

4-21-2015

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Recommended Citation

Davis, Jeremy M.; Coogan, Laura E.; and Papaj, Daniel R., "Big Maggots Dig Deeper: Size-Dependent Larval Dispersal in Flies" (2015). *SIAS Faculty Publications*. 554.

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1 Big maggots dig deeper: Size dependent larval dispersal in flies

2

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4

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10

11 Running title: Size-dependent larval dispersal

12 Keywords: Condition-dependent dispersal, overwintering site selection, silver spoon

13 effect, Tephritidae, *Rhagoletis*

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21 Author Contributions: JD conceived of the experiments and analyzed the data. JD
22 and DP designed the experiments. JD and LC performed the experiments and
23 wrote the manuscript and DP provided editorial advice.

24

25 Summary

26

27 The ability of individual animals to select habitats optimal for development and survival
28 can be constrained by the costs of moving through the environment. Animals that seek
29 overwintering sites underground, for example, may be constrained by the energy required
30 to burrow into the soil. We conducted field and laboratory studies to determine the
31 relationship between individual size and overwintering site selection in the tephritid flies,
32 *Rhagoletis juglandis* and *R. suavis*. We also explored the effect of site selection on pupal
33 mortality, parasitism, and the ability to emerge from overwintering sites after eclosion.
34 In both species, and in both lab and field tests, larger pupae were found at deeper soil
35 depths. In addition, marginally non-significant trends indicated pupae in deeper sites
36 were 48% more likely to survive the overwintering period. Finally, larger individuals
37 were more likely to eclose and emerge from the soil at a given depth, but flies in deep
38 overwintering sites were less likely to emerge from those sites than flies in shallow sites.
39 Our data indicates that overwintering site selection represents a trade-off between
40 avoiding predators and parasites that occur at shallow sites, and the energetic and
41 mortality costs of burrowing to, overwintering in, and emerging from, deeper sites.
42 The size-dependent overwintering site selection demonstrated here has implications for
43 population dynamics and pest control strategies. Some fly control measures, such as the
44 introduction of parasites or predators, will be mitigated when the deepest and least
45 accessible overwintering pupae represent a disproportionately large amount of the
46 population's reproductive capacity.

47 The dispersal and habitat choices of animals are expected to be under strong
48 selection since they affect the microclimate, food, mates, predators, and parasites
49 that individuals encounter post-settlement. However, several factors can maintain
50 phenotypic variation in habitat choice, even under conditions where a single habitat
51 type is most suitable for all individuals (Bolnick et al. 2003). For example,
52 phenotypically variable traits, such as body size, can influence individuals' capability
53 to search for, assess, and/or defend optimal habitats, forcing some subset of
54 individuals to use suboptimal habitats (Stamps 2006, Davis 2007, Benard and
55 McCauley 2008).

56 In holometabolous insects, such as flies, there are typically two types of dispersal
57 that end in habitat choice. After natal dispersal, adult females must choose where to
58 oviposit; a decision that frequently determines the habitat in which larvae will live until
59 just prior to pupation. Then, after feeding, larval offspring must choose where to pupate
60 and, in many cases, overwinter. Although oviposition decisions have been well studied
61 in many species, larval overwintering site selection is relatively under-studied.

62 For insects that pupate and overwinter in the soil, deeper sites provide at least two
63 potential advantages: protection from soil predators, and moderate temperatures. Both
64 ant predation and wasp parasitism on tephritid fly pupae have been shown to be more
65 prevalent near the soil surface (ant predation: Aluja et al. 2005; *Coptera* wasp parasitism:
66 Baeza-Larios et al. 2002; Guillén et al. 2002). Indeed, larvae of the Mexican fruit fly
67 *Anastrepha ludens*, respond to ant attacks by burrowing deeper into the soil (Aluja et al.
68 2005).

69 Winter temperatures are higher and less variable deeper in the soil than they are
70 near the surface (Leather et al. 1993). These patterns can be either advantageous or
71 disadvantageous to pupae. On the one hand, temperature variation, sub-freezing
72 temperatures or heat stress can kill some overwintering insects (Leather et al. 1993) or
73 delay diapause (Teixeira and Polavarapu 2005). On the other hand, the colder
74 temperatures near the soil surface can reduce the metabolic rates of pupae, conserving
75 limited energy reserves until spring emergence (Irwin and Lee 2003). Moreover, digging
76 too deeply may isolate pupae from the cold temperatures needed to break diapause, and
77 may make it difficult for teneral adults to reach the soil surface after emergence (Leather
78 et al. 1993).

79 The energetic expenditure required for locomotion in fly larvae is among the
80 highest recorded for terrestrial locomotion (Berrigan and Lighton 1993). Thus, while
81 there are advantages to pupating deep in the soil, the metabolic costs associated with
82 digging may prevent some individuals from reaching deeper pupation sites. Among
83 legless burrowing invertebrates, larger individuals have larger energy stores, can crawl
84 faster (Berrigan and Pepin 1995), use less energy per unit mass (Chown and Nicholson
85 2004), and apply more force (Quillin 2000). These factors should allow larger larvae to
86 dig deeper and maintain a higher overwintering metabolic rate. We therefore predict that
87 larger larvae will choose deeper pupation sites than small larvae. This pattern has
88 previously been observed in the overwintering larvae of the Colorado potato beetle
89 (*Leptinotarsa decemlineata*) (Noronha and Cloutier 1998).

90 In this study, we used field collections and laboratory experiments to test for a
91 relationship between body size and the depth at which larvae of two members of the

92 *Rhagoletis suavis* species group pupate. We also assessed survival and parasitism rates in
93 pupae collected from different soil depths. Finally, we tested whether body size
94 influenced the ability of flies to emerge from their pupation site after eclosion. We
95 predicted that larger individuals would be able to reach and later emerge from deeper
96 overwintering sites, potentially providing them fitness advantages over smaller
97 individuals.

98 MATERIALS AND METHODS

99 Species Descriptions

100 *Rhagoletis juglandis* (Cresson) and *Rhagoletis suavis* (Loew) are sister species
101 within the *Rhagoletis suavis* species group, a group of tephritid fly species that are larval
102 specialists on the husks of walnut fruits. Within population size variation is pronounced
103 in this group, making it ideal for studies on the consequences of size. *R. suavis* primarily
104 utilizes the husk of the black walnut (*Juglans nigra*) throughout the eastern United States,
105 while *R. juglandis* specializes on the Arizona walnut (*J. major*) in the southwestern
106 United States and Mexico (Bush 1966). Both species are univoltine, and adult flies
107 emerge from soil beneath their natal tree in the midsummer prior to the ripening of
108 walnut husks. In *R. juglandis*, larval survival is higher and final size is larger when
109 larvae develop in larger, less infested, walnuts (Nufio and Papaj 2000; Nufio et al. 2004).
110 Development is usually completed after the fruit fall to the ground, with larvae burrowing
111 into the soil prior to pupation. After pupating in the soil, pupae may be parasitized by
112 wasps in the genus *Coptera*, and predated upon by ants (Buckingham 1975, *pers obs*).

113

114 Field Collections (*R. juglandis* and *R. suavis*)

115 Pupae of *R. juglandis* and *R. suavis* were collected from the soil beneath walnut
116 trees to determine if large pupae were found at deeper depths than small pupae. We
117 categorized soil type using the USDA web soil survey tool
118 (<http://websoilsurvey.nrcs.usda.gov>). *R. juglandis* pupae were collected underneath trees
119 at 5 sites in southern Arizona (Ash Creek (loamy sand): 32° 3'42.45"N 110°22'3.21"W,
120 Adobe Canyon (sandy loam): 31°38'34.81"N 110°43'5.00"W, Hog Canyon (sandy
121 loam): 31°39'16.16"N 110°42'45.57"W, Ft. Huachuca (gravelly fine sandy loam):
122 31°32'38.08"N 110°19'51.72"W, Garden Canyon (sandy loam): 31°28'54.50"N
123 110°20'1.08"W) in the spring of 2008. We used a hand trowel to collect soil from 10 cm
124 diameter holes in three increments, 5 cm in depth. We sampled between 5 and 20 holes
125 underneath each tree.

126 *R. suavis* pupae were collected in late spring and early summer of 2009. We
127 collected 22 samples from under 5 trees on Vassar College Farm (silty clay loam)
128 (41°39'49.8"N 73°54'08.3"W). We used a PVC corer 10 cm in diameter and 20 cm deep
129 to collect 15 cm soil cores. Cores were cut into 5 cm sections in the laboratory and then
130 examined for pupae.

131

132 Parasitism and Overwintering Survival (*R. suavis* only)

133 After measurement (see below), *R. suavis* pupae collected in the field during the
134 spring and summer of 2009 were held individually in 96 well plate cells and maintained
135 at room temperature (approximately 23°C) as adults emerged. In the late fall, we
136 checked the status of each pupa. Pupae were found in one of 4 states: A) Adult fly
137 emerged, B) pupa white and moist (alive but not metamorphosed), pupa brown and/or dry

138 (dead), D) pupa killed by a *Coptera* wasp found in the puparium or having emerged
139 (parasitized).

140 We tested whether survival (A and B vs. C and D) and parasitism (D vs. A, B, or
141 C) were related to size and depth using a generalized linear mixed model with a binomial
142 logistic link function (SPSS v20). Depth was considered a fixed factor, size was a
143 covariate, and soil core was included as random nuisance variable.

144

145 Laboratory Burrowing Experiment (*R. juglandis* and *R. suavis*)

146 In order to test for a relationship between larval size on pupation site in a
147 laboratory environment in which confounding variables present in the wild, such as size-
148 dependent predation and temperature variation (see Gomes, *et al* 2009), were controlled,
149 we set fly-infested walnuts atop columns of sand, allowing migrating larvae to burrow
150 prior to pupation. The experiment was conducted on *R. juglandis* in the summer of
151 2007. We collected 42 uninfested *J. major* fruit from Patagonia, AZ (31°32'23.43"N,
152 110°45'16.77"W) and allowed a single mated female to lay a clutch of eggs in each fruit.
153 Fruit were then placed atop a 15 cm column of sand held in PVC pipe (5 cm diameter, 20
154 cm length), with slits cut halfway through the tubes at five and ten centimeters from the
155 bottom. These slits were covered with duct tape to prevent sand and migrating larvae
156 from escaping.

157 For *R. suavis*, 29 infested *J. nigra* fruit were collected from underneath trees at
158 Bowdoin State Park (41°36'04N; 73°57'35W) in Poughkeepsie, NY in the late summer of
159 2008. Walnuts were set atop 20 cm columns of sand similar to those described for *R.*

160 *juglandis*, except that 10 cm diameter PVC pipes were used to accommodate the larger
161 black walnut fruit.

162 In both experiments, after four weeks, cardboard inserts were placed into the slits
163 in each tube and the pupae in each section were counted and removed for measurement.
164 Walnut husks were also dissected and all pupae were collected.

165

166 Pupal Size Measurement and Analysis (*R. juglandis* and *R. suavis*)

167 In the laboratory, digital images were obtained of each individual puparium
168 positioned on its ventral side next to a stage micrometer under stereoscopy. With ImageJ
169 software, (NIH) we estimated pupal size using the maximum transverse sectional area of
170 the puparia,.

171 We tested for a relationship between larval/pupal size and the depth category at
172 which pupae were collected using generalized linear mixed models with size as covariate
173 and with soil core (for field samples) or tube (for laboratory experiments) as random
174 nuisance variables. Because field sampling yielded unbalanced data, we used
175 Satterthwaite approximation when calculating degrees of freedom. For field-collected *R.*
176 *suavis*, only 1 pupa was found deeper than 10 cm, so we reduced the data to two depth
177 categories (shallow (0-5 cm) and deep (>5 cm)) and used a binary logistic link function in
178 our GLMM. In the remaining 3 experiments, pupae were found at all depths collected,
179 but found most often at shallower sites, so we used a GLMM with a multinomial
180 distribution and a cumulative negative log-log link (SPSS v20). In laboratory
181 experiments, some pupae remained in walnut husks. This was considered the shallowest
182 depth category for these experiments.

183

184 Effect of Size on Emergence from Soil (*R. juglandis* only)

185 In this experiment, we tested the effect of imago size and soil depth on the ability
186 of walnut fly imagoes to dig up through the soil and emerge from their overwintering
187 sites. *R. juglandis* were collected as larvae inhabiting fruit that had fallen from *J. major*
188 trees in southern Arizona. Infested fruit were placed in bins with holes drilled in the
189 bottom, and those bins were placed above bins containing moist sand. Migrating larvae
190 entered the lower bin to pupate in the sand. After pupation, flies were kept at 4°C for at
191 least 9 months and warmed to room temperature 4-6 weeks prior to each experiment. We
192 sorted pupae into tertiles based on size and used large (Mean Size: 5.6 mm² transverse
193 sectional area, SD: 1.0) and small (Mean Size: 2.9 mm², SD: 0.8) pupae in this
194 experiment.

195 Eclosion from pupae and emergence from pupation sites were tested in 72 glass
196 tubes 1 cm in diameter and 15 cm in height. Four pupae and 15 mL of dry play sand
197 were arranged in each tube such that the pupae were buried in either 15 cm or 1 cm of
198 sand. Tubes were capped with cotton fabric secured with rubber bands. We checked the
199 tubes daily for emergence until no flies were observed for a week. We recorded the sex
200 of all flies that emerged from the sand. We then sifted the pupae, empty puparia and any
201 dead adults from the sand. This experiment was conducted in 3 blocks (288 pupae per
202 block).

203 We tested for effects of pupal size, soil depth, and their interaction on eclosion
204 and emergence using generalized linear mixed models with binomial link functions.
205 Individuals that did not eclose were not included in the analysis of emergence. Size and

206 depth were fixed factors, while block and tube were included as random nuisance
207 variables.

208

209 RESULTS

210 Field Collections

211 *R. juglandis*: The depth at which pupae were found underneath walnut trees was
212 related to pupa size (GLMM: $F_{1,135}=15.35$, $p<0.001$). Larger pupae were relatively more
213 likely to be found deeper in the soil (Figure 1A).

214 *R. suavis*: Larger pupae were found deeper than 5 cm in the soil more often than
215 smaller pupae (GLMM: $F_{1,56}=6.642$, $p=0.016$; Figure 1B).

216 *R. suavis* survival: A larger proportion of *R. suavis* pupae from deeper sites
217 survived the winter (0-5 cm: 75 of 151 survived; >5 cm: 30 of 47 survived). After
218 controlling for size, our model indicated that pupae in deeper sites had a marginally non-
219 significant 48% improved chance of surviving the winter (95% CI: 0.0 – 73.1%; GLMM:
220 $F_{1,189}=3.866$, $p=0.051$). There was also an independent non-significant trend for large
221 individuals to survive overwintering at a higher rate than small individuals (GLMM:
222 $F_{1,189}=3.716$, $p=0.066$). Parasitism rates were too low to warrant statistical analysis, but
223 the numerical trend indicated similar-sized advantage to pupae deeper in the soil (0-5 cm:
224 12 of 151 (8.0%) parasitized; >5 cm: 2 of 47 (4.3%) parasitized).

225

226 Laboratory Burrowing Experiment (*R. juglandis* and *R. suavis*)

227 In both *R. juglandis* and *R. suavis*, large individuals were found deeper in the sand
228 than were small individuals (*juglandis*: GLMM: $F_{1,616}=46.025$, $p<0.001$; *suavis*: GLMM:
229 $F_{1,650}=74.907$, $p<0.001$, Figure 2).

230

231 Effect of size on emergence from soil in *R.juglandis*:

232 Eclosion: There was no main effect of position in the sand column on the
233 probability of eclosion (Bottom: 72% eclosed, Top: 73% eclosed; GLMM, $F_{1,841}=0.51$,
234 $p=0.45$). Large flies were more likely to eclose than small flies (Large: 83% eclosed,
235 Small: 61% eclosed; GLMM, $F_{1,841}=55.83$, $p<0.0001$). There was a small but significant
236 interaction between position and size (GLMM, $F_{1,841}=5.15$, $p=0.024$) in which large
237 individuals were slightly less likely to eclose when placed deep in the sand, while the
238 opposite was true for small individuals (Figure 3A).

239 Emergence: There was an effect both of size and position on the probability that
240 eclosed adults emerged from the column of sand. Large individuals were slightly more
241 likely to emerge than small (Large: 75% emerged, Small: 70% emerged; GLMM,
242 $F_{1,607}=6.66$, $p=0.010$) and all flies were much more likely to emerge when they were
243 placed only 1 cm from the surface (Bottom: 55%, Top: 91%; GLMM, $F_{1,607}=61.93$,
244 $p<0.0001$). There was no interaction between size and position (GLMM, $F_{1,607}=2.01$,
245 $p=0.16$) indicating that large flies do not have any additional advantage emerging from
246 deeper overwintering sites (Figure 3B).

247 Sex: Among flies that emerged from the sand (and were thus easily sexed), large
248 pupae were significantly more likely to be female (Large pupae: 59% female, Small
249 pupae: 47% female; Mantel–Haenzel $\chi^2=4.267$, $df=1$, $p=0.039$).

250

251 DISCUSSION

252 Our results consistently demonstrated (in both species tested and in both
253 laboratory and field contexts) that large fly larvae are found at significantly deeper
254 overwintering sites than small larvae. While patterns in the field could hypothetically
255 derive from size selective predators removing large pupae from shallow soils, this seems
256 unlikely since laboratory patterns were similar. The consistency between species seen in
257 our results is in contrast to the equivocal results of the only previous study on size-
258 dependent larval dispersal in flies of which we are aware (Gomes et al. 2005), in which a
259 weak positive relationship was observed in one calliphorid species, while a weak
260 negative relationship was detected in its congener. The positive relationship between size
261 and dispersal distance we observed in *Rhagoetis* larvae is consistent with patterns
262 observed in intra- and interspecific comparisons in vertebrates and adult insects (Benard
263 and McCauley 2008).

264 The effects of overwintering depth on fitness components are similar to those seen
265 in other fly species in which pupae overwinter in the soil. In *R. suavis*, we found a
266 marginally non-significant trend ($p=0.051$) that indicated that, after controlling for size,
267 pupae from deeper sites in the field were more likely to survive to eclosion. This trend
268 most likely underestimates the survival benefits of deep pupation, since previous studies
269 (Aluja, et al 2005, Judd and Maw 1996) indicate that fly pupae at shallow sites are
270 removed by predators and therefore would be absent from our samples. In our laboratory
271 experiments on *R. juglandis*, we found that while large individuals were more likely to
272 eclose from their pupae, all individuals that were placed deep in artificial overwintering

273 sites suffered higher mortality after eclosion. This negative fitness consequence of deep
274 overwintering has been observed in other fly species (Finch and Skinner, 1980; Renkema,
275 *et al.* 2012).

276 The depth at which larvae stop burrowing is most likely the product a trade off
277 between the energy costs and mortality risks associated with burrowing and extrication
278 and the survival benefits associated with deep sites. Similar trade offs are reported in
279 burrowing bivalves (which also demonstrate size dependent burrow depth; Zaklan and
280 Ydenberg, 1997), and could play a role in adaptive site selection in many burrowing
281 species but are generally under-studied. Based on the available studies, we predict
282 deeper site selection in drier, colder environments, with many predators (Aluja, *et al.*
283 2005; Zaklan and Ydenberg, 1997) but that this will be affected by the density and type
284 of substrate (Renkema *et al.* 2012, Thomson and Gannon, 2013), the need to access
285 surface resources (de Goiej and Luttikhuizen 1998), and the ability to pay energy and
286 damage costs of digging.

287 More research is needed to determine whether size-dependent larval burrowing is
288 an adaptive response to this trade-off in *Rhagoletis*. Stabilizing selection might favor
289 some average size-independent time or energy investment in dispersal based on local
290 ecological conditions, but larger larvae may simply move more quickly or efficiently
291 through the soil. Alternatively, if larger individuals are better able to pay dispersal or
292 post-dispersal costs associated with deep overwintering site, size-dependent dispersal
293 may be an adaptation. For example, large individuals could be investing their larger
294 energy budget in dispersal when deep sites are better protected from temperature
295 extremes and natural enemies. Similarly, after dispersal, larger individuals may be better

296 able to pay the higher metabolic cost of overwintering in warmer temperatures, or the
297 higher energetic cost of emerging from deep sites.

298

299 Implications

300 These results may have implications for both pest control efforts and life history
301 evolution. Many tephritid species are pests of economic important fruit crops. Any
302 practice that aims to control fly populations by targeting overwintering pupae, such as the
303 use of pupal parasitoids (e.g. Sivinski et al. 1998; Baeza-Larios et al. 2002; Guillén et al.
304 2002) or microbial agents (e.g. Yee et al. 2009), will underperform if the agent only
305 reaches pupae at shallow depths. Insect size is tightly correlated with fecundity (Honek
306 1993), survival (Smith 2002, Moraiti 2012), and in this study, sex, so the proportion of a
307 population's potential fecundity that is eliminated by pupal control efforts will be smaller
308 than the proportion of pupae killed by the control practice. Moreover, pupal control
309 efforts that only effectively target individuals buried at shallower depths may select for
310 deeper pupation, and indirectly, for larger size at dispersal.

311 While the fitness effects of overwintering site selection are not entirely clear, the
312 consistent relationship between larval size and burrowing depth means that any selective
313 forces that are experienced during dispersal and overwintering may shape life history
314 traits that determine size at dispersal. For example, increases in winter temperatures in
315 temperate regions may favor a reduced overwintering site depth and, indirectly, a
316 reduction in size at dispersal. Similarly, an increase in soil predators or a reduction in
317 soil moisture may favor deeper burrowing and therefore select for larvae that continue to
318 feed longer in their natal fruit prior to dispersal.

319 Moreover, because larval feeding habitat is restricted to the fruit in which a
320 female oviposits, our results indicate that female oviposition decisions influence the
321 habitats their offspring experience even after they leave the host. Therefore, in
322 environments where deep overwintering is favored (e.g., in the presence of predatory
323 ants), selection for larger larvae will favor females who lay smaller clutches (Nufio et al
324 2000), avoid superparasitism (Nufio and Papaj, 2012), and are selective with regards to
325 host size or type (Yee *et al.* 2011).

326 These results add to a growing literature on the effect of individual phenotype on
327 the ability of animals to find and/or defend habitats (Stamps 2006; Benard and McCauley
328 2008). Moreover, they highlight that the habitat decisions at each stage of life may be
329 dependent not only on decisions made by the preceding life stage, but by previous
330 generations as well.

331

332 ACKNOWLEDGEMENTS

333 We would like to thank Jose Hoyos, Noel Eloriega, Annamarie Pasqualone, Manasi
334 Jiwrajka and Joe Rozek for their assistance with data collection. *R. juglandis* research
335 was supported by National Institutes of Health (GM00708) to the Center for Insect
336 Science, University of Arizona.

337

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438

439 Figure 1. Relationship between size of A) *R. juglandis* and B) *R. suavis* pupae and the
440 depth of their overwintering site. Each diamond represents a single pupa collected from
441 the soil under walnut trees. Black circles represent the mean pupal size (area of
442 transverse section) at each soil depth.

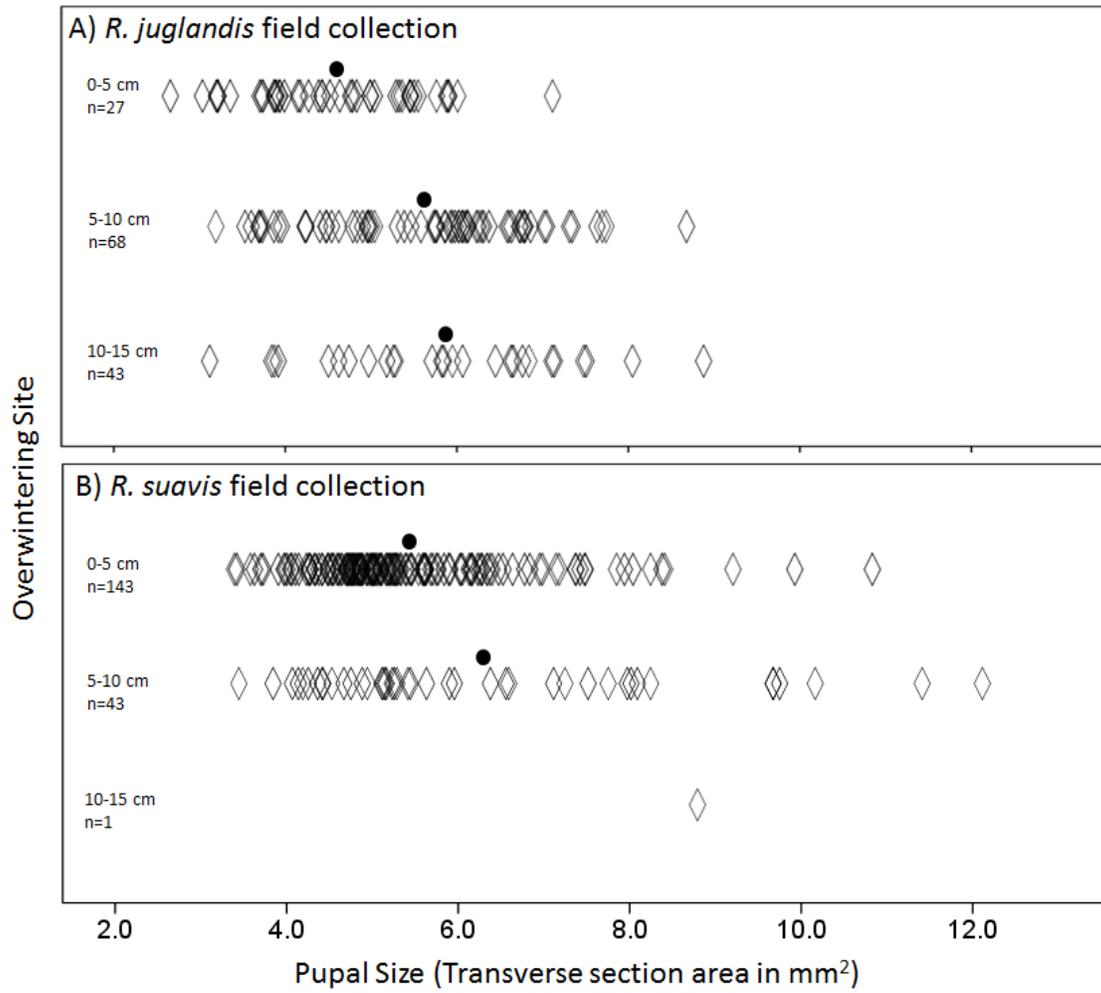
443

444 Figure 2. Relationship between size of A) *R. juglandis* and B) *R. suavis* larvae and the
445 distance that they burrow into sand under laboratory conditions. Each diamond
446 represents a single pupa collected from columns of sand into which larvae were allowed
447 to burrow prior to pupation. Black circles represent the mean pupal size (area of
448 transverse section) at each depth.

449

450 Figure 3. Pupal size and position affects eclosion (A) and emergence (B) of adult
451 *Rhagoletis juglandis*. Large and small pupae were set at different depths in a column of
452 sand (top=1 cm under sand, bottom = 15 cm under sand) 1-2 weeks prior to expected
453 eclosion. Columns were checked daily for emerged flies. Only flies that eclosed were
454 included in the dataset for emergence.

455 Figure1.



456

Figure 2.

