupied) and experimental (removal) tree with a
125 coin toss. The removal tree controlled for temporal differences in hunger within the goats. Five
126 days before conducting the feeding trials, we removed the ants from experimental trees by
127 disturbing the trees and misting them with short-lived, pyrethrin based insecticide (0.6% alpha-
128 cypermethrine) and wrapped the trunks in duct tape and Tanglefoot (Contech, Spartanburg,
129 South Carolina, USA) to prevent ground recolonization by ants.

130 Over two days, we brought goats to the site and allowed them to feed on experimental
131 trees at three times that spanned a range of temperatures: 6:30am, 9:30am, and 12:30pm. A pair
132 of goats was assigned to each pair of trees (removal and occupied). Both goats fed from the same
133 tree simultaneously so they experienced the same level of ant aggression. The first tree the pair
134 of goats fed from was randomized by another coin flip. We allowed the goats to feed from the
135 trees for a maximum of five minutes and counted the bites taken and total time spent feeding.
136 Total feeding time was defined as the time from the first bite to the last bite before the goat

137 refused to continue feeding. For each goat, we calculated the difference in total number of bites
138 taken and total feeding time for ant-removal and ant-occupied trees, and used this difference for
139 our analyses.

140 *Statistical Analyses*

141 *Behavioral observations* - To assess for temperature dependence in baseline ant activity
142 and response to a simulated herbivore, we performed a generalized linear mixed model (GLMM)
143 with a negative binomial distribution due to over-dispersion. We constructed models using
144 temperature, species, and the interaction of the two as fixed effects. Each model included the
145 individual tree identity and time block as random effects to account for repeated measures and
146 variation between individual trees. The models were compared using Akaike Information
147 Criterion (AICc) scores. We designated the best model as the one with the lowest AICc that did
148 not produce a convergence error.

149 *Goat experiment* – We used a linear mixed-model to assess the effect of temperature on
150 *C. mimosae*'s efficacy in deterring goat herbivory. We modeled the difference in bites taken and
151 time spent feeding between the two treatments (removal tree-occupied tree) with temperature as
152 the only fixed effect; the random effects were tree identity and goat identity, nested within tree
153 identity.

154 All statistical analyses were conducted in R 3.4.2 (R Core Team 2017). We used the lme4
155 package (Bates et al. 2015) to perform the generalized linear mixed models, the lmerTest
156 package to calculate p values for parameter estimates (Kuznetsova et al. 2017), and the bbmle
157 packaged was used to calculate and compare AICc scores (Bolker 2017).

158

159 **Results**

160 *Surface temperature and behavioral observations*

161 Over the course of the day, surface temperatures ranged from 9°C to 49°C, displaying a
162 strong parabolic relationship with time (Figure 1). The average surface temperature was highest
163 during the 10:31-12:30 period. The variance in surface temperature between trees increased with
164 time, peaking at the 12:30 to 14:30 time period, and declining thereafter. Scattered cloud cover
165 during the middle of the day appeared to cause the variance in surface temperature during the
166 middle of the day. For baseline activity, our model with the lowest AICc included species,
167 temperature but not an interaction term (Figure 2). Temperature was positively associated with
168 baseline activity but not significantly so (Temperature: $b = 0.0067$, $SE = 0.0084$, $p = 0.423$), and
169 the more aggressive ants (*C. mimosae* and *C. nigriceps*) had higher levels of activity compared to
170 *C. sjostedti* (*C. mimosae*: $b = 1.6138$, $SE = 0.197346$, $p \ll 0.01$; *C. nigriceps*: $b = 1.5564$, $SE =$
171 0.1977 , $p \ll 0.01$). *Tetraponera penzigi* had the lowest level of baseline activity measured on the
172 branches ($b = -0.5445$, $SE = 0.2167$, $p = 0.012$). While temperature was not associated with the
173 number of ants moving through the branch segment, the speed of the ants appeared to greatly
174 increase with surface temperature.

175 In examining the relationship between defensive response and temperature, our model
176 including temperature and species had the lowest AICc (Figure 2). Temperature was positively
177 correlated with the number of ants responding to simulated herbivory (Temperature: $b = 0.0251$,
178 $SE = 0.0068$, $p \ll 0.01$). Again, *C. mimosae* and *C. nigriceps* were more aggressive than *C.*
179 *sjostedti* (*C. mimosae*: $b = 3.1151$, $SE = 0.2088$, $p \ll 0.01$; *C. nigriceps*: $b = 2.7252$, $SE =$
180 0.2094 , $p \ll 0.01$), but *T. penzigi* was also a more aggressive defender ($b = 0.9894$, $SE = 0.2133$,
181 $p \ll 0.01$). Across all temperature, *C. mimosae* and *C. nigriceps* released an acrid odor when

182 responding to the disturbance. At the lowest temperatures, many of the ants fell off of the tree
183 when responding to simulated herbivory.

184 *Defense against the Model Herbivore*

185 Surface temperature was a significant predictor of the time spent feeding and bites taken
186 by goats (Figure 3). The difference in bites that the goats took from the ant-removal trees
187 compared to the ant-occupied tree increased with branch surface temperature ($b = 1.554$, $SE =$
188 0.3489 , $p \ll 0.01$). Similarly, goats spent more time feeding from the removal trees compared to
189 the occupied trees as branch surface temperature increased ($b = 5.320$, $SE = 1.351$, $p = 0.0017$).
190 The time spent feeding and total number of bites that each goat took from ant-removal trees did
191 not significantly differ across different temperatures. When introduced to experimental plants,
192 goats rapidly began feeding during all time intervals each day, suggesting that did not learn
193 avoidance of ant-occupied trees, consistent with an earlier experimental study of goat browsing
194 on *A. drepanolobium* by Stapely (1998). As the goats began feeding from the occupied trees, the
195 ants crawled onto the goat's head and began biting near the eyes and nose. The goats responding
196 by scratching at their face and snorting violently. These behaviors were not observed while the
197 goat fed from the removal trees.

198 **Discussion**

199 Our results demonstrate that surface temperature plays a key role in mediating the
200 intensity of the ant defenders to both simulated herbivory and a model herbivore increases with
201 branch surface temperature. The increase in defensive activity was observed across the four ant
202 species, but the differences in defensive intensity between the species was accentuated by
203 increasing temperature as the more aggressive ant species (*C. mimosae* and *C. nigriceps*)
204 increased their swarming response to a greater extent. Within *C. mimosae*, we found that

205 increased surface temperature produced a functional defensive response, as goats became
206 increasingly hesitant to feed from ant-occupied trees than ant-removal trees with increasing
207 temperature.

208 When the thermal niche of the ant defenders does not align with that of the herbivores,
209 host plants are at increased risk of damage (Fitzpatrick et al. 2013, 2014). In sub-Saharan Africa,
210 many browsers, including those that feed on *A. drepanolobium*, preferentially feed during cooler
211 periods of the day such as dawn and dusk (i.e. steenbok, impala, greater kudu, giraffe, Du toit
212 and Yetman 2005; african elephant, Shannon et al. 2008), the same periods with lower levels of
213 ant activity. Unlike the barrel cactus system, the four *A. drepanolobium* mutualist are obligate
214 mutualist rather than facultative (Stanton et al. 2002), where the survival of the colony closely
215 intertwines with the fitness of their host (Agrawal and Rutter 1998).

216 Thus, the discordance between the temperature preferences of the ants and vertebrate
217 herbivores is surprising, though several hypotheses may explain our observations. First, goats
218 may serve as poor model browsers in a landscape dominated by mega-herbivores (Goheen and
219 Palmer 2010). They have not evolved alongside the myrmecophyte and may not respond to the
220 visual and chemical cues of the ants (Wood et al. 2002 and 2006). Ant pheromones have been
221 shown to repel insect herbivores (reviewed in Offenberg 2014), and Goheen and Palmer (2010)
222 have suggested that the smell of the aggressive acacia ants is enough to deter elephants. Goat
223 feeding also varies markedly from elephant or giraffe feeding and may not elicit the proper
224 response from the ants early in the morning. For instance, we observed the goats pick at
225 individual leaflets between the thorns. Conversely, elephants destroy large sections of the canopy
226 or knock the tree over entirely (Stanton and Palmer 2011) and giraffes strip entire branches of the
227 leaves, thorns, and bark (Milewski et al. 1991). By engaging larger areas of the tree, the larger

228 herbivores potentially cause more ants may to respond to the disturbance. Second, the seemingly
229 redundant spines could function as the plant's primary defense during times when ant activity is
230 depressed, reducing selective pressure for ants active at lower temperatures. The spines have
231 been shown to reduce or at least slow herbivory in *A. drepanolobium* and other *Acacia species*
232 (Milewski et al. 1991, Stapley 1998), though this effect is countered by longer feeding time.
233 Lastly, the daily pattern of ant activity and our short term observations may not capture the long-
234 term consequences of the mutualism. For example, Stanton and Palmer (2011) have argued that
235 short term experiments will underestimate the benefits of the ant mutualists as the costs of
236 hosting ants are relatively continuous and the benefits are sporadic. Here, we examined only a
237 single aspect of the mutualism that contributes to the plant's fitness over a few days, but
238 neglected other aspects such as the costs associated with hosting the ants (i.e. branch pruning,
239 Stanton et al. 1999; nectar induction and tending scale insects, Prior et al. 2018; but see
240 Bronstein 2001). The plants may tolerate the cyclical pattern of activity because it also reduces
241 the costs associated with the mutualism.

242 As global temperatures continue to rise, scientists continue to predict how climate change
243 will affect the fate of species (Thomas et al. 2004, Keith et al. 2008, Urban 2015) and species
244 interactions (Walther et al. 2002, Wong and Candolin 2015). Unlike other studies (Cerdá et al.
245 1998, Fitzpatrick et al. 2013 and 2014), we did not find that higher temperatures limited ant
246 activity. Thus, a 1 – 3°C rise (Collins et al. 2013) may reinforce the ant-*Acacia* mutualism rather
247 than disrupt it. Higher temperatures, especially during the early periods of the day, increased the
248 quality of defense that the mutualists are supposed to provide and may improve the fitness of the
249 host tree as a result. However, the fitness improvement likely depends on the physiological
250 constraints of the ants and a complicated network of interactions between the different members

251 of the mutualism network (Berg et al. 2010). In order to predict the future of keystone species
252 interactions (such as mutualisms) in the face of climate change, a strong understanding in the
253 role that temperature plays in each aspect of the interactions is vital.

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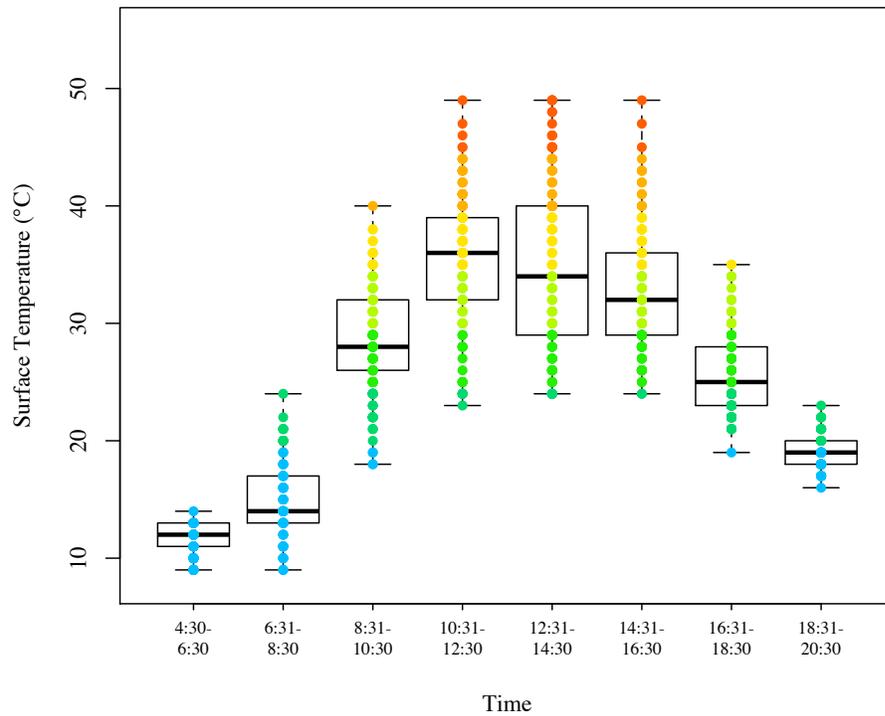
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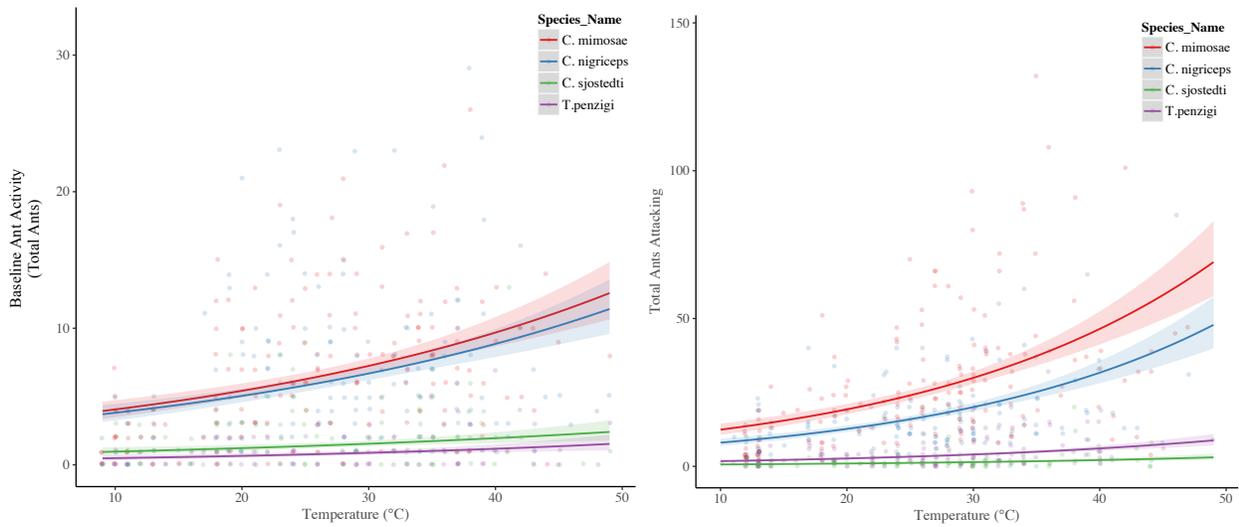
361

362 *Figure 1.* Outer canopy surface temperatures (°C) throughout the day on *A. drepanolobium*

363 occupied by any of the four ant symbionts. Temperatures were measured to the nearest degree,

364 so each point in the figure can represent several observations. Boxplots represent the interquartile

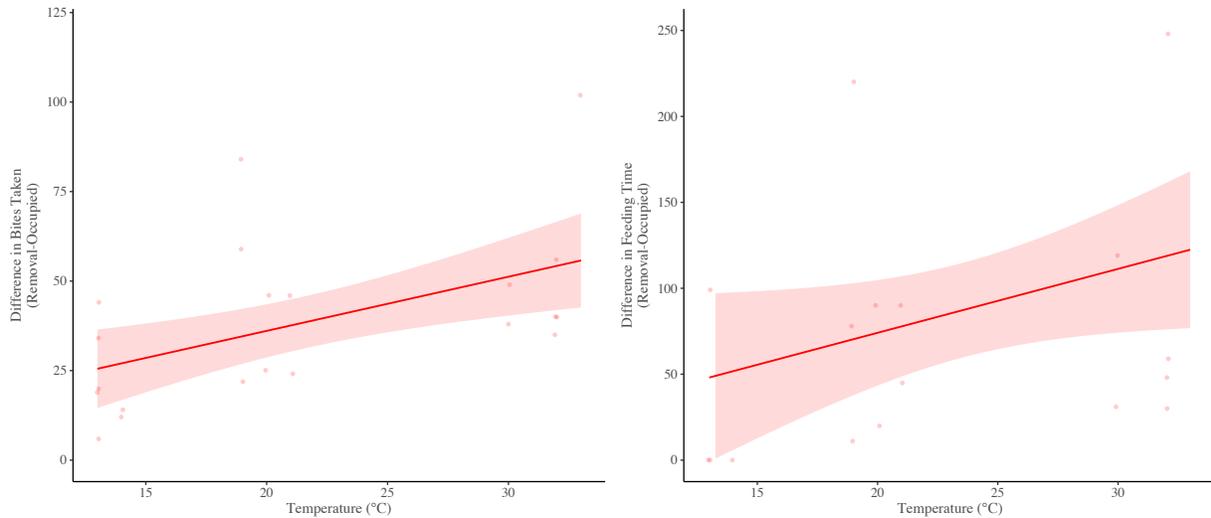
365 ranges of temperature during each measurement period.



366

367 *Figure 2:* Predictions of our best model for the relationship between surface temperature and
 368 baseline activity of an ant colony (left) and defensive response to a simulated herbivore (right).

369 Baseline activity was measured by the number of ants passing in or out of two 5cm segments of a
 370 branch for 30 seconds. The positive relationship between baseline and activity was not
 371 significant once we controlled for colony identity and measurement periods. The defensive
 372 response was measured by the number of ants swarming onto two gloves after the tree was
 373 disturbed. The positive relationship between temperature and defensive activity was significant
 374 while controlling for colony identity and measurement periods.



375

376 *Figure 3.* Predictions for the relationship between surface temperature and goat feeding behavior.

377 Goats fed for up to five minutes from trees with ants (occupied trees) and trees where the ants

378 were removed (removal trees) at three different times. Goats took more bites from removal trees

379 compared to occupied trees as temperature increased (left), measured by the difference of bites

380 taken from removal trees and occupied trees. Goats also spent more time feeding from removal

381 trees compared to occupied trees as temperature increased (right), measured by the the difference

382 in feeding time of removal trees and occupied trees. Feeding time was measured as the time from

383 the first bite to the time when the goat refused to continue feeding. Both relationships were

384 significantly positive when controlling for the individual goat and tree pair.