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Manuscripts

1   **Leaf herbivory induces resistance against florivores in *Raphanus sativus***

2

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23

24   **Abstract**

25   Florivory can have significant negative effects on plant fitness, driving selection for

26   resistance traits in flowers. In particular, herbivory to leaves may induce resistance

27   in flowers because herbivores on leaves often become florivores on flowers as plant

28   ontogeny proceeds. The literature on inducible resistance in floral tissues is limited,

29   so we used a series of experiments to determine if prior leaf damage by *Spodoptera*

30   *exigua* caterpillars affected florivore preference and performance on wild radish

31   (*Raphanus sativus* L.). We found that *S. exigua* larvae preferred petals from control

32   plants versus petals from plants exposed to prior leaf damage, and that larvae

33   gained more weight on petals from control plants, although this depended on the

34   presence of anthocyanins in the petals. Our results suggest that leaf damage can

35   induce changes in petals that reduce *S. exigua* larval fitness.

36   **Key words:** anthocyanins, floral herbivory, florivory, induced resistance, *Raphanus*

37   *sativus*

38

Introduction

40 Many different types of herbivores may feed on a plant during its growth and

41 development. Some of these herbivores specialize on particular parts of the plant,

42 while others are more general in their preferences. For example, some herbivores

43 primarily feed on roots, some on shoots, and others on floral tissues. Florivores,

44 those animals feeding on floral tissue, are some of least studied among herbivores

45 (McCall and Irwin 2006). These organisms consume floral tissues before

46 maturation of the seed, and thus can have direct and indirect effects on overall plant

47 fitness.

48

49 Florivores may eat or damage the primary reproductive organs in a flower

50 (Krupnick and Weis 1999). This can result in nearly complete seed loss in some

51 plant populations (Washitani et al. 1996). Other types of damage may affect tissues

52 involved in pollinator signaling, like petals (McCall 2008). Damage to these tissues

53 can result in reduced reproduction through reduced pollinator service and/or seed

54 set (Karban and Strauss 1993; Lohman et al. 1996; Cardel and Koptur 2010; see

55 González-Browne et al. 2016 for a meta-analysis). Given the potential fitness costs

56 of florivory, plants may have evolved to limit the amount of damage caused by

57 florivores through physical and/or chemical resistance. One framework that can

58 explain the distribution of these traits is the Optimal Defense Theory (ODT) (McKey

59 1974; Rhoades 1979). ODT assumes that there is a substantial cost to producing

60 resistance traits, such that all tissues cannot be maximally protected at all times.

61 Given this tradeoff, ODT predicts that resistance should be higher in tissues that are

62   more valuable, more likely to be attacked by herbivores, or are less costly to defend

63   (McKey 1974; Rhoades 1979).

64

65   Plant resistance may also vary depending on herbivore activity.   Constitutive

66   resistance is active without an initial trigger, whereas induced resistance is only

67   activated after an initial bout of herbivore damage or some other trigger (Karban

68   and Baldwin 1997).   Induced resistance is thought to be less costly than constitutive

69   resistance because it is not activated if initial damage does not occur. Thus, ODT

70   predicts that less valuable tissues will be protected with inducible resistance,

71   whereas more valuable tissues would be protected with constitutive resistance, all

72   other factors being equal (Karban and Nagasaka 2004).   Induced resistance in

73   flowers is not without costs, though, as induction by leaf damage can reduce

74   pollinator attractiveness in *Solanum peruvianum* (Kessler et al. 2011). In another

75   example, natural leaf damage to *Sinapis arvensis* affected both pollinator preference

76   and the pollinator-plant community networks that included *S. arvensis*, possibly due

77   to changes in floral volatiles (Hoffmeister et al. 2016).

78

79   Flowers might be expected to have high levels of constitutive resistance because

80   they are directly involved in fitness and are usually highly apparent to visual

81   herbivores (Zangerl and Rutledge 1996; Strauss et al. 2004; McCall and Irwin 2006).

82   Researchers have found inducible responses in flowers. In particular, damage to

83   leaves can lead to increased resistance in flowers. This mechanism may be adaptive

84   if the same leaf herbivores also eat flowers, which has been reported in the

85   literature (*e.g.* Agerbirk et al. 2010; Merwin and Parrella 2014; Abdalsamee and

86   Müller 2015). In some systems, a leaf herbivore first eats leaves and then moves up

87   a stem to feed on flowers or inflorescence tissue (Smallegange et al. 2007; Bandeili

88   and Müller 2010). Under these circumstances, a plant may be selected to defend its

89   reproductive tissues after initial damage to its leaves because leaf damage would be

90   a reliable predictor of floral damage. For example, leaf damage can increase floral

91   nicotine levels in *Nicotiana attenuata* (Euler and Baldwin 1996). McCall and Karban

92   (2006) showed that artificial leaf damage and methyl jasmonate were able to deter

93   florivory in *N. attenuata*. In *Raphanus sativus,* leaf damage increased total

94   glucosinolate concentrations in flowers (Strauss et al. 2004). Researchers have also

95   found inducible chemicals in floral nectar. Halpern et al. (2010) showed that leaf

96   damage can increase nicotine concentrations in *Nicotiana quadrivalvis* nectar and

97   Adler et al. (2006) showed that leaf damage increased alkaloid levels in *Nicotiana*

98   *tabacum* nectar.

99

100   Despite these gains, past studies focused solely on tissue chemistry, and no research

101   to date has addressed whether induced resistance in floral tissue has an actual effect

102   on florivore preference and performance. For selection to favor induced resistance

103   in flowers, there should be a negative effect of induction on florivores, especially if

104   the florivores are also leaf herbivores early in plant ontogeny. As of yet, there is no

105   work investigating if induced resistance, triggered by leaf damage, can negatively

106   affect florivore performance.

107

108   Genetic variation within populations is also necessary for natural selection of

109   induced resistance in flowers. Researchers have found significant genetic variation

110   in induced resistance in leaves, although few have investigated how genetic

111   variation affects induced resistance in floral tissues. In a rare example, Strauss et al.

112   (2004) found that although different families differed in glucosinolate

113   concentrations in *Raphanus sativus*, the strength of induction across petals and

114   leaves did not depend on maternal family. Because of the paucity of studies on the

115   variation in floral induction strength within populations, we investigated whether

116   induced resistance differs within maternal families in a single population.

117

118   The above studies considered genetic variation within single populations, but the

119   strength of induction observed in one particular population may not be

120   generalizable to other populations. In fact, there is reason to expect induction to

121   vary across plant populations, especially if the type of herbivores and the severity of

122   their damage varies across those populations (Karban and Nagasaka 2004). To our

123   knowledge, no one has investigated whether different plant populations differ in

124   their strength of induced resistance in floral tissue or if induced resistance, in

125   general, affects florviore performance.

126

127   Using the model species *Raphanus sativus,* we consider several questions related to

128   the aforementioned gaps in our knowledge about induced resistance in flowers.

129   First, we ask whether prior damage to leaves affects florivore preference of petals.

130   Second, we ask if prior leaf damage affects florivore performance, as measured by

131   weight, on petals among maternal families within a single population. Third, we ask

132   if the effects of leaf damage on florivore performance differ among several plant

133   populations and if petal anthocyanin presence affects induced resistance.

134

135

136

137

138   **Methods**

139   **Plant system**

140   *Raphanus sativus* L. is an annual plant produced from crosses between *R.*

141   *raphinistrum* and agronomic *R. sativus* cultivars (Hegde et al. 2006). Its seeds

142   germinate during the first part of the wet season in California's Mediterranean

143   climate (October–December), with plants flowering between March and July. Floral

144   color phenotype (bronze, pink, white, and yellow) is controlled by two

145   independently segregating loci (Panestsos 1964; Irwin et al. 2003). Leaf herbivores

146   include *Plutella xylostella* (Plutellidae) and *Platyprepia virginalis* (Erebidae), both of

147   which also can feed on flowers. On a single plant, *Pl. virginalis* and *P. xylostella*

148   individuals can be both leaf herbivores and florivores (A. McCall, personal

149   observation).

150   **Florivore choice and performance procedures**

151   We used the same general procedure to test florivore preference and performance

152   for the three experiments described below. We attempted to induce resistance

153   responses in *R. sativus* flowers by damaging the leaves of experimental plants with

154   *Spodoptera exigua* larvae, a generalist herbivore often used for bioassays. Half of the

155   plants were randomly chosen to receive herbivore damage by caging a neonate *S.*

156   *exigua* larva on the third true leaf. Control plants received a cage without any

157   larvae. We allowed the larvae on the experimental plants to feed until half of the

158   leaf area was consumed. Thus, every damaged plant received the same relative

159   amount of damage. We then removed all cages from control and experimental plants

160   and allowed the plants to grow until they flowered about 2 weeks later. Very little

161   work on the duration of induction in *Raphanus* has been conducted, but Agrawal

162   (1999) showed that induction of resistance in leaves may last 6 weeks in *R.*

163   *raphinistrum.*

164

165   For the preference test, a single petal from a randomly-chosen control and

166   experimental plant of the same color morph was selected and placed in a 120 mm

167   Petri dish, along with a moist ball of tissue paper to ensure that the petals would

168   remain fresh for consumption. For the performance tests, we selected two petals

169   from the newest flower on a plant and placed them in the arena with a moist tissue

170   paper ball and a neonate larva. Petals and tissue paper balls were replaced every

171   two days and petals always came from the same plant for each arena. The only

172   exception to this procedure was if the larvae ate all of the available petal tissue

173   before 2 days. If this happened, we added 2 more petals when the food supply was

174   exhausted.   During the replacement of tissue, we weighed the petals to ensure that

175   constant weights were maintained among and within individual arenas. If weights

176   were not within 0.005g of each other on successive days of the tests, we selected

177   new petals until the desired mass was achieved. We allowed the larvae to feed for

178   24h for the preference test and for 10 days for the performance tests.

179

180   **Preference**

181   Forty *R. sativus* plants from five dams in the Jepson Prairie population (Table 1)

182   were grown in a greenhouse in September of 2008. After growing the experimental

183   and control plants as described above, we set up feeding trials where each larva

184   could feed on petals obtained from damaged and undamaged plants. We allowed the

185   larvae to feed for 24h, after which we scored which flower disk had the most

186   damage. One larva escaped and we were unable to determine which disk had the

187   most damage in one arena, leaving us with 18 total replicates. We tested preference

188   using a Chi-square goodness-of-fit test.

189

190   **Performance: single population**

191   We also wanted to know if induction of resistance in petals or anther tissue could

192   affect *S. exigua* performance, measured as total weight gain.   We employed a

193   bioassay method using *S. exigua* and plants from a population at Jepson Prairie,

194   California (Lat.: 38.303°; Long.: 121.824°). On May 15, 2009, seeds from this

195   population were collected by placing a 20m linear transect along the side of the road

196   where the bulk of the population was located. Silique-bearing plants at every 2m

197   were selected for collection, although there were only eight total plants that set fruit

198   in that year.   Approximately 30 siliques from each maternal plant were haphazardly

199   collected and placed in paper bags at room temperature until germination for the

200   experiment. On November 10, 2009, five seeds from five different siliques per

201   maternal plant were planted in potting soil. Plants were grown under a 16h day: 8h

202   night regime in a pest-free greenhouse until the third true leaf was fully expanded

203   on each plant, when we applied the initial damage treatment.   As the plants

204   flowered we also noted whether they expressed anthocyanins in their petals

205   (bronze and purple) or if they did not (white and yellow).

206

207   We tested the performance of neonate *S. exigua* larvae using the methods described

208   above, feeding larvae petals from control or treatment plants. Initially we planted

209   70 plants, with 36 plants randomly assigned to the control treatment and 34 plants

210   randomly assigned to the induction treatment. During the course of the feeding

211   trials 10 larvae died, resulting in 60 total replicates. There was no association of

212   larval death with a particular treatment (Chi-square = 1.611, *P* = 0.20). Maternal

213   family 1 had 14 replicates, family 2 had 16 replicates, family 3 had 11 replicates, and

214   family 4 had 19 replicates. Fifty larvae were reared on pink-flowering plants and

215   ten were reared on white-flowering plants.

216

217   The data exhibited homogeneity of variances and were normally distributed, so we

218   performed a mixed-model ANOVA with the dependent variable of larval weight and

219   the independent variables of planting block, maternal family, damage treatment, and

220   the interaction between maternal family and damage treatment. We considered

221   planting block as a random effect and maternal family a fixed effect because we

222   sampled a large proportion (approximately 50%) of this population.

223

224   **Performance: multiple populations**

225   In order to test whether the effects of prior leaf damage are dependent on the

226   population being tested and to see if the presence of anthocyanins affected the

227   results, we conducted an experiment where we manipulated leaf damage on plants

228   from several populations of *R. sativus.* These populations were a subset of those

229   used in an earlier experiment that investigated whether variance in leaf damage was

230   related to inducibility of defense in leaves (Karban and Nagasaka 2004). Those

231   populations in Karban and Nagasaka (2004) were originally chosen because they

232   spanned a climatic gradient from wet areas to dry areas in Northern California.

233

234   We planted 10 seeds from each of 12 *R. sativus* populations in a pollinator-free

235   greenhouse. Nine seeds failed to germinate, leaving us with 111 plants for the

236   experiment. We again produced experimental and control plants using the general

237   procedure outlined above.   During the course of the experiment 34 larvae died, so

238   we were left with 77 living larvae at the end of 10 days. There was no difference in

239   the proportion of larvae that died between the damage treatments (X^2 = 1.54, 1df, *P*

240   = 0.22). We then reduced the data set further by excluding those populations

241   (CHER, DIXO, NAPA, PLIN, STRW, see Table 1) that did not have at least two

242   replicates of each treatment or anthocyanin morph. This left us with 58 total plants

243   in seven populations for the final analysis (Table 1). There were 34 control plants

244   and 24 experimental plants and 28 with anthocyanins present in their flowers

245   versus 30 plants with anthocyanins absent from their flowers.

246

247   The data exhibited homogeneity of variances and residuals were normally-

248   distributed, so we performed a mixed-model ANOVA to test if planting block,

249   population, anthocyanin presence in petals, population by treatment or population

250   by anthocyanin had significant effects on larval weight gain after 10 days of feeding.

251   We considered the population, population by treatment, and planting block as

252   random effects. We determined the significance of random factors by examining the

253   95% CI; if these intervals included zero, then the random effect was not considered

254   significant at the α = 0.05 level. We included anthocyanin presence in the petals

255   (A+/A-) and the interaction between anthocyanin presence and the treatment effect

256   because different color morphs are known to induce different levels of

257   glucosinolates (Strauss et al. 2004).

258

259   All analyses were performed using JMP Pro v.12 (SAS Institute, Cary, NC USA)

260

261

262   **Results**

263   **Preference**

264   *S. exigua* larvae preferred petals from undamaged plants in 15 out of 18 (83.3%)

265   trials. This number was significantly different from the numbers expected from the

266   null hypothesis of no preference between the treatments ($X^2 = 8.0$, 1df, $P = 0.005$).

267

268   **Performance: single population**

269   For insects reared on petals, there was a significant effect of planting block

270   (variance component = 1.01e-8, $P < 0.05$) and no significant effect of the damage

271   treatment ($F_{1,51} = 2.96$, $P = 0.09$), or maternal family ($F_{3,51} = 0.38$, $P = 0.77$) on larval

272   weight after ten days.   There was a strong trend for an interaction between damage

273   treatment and maternal family ($F_{3,51} = 2.76$, $P = 0.051$). On examining the simple

274   effects of damage treatment within each maternal family, there were no significant

275   effects of damage in families 1 ($F_{1,51} = 2.42$, $P = 0.13$), 2 ($F_{1,51} = 0.77$, $P = 0.38$), or 4

276   ($F_{1,51} = 2.38$, $P = 0.12$), but larvae from control treatments weighed 79% more than

277   larvae from damage treatments in family 3 ($F_{1,51} = 4.69$, $P = 0.03$, see Figure 1).

278

279   **Performance: multiple populations**

280   Planting block in the greenhouse had a significant effect on larval weight (variance

281   component = 1.51e-8, $P < 0.05$). There was no significant effect of population

282   (variance component = 8.19e-9, $P > 0.05$), and no significant population by treatment

283   effect (variance component = -1.00e-8, $P > 0.05$). For fixed effects, there was a

284   significant effect of the leaf damage treatment, such that larvae on damaged plants

285   weighed 18% less than larvae on undamaged plants ($F_{1,9.01} = 6.42$, $P = 0.03$) and no

286   significant effect of petal anthocyanins on larval weight ($F_{1,37.18} = 0.84$, $P = 0.36$).

287   There was a significant leaf damage by petal anthocyanin interaction ($F_{1,13.3} = 5.59$, P

288    $= 0.03$). Examining the simple effects within anthocyanin-free morphs, we found

289   that larval weights were significant lower on petals from damaged plants than on

290   petals from undamaged plants ($F_{1,19.5} = 9.66$, $P = 0.006$). In contrast, there were no

291   significant effects of leaf damage on larval weight in morphs containing

292   anthocyanins in their petals ($F_{1,5.43} = 0.012$, $P = 0.92$, see Figure 2).

293

294

295

296

297

298   **Discussion**

299   If induced resistance occurs in petals, we might expect that florivores to choose

300   petals from undamaged versus damaged plants. We found evidence that leaf damage

301   can alter the preference for petals. Specifically, florivores preferred petals from

302   control plants versus petals from plants with prior leaf damage. This finding is

303   consistent with the idea that insects can initially reject petals due to induced

304   secondary compounds in the flowers, physical changes in petal structure, or changes

305   in the nutritional composition of the flowers. To our knowledge, this is the first

306   evidence showing that florivores show a preference for petals from undamaged

307   versus damaged plants. Likewise, larvae reared on petals from damaged plants

308   gained significantly less weight than those larvae reared on petals from control

309   plants. We observed this both within a single population and across multiple

310   populations, although this depended on the anthocyanin content in the latter case.

311   These results suggest that either signals from damaged leaves or defensive

312   chemicals themselves are able to travel to flowers after initial herbivore damage

313   and can have negative effects on florivore health.

314

315   Other researchers have found that prior damage to plant tissue can also affect floral

316   visitors, although few have documented a negative fitness effect on florivores. For

317   example, McCall and Karban (2006) found that both insect damage and application

318   of methyl jasmonate on leaves decreased florivore damage in *Nicotiana attenuata*.

319   In *Nemophila menziesii*, McCall (2006) showed that initial damage to flowers could

320   reduce florivory in later flowers, but they did not examine florivore performance.

321   Our current work suggests that plants can employ less costly inducible resistance in

322   petals. Our results could be explained by ODT if flowers, and petals in particular, are

323   relatively cheap to produce or if more constitutive resistance may negatively affect

324   pollination (McCall and Irwin 2006). *Raphanus sativus* plants often make hundreds

325   of flowers (A. M., personal observation) so that the sheer number of flowers

326   available for reproduction may mitigate the cost of losing petal tissue when

327   florivore pressure is relatively low. This, in turn, might preclude the selection for

328   costly constitutive chemical defenses in the flowers. Another reason that *R. sativus*

329   might be selected to induce resistance in flowers is because induction is adaptive

330   with respect to florivore behavior. For example, if florivores start their lives on

331   basal leaves, they can move acropetally to the top of the plant, where inflorescences

332   will be at risk for damage. This type of movement has been observed in larvae

333   feeding on other Brassicaceae (Smallegange et al. 2007; Bandeili and Müller 2010),

334   so it is possible that *R. sativus* may induced resistance against similar florivores.

335

336   We also found evidence that induction of resistance varies with maternal family in

337   the Jepson population. This may not be surprising, as Agrawal et al. (2002) found

338   significant additive genetic variation in the strength of induction in *Raphanus*

339   *raphanistrum* leaves. It would be interesting to investigate whether those

340   genotypes that display high levels of induced resistance in leaves also have high

341   levels of induction in flowers.

342

343   Interestingly, we found no evidence that induced resistance in flowers varied

344   among populations of *R. sativus*, even though the type and intensity of herbivory did

345   vary across the populations (see Table 1). This is in contrast to Karban and

346   Nagasaka's (2004) findings that populations of *R. sativus* along a climatic gradient in

347   Northern California exhibited significant variation in induction of resistance in

348   leaves. Our results may differ from this study for at least two reasons. First, our

349   sample sizes per population were much lower, with an average of 8 plants per

350   population, so it is possible that we did not have enough statistical power to detect

351   differences in induction. Second, it is possible that the variation in induced

352   responses in flowers does not necessarily reflect the amount of variation in

353   induction for leaves across these populations. This may be true in *R. sativus,* where

354   researchers found that floral glucosinolates were not as inducible as leaf

355   glucosinolates within a population (Strauss et al. 2004).

356

357   We also found that induction of resistance in petals depended on the presence of

358   anthocyanins in petals in the multiple population experiment. In particular, induced

359   resistance was only found in anthocyanin-free petals. This could be due to

360   pleiotropic action of genes involved in producing glucosinolates and the precursors

361   to anthocyanins (Hemm et al 2003). Strauss et al. (2004) found that white and pink

362   flowers had similar constitutive concentrations of gluosinolates, but that

363   glucosinolates were induced at higher levels in pink *R. sativus* flowers than in the

364   white morphs.   This is in contrast to our findings, and could be explained because

365   we used different populations of *R. sativus* than Strauss et al. (2004) or that our

366   methods of induction were not identical. Alternatively, glucosinolate induction in

367   pink morphs as shown by Strauss et al. (2004) may not necessarily translate into

368   reduced performance of florivores relative to the other color morphs.

369

370   Although we showed that leaf damage has negative effects on florivores, we cannot

371   necessarily conclude that induction is an adaptation to reduced flower damage in *R.*

372   *sativus*. Alternatively, damage to leaves may increase levels of defensive chemicals,

373   reduce nutrient concentration or availability, or affect some other physical attribute

374   in the entire plant, with effects on florivores being a nonadaptive side effect of the

375   leaf damage. This hypothesis would mirror Adler's (2000) nonadaptive hypothesis

376   regarding correlated concentrations of resistance compounds in leaves and nectar.

377   Manson et al. (2012) found that pooled concentrations of cardenolides across 12

378   *Asclepias* species were positively correlated in nectar and leaves, possibly giving

379   support to the nonadaptive hypothesis. Alternatively, the authors also found that

380   the qualitative profiles of cardenolides were significantly different among nectar

381   and leaf samples.

382

383   **Conclusions**

384   We show that *S. exigua* larvae both prefer petals from control plants and perform

385   better on control plant petals than on damaged plant petals. This effect is dependent

386   on maternal family identity in at least one population, and across multiple

387   populations the effect was only seen in plants without anthocyanins in their petals.

388   These results suggest that plants may protect themselves from leaf and floral

389   damage through induction of resistance stimulated by relatively small levels of

390   initial leaf damage. Whether or not this effect can increase fitness of *R. sativus* under

391   field conditions remains an open, and important, question.

392

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394
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396

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514

515   **Table 1.** Locations of populations of *Raphanus sativus* where seed was originally
516   collected. Bolded populations indicate those populations that were used in the
517   multiple populations experiment. The remaining populations did not have enough
518   variation in petal anthocyanins to include in the final analysis.
519

Population	Latitude	Longitude
CHER	38.336	121.022
CONS	**38.2575**	**121.441**
DIXO	38.361	121.824
HOOD	**38.364**	**121.510**
NAPA	38.221	122.238
PEDR	**38.511**	**121.804**
PLIN	38.584	121.732
SNOD	**38.2772**	**121.5008**
SONO	**38.2356**	**122.5292**
STON	**38.4083**	**121.4892**
STRW	38.563	121.800
YOLO	**38.557**	**121.672**

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523   **Figure Legends**

524

525   **Fig. 1** The effects of maternal family and leaf damage treatment on the weights of

526   *Spodoptera exigua* larvae after 10 days of feeding on petals. Black bars represent

527   larval weights from control plants and grey bars represent larval weights from the

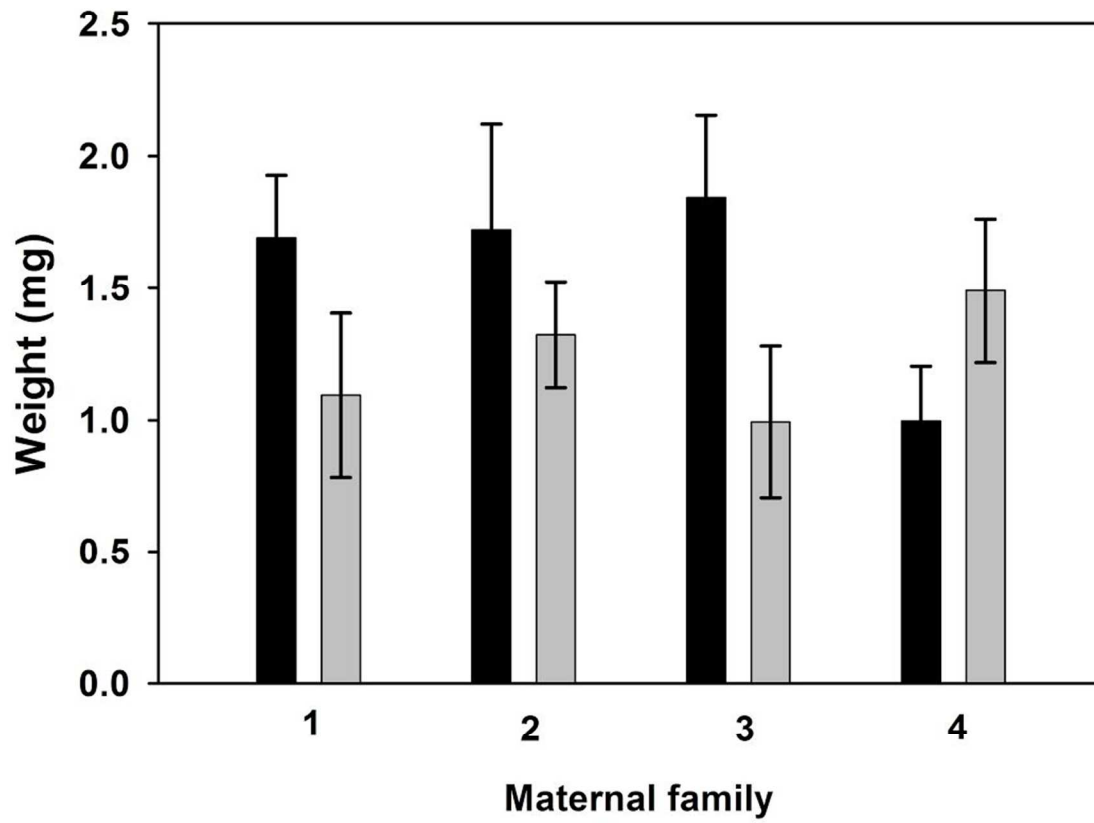528   leaf damage treatment. Bars represent means ± 1 SE.

529

530   **Fig. 2** The effect of leaf damage and petal anthocyanin content on *Spodoptera exigua*

531   weight after 10 days of feeding on petals. Plants were grown from seven different

532   populations in Northern California (see Table 1). Black bars represent larval

533   weights from control plants and grey bars represent larval weights from the leaf

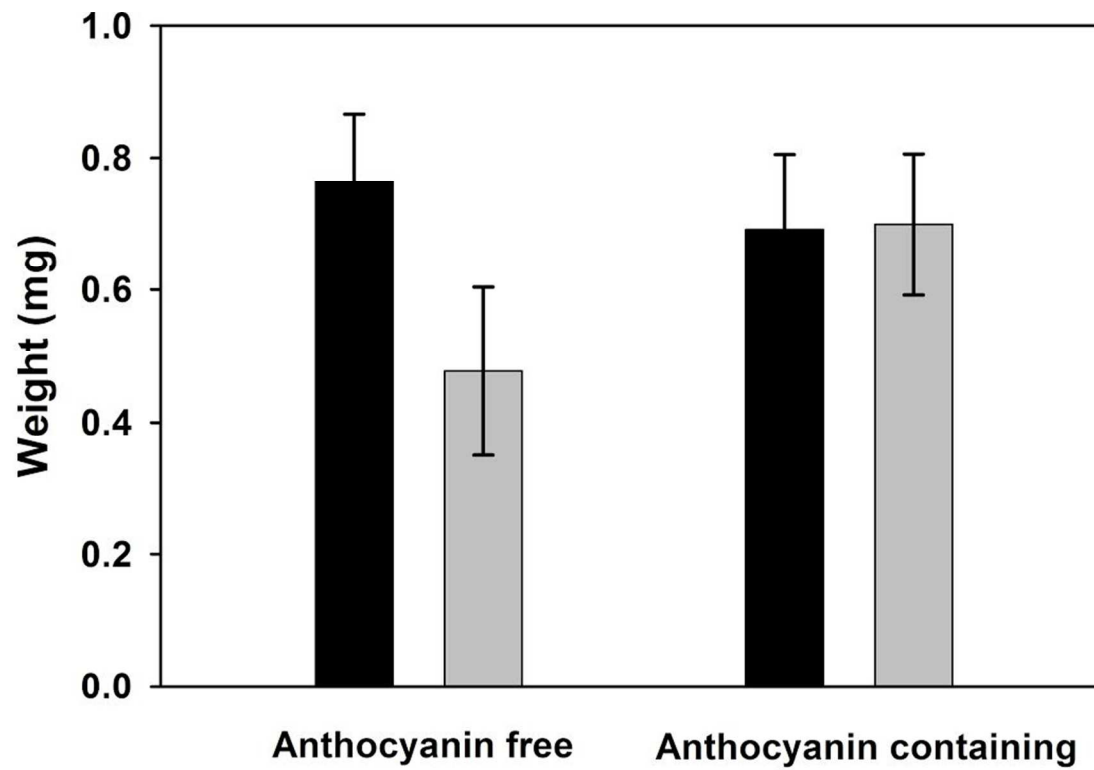534   damage treatment. Bars represent means ± 1 SE.

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537   **Fig. 1**

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540   **Fig. 2**

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