1   **Title: Drosophila species learn dialects through communal living**

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3   **Authors:** Balint Z Kacsoh, Julianna Bozler and Giovanni Bosco*

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5   **Affiliation:**

6   Department of Molecular and Systems Biology, Geisel School of Medicine at Dartmouth,

7   Hanover, NH, 03755, USA.

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9   *Corresponding author: (GB) giovanni.bosco@dartmouth.edu

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12   **One sentence summary**: Fruit flies learn dialects following co-habitation with other species.

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24   **ABSTRACT**

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26   **Many species are able to share information about their environment by communicating**

27   **through auditory, visual, and olfactory cues. In Drosophila melanogaster, exposure to**

28   **parasitoid wasps leads to a decline in egg laying, and exposed females communicate this**

29   **threat to naïve flies, which also depress egg laying. We find that species across the genus**

30   **Drosophila respond to wasps by egg laying reduction, activate cleaved caspase in oocytes,**

31   **and communicate the presence of wasps to naïve individuals. Communication within a**

32   **species and between closely related species is efficient, while more distantly related species**

33   **exhibit partial communication. Remarkably, partial communication between some species**

34   **is enhanced after a cohabitation period that requires exchange of visual and olfactory**

35   **signals. This interspecies "dialect learning" requires neuronal cAMP signaling in the**

36   **mushroom body, suggesting neuronal plasticity facilitates dialect learning and memory.**

37   **These observations establish Drosophila as genetic models for inter-species social**

38   **communication and evolution of dialects.**

39   The ability to interpret environmental information is a phenomenon found throughout all

40   life forms. From bacteria to plants and to mammals, communication occurs within as well as

41   between species. In some cases, information that is being shared can be highly specific, such as

42   in the case of honeybees communicating instructions on where to find nectar (1-3) . In other

43   cases, opportunistic bystanders can also benefit from general information. For example, predator

44   alarm calls generated as a warning are observed, where multiple species participate in repeating

45   the alarm throughout the community (4-8) . In all cases, the information that is shared can be

46   dependent on local environmental cues and experiences and the manner in which information is

47   communicated is strongly influenced by past experiences of each individual. For example, birds,

48   which live in geographically distinct populations, manifest unique song variants or regional

49   dialects that can last for decades, but these animals are nevertheless still able to communicate

50   with others of their species (9-11) . Because dialects are learned and therefore influenced (12)

51   by specific local environmental differences, it suggests that both social and non-social

52   experiences can have dramatic effects on cognitive development (13) .

53   It is proposed that a myriad of environmental cues, both social and non-social, are critical

54   to animal development in determining the ability to convey and receive specific types of

55   information. However, there are many outlying questions as a result of this proposition: What

56   cues are important? When are these cues important? How can environmental cues interact with

57   genetically determined developmental programs? Although social communication is most

58   extensively documented in more derived organisms such as mammals and birds, insects can also

59   display a broad range of behavioral tasks. Bees are known to be able to learn from non-natural

60   sources in order to obtain a reward through social learning. Such information can be passed on to

61   naïve, student bees through the use of visual cues (14,15) . Insect social learning extends to the

62   genetic model system of Drosophila, where student, observer flies learn from a trained, teacher-

63   fly, using visual cues. This has been shown in communication involving food sources and

64   predator threats (16,17) .

65   Chemical cues can serve as intra- and inter-species signals, such as fox and guinea pig

66   urine affecting not only conspecific behavior, but also the behavior of other animals (18-20) .

67   Sound can also be used, such as in bats and bottlenose dolphins, which are able to distinguish

68   members of the community through the use of echolocation pitch recognition (21,22) . Plants

69   have a vast arsenal of responses to pathogens (23) , including communicating a threat to

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70   neighboring plants through the use of volatile organic compounds (24) . Plant inter-species (25-

71   31) and intra-species (32-34) communication occurs both in laboratory settings and in the wild

72   (30,35) .

73   *Drosophila melanogaster* and other Drosophila species have provided insights into

74   mechanisms of learning, memory, and complex behaviors (36,37) . However, these behaviors

75   and phenotypes have been studied almost exclusively in domesticated *D. melanogaster* lab

76   monocultures, while *D. melanogaster* wild populations are surrounded by a broad range of

77   predators, microbes, and other Drosophilids, highlighting a communal component of the

78   organism's life cycle (38) . This raises the possibility of behavioral phenomenon that have yet to

79   be discovered and analyzed in domesticated lab monocultures (39-41) . Given the vast range of

80   environmental inputs on a wild Drosophilid, a fly must be able to discern important information

81   from extraneous inputs, while interacting with conspecifics and a variety of other species.

82   Although modes of intra- and inter-species communication are likely to be genetically

83   limited, there is also value in learning to interpret signals from variable, local environments that

84   may provide immediate survival benefits. How do genetically constrained neurological features

85   and variable environmental factors interact to produce context-dependent, meaningful

86   information? Under which environmental factors would information sharing between different

87   species occur and be beneficial? In this study, we sought to begin to address these questions in

88   the Drosophila model system by using a pan-Drosophila predator known to elicit social

89   communication (17,42) . *D. melanogaster* presented with parasitoid wasps have multiple

90   behavioral responses, including a reduction in oviposition (egg laying) through an increase in

91   ovarian apoptosis (17,43-46) . After removal of the wasp, a wasp-exposed "teacher" fly can

92   instruct a naïve "student" fly about the presence of the wasp threat through the exclusive use of

93   visual cues, such that students now reduce their own oviposition by triggering ovarian apoptosis.

94   Using this fly-fly social communication paradigm we asked (1) whether social communication is

95   conserved among other Drosophila species, (2) if Drosophilids engage in interspecies

96   communication, and (3) what environmental and genetic factors are required for interspecies

97   communication.

98   **RESULTS**

99   **INTRA- AND INTER-SPECIES COMMUNICATION**

100   We utilized the fly duplex, an apparatus with two transparent acrylic compartments to test

101   whether different species respond to seeing predators (acute response) and if exposed "teacher"

102   female flies can communicate this threat to naïve unexposed "student" female flies (17) . The

103   duplex allows flies to see other flies or wasps in the adjacent compartment, without direct

104   contact, making all communication only visual (Fig. 1A). Ten female and two male flies are

105   placed into one duplex compartment, with an adjacent compartment containing twenty female

106   wasps. Following a 24-hour exposure, wasps are removed and acute response is measured by

107   counting the number of eggs laid in the first 24-hour period in a blinded manner. Flies are shifted

108   to a new duplex, with ten female and two male naïve student flies in the adjacent compartment

109   (Fig. 1 A, see methods). Following a second 24-hour period, all flies are removed and the

110   response of both teacher and student is measured by counting the number of eggs laid in a

111   blinded manner. The 24-48-hour period measures memory of teachers having seen the wasps and

112   students having learned from the teachers. Using wild-type *D*. *melanogaster*, we find both an

113   acute response and a memory response to the wasp in teacher flies and a learned response in

114   naïve student flies (Fig. 1 B, Fig. S 1 A) (17,45,46) .

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115 We then asked whether the acute, memory, and student social learning behaviors are

116 conserved in other Drosophila species, with varying relatedness to *D. melanogaster* ranging from

117 sister species, such as *D. simulans*, to very distantly related species, such as *D. virilis*. For each

118 species, we tested a sister species as an additional way to validate our observations. Across a

119 broad span of the genus Drosophila, we find the conservation of both the acute and memory

120 responses in teacher flies in addition to the ability of teachers to communicate to student flies.

121 (Fig. 1 C, Fig. S 1 B-H). Some of these species have been previously shown to depress

122 oviposition during wasp exposure (46) . Our experimental design allows for only visual cues to

123 be detected from the wasps and from teachers to student flies. Thus, in all species tested, visual

124 cues are sufficient for flies to detect wasps and for naïve flies to learn from wasp-exposed

125 teacher flies. Conservation of these behaviors is especially impressive as the species tested are

126 separated by millions of years of evolution, yet the basic behaviors observed in *D. melanogaster*

127 are maintained. Moreover, this conservation further underscores the importance this innate

128 behavior must have since even laboratory cultures that have not experienced wasp for many

129 generations nevertheless exhibit a robust response.

130 Oviposition reduction is modulated in part by the effector caspase Dcp-1 (17) . In *D.*

131 *melanogaster*, we observe overlapping staining of activated Dcp-1 with a punctate pattern of

132 DNA staining with 4', 6-diamidino-2-phenylindole (DAPI), indicative of oocyte specific

133 apoptotic activity (Fig. 1 D-K, Fig. S 2). We performed immunofluorescence of activated Dcp-1

134 across a broad range of Drosophila species, revealing cleaved caspase following wasp exposure

135 in all 15 Drosophila species tested (Fig. S 3). We demonstrate an increase in positive cleaved

136 caspase oocytes following wasp exposure (Fig. S 4), along with a decrease in total number of egg

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137   chambers (Fig. S 5), suggestive of ovarian apoptosis and elimination of oocytes (17) .

138   Phylogenetic trees shown are adapted from previous work (47) .

139   Following the observation that an acute response to wasps and intra-species

140   communication is conserved across the genus, we asked whether the wasp threat could be

141   communicated between two different species. We utilized 15 Drosophila species that respond to

142   wasps to answer this question (Fig. S 3,4). The species were selected to span the phylogeny with

143   different degrees of relatedness to *D. melanogaster* (47) . We find that *D. melanogaster* are able

144   to communicate the threat to and receive communications from closely related species, such as

145   *D. simulans* and *D. yakuba*, with oviposition of students paired with wasp-exposed teachers

146   being ~10-30% compared to unexposed (Fig. 2 A-B, Fig. S 6 A-F). Interestingly, species more

147   distantly related to *D. melanogaster*, such as *D. ananassae* and its sister species, elicit a partial

148   communication phenotype, with oviposition depression of students paired with wasp-exposed

149   teachers being ~50-65% of unexposed flies (Fig. 2 C-F, Fig. S 6 G-J). A second strain isolate of

150   *D. ananassae* also show partial communication with *D. melanogaster* (Fig. 2 C-F, Fig. S 6 G-J).

151   Species more distantly related to *D. melanogaster*, such as *D. willistoni* and *D. virilis*, cannot

152   communicate with *D. melanogaster* (Fig. 2 G-J, Fig. S 6 K-P). Collectively, the data suggest that

153   evolutionary distance contributes the to the efficiency of interspecies communication. *D.*

154   *ananassae* show varying communication phenotypes with other Drosophila species, though the

155   pattern of communication is different. For example, *D. ananassae* exhibit partial communication

156   with *D. simulans* (Fig. S 7 A-B), strong communication with its sister *D. kikkawai* (Fig. S 7 C-

157   D), and partial communication with *D. equinoxialis* and *D. willistoni* (Fig. S 7 E-H). D.

158   *ananassae*, in addition to *D. melanogaster*, are unable to communicate with the distantly related

159   *D. mojavensis* and *D. virilis* (Fig. 2 I-J, Fig. S 7 I-L). Species such as *D. virilis*, which were

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160   unable to communicate with *D. melanogaster* and *D. ananassae*, can communicate with other

161   species, such as its sister species *D. mojavensis* (Fig. 2 K-L). Thus, although all species tested

162   are capable of intra-species communication, there is a fundamental, species-specific difference in

163   "fly language" or in strategy for communicating wasp threat.

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165   **DIALECT LEARNING**

166   Given that closely related species can communicate the threat of a wasp, we examined

167   the environmental factors that could be contributing to such interspecies communication.

168   Specifically, we tested whether a period of cohabitation with frequent contact between two

169   poorly communicating species could improve interspecies communication. *D. melanogaster*

170   were cohabitated with species capable of only partial communication (*e.g. D. ananassae*) (Fig. 2

171   C-D) for one week in a single container, allowing for frequent and multiple channels of sensory

172   interactions. Following a weeklong cohabitation period, the two species were separated and used

173   as students paired with teachers of the other species (Fig. 3 A). In all experiments teachers had

174   existed only as a monoculture, while all flies experiencing an interspecies cohabitation period

175   were subsequently used only as students.

176   We find that cohabitation can greatly enhance communication between some species,

177   suggesting that some form of training occurs during this period. After cohabitation, *D. ananassae*

178   learn very efficiently from *D. melanogaster* teachers, demonstrating that cohabitation of two

179   species yields an expanded communication repertoire (Fig. 3, Fig. S8). This observation

180   indicates that poorly communicating species are not limited by structural barriers such as wing

181   shape or olfactory capacity. Instead this suggests that, similar to local dialects in bird songs,

182   Drosophila species-specific cues can be learned simply by repeated exposure to the "dialect". We

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183   observed "dialect learning" in two different *D. ananassae* strain isolates, and two additional

184   sister species (Fig. 3 B-E, Fig. S 8 A-G), indicating that dialect learning is likely to be a wide-

185   spread phenomenon in Drosophila. Interestingly, some distantly related species that were unable

186   to communicate with *D. melanogaster* (i.e. *D. willistoni*, *D. equinoxialis*) acquired the ability to

187   partially communicate following a cohabitation-training period (Fig. 3 F-I, Fig. S 8 H-I). This

188   was not the case for very distantly related species (i.e. *D. virilis*, *D. mojavensis*), which showed

189   no ability to communicate with *D. melanogaster* even after training (Fig. 3 J-K, Fig. S 8 J-M).

190   We also tested a transgenic *D. melanogaster*, to see if it was capable of teaching and dialect

191   learning, and find such flies can teach their dialect to and learn the dialect from *D. ananassae*

192   (Fig. S 8 N-O).

193   Additionally, we tested whether *D. ananassae* communication could benefit from

194   cohabitation-training with species other than *D. melanogaster*. We find efficient communication

195   between *D. simulans* (Fig. S 9 A-B), *D. equinoxialis* (Fig. S 9 C-D), and *D. mojavensis* (Fig. S 9

196   E-F) with *D. ananassae* following a cohabitation-training period. In contrast to the *D.*

197   *melanogaster* results, we find communication with more distantly related species is altered after

198   dialect training. *D. mojavensis and D. virilis* are able to dialect-learn following a cohabitation-

199   training period with *D. ananassae*. With these species, in the untrained states we observe no

200   ability to communicate (Fig. S 7 I-L), but find a partial communication phenotype following

201   cohabitation (Fig. S 9 G-J). Therefore, *D. virilis* and *D. mojavensis*, although capable of inter-

202   species communication and dialect learning, cannot learn the *D. melanogaster* dialect, but can

203   learn *D. ananassae* dialect. These results suggest that some inter-species communication barriers

204   do exist while others can be overcome by a period of dialect training and cohabitation.

205   Given our observation that two species can learn dialects following a cohabitation-

206 training period, we wondered whether having more species present during the dialect training

207 period influences dialect learning. In nature, flies are exposed to many different species of

208 Drosophila. Given this, we hypothesized that neuronal plasticity exists in the fly brain to allow

209 flies to learn multiple dialects from a given training period that includes multiple species as

210 inputs. To probe this question, *D. melanogaster* were cohabitated with species capable of only

211 partial communication or no communication in the untrained state, but show efficient and partial

212 communication after dialect training (*i.e*. *D. ananassae* and *D. willistoni*, respectively). These

213 three species were cohabitated for one week in a single container. We then used the trained *D.*

214 *melanogaster* as students to *D. ananassae* and *D. willistoni* teachers (Fig. 4 A). We find that

215 trained *D. melanogaster* are able to efficiently communicate with *D. ananassae* and partially

216 communicate with *D. willistoni* (Fig. 4. B-C). These results mirror assays where these species

217 were individually trained (Fig. 3 B-C, F-G), suggesting that flies can simultaneously make use of

218 multiple inputs from multiple species and be able to learn and remember each unique dialect they

219 encounter. Additionally, we tested *D. ananassae* and *D. willistoni* as students that were

220 cohabitated with *D. melanogaster*. We find that *D. ananassae* can communicate efficiently with

221 *D. melanogaster* and *D. willistoni* (Fig. 4 D-E), and that *D. willistoni* can partially communicate

222 with *D. melanogaster* and effectively communicate with *D. ananassae* (Fig. 4 F-G). These data

223 also mirror individual training (Fig. 3 B-C, F-G, Fig. S 9 E-F). Collectively, these data

224 demonstrate that a fly can have vast communication repertoires consisting of multiple dialects

225 that it acquires.

226 **DIALECT LEARNING INPUTS**

227   In order to better understand dialect learning, we tested the roles of sensory cues and

228   genetic factors during the dialect-learning period. We measured dialect learning by quantifying

229   improvement in interspecies partial communication between *D. melanogaster* and *D. ananassae*

230   that normally exhibit efficient communication only after cohabitation. Given that in *D.*

231   *melanogaster*, and in other species tested, we found visual cues to be sufficient for the teacher-

232   student dynamic (Fig. 1) (17) , we asked if visual cues are sufficient and/or necessary for dialect

233   learning. We approached this question by performing the dialect training in the fly duplex, such

234   that the two species could only see each other (Fig. 5 A), or by performing the training in the

235   dark, so that the two species could physically interact, but lacked visual cues (Fig. S 10 A). We

236   find that visual cues alone are not sufficient (Fig. 5 B-C), but are necessary (Fig. S 10 B-C) for

237   dialect learning. The observation that visual cues are necessary but not sufficient makes the

238   dialect learning phenomena fundamentally different from the teacher-student dynamic that

239   requires only visual cues (17) . Furthermore, we wondered if seeing another species altered the

240   behavior of a fly to facilitate dialect learning. Blind *D. melanogaster ninaB* mutants do not

241   function as students. Surprisingly, *D. ananassae* cohabitated with blind *D. melanogaster* do not

242   learn the *D. melanogaster* dialect (Fig. S 10 D-E). We also performed cohabitation training

243   under two different, monochromatic light sources, and this resulted in only a partial

244   communication between *D. melanogaster* and *D. ananassae*, (Fig. 5 D-E, Fig. S 10 F-G). To

245   exclude the possibility of a dimmer light source inhibiting dialect training under monochromatic

246   settings, we repeated cohabitation-dialect-training in a full spectrum, lower light intensity setting,

247   and found both species were able to learn the dialect (Fig. S 10 H-I). Thus, full spectrum light is

248   essential in dialect learning. Importantly, the observation that blind *D. melanogaster* do not allow

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249   wild-type *D. ananassae* to dialect-learn suggests that species must see each other in order to alter

250   their behavioral/chemical outputs required to facilitate dialect-learning.

251   Wing movement was shown to be required for teacher flies to instruct students in the

252   teacher-student dynamic (17) , raising the possibility that wing movement was also important for

253   dialect learning. Therefore, we tested flies mutant in the erect wing gene (*ewg*), which impairs

254   wing movement while maintaining morphologically normal wings. The allele tested has wild-

255   type EWG protein expression in the nervous system, thus is only deficient in its non-neuronal

256   functions, such as flight muscles (48) . We find that *D. ananassae* cannot dialect learn from

257   *ewg*^{NS4} flies (Fig. 5 F), although *ewg*^{NS4} mutants have no dialect learning impairment (Fig. 5 G).

258   This suggests that dialect learning by *D. ananassae* requires *D. melanogaster* to have mobile

259   wings.

260   To test if olfactory cues play a role in dialect learning, we utilized *D. melanogaster*

261   mutants defective in chemosensory signaling. The majority of olfactory receptors require a co-

262   receptor for wild-type function, including *Orco* (Or83b) for odorant receptors (49) and *Ir8a* or

263   *Ir25a* for ionotropic receptors (50) . Ir8a olfactory sensory neurons (OSNs) primarily detect acids

264   and Ir25a OSNs detect amines, allowing us to probe specificity of detection. We find that *D.*

265   *ananassae* are able to learn dialect from *Orco*^{1}, *Ir8a*^{1}, *Ir25a*^{2}, single and *Ir8a*^{1};*Ir25a*^{2};*Orco*^{1}

266   triple mutants and RNAi expressing *D. melanogaster* targeting each of these gene products (Fig.

267   4 H,J, Fig S 9 A-L). By contrast only *Ir25a*^{2} mutant and RNAi knockdown *D. melanogaster*

268   were able to learn the *D. ananassae* dialect (Fig. 5 I,K, Fig S 11 A-L). These data demonstrate

269   that Orco- and Ir8a-mediated olfactory inputs are required for dialect-learning. This further

270   suggests that multiple olfactory cues play important roles in the dialect-learning period. We also

271   find that *D. melanogaster* males and females are both required for dialect-training *D. ananassae*

272   (Fig. 5 L-M, Fig. S 11 M-N) and that the length of the training period is also critical, as 24 hours

273   is insufficient a period for dialect-learning (Fig. S 11 O-P). Thus, although the exact olfactory

274   molecule(s) critical during a dialect-learning period are yet to be identified, we speculate that

275   dialect-learning is a complex process requiring visual, olfactory and sex specific cues.

276   To examine the possibility that dialect training involves active learning mediated by

277   neurons of the mushroom body, we utilized the GAL4 Gene-Switch system to transiently express

278   a transgene specifically in the mushroom body (MB). Using the GAL4 Gene-Switch ligand

279   system, RU486 (51) activates the GAL4 transcription factor, while administration of the vehicle

280   (methanol) does not (51) . RU486 was administered during the cohabitation period (or methanol

281   for control), but not when flies were used as students post-dialect training (Fig. 6 A). Feeding of

282   RU486 to the MB switch driver line does not impair dialect learning (Fig. S 12 A). We expressed

283   the Tetanus toxin light chain (UAS-TeTx) specifically in the MB of *D*. *melanogaster* (to inhibit

284   synaptic transmission during dialect training). We find that *D*. *ananassae* are able to learn the

285   dialect of these MB inhibited flies (Fig. 6 B). However, *D*. *melanogaster* in which MB synaptic

286   transmission is inhibited during the training period are unable to learn the *D*. *ananassae* dialect

287   (Fig. 6 C). Control methanol only conditions (i.e. no RU486 ligand) with flies of identical

288   genotypes do not show this defect (Fig. S 12 B). These data collectively indicate that visual and

289   olfactory cues are required and possibly relayed to the MB, either directly or indirectly, to

290   facilitate dialect learning. By contrast MB function does not appear to be important for *D*.

291   *melanogaster* behavior(s) that enable *D*. *ananassae* to learn a dialect (Fig. S 6 B). Consistent

292   with this idea, although $Orb2^{AQ}$ mutants cannot function as students (Fig. 6 E) (17) , *D*.

293   *ananassae* nevertheless learns the *D*. *melanogaster* dialect from $Orb2^{AQ}$ mutants (Fig. 6 D).

294   Because MB function is necessary for dialect learning during dialect training, we tested

295   the long-term memory proteins Orb2, FMR1, and phosphatase and tensin homolog (PTEN)

296   (52,53) that are known to be required in the MB for memory formation. PTEN has been

297   implicated in murine social learning models, though it has not been tested in a social learning

298   assay in Drosophila (54) . We used the MB Gene-Switch to knockdown expression only during

299   the cohabitation period, after which expression was allowed to resume. *D. ananassae* learn the

300   dialect of each of these three knockdown lines, again suggesting that MB mediated processes in

301   *D. melanogaster* are not necessary for *D. ananassae* dialect-training (Fig. S 12 C-G). However,

302   under these conditions we find that functional Orb2 and PTEN are required for dialect learning

303   in *D. melanogaster*, but FMR1 is dispensable (Fig. 6 F-H). Orb2 and FMR1 were previously

304   shown to be important in the teacher-student transmission of a wasp threat, and knockdown of

305   either gene completely ablated students learning from teacher flies. In this case, partial

306   communication between *D. ananassae* teachers and *D. melanogaster* students can occur because

307   Orb2 and PTEN expression is restored after the dialect-training period, thus functioning as wild-

308   type *D. melanogaster*. *D. melanogaster* flies having undergone knockdown of Orb2 and PTEN

309   only during dialect-training are able to communicate with and function as students to wild-type

310   *D. melanogaster* after the cohabitation period is completed, suggesting the partial

311   communication phenotype observed with *D. ananassae* teachers is a result of gene knockdown

312   during cohabitation and not a by-product of irreversible cellular damage or death caused by the

313   RNAi treatments (Fig. S 12 H-L). Collectively, these data show critical gene products are

314   required to function in the MB for dialect learning during the training period. Importantly, MB

315   function and active learning are not necessary in *D. melanogaster* in order to in turn provide cues

316   enabling dialect learning by a wild-type *D. ananassae* student.

317

318 **DISCUSSION**

319   In this study, we present an evolutionarily conserved response to predatory wasps across

320 the genus Drosophila, manifesting as oviposition depression coincident with an activated effector

321 caspase, Dcp-1. We have shown that flies communicate a wasp threat through visual cues. Inter-

322 species communication occurs to varying degrees, likely dependent on evolutionary relatedness.

323 Closely related species, such as *D. melanogaster* and *D. simulans*, *D. ananassae* and *D.*

324 *kikkawai*, and *D. mojavensis* and *D. virilis*, communicate as effectively as conspecifics. Species

325 more distantly related to *D. melanogaster* exhibit only partial communication or lack the ability

326 to confer predator information with *D. melanogaster*. When two species are only able to partially

327 communicate, they can learn each other's dialect after a period of cohabitation, yielding inter-

328 species communication enhanced to levels normally observed among conspecifics. Although

329 dialect learning facilitates inter-species communication across broad evolutionary distances, the

330 ability to learn a specific dialect is dependent on relatedness of the two species (Fig. 7 A). This

331 observation of the role of phylogenetic distance influencing dialect learning is true in cases both

332 utilizing *D. melanogaster* and *D. ananassae* in combination with other species tested (Fig. 7 A,

333 Fig. S 13). The observation that different strains of the same species exhibit this partial

334 communication that can then be enhanced by cohabitation, suggests that both social

335 communication and dialect learning are innate behaviors conserved among all Drosophilids

336 tested here (Fig. 7 A, Fig. S 13). Multiple strains of *D. melanogaster* reared in the laboratory for

337 many decades exhibit this behavior, supporting the idea that this is an innate behavior. Thus,

338 adult Drosophila neuronal plasticity allows for learning of dialects, but the specific dialect

339 learned is dependent on social interactions specific to a communal environmental context that

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340   provides both visual and olfactory inputs. This same plasticity allows for the learning of multiple

341   dialects in a given environment. It is remarkable that communal rearing of two species can

342   enhance communication about a predator that is yet to be experienced by either species.

343   Furthermore, dialect-learning does not trigger Dcp-1 activation and oviposition depression,

344   suggesting that social communication about predator presence is different from social

345   interactions that enable dialect-learning that later enhances predator presence communication.

346   We propose dialect-learning to be a novel behavior requiring visual and olfactory inputs,

347   perhaps integrated in and relayed through the MB, resulting in the ability to more efficiently

348   receive information about a common predator. Without dialect learning, this information would

349   otherwise be lost in translation or muddled, resulting in an inefficient behavioral response with

350   significant survival disadvantages. Inhibiting synaptic transmission and knockdown of key

351   learning and memory genes in the MB demonstrates that these inputs must be processed and

352   consolidated in the MB, although input neuronal signaling is initiated from the visual and

353   olfactory systems (Fig. 7 B). Given the need for multiple sensory inputs, dialect learning is

354   fundamentally different from the previously described teacher-student paradigm, where visual

355   cues are necessary and sufficient for information exchange (17) . Additionally, we suggest that

356   this study also points to previously unappreciated functions of the Drosophila MB in integrating

357   information from multiple olfactory and visual inputs. Such cognitive plasticity that allows for

358   dialect learning from many different species hints that adult behaviors could only emerge in a

359   manner that is dependent on previous social experiences where relevant ecological pressures are

360   ever present and multiple species co-exist in nature. Thus, there is a real benefit to cognitive

361   plasticity, where sharing of information directly, or by coincident bystanders, could result in

362   behavioral immunity to pan-specific threats.

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363   The specific information shared by different species during dialect learning is not known.

364   This study, however, provides important clues as the complex suite of sensory systems and cues

365   that may be required for efficient dialect learning. Visual sensory input is critical in dialect

366   learning and it is intriguing that both wing movement and full spectrum light are essential. This

367   observation raises the very interesting possibility that dialect learning may require wing

368   interference patterns (WIPs) *via* wing movement in the presence of full spectrum light (55,56)

369   (Fig. 7 B). WIPs are known to be produced by species-specific wing patterns and light

370   diffraction abilities and in Drosophila are a source of information for making mate choice

371   decisions (CITE). Given that visual, wing movement based cues are required for dialect learning,

372   we speculate that in full spectrum light WIPs could facilitate dialect learning in closely related

373   species, while more divergent WIPs could also prohibit distantly related species from

374   communicating at all.

375   We have presented an example of how inter-species social communication and dialect

376   learning in Drosophila can lead to changes in germline physiology and reproductive behavior.

377   What other ethological behaviors are modulated by MB functions and social interactions

378   typically not revealed in laboratory monocultures? We suggest that the Drosophila MB may

379   integrate a myriad of social and environmental cues in order to produce ethologically relevant

380   behavior that is responsive and useful to local environmental conditions.

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513   **FIGURE LEGENDS**

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535   **Figure 1.** A predator threat is communicated through visual cues within species across the genus

536   Drosophila, modulating reproductive behavior and caspase activation.

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538   (A) Standard experimental design. (B) Percentage of eggs laid by exposed flies normalized to

539   eggs laid by unexposed flies is shown. Wild-type *D. melanogaster* (Canton S) exposed to wasps

540   lay fewer eggs than unexposed flies. (C) Phylogeny of 8 species tested across the genus

541   Drosophila that demonstrate the ability to communicate through visual cues. Green boxes

542   indicate social learning is present in species tested. Representative ovary of control and wasp

543   exposed Drosophila showing caspase activation (*D. melanogaster*). DAPI (D, H), activated Dcp-

544   1 (E, I), WGA (F,J), and the merged images (G, K) are shown. Arrows denote apoptotic egg

545   chambers. Error bars represent standard error (n = 12 biological replicates) (*p < 0.05).

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558   **Figure 2**. Interspecies communication of predator threats.

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560   Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

561   Flies exposed to wasps lay fewer eggs than unexposed flies. Communication between *D.*

562   *melanogaster* and: *D. simulans* (A, B), *D. ananassae* (C, D), *D. kikkawai* (E,F), *D. willistoni*

563   (G,H), and *D. virilis* (I,J) shows varying communication abilities.. Communication between *D.*

564   *virilis* and *D. mojavensis* occurs (K,L). Error bars represent standard error (n = 12 biological

565   replicates) (*p < 0.05).

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581   **Figure 3**. Species cohabitation enables inter-species communication.

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583   (A) Experimental design of dialect training for flies that are used as students. Two species are

584   cohabitated for one week prior to being used as students for naive, untrained teacher flies of the

585   opposite species. Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed

586   flies is shown. Communication between trained students *D. melanogaster* and: *D. ananassae*

587   showing strong communication following cohabitation (B, C), *D. kikkawai* showing strong

588   communication following co-incubation (D, E), *D. willistoni* showing partial communication

589   following co-incubation (F,G), *D. equinoxialis* showing partial communication following co-

590   incubation (H,I), and *D. virilis* showing no communication following co-incubation (J,K). Error

591   bars represent standard error (n = 12 biological replicates) (*p < 0.05).

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604   **Figure 4**. Flies can learn multiple dialects.

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606   (A) Experimental design of dialect training for flies that are used as students using multiple three

607   unique species Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed

608   flies is shown. Communication between *D. melanogaster* students trained by *D. ananassae* and

609   *D. willistoni*, shows that *D. melanogaster* learn each species dialect even in the presence of more

610   than one species (B, C). Communication between *D. ananassae* students trained by *D.*

611   *melanogaster* and *D. willistoni*, shows that *D. ananassae* learn each species dialect even in the

612   presence of more than one species (D, E). Communication between *D. willistoni* students trained

613   by *D. melanogaster* and *D. ananassae*, shows that *D. willistoni* learn each species dialect even in

614   the presence of more than one species (F, G). Error bars represent standard error (n = 12

615   biological replicates) (*p < 0.05).

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627 **Figure 5**. Dialect training requires multiple sensory inputs.

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629 (A) Experimental design of dialect training for flies that are used as students using only visual

630 cues (panels B,C). Flies only see each other through the duplex, with no direct interaction. Two

631 species are co-incubated for one week prior to being used as students. Percentage of eggs laid by

632 exposed flies normalized to eggs laid by unexposed flies is shown. Communication between

633 trained students *D*. *melanogaster* and *D*. *ananassae* with training through visual cues only,

634 shows that visual cues are not sufficient (B, C). Communication between trained students *D*.

635 *melanogaster* and *D*. *ananassae* with training through monochromatic, red light only, shows a

636 lack of dialect training (D, E). Communication between trained students ewg^{NS4}, mutant flies,

637 and *D*. *ananassae* shows that moving wings are necessary (F, G). Communication between

638 trained students $Orco^{1}$ and *D*. *ananassae* shows that olfactory cues are necessary (H, I).

639 Communication between trained students $Ir8a^{1}$ and *D*. *ananassae* shows that *Ir8a* is a necessary

640 receptor (J, K). Communication between trained students *D*. *melanogaster* and *D*. *ananassae*

641 with training by male *D*. *melanogaster* only or by female *D*. *melanogaster* only, is not sufficient

642 for dialect training (L,M). Error bars represent standard error (n = 12 biological replicates) (*p <

643 0.05).

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649   **Figure 6**. Genetic perturbations reveal a critical role of the mushroom body and memory

650   proteins for dialect learning.

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652   (A) Experimental design of dialect training for flies being fed RU486 or methanol that are used

653   as students. Both species are fed either RU486 or methanol during dialect training. Two species

654   are co-incubated for one week prior to being used as students for naive, untrained teacher flies of

655   the opposite species. Standard Drosophila media is used once the training period is over.

656   Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

657   Communication between trained students *D. melanogaster* and *D. ananassae* trained by flies

658   expressing tetanus toxin (UAS-TeTx) in the mushroom body (MB) shows that the MB serves a

659   critical role during the training period. *D. ananassae* learn from *D. melanogaster* with an

660   inhibited MB, demonstrating that a functional MB is not needed to confer information during the

661   training period (B, C). Communication between trained students $Orb2^{\Delta Q}$ and *D. ananassae*

662   shows that Orb2 is required in students, but is dispensable for teachers to *D. ananassae* (D, E).

663   Communication between *D. ananassae* and students co-incubated with *D. ananassae* that have

664   RNAi-mediated Orb2 knockdown in the MB through RU486 feeding shows that the MB requires

665   Orb2 during the training period (F). Communication between *D. ananassae* and students co-

666   incubated with *D. ananassae* that have RNAi-mediated FMR1 knockdown (strain #24944) in the

667   MB through RU486 feeding shows that FMR1 is not required in the MB during the training

668   period (G). Communication between *D. ananassae* and students co-incubated with *D. ananassae*

669   that have RNAi-mediated PTEN knockdown in the MB through RU486 feeding shows that

670   PTEN is required in the MB during the training period (H). Error bars represent standard error (n

671   = 12 biological replicates) (*$p < 0.05$).

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672   **Figure 7**. Phylogenetic summary of dialect learning and pathway model for interspecies social

673   learning.

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675   Utilizing species across the genus Drosophila (A) demonstrates conservation of oviposition

676   depression following wasp exposure, mediated by activated Dcp-1 to varying degrees and with

677   varying expression patterns. The ability to communicate with *D. melanogaster* and the ability to

678   demonstrate interspecies communication varies across the genus, with species closely related to

679   *D. melanogaster* able to communicate without barriers. More distantly related species have

680   difficulty communicating, though the barrier can be alleviated with dialect training. Finally,

681   some species are too distantly related to communicate even after dialect training. Double boxes

682   in a given row and column indicate multiple wild-type strains were tested. Interspecies

683   communication is dependent on the presence of both male and female flies, the visual and

684   olfactory systems, the mushroom body, and various long-term memory gene products (B). This

685   model is based of the use of *D. melanogaster* and *D. ananasse*. Alleles tested in (B) are: Orco[1],

686   Ir8a[1];Ir25a[2];Orco[1], Ir8a[1], Ir25a[2], ninaB[P315], Orb2ΔQ, ewg[NS4], Orb2[RNAi],

687   PTEN[RNAi], FMR1[RNAi] , and UAS-TeTx.

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695   **SUPPLEMENTARY FIGURE LEGENDS**

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718   **Supplementary Figure 1**. Intra-species communication is present across the genus Drosophila.

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720   Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

721   Species shown are (A) *D. melanogaster* (Oregon-R), (B) *D. simulans*, (C) *D. ananassae*, (D) *D.*

722   *kikkawai*, (E) *D. willistoni*, (F) *D. equinoxialis*, (G) *D. mojavensis*, and (H) *D. virilis*. Error bars

723   represent standard error (n = 12 biological replicates) (*p < 0.05).

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741   **Supplementary Figure 2.** Activated Dcp-1 is indicative of apoptotic events in *D. melanogaster*.

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743   Magnified images from Figure 1 (H-K) showing apoptotic egg chamber displaying activated

744   caspase. DAPI (A), activated Dcp-1 (B), WGA (C) and merge are shown (D). Additional

745   representative ovaries of unexposed and wasp-exposed *D. melanogaster* are shown. DAPI (E, I,

746   M, Q, U), activated Dcp-1 (F, J, N, R,V), WGA (G, K, O, S, W), and the merged images (H, L,

747   P, T, X) are shown. Arrows denote apoptotic egg chambers.

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763   **Supplementary Figure 3**. Increases in activated caspase are observed in the ovary across the

764   genus Drosophila following wasp exposure.

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766   Representative images of unexposed and wasp-exposed ovaries stained for activated Dcp-1 for

767   *D. yakuba* (A-F), *D. tsacasi* (G-L), and *D. equinoxialis* (M-R). DAPI, Dcp-1, and the merged

768   images are shown. The broad range of staining patterns observed in these species is

769   representative of other species tested.

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786   **Supplementary Figure 4**. Increases in activated caspase are quantified in the ovary across the

787   genus Drosophila following wasp exposure.

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789   Proportion of egg chambers with Dcp-1 signal shown for (A) *D. melanogaster*, (B) *D. simulans*,

790   (C) *D. mauritiana*, (D) *D. sechellia*, (E) *D. yakuba*, (F) *D. tsacasi*, (G) *D. kikkawai*, (H) *D.*

791   *ananassae*, (I) *D. pseudoobscura*, (J) *D. neocordata*, (K) *D. equinoxialis*, (L) *D. willistoni*, (M)

792   *D. immigrans*, (N) *D. mojavensis*, and (O) *D. virilis*. Error bars represent standard error (n = 36

793   ovaries) (*p < 0.05).

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808   **Supplementary Figure 5**. A decrease in egg chamber numbers are quantified in the ovary across

809   the genus Drosophila following wasp exposure.

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811   Total number of egg chambers shown for (A) *D. melanogaster*, (B) *D. simulans*, (C) *D.*

812   *mauritiana*, (D) *D. sechellia*, (E) *D. yakuba*, (F) *D. tsacasi*, (G) *D. kikkawai*, (H) *D. ananassae*,

813   (I) *D. pseudoobscura*, (J) *D. neocordata*, (K) *D. equinoxialis*, (L) *D. willistoni*, (M) *D.*

814   *immigrans*, (N) *D. mojavensis*, and (O) *D. virilis*. Error bars represent standard error (n = 36

815   ovaries) (*p < 0.05).

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831   **Supplementary Figure 6**. Interspecies communication of predator threats

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833   Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

834   Communication between *D. melanogaster* and: *D. sechellia* (A, B), *D. mauritianna* (C, D), *D.*

835   *yakuba* (E, F), *D. tsacasi* (G, H), *D. pseudoobscura* (I, J), *D. neocordata* (K, L), *D. immigrans*

836   (M, N), and *D. mojavensis* (O, P), shows varying communication abilities.. Error bars represent

837   standard error (n = 12 biological replicates) (*p < 0.05).

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854   **Supplementary Figure 7**. Interspecies communication of predator threats using *D. ananassae*.

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856   Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

857   Communication between *D. ananassae* and: *D. simulans* (A, B), *D. kikkawai* (C, D), *D.*

858   *equinoxialis* (E, F), *D. willistoni* (G, H), *D. mojavensis* (I, J), and *D. virilis* (K, L), shows varying

859   communication abilities. Error bars represent standard error (n = 12 biological replicates) (*p <

860   0.05).

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876 **Supplementary Figure 8.** Cohabitation of additional species with *D. melanogaster* allows for

877 interspecies communication.

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879 Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

880 Communication between trained students *D. melanogaster* and: *D. ananassae* (second line) (A-

881 C), *D. tsacasi* (D, E), *D. pseudoobscura* (F, G), *D. neocordata* (H, I), *D. immigrans* (J, K), and

882 *D. mojavensis* (L, M). (C) An additional *D. melanogaster* line (w^{1118}) learns from w^{1118} trained

883 *D. ananassae*. Communication between *D. ananassae* and a transgenic *D. melanogaster*

884 (Histone-RFP) occurs following training period (N, O). Error bars represent standard error (n =

885 12 biological replicates) (*$p < 0.05$).

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898   **Supplementary Figure 9.** Cohabitation of additional species with *D. ananassae* allows for

899   interspecies communication.

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901   Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

902   Communication between trained students *D. ananassae* and: *D. simulans* (A,B), *D. equinoxialis*

903   (C,D), *D. willistoni* (E,F), *D. mojavensis* (G,H), and *D. virilis* (I,J). Error bars represent standard

904   error (n = 12 biological replicates) (*p < 0.05).

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920   **Supplementary Figure 10**. Additional evidence demonstrating that dialect training requires

921   visual cues.

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923   (A) Experimental design of dialect training for flies that are used as students using no visual cues

924   by running the dialect training period in the dark (B,C). Flies do not see each other, but still

925   interact and innervate other sensory inputs. The two species are co-incubated for one week prior

926   to being used as students for naive, untrained teacher flies of the opposite species. Percentage of

927   eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown. Communication

928   between trained students *D*. *melanogaster* and *D*. *ananassae* with training involving no visual

929   cues (dark-trained), shows that visual cues necessary for dialect learning (B, C). Communication

930   between trained students *D*. *ananassae* and the mutant *ninaB* (D,E). Communication between

931   trained students *D*. *melanogaster* and *D*. *ananassae*, with training in monochromatic blue light

932   only, shows a lack of dialect training (F, G). Communication between trained students of *D*.

933   *ananassae* and *D*. *melanogaster* at 4.0_8 light intensity shows communication (H,I). Error bars

934   represent standard error (n = 12 biological replicates) (*p < 0.05).

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943 **Supplementary Figure 11**. Further evidence demonstrating that dialect training requires

944 multiple sensory inputs including olfactory cues and duration of training.

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946 Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

947 Communication between naïve *D. ananassae* and *Ir8a^1* mutant flies shows partial

948 communication (A). Communication between naive students of *Ir8a* knockdown in Ir8a-

949 expressing neurons and *D. ananassae* shows partial communication (B). Communication

950 between trained students *Ir8a^{RNAi}* knockdown in Ir8a expressing neurons and *D. ananassae*

951 shows that IR8a receptor-mediated cues are necessary (C, D). Communication between naïve

952 *Ir25a^2* mutants and *D. ananassae* shows partial communication (E). Communication between

953 trained students *Ir25a^2* mutants and *D. ananassae* shows communication suggesting that IR25a

954 receptors are not required for dialect training (F,G). Communication between naïve *Ir25a*

955 knockdown in Ir25a-expressing neurons and *D. ananassae* shows partial communication (H).

956 Communication between trained students *Ir25a^{RNAi}* knockdown in Ir25a-expressing neurons and

957 *D. ananassae* shows communication suggesting that IR25a receptors are not required for dialect

958 training (I, J). Communication between trained *Ir8a^1;Ir25a^2;Orco^1* students and *D. ananassae*

959 shows that olfactory and IR-receptor mediated cues are necessary (K, L). Communication

960 between students *D. melanogaster* and *D. ananassae*, with training by males only or by females

961 only, shows partial communication, suggesting that both male and female flies are required for

962 dialect learning (M, N). Communication between trained students *D. melanogaster* and *D.*

963 *ananassae*, with training for only one day, shows that 24 hours is not sufficient for dialect

964 training (O, P). Error bars represent standard error (n = 12 biological replicates) (*p < 0.05).

965 **Supplementary Figure 12**. Further evidence showing a critical role for the mushroom body and

966 memory proteins for dialect learning.

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968 Percentage of eggs laid by exposed flies normalized to eggs laid by unexposed flies is shown.

969 Communication between trained *D. melanogaster*, MBswitch/+ (outcrossed to *Canton S*)

970 students and *D. ananassae* teachers fed RU486 during the training period shows communication

971 between the two species, demonstrating that RU486 feeding does not perturb dialect learning

972 (A). Communication between trained students *D. melanogaster* and *D. ananassae*, with training

973 by flies not expressing tetanus toxin (UAS-TeTx) in the mushroom body (MB) (i.e. methanol

974 fed), shows communication between the species (B). Communication between *D. ananassae* and

975 students trained with *D. ananassae* with no RNAi-mediated Orb2 knockdown in the MB (i.e.

976 methanol fed) shows communication between the species (C). Communication between *D.*

977 *ananassae* and students trained with *D. ananassae* with RNAi-mediated FMR1 knockdown

978 (strain #34944) in the MB (i.e. RU486 fed) shows that FMR1 is not required in the MB during

979 the training period (D). Communication between *D. ananassae* and students trained with *D.*

980 *ananassae* with no FMR1 knockdown (strain #24944) in the MB (i.e. methanol fed) shows wild-

981 type behavior (E). Communication between *D. ananassae* and students trained with *D.*

982 *ananassae* with no FMR1 knockdown (strain #24944) in the MB (i.e. methanol fed) shows wild-

983 type behavior (F). Communication between *D. ananassae* and students trained with *D.*

984 *ananassae* with no PTEN knockdown in the MB (i.e. methanol fed) shows wild-type behavior

985 (G). Error bars represent standard error (n = 12 biological replicates) (*p < 0.05).

986 Communication between various *D. melanogaster* lines trained by *D. ananassae* show wild-type

987 communication with *D. melanogaster* (*Canton S*). Lines shown are MB switch expressing TeTx

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988   (H), Orb2^{RNAi} (I), FMR1^{RNAi} (strain number 27484) (J), FMR1^{RNAi} (strain number 34944) (K),

989   PTEN^{RNAi} (L), and were fed RU486 during cohabitation with *D*. *ananassae*.

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1011 **Supplementary Figure 13**. Phylogenetic summary of dialect learning for *D. ananassae*.

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1013 We utilize species across the genus Drosophila to show communication ability of *D. ananassae*

1014 (A). We observe the ability to demonstrate interspecies communication, which varies across the

1015 genus, with species closely related to *D. ananassae* able to communicate without barriers. More

1016 distantly related species have difficulty communicating, though the barrier can be alleviated with

1017 dialect training. Double boxes in a given row and column indicate multiple wild-type strains

1018 were tested.

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1034   **SUPPLEMENTARY TABLE LEGENDS**

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1057   **Supplementary Table 1**. Fly lines and species used in this study.

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1080 **MATERIALS AND METHODS**

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1082 <u>Insect Species/Strains</u>

1083   The *D. melanogaster* strains Canton-S (CS), Oregon-R (OR), white^{1118}(w^{1118}), and

1084 transgenic flies carrying Histone H2AvD-RFP (His-RFP) were used as wild-type strains.

1085 Experiments were primarily performed using CS as wild type flies except where otherwise

1086 indicated. $Orco^1$($Or83b^1$), UAS-TeTx, UAS-Orb2^{RNAi}, UAS-FMR1^{RNAi}, UAS-FMR1^{RNAi}, UAS-

1087 PTEN^{RNAi}, UAS-Ir8a^{RNAi}, UAS-Ir25a^{RNAi}, $ninaB^{P315}$ were acquired from the Bloomington

1088 Drosophila Stock Center (stock numbers 23129, 28838, 27050, 27484, 34944, 25841, 25813,

1089 43985, and 24776 respectively). Drosophila species were acquired from the Drosophila Species

1090 Stock Center (DSSC) at the University of California, San Diego. Flies and their respective stock

1091 numbers are listed: *D. simulans* (14021-0251.196), *D. mauritiana* (14021-0241.01), *D. sechellia*

1092 (14021-0248.25), *D. yakuba* (14021-0261.01), *D. tsacasi* (14028-0701.00), *D. kikkawai* (14028-

1093 0561.00), *D. ananassae* (14024-0371.13 and 14024-0371.11), *D. pseudoobscura* (14011-

1094 0121.00), *D. neocordata* (14041-0831.00), *D. equinoxialis* (14030-0741.00), *D. willistoni*

1095 (14030-0811.00), *D. immigrans* (15111-1731.08), *D. mojavensis* (15081-1352.22), and *D. virilis*

1096 (15010-1051.87). All experiments with *D. ananassae* used strain number 14024-0371.13 unless

1097 otherwise noted (Table S 1).

1098   The ewg^{NS4} mutant line was kindly provided by Yashi Ahmed (Geisel School of

1099 Medicine at Dartmouth). The mushroom body Gene-Switch line was kindly provided by Greg

1100 Roman (Baylor College of Medicine). $Ir8a^1$, $Ir25a^2$, $Ir8a{>}GAL4$, $Ir25a{>}GAL4$ and

1101 $Ir8a^1;Ir25a^2;Orco^1$ lines were kindly provided by Greg S. B. Suh (Skirball Institute at NYU).

1102 Flies aged 3-6 days post-eclosion on fresh Drosophila media were used in all experiments. Flies

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1103   were maintained at room temperature with approximately 30% humidity. All species and strains

1104   used were maintained in fly bottles (Genesse catalog number 32-130) containing 50 mL of

1105   standard Drosophila media. Bottles were supplemented with 3 Kimwipes rolled together and

1106   placed into the center of the food. Drosophila media was also scored to promote oviposition. Fly

1107   species stocks were kept separate to account for visual cues that could be conferred if the stocks

1108   were kept side-by-side.

1109   The Figitid larval endoparasitoid *Leptopilina heterotoma* (strain Lh14) was used in all

1110   experiments. *L. heterotoma* strain Lh14 originated from a single female collected in Winters,

1111   California in 2002. In order to propagate wasp stocks, we used adult *D. virilis* in batches of 40

1112   females and 15 males per each vial (Genesse catalog number 32-116). Adult flies were allowed

1113   to lay eggs in standard Drosophila vials containing 5 mL standard Drosophila media

1114   supplemented with live yeast (approximately 25 granules) for 4-6 days before being replaced by

1115   adult wasps, using 15 female and 6 male wasps, for infections. These wasps deposit eggs in

1116   developing fly larvae, and we gave them access specifically to the L2 stage of *D. virilis* larvae.

1117   Wasp containing vials were supplemented with approximately 500 µL of a 50% honey/water

1118   solution applied to the inside of the cotton vial plugs. Organic honey was used as a supplement.

1119   Wasps aged 3-7 days post eclosion were used for all infections and experiments. Wasps were

1120   never reused for experiments.

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1122   Fly Duplexes

1123   Briefly, fly duplexes were constructed (Desco, Norfolk, MA) by using three standard

1124   25mm x 75mm pieces of acrylic that were adhered between two 75mm x 50mm x 3mm pieces of

1125   acrylic. Clear acrylic sealant was used to glue these pieces together, making two compartments

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1126   separated by one 3mm thick acrylic piece. Following sealant curing, each duplex was soaked in

1127   water and Sparkleen detergent (Fisherbrand™ catalog number 04-320-4) overnight, then soaked

1128   in distilled water overnight and finally air-dried. The interior dimensions of each of the two units

1129   measured approximately 23.5mm (wide) x 25mm (deep) x 75mm (tall).

1130   For experiments using Fly Duplexes (teacher-student interaction), bead boxes (6 slot

1131   jewelers bead storage box watch part organizer sold by FindingKing) were used to accommodate

1132   12 replicates of each treatment group. Each compartment measures 32 x 114 mm with the tray in

1133   total measuring 21 x 12 x 3.5 mm. Each compartment holds 2 duplexes, and the tray in total

1134   holds 12 duplexes. Empty duplexes were placed into the bead box compartments. 50 mL

1135   standard Drosophila media in a standard Drosophila bottle (Genesse catalog number 32-130) was

1136   microwaved for 39 seconds. This heated media was allowed to cool for 2 minutes on ice before

1137   being dispensed. Each duplex unit was then filled with 5 mL of the media and further allowed to

1138   cool until solidification. The open end of the Fly Duplex was plugged with a cotton plug

1139   (Genesse catalog number 51-102B) to prevent insect escape. 10 female flies and 2 male flies

1140   were placed into one chamber of the Fly Duplex in the control, while 20 female Lh14 wasps

1141   were placed next to the flies in the experimental setting for 24 hours. After the 24-hour exposure,

1142   flies and wasps were removed by anesthetizing flies and wasps in the Fly Duplexes. Control flies

1143   underwent the same anesthetization. Wasps were removed and replaced with 10 female and two

1144   male "student" flies. All flies were placed into new clean duplexes for the second 24-hour

1145   period, containing 5 mL Drosophila media in a new bead box. For fly duplexes containing a

1146   subset of species, specifically *D*. *mojavensis*, *D*. *immigrans*, and *D*. *virilis*, 10 yeast granules

1147   were added to the standard Drosophila media after solidification of the food. This activated yeast

1148   was added to promote oviposition. Flies showed minimal oviposition in food lacking yeast. We

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1149   speculate this was observed due to the fly food being optimized for *D. melanogaster*. Plugs used

1150   to keep insects in the duplex were replaced every 24 hours to prevent odorant deposition on

1151   plugs that could influence behavior. The oviposition bead box from each treatment was replaced

1152   24 hours after the start of the experiment, and the second bead box was removed 48 hours after

1153   the start of the experiment. Fly egg counts from each bead box were made at the 0-24 and 24-48-

1154   hour time points.

1155   All experimental treatments were run at 25ºC with a 12:12 light:dark cycle at light

1156   intensity 16_7, using twelve replicates at 40% humidity unless otherwise noted. Light intensity

1157   was measured using a Sekonic L-308DC light meter. The light meter measures incident light and

1158   was set at shutter speed 120, sensitivity at iso8000, with a 1/10 step measurement value (f-stop).

1159   Fly duplexes and bead boxes soaked with distilled water mixed with Sparkleen after every use

1160   for 4 hours at minimum and subsequently rinsed with distilled water and air-dried. All egg plates

1161   were coded and scoring was blind as the individual counting eggs was not aware of treatments or

1162   genotypes.

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1164   <u>Dialect Exposure</u>

1165   Species were cohabitated in standard Drosophila bottles (Genesee catalog number 32-

1166   130) containing 50 mL standard Drosophila media. Three Kimwipes were rolled together and

1167   placed into the center of the food. Batches of 3 bottles were made per treatment. Two species

1168   were incubated in each bottle with 100 female and 20 males of each species per bottle. Every two

1169   days, flies were placed into new bottles prepared in the identical manner. Flies were cohabitation

1170   for approximately 168 hours (7 days), unless otherwise noted. Following cohabitation, flies were

1171   anesthetized and the two species were separated. The flies were then used as students to wasp or

1172   mock exposure teachers of the opposite species. For example, we cohabitated *D. melanogaster*

1173   and *D. ananassae* for one week. Following the weeklong cohabitation, we separated the dialect-

1174   trained flies. Trained *D. melanogaster* were placed in duplexes next to *D. ananassae* either mock

1175   or wasp exposed. Trained *D. ananassae* were placed in duplexes next to *D. melanogaster* either

1176   mock treated or wasp exposed.

1177   For experiments utilizing more than two species for dialect learning, species were

1178   cohabitated in standard Drosophila bottles (Genesee catalog number 32-130) containing 50 mL

1179   standard Drosophila media. Three Kimwipes were rolled together and placed into the center of

1180   the food. Batches of 3 bottles were made per treatment. The three species were incubated in each

1181   bottle with 100 female and 20 males of each species per bottle. Every two days, flies were placed

1182   into new bottles prepared in the identical manner. The three-fly species were cohabitation for

1183   approximately 168 hours (7 days), unless otherwise noted. Following cohabitation, flies were

1184   anesthetized and one of the three species was tested by pairing them with teachers of the other

1185   two species. For example we cohabitated *D. melanogaster*, *D. ananassae,* and *D. willistoni* for

1186   one week. Following the weeklong cohabitation, we separated the dialect-trained flies. Trained

1187   *D. melanogaster* were placed in duplexes next to either *D. ananassae* or *D. willistoni*, mock or

1188   wasp exposed.

1189   For cohabitation experiments where two species were allowed visual only cues, the Fly

1190   Duplex was utilized. The two species were co-incubated side-by-side with 100 female and 20

1191   males of each species per unit using the two chambers of the fly duplex such that the flies could

1192   only see each other. The fly duplex was placed into bead boxes, with each unit of the duplex

1193   containing 5 mL of standard Drosophila media. Every two days, flies were placed into new fly

1194   duplexes with fresh 5 mL standard Drosophila media. Following the weeklong co-incubation,

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1195   flies were anesthetized and the two species were separated. The flies were then used as students

1196   to wasp or mock exposure teachers of the opposite species.

1197   For cohabitation experiments where the two species did not have visual cues, the two

1198   species were incubated in bottles with 100 female and 20 males of each species per bottle in

1199   complete darkness. The only difference between this method and other training sessions was the

1200   lack of light—meaning flies were subject to 25ºC with 40% humidity. Every two days, flies were

1201   placed into new bottles prepared in the identical manner. Flies were exposed to light for less than

1202   30 seconds, during which they were placed into a new bottle, and immediately returned to the

1203   dark. Following the weeklong dark-cohabitation, flies were anesthetized and the two species

1204   were separated. The flies were then used as students to wasp or mock exposure teachers of the

1205   opposite species.

1206   For cohabitation experiments under monochromatic light settings, batches of 3 bottles

1207   with 100 female and 20 males of each species were placed into 27.9cm x 16.8cm x 13.7cm

1208   plastic boxes (Sterilite 1962 Medium Clip Box with Blue Aquarium Latches sold by Flikis).

1209   These boxes were externally wrapped with colored cellophane wrap, allowing only a certain

1210   wavelength of light to be transmitted into the boxes. Red and blue cellophane wraps were

1211   purchased from Amscam (Amscan Party Supplies for Any Occasion Functional Cellophane

1212   Wrap, 16' x 30", Rose Red and Spanish Blue). Cellophane wrapped boxes with bottles containing

1213   flies were subject to 25ºC with 40% humidity under the same light intensity as previous

1214   experiments. Light intensity within the red wrapped box was 11_2 and within the blue wrapped

1215   box was 11_5 measured using the Sekonic L-308DC light meter. Every two days, flies were placed

1216   into new bottles prepared in the manner described previously. Flies were exposed to broad-

1217   spectrum light for less than 30 seconds, during which they were placed into a new bottle, and

1218   immediately returned to monochromatic light. Following the weeklong monochromatic-light-

1219   cohabitation, flies were anesthetized and the two species were separated. The flies were then

1220   used as students to wasp or mock exposure teachers of the opposite species.

1221   For the one-day cohabitation experiments, batches of 3 bottles with 100 female and 20

1222   males of each species were placed at 25ºC with 40% humidity for 24 hours. Following the 24-

1223   hour cohabitation, flies were anesthetized and the two species were separated. The flies were

1224   then used as students to wasp or mock exposure teachers of the opposite species.

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1226   RU486 feeding

1227   RU486 (Mifepristone) was used from Sigma (Lot number SLBG0210V) as the ligand for

1228   Gene-Switch experiments. Dialect training bottles were prepared by directly pipetting an RU486

1229   solution onto the 3 Kimwipes in the bottle. The solution was prepared by dissolving 3.575 mg of

1230   RU486 in 800µL methanol (Fisher Scientific Lot number 141313). This solution was added to

1231   15.2 mL of distilled water. The total solution (16 mL) was thoroughly mixed and 4000 µL was

1232   pipetted onto the Kimwipe in each bottle. For bottles containing no RU486 (methanol only)

1233   800µL methanol was mixed with 15.2 mL of distilled water. The total solution (16 mL) was

1234   thoroughly mixed and 4000 µL were pipetted onto the Kimwipe in each bottle. Flies were shifted

1235   to new bottles prepared in the exact same manner every two days. Flies were cohabitated for

1236   approximately 7 days. Following cohabitation, flies were anesthetized and the two species were

1237   separated. The flies were then used as students to wasp or mock exposure teachers of the

1238   opposite species.

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1240   Immunofluorescence

1241   Ovaries were collected from flies that were placed in vials along with female wasps for

1242   experimental or no wasps for control settings. Flies were placed in batches into standard vials

1243   (Genesee catalog number 32-116) of 20 females, 2 males along with 20 female wasps for

1244   exposed vials, or simple placing 20 female and 2 male flies in vials for the unexposed treatments.

1245   Three vials were prepared to produce three replicates to account for batch effects. We observed

1246   no batch effects so each of the 12 ovaries imaged from each treatment were then counted as a

1247   replicate, thus providing an n of 36. Ovaries that were prepared for immunofluorescence were

1248   fixed in 4% methanol-free formaldehyde in PBS with 0.001% Triton-X for approximately five

1249   minutes. The samples were then washed in PBS with 0.1% Triton-X, and blocked with 2%

1250   normal goat serum (NGS) for two hours. The primary antibody, cleaved Drosophila Dcp-1

1251   (Asp216) (Cell Signaling number 9578) at a concentration of 1:100, was used to incubate the

1252   ovaries overnight at 4° C in 2% normal goat serum (NGS). The secondary antibody used was

1253   Fluorescein isothiocyanate (FITC) conjugated (Jackson Immunoresearch), and used at a

1254   concentration of 1:200 for a two-hour incubation at room temperature. This was followed by a

1255   10-minute nuclear stain with 4', 6-diamidino-2-phenylindole (DAPI). For confocal imaging of *D*.

1256   *melanogaster* ovaries, wheat germ agglutinin (WGA) was also used as a membrane marker (Fig.

1257   1 F,J, Fig. S 2).

1258

1259   Imaging

1260   A Nikon A1R SI Confocal microscope was used for imaging activated Dcp-1 caspase

1261   staining in *D*. *melanogaster* (Fig. 1 D-K, Fig. S 2). Image averaging of 4x during image capture

1262   was used for all images. A Nikon E800 Epifluorescence microscope with Olympus DP software

1263   was used to image Dcp-1 caspase staining on all other Drosophila species tested. This

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1264 microscope was also used to quantify egg chambers with Dcp-1 signal and total number of egg

1265 chambers in all species tested.

1266

1267 <u>Statistical analysis</u>

1268 Statistical tests were performed in Microsoft Excel. Welch's two-tailed t-tests were performed

1269 for data. P-values reported were calculated for comparisons between paired treatment-group and
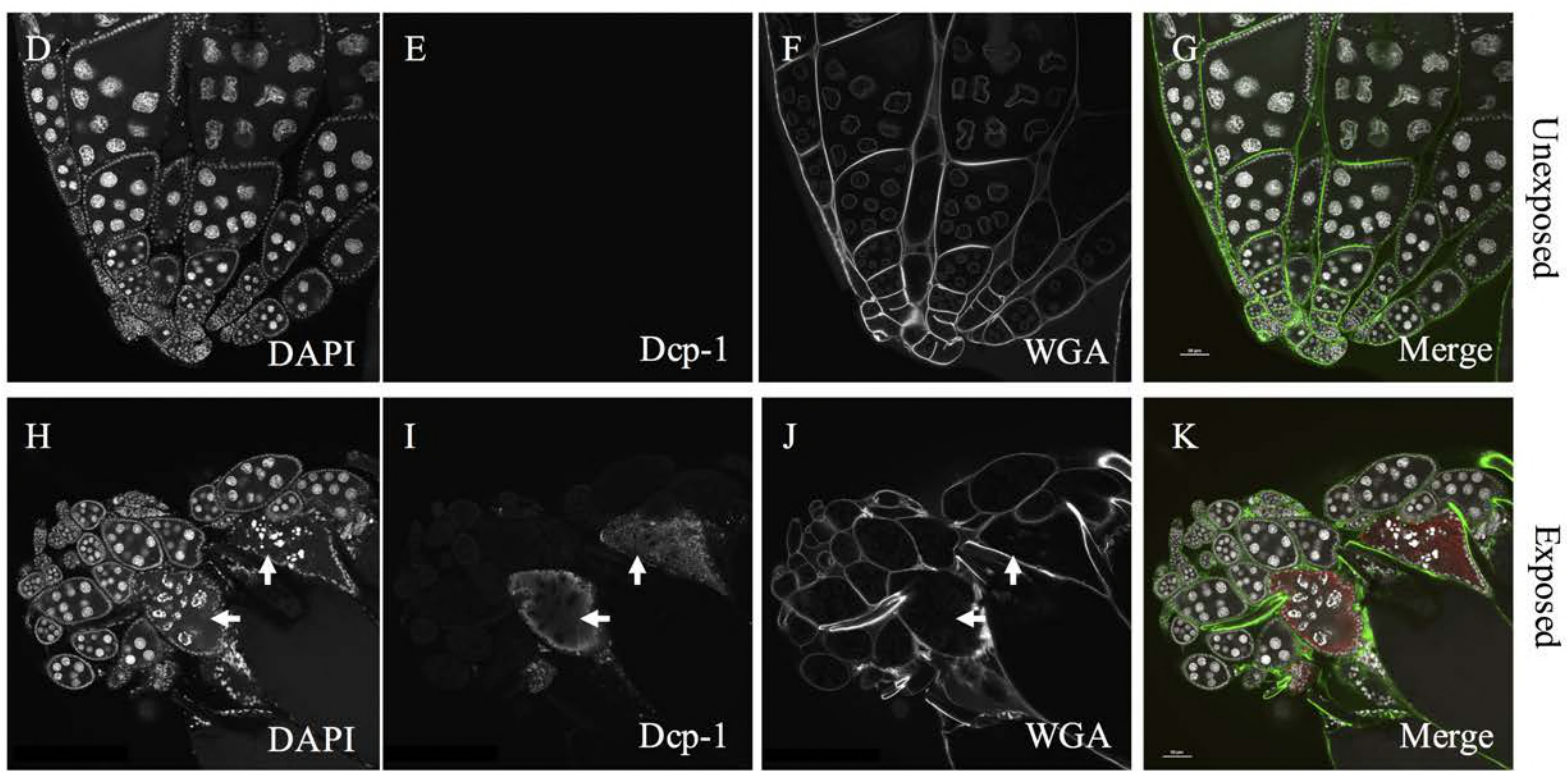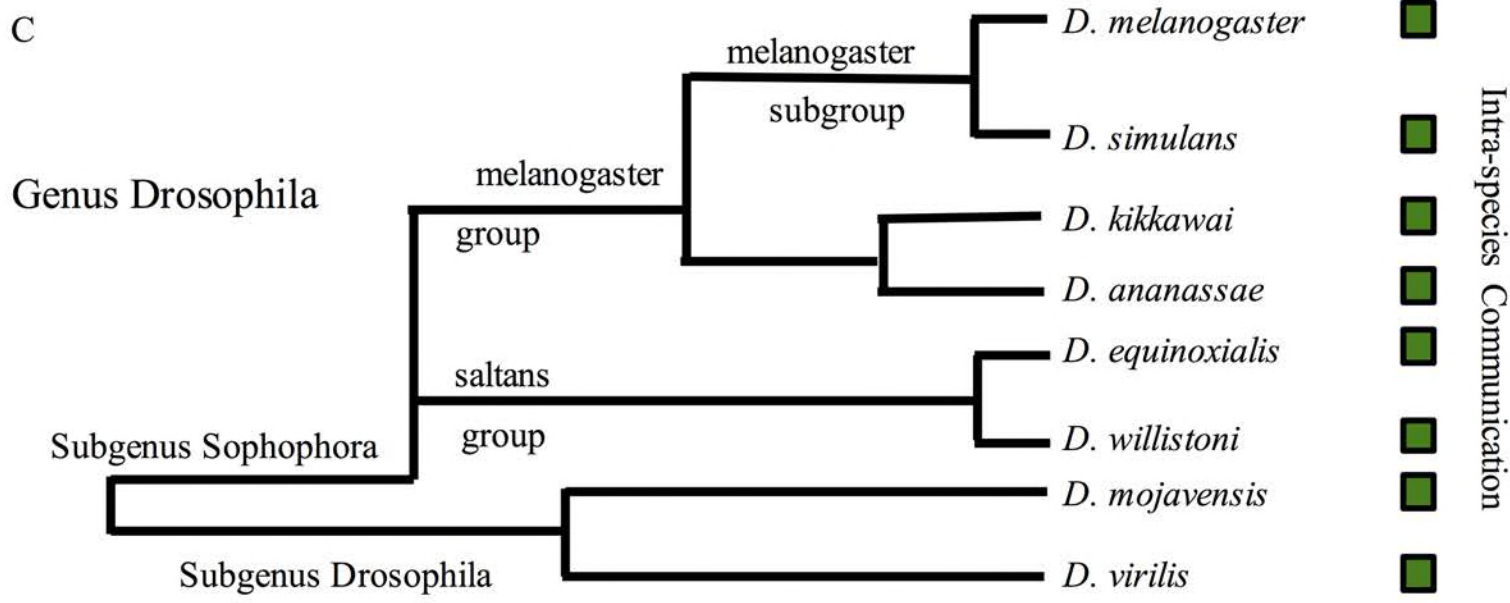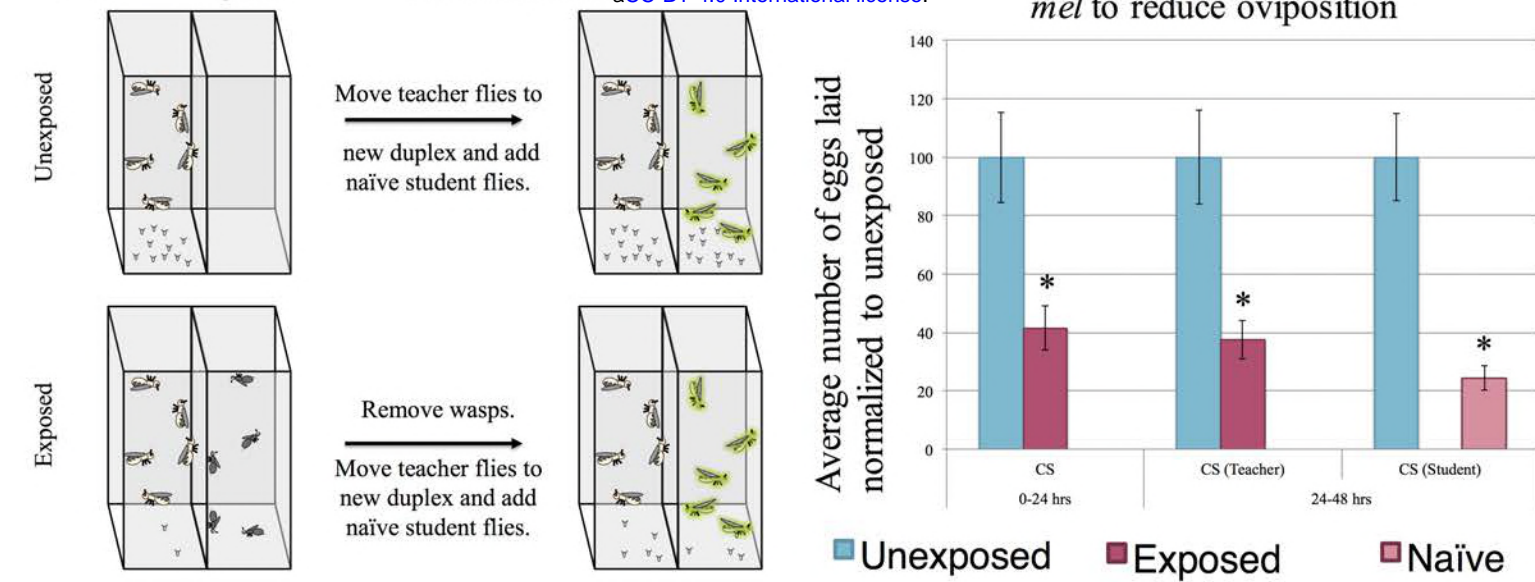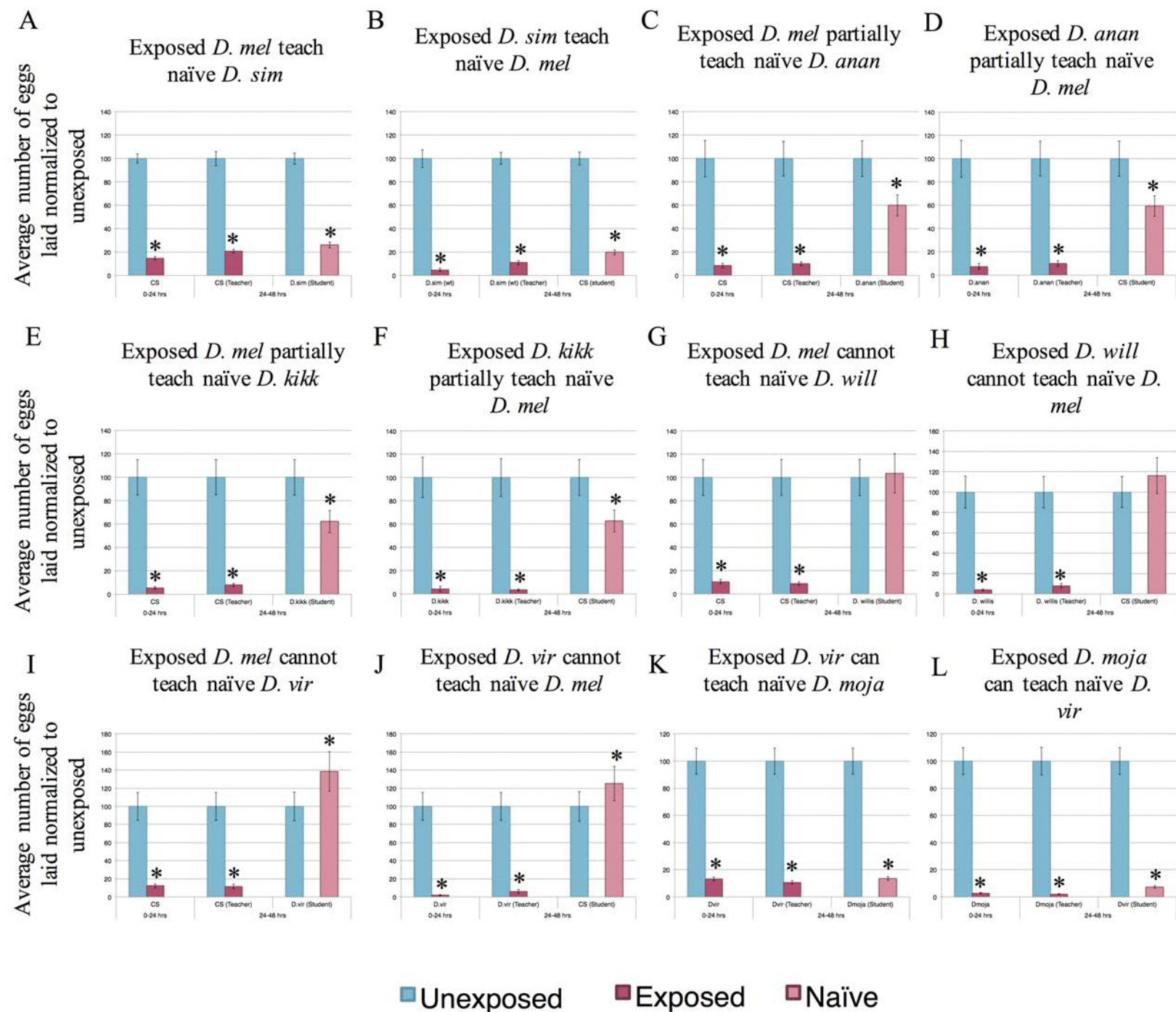
1270 unexposed.

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A Time (hrs) — acute response period — 24 — social learning and learned response period — 48

Unexposed
Move teacher flies to new duplex and add naïve student flies.

Exposed
Remove wasps.
Move teacher flies to new duplex and add naïve student flies.

B Wasp exposed *D. mel* teach naïve *D. mel* to reduce oviposition

Unexposed   Exposed   Naïve

CS (0-24 hrs)   CS (Teacher)   CS (Student) (24-48 hrs)

C

Genus Drosophila

Subgenus Sophophora

Subgenus Drosophila

melanogaster subgroup — *D. melanogaster*, *D. simulans*
melanogaster group — *D. kikkawai*, *D. ananassae*
saltans group — *D. equinoxialis*, *D. willistoni*
D. mojavensis
D. virilis

Intra-species Communication

Unexposed: **D** DAPI, **E** Dcp-1, **F** WGA, **G** Merge

Exposed: **H** DAPI, **I** Dcp-1, **J** WGA, **K** Merge

Figure with panels A–L showing average number of eggs laid normalized to unexposed.

A Exposed *D. mel* teach naïve *D. sim*

B Exposed *D. sim* teach naïve *D. mel*

C Exposed *D. mel* partially teach naïve *D. anan*

D Exposed *D. anan* partially teach naïve *D. mel*

E Exposed *D. mel* partially teach naïve *D. kikk*

F Exposed *D. kikk* partially teach naïve *D. mel*

G Exposed *D. mel* cannot teach naïve *D. will*

H Exposed *D. will* cannot teach naïve *D. mel*

I Exposed *D. mel* cannot teach naïve *D. vir*

J Exposed *D. vir* cannot teach naïve *D. mel*

K Exposed *D. vir* can teach naïve *D. moja*

L Exposed *D. moja* can teach naïve *D. vir*

Legend: Unexposed, Exposed, Naïve

Figure — A: Schematic of dialect training procedure. Unexposed and Exposed conditions. "Move teacher flies to new duplex." / "Remove wasps. Move teacher flies to new duplex." "Use naïve, untrained flies" / "Dialect training complete. Each species is now used as a student paired with an untrained teacher of the opposite species." "Two different, naïve fly species incubated side-by-side for one week. Only visual cues are transferred."

B. Exposed *D. mel* partially teach visually trained *D. anan*

C. Exposed *D. anan* partially teach visually trained *D. mel*

D. Exposed *D. mel* partially teach red light-trained *D. anan*

E. Exposed *D. anan* partially teach red light-trained *D. mel*

F. Exposed *D. mel* partially teach ewg^{NS4} –trained *D. anan*

G. Exposed *D. anan* teach trained ewg^{NS4}

H. Exposed *D. mel* teach $Orco^1$ –trained *D. anan*

I. Exposed *D. anan* partially teach trained $Orco^1$

J. Exposed *D. mel* teach $Ir8a^1$ –trained *D. anan*

K. Exposed *D. anan* partially teach trained $Ir8a^1$

L. Exposed *D. mel* partially teach ♂– trained *D. anan*

M. Exposed *D. mel* partially teach ♀– trained *D. anan*

Y-axis (B–M): Average number of eggs laid normalized to unexposed

Legend: ■ Unexposed ■ Exposed ■ Naïve

Exposed

Unexposed

Unexposed

Exposed

Exposed

Exposed

DAPI

Dcp-1

WGA

Merge

DAPI
Dcp-1
Merge

Figure legend: A. Time (hrs) 0 — acute response period — 24 — social learning and learned response period — 48 | 168 — dialect training period — 0 Time (hrs). Unexposed: Use naïve, untrained flies; Move teacher flies to new duplex. Exposed: Remove wasps. Move teacher flies to new duplex. Dialect training complete. Each species is now used as a student paired with an untrained teacher of the opposite species. Two different, naïve fly species cohabitated for one week in complete darkness.

B. Exposed *D. mel* partially teach dark—trained *D. anan*
C. Exposed *D. anan* partially teach dark—trained *D. mel*
D. Exposed *D. anan* cannot teach *D. anan*—trained *ninaB*
E. Exposed *D. mel* partially teach *ninaB*—trained *D. anan*
F. Exposed *D. mel* partially teach blue light—trained *D. anan*
G. Exposed *D. anan* partially teach blue light—trained *D. mel*
H. Exposed *D. mel* teach *D. mel*—trained *D. anan*
I. Exposed *D. anan* teach *D. anan*—trained *D. mel*

Y-axis: Average number of eggs laid normalized to unexposed

Legend: Unexposed | Exposed | Naïve

A

Naïve Communication with D. ananassae

Dialect Learned Communication with D. ananassae

Genus Drosophila

melanogaster subgroup

melanogaster group

D. melanogaster

D. simulans

D. kikkawai

D. ananassae

saltans group

D. equinoxialis

D. willistoni

Subgenus Sophophora

D. mojavensis

Subgenus Drosophila

D. virilis

KEY   ■ *Strong oviposition reduction response*   ☐ *Intermediate oviposition reduction response*   ■ *No oviposition reduction response*