The predatory mite *Neoseiulus womersleyi* (Acari: Phytoseiidae) follows extracts of trails left by the two-spotted spider mite *Tetranychus urticae* (Acari: Tetranychidae)

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Abstract As it walks, the two-spotted spider mite *Tetranychus urticae* Koch (Acari: Tetranychidae) spins a trail of silk threads, that is followed by the predatory mite, *Neoseiulus womersleyi* Schicha (Acari: Phytoseiidae). Starved adult female *N. womersleyi* followed *T. urticae* trails laid down by five *T. urticae* females but did not follow a trail of one *T. urticae* female, suggesting that the amount of spun threads and their chemical components should correlate positively with the number of *T. urticae* individuals. To examine whether chemical components of *T. urticae* trails are responsible for the predatory mite's trail following, we collected separate *T. urticae* threads from the exuviae and eggs, and then washed the threads with methanol to separate chemical components from physical attributes of the threads. Female *N. womersleyi* did not follow *T. urticae* trails that had been washed with methanol but contained physical residues, but they did follow the direction to which the methanol extracts of the *T. urticae* trails was applied. These results suggest that the predatory mite follows chemical, not physical, attributes of *T. urticae* trails.

Key words *Tetranychus urticae* ▪ *Neoseiulus womersleyi* ▪ Silk thread ▪ Methanol extracts ▪ Trail following

Introduction

Predatory mites are promising biological control agents against tetranychid mites (e.g. McMurtry 1992; Croft and Slone 1997). For effective use of predatory mites as biological control agents, the prey-searching cues of the mites must be elucidated. Although volatiles produced by spider-mite-infested plants are thought to attract predatory mites (Sabelis and Van de Baan 1983; Dicke et al. 1990), recent observations in open environments suggest that spider mite patches on plant leaves do not attract predatory mites at a distance on the plant (Zemek et al. 2008; Yano and Osakabe 2009). On the other hand, Yano and Osakabe (2009) showed that the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae) follows trails left by adult female *Tetranychus urticae*, suggesting that the spider mite trail could be a reliable prey-searching cue for spider patches at a distance.

Tetranychus urticae feeds on various host plant species and is considered an important

48 agricultural pest globally (Jeppson et al. 1975). This mite often exhausts its host plant's resources,

49 and mated adult females then disperse to new hosts, primarily by walking (Margolies and Kennedy

50 1985). Ambulatory dispersing adult female *T. urticae* follow the trails left by preceding females,

51 which results in aggregation at a new colony site (Yano 2008). *Tetranychus urticae* spins a trail of

52 silk threads when walking (Saito 1977). Silk threads spun by *T. urticae* have been reported to retain

53 predatory mites and elicit typical prey-searching behavior (Hislop et al. 1978; Hoy and Smilanick

54 1981; Hislop and Prokopy 1981). Hoy and Smilanick (1981) reported that the predatory mite

55 *Metaseiulus* (=*Typhlodromus*) *occidentalis* Nesbitt (Acari: Phytoseiidae) detects silk and other

56 residuals of *T. urticae* on spider-mite-infested leaves, which can be removed by washing the leaves

57 with water. Although they suggested that water-soluble extracts of deposits are likely to serve as a

58 search cue for the predatory mite, important cues may be physically washed off. Hislop and Prokopy

59 (1981) showed that although the predatory mite *Neoseiulus fallasis* (Garman) (Acari: Phytoseiidae)

preferred methanol extracts of *T. urticae* silk threads placed on filter paper, it showed a stronger preference for intact silk. They concluded that *N. fallasis* may detect the chemical components of *T. urticae* silk, although the physical stimulus of silk threads may also be involved.

However, these studies reported on the retention of predatory mites within a spider mite colony, not on how they were guided toward the colony. Moreover, extracts of *T. urticae* silk contain residuals, such as feces, exuviae, and even eggs, the latter of which are food of the above predatory mites. Hence, these extracts may differ substantially from those of spider mite trails outside colonies. To determine if predatory mites use the chemical components of spider mite trails to orient themselves toward spider-mite-infested leaves, residual deposits that are apparently not associated with trails outside the colony must be excluded.

In this study, we examined the prey-searching behavior of the endemic predatory mite *Neoseiulus womersleyi*, which is an important predator of tetranychid mites in Japan (Hamamura

72 1986; Ehara and Shinkaji 1996). We collected *T. urticae* silk threads separately from their residuals.

73 Using extracts of the collected threads, we examined the hypothesis that *N. womersleyi* can detect

74 chemical components of *T. urticae* trails.

75

76 **Materials and methods**

77 Mites

78 The *T. urticae* study population was collected from a rose garden in Kyoto, Japan, and maintained on

79 individual discs of kidney bean *Phaseolus vulgaris* L. (Leguminosae) leaves pressed onto

80 water-saturated cotton in Petri dishes (90-mm diameter, 14 mm deep). The *N. womersleyi* study

81 population was also collected in Kyoto and was maintained on kidney bean leaf discs that were

82 heavily infested with *T. urticae* as prey. The leaf discs were maintained at 25 ± 2°C with 50 ± 5 %

83 relative humidity and a photoperiod of 16 : 8, light: dark (hereafter described as “laboratory

84 conditions").

85

86 Preference of *N. womersleyi* for *T. urticae* trails

87 We first examined whether female *N. womersleyi* could follow trails laid down *by T. urticae*. The

88 term "trail", as used in this study, refers to the silk threads and/or other chemical compound(s)

89 deposited by mated adult female *T. urticae* (hereafter described as "female *T. urticae*"). To conduct

90 dual-choice experiments under laboratory conditions, we connected one Parafilm square (Parafilm

91 M, American National Can Group, Chicago, IL, USA) and two bean leaf squares (10 × 10mm) with a

92 T-shaped Parafilm pathway on water-saturated cotton in Petri dishes (90-mm diameter, 14mm deep).

93 To induce a spider mite trail, we blocked a randomly selected branch with a piece of wet filter paper,

94 and then introduced either one or five 2– to 4-day-old female *T. urticae* onto the Parafilm square

95 (Fig.1a). One to five adult female *T. urticae* correspond to a typical colony size of the mite in the

96 wild (Yano, unpublished results). After 1h, when all of the *T. urticae* females had moved to the

97 available bean leaf square, we removed the wet filter paper and two bean squares together with the *T.*

98 *urticae* females from each disc, leaving only a trail on the Parafilm (Fig.1b).

99 We used 1-day-old mated adult female *N. womersleyi* (hereafter described as "female *N.*

100 *womersleyi*") that had been previously starved since late deutonymph period. To prepare these

101 females, we isolated an old deutonymph female and an adult male *N. womersleyi* in a 1.5-ml

102 microtube (Treff AG, Degersheim, Switzerland) with a water droplet. We had previously confirmed

103 that old deutonymph females mature without feeding and that these females most intensively follow

104 spider mite trails (Yano, unpublished results). To avoid cannibalism and unsuccessful mating, we

105 used mature females only when both the female and male were alive after 48h. We introduced

106 individual female mites to the bottom of the T-shaped pathway using a fine brush (Fig.1b) and

107 recorded which branch they followed to the far end. We used each female *N. womersleyi* and

T-shaped Parafilm path only once. The female *N. womersleyi* that did not reach either end within 5min were not included in the analysis. The number of replicates was 25 for trails made by five females and 30 for trails made by one female. Experimental outcomes were compared using the binomial tests (Sokal and Rohlf, 1995), with the common null hypothesis that a female *N. womersleyi* would chose either of the two branches with equal probability (i.e. 0.5).

Preference of *N. womersleyi* for *T. urticae* trails washed with methanol

To conduct dual-choice experiments, trails made by five *T. urticae* females were induced on a Parafilm pathway in the manner described above. We then transferred the Parafilm to a Petri dish filled with methanol (Wako Pure Chemical Industries Ltd, Osaka, Japan, min. 99.5%) and gently shook the dish for 5min. The Parafilm was then completely dried at room temperature and placed on water-saturated cotton in another Petri dish (Fig.1b). To confirm whether threads were still present

120 on Parafilm pathways after they were washed with methanol, we observed threads on the surfaces of

121 randomly sampled Parafilm pathways using a scanning electron microscope (3D Real Surface View

122 Microscope VE-8800, Keyence, Osaka, Japan).

123 We then introduced a starved 1-day-old female *N. womersleyi* to the base point of the T-shaped

124 Parafilm in the manner described above (Fig.1b) and recorded the branch that the female first

125 followed to the far end. Each female *N. womersleyi* female and T-shaped Parafilm pathway was used

126 only once. Female *N. womersleyi* that did not reach an end point within 5min were not included in

127 the analysis. The number of replicates was 59. The numbers of females were compared using a

128 binomial test in the same manner described above.

129

130 Preference of *N. womersleyi* for *T. urticae* trail extracts

131 To collect *T. urticae* silk threads separately from residuals such as exuviae and eggs, we confined 10

132 *T. urticae* females within 6h of maturation in each of ten 1.5-ml microtubes (i.e. 100 females in total)

133 with a water droplet. We had previously confirmed that *T. urticae* females within 6h of maturation do

134 not oviposit when deprived of food.

135 To allow the females to deposit silk threads inside the microtubes, the tubes were kept under

136 laboratory conditions for 48h. Each tube was then opened onto a bean leaf disc to release the females.

137 After 30min, when all the females had exited, 80μl methanol was added to each tube and the tubes

138 were shaken gently for 20min using a constant temperature shaker (Synthetech Oven SO-1G, Nippon

139 Genetics Co., Tokyo, Japan) at 30°C. Extracts from the 10 tubes (c. 800μl total) were consolidated

140 into another tube and centrifuged at 12,000 r.p.m. for 5min. To exclude residual deposits, the top

141 clear layer in the tube was collected into another tube. To evaporate the methanol, the tube was

142 opened and placed in the shaker at 30°C for 60min. The final volume of the concentrated extracts was

143 c.400μl. For the control solvent, the same amount of methanol was poured into new microtubes,

144 which were treated in the manner described above.

145 To conduct the dual-choice experiments, we constructed T-shaped pathways of filter paper

146 (35 × 35mm, 2mm wide, Fig.2). Methanol extract of about 40 spider mite's trails was applied to the

147 trunk and a randomly selected branch of each constructed pathway, with the control methanol applied

148 to the other branch. The two solutions were applied uniformly to both sides of the filter paper. Since

149 a starved *N. womersleyi* female is 0.25mm wide (1/8 of 2mm wide pathway) at most, *N. womersleyi*

150 advancing along the pathway would not contact more than 1/8 of the applied extracts. Since 1/8 of 40

151 *T. urticae* females is 5 females, this corresponds to the number of spider mites used in the first

152 experiment. Using 10μl micropipettes (Calibrated Pipets, Drummond Scientific Co., PA, USA), we

153 applied each solution little by little at the junction point to minimize mixing. The pathways were

154 completely dried at room temperature and then placed on three 15 x 15mm cardboard pieces fixed to

155 a Petri dish (Fig.2). We then introduced a starved 1-day-old female *N. womersleyi* to the base of the

156 T-shaped filter paper and recorded the branch that the females first followed to the far end. Females

157 that walked on the backside of the filter paper were observed via a 100 × 100-mm mirror placed

158 under the Petri dish (Fig.2). Each female *N. womersleyi* and T-shaped filter paper was used only once.

159 Female *N. womersleyi* that did not reach an end point within 5min were not included in the analysis.

160 The number of replicates was 54. The numbers of females were compared using a binomial test in the

161 same manner described above.

162

163 **Results**

164 Preference of *N. womersleyi* for *T. urticae* trails

165 Female *N. womersleyi* followed trails created by five female *T. urticae* (trail : control, 19 : 6;

166 *P*=0.0073, binomial test, Fig. 3a), but did not follow the trail made by one female *T. urticae* (trail :

167 control, 19 : 11; *P* = 0.100, binomial test, Fig. 3b). The latter statistically insignificant result,

however, may in part be due to the low number of replicates.

Preference of *N. womersleyi* for *T. urticae* trails washed with methanol

Although we found *T. urticae* threads on the Parafilm surfaces after they were washed with methanol, the female *N. womersleyi* showed no preference for washed *T. urticae* trails (trail : control, 29 : 30; $P = 0.60$, binomial test, Fig. 3c).

Preference of *N. womersleyi* for *T. urticae* trail extracts

Neoseiulus womersleyi females preferred trail branches with the methanol extract of *T. urticae* trails to those with only methanol (trail extracts : methanol, 35 : 19; $P = 0.020$, binomial test, Fig. 3d), suggesting that chemical components of *T. urticae* trails are used as a prey-searching cue. In addition, we observed that some female *N. womersleyi* slowed down on the filter paper when they encountered

180 the trail extract. We also observed that some turned back from a trail-extract branch and retraced

181 their steps to the other, methanol-only branch. These behaviors were not observed during the

182 previous experiment that presented female *N. womersleyi* with *T. urticae* trails washed with

183 methanol.

184

185 **Discussion**

186 Not surprisingly, starved female *N. womersleyi* followed *T. urticae* trails in a density-dependent

187 fashion, as the amount of spun threads and their chemical components should correlate positively

188 with the number of *T. urticae* individuals used. Conspecific adult females (Yano 2008) and the

189 predatory mite *Phytoseiulus persimilis* (Yano and Osakabe 2009) also follow *T. urticae* trails in a

190 density-dependent fashion.

191 Because *T. urticae* spins silk threads while walking (Saito 1977), large quantities of threads can

192 be collected from long-settled mite colonies. However, residual deposits, such as exuviae, feces,

193 carcasses, and all stages of living mites, cannot be excluded from these samples. Moreover, as the

194 attractiveness of *T. urticae* threads appears to decline with time (Yano 2008), the properties of fresh

195 threads may differ from those collected at spider-mite colonies, which contain old threads. Therefore,

196 we examined *T. urticae* threads produced within 48 h, although some feces may have been included

197 in the extract.

198 The predatory mite did not follow *T. urticae* trails that had been washed with methanol, whereas

199 they did follow extracts of *T. urticae* trails. These results suggest that chemical, not physical

200 attributes of *T. urticae* trails are responsible for trail following by *N. womersleyi,* although the

201 washing treatment may have altered the physical properties of *T. urticae* threads. It is possible that

202 substances not soluble in methanol may be also responsible for the predatory mite's trail following.

203 However, *N. womersleyi* females did not follow washed *T. urticae* trails where substances not soluble

204 in methanol should remain, suggesting that such substances are unimportant. Some *N. womersleyi*

205 females slowed down and exhibited typical prey-searching behavior (Hoy and Smilanick 1981) on

206 filter paper painted with extracts of *T. urticae* trails, but no predator engaged in this behavior on

207 trails washed with methanol. These observations further suggest that the predatory mite responds

208 positively to chemical components in the *T. urticae* trails as a prey-searching cue. Therefore,

209 predatory mites most likely use the chemical cues of *T. urticae* trails to find prey colonies, and then

210 use both physical and chemical trail cues after arriving at the colonies, as suggested by Hislop and

211 Prokopy (1981).

212 To efficiently attract and retain predatory mites of tetranychid mite colonies, the chemical

213 components responsible for trail following by predatory mites must be clarified. Methods to collect

214 efficiently the unadulterated spider-mite threads should also be improved.

215

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271

272 **Figure Legends**

273 **Fig.1** Experimental designs for testing the preference of *N. womersleyi* females for *T. urticae* trails

274 and trails washed with methanol. a) To guide *T. urticae* females in one direction, a piece of wet filter

275 paper blocked the other path branch; b) A 1-day-old female *N. womersleyi* was placed on the base

276 point of the T-shaped Parafilm. We recorded the branch that the female first followed to the far end.

277

278 **Fig.2** Experimental design for testing the preference of *N. womersleyi* for a methanol extract of *T.*

279 *urticae* trails. The methanol trail extract was applied to the trunk of a filter-paper path and a

280 randomly selected branch; pure methanol was applied as a control to the other branch.

281

282 **Fig.3** Summary of the dual-choice experiments. Female *N. womersleyi* followed *T. urticae* trails laid

283 down by a) five females but did not follow a trail of b) one *T. urticae* female. Moreover, female *N.*

womersleyi c) did not follow *T. urticae* trails that had been washed with methanol but d) did follow the methanol extracts of the *T. urticae* trails.

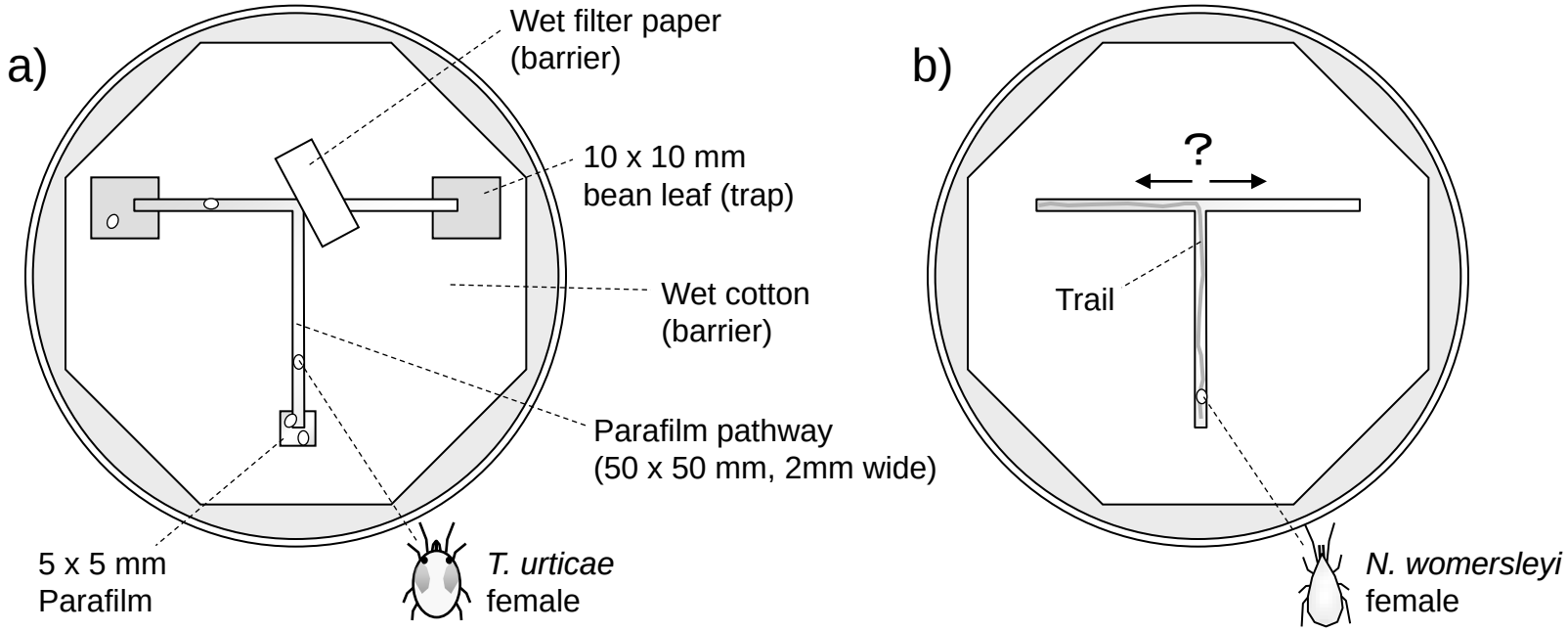
a)
Wet filter paper
(barrier)
10 x 10 mm
bean leaf (trap)
Wet cotton
(barrier)
Parafilm pathway
(50 x 50 mm, 2mm wide)
5 x 5 mm
Parafilm
T. urticae
female
b)
?
Trail
N. womersleyi
female

Fig. 1

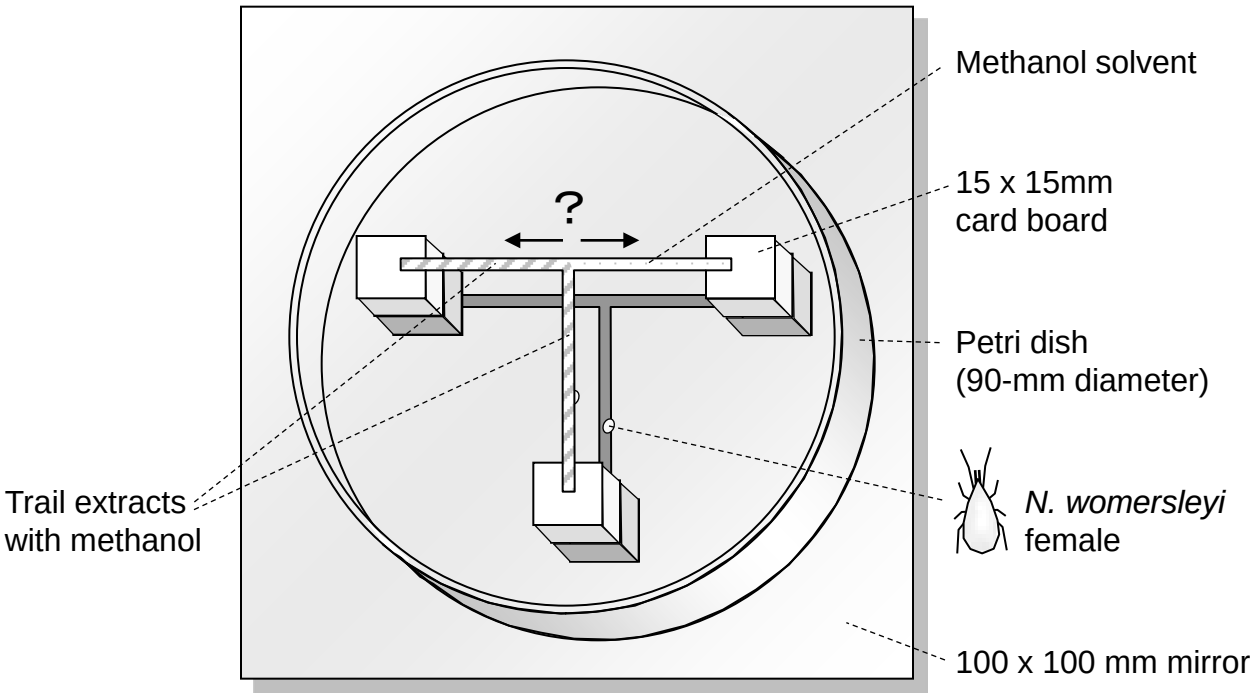
?
Methanol solvent
15 x 15mm
card board
Petri dish
(90-mm diameter)
Trail extracts
with methanol
N. womersleyi
female
100 x 100 mm mirror

Fig. 2

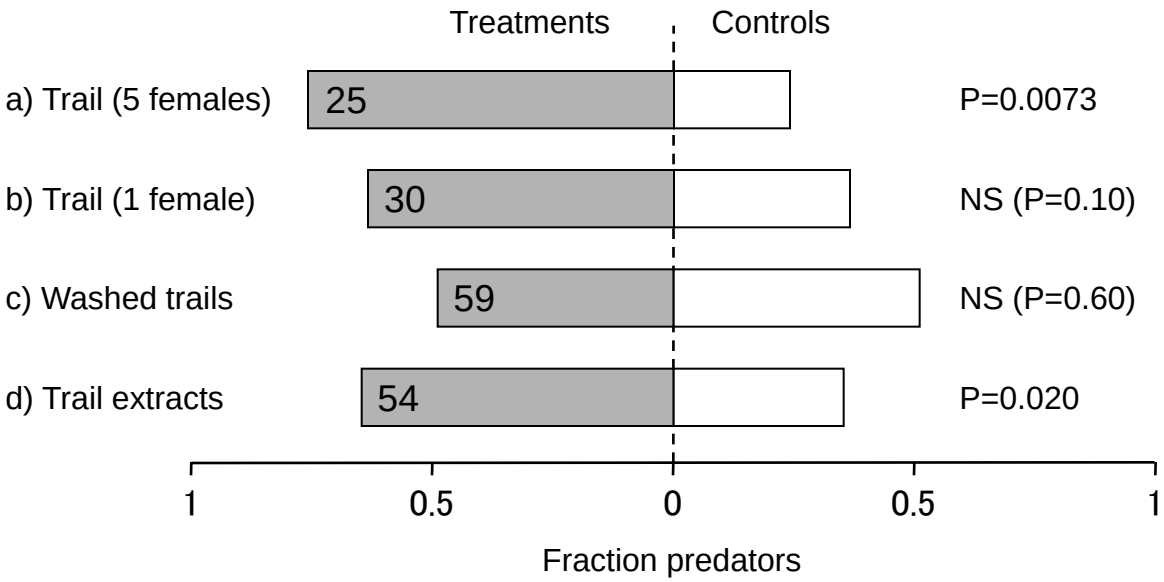
Treatments
Controls
a) Trail (5 females)
25
P=0.0073
b) Trail (1 female)
30
NS (P=0.10)
c) Washed trails
59
NS (P=0.60)
d) Trail extracts
54
P=0.020
1
0.5
0
0.5
1
Fraction predators

Fig. 3