Title: Rapid evolution of learning and reproduction in natural populations of *Drosophila melanogaster*

Authors: Emily L. Behrman[1,2,3], Tadeusz J. Kawecki[2*] and Paul Schmidt[1*]

Author Affiliations:
1. Department of Biology, University of Pennsylvania, 433 S. University Ave. Philadelphia, PA 19104
2. Department of Ecology and Evolution, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland
3. Current address: Janelia Research Campus, Howard Hughes Medical Institute, 19700 Helix Drive Ashburn, VA 20147
* Authors contributed equally

Orcid IDs:
Emily Behrman: 0000-0002-2472-9635
Tadeusz Kawecki: 0000-0002-9244-1991
Paul Schmidt: 0000-0002-8076-6705

Running head: Rapid evolution of learning

Corresponding Author:
Emily L. Behrman
Email Address: Bemily@sas.upenn.edu
Mailing address: 19700 Helix Drive
Ashburn, VA 20147
Phone: 571.209.4000

Keywords
Rapid adaptation, learning, fecundity, trade-offs, *Drosophila melanogaster, couch potato*

Abstract

Learning is a general mechanism of adaptive behavioural plasticity whose benefits and costs depend on the environment. Thus, seasonal oscillations in temperate environments between winter and summer might produce cyclical selection pressures that would drive rapid evolution of learning performance in multivoltine populations. To test this hypothesis, we investigated the evolutionary dynamics of learning ability over this rapid seasonal timescale in a natural population of *Drosophila melanogaster*. Associative learning was tested in common garden-raised flies collected from nature in the spring and fall over three consecutive years. The spring flies consistently learned better than fall flies, revealing seasonal evolution of improved learning performance in nature. Fecundity showed the opposite seasonal pattern, suggesting a trade-off between learning and reproduction. This trade-off also held within population: more fecund individual females learned less well. This trade-off is mediated at least in part by natural polymorphism in the RNA binding protein *couch potato* (*cpo*), with a genotype favoured during summer showing poorer learning performance and higher fecundity than a genotype favoured over winter. Thus, seasonal environments can drive rapid cyclical evolution of learning performance, but the evolutionary dynamics may be driven by trade-offs generated by pleiotropic effects of causative alleles selected for other reasons.

1. Introduction

Learning is a powerful mechanism of plasticity that allows animals to behaviourally respond to changing environments without genetically-based evolutionary change [1,2]. Learning plays an important role in many aspects of animal biology including foraging, spatial orientation, predator avoidance, aggression, social interactions, and sexual behaviour [1,3–7], and thus is likely to have manifold effects on fitness.

The ability to learn is itself a product of evolution, but we know very little about how rapidly characteristics of the learning process evolve in nature. Examples of genetic differences in learning performance have been reported between closely related species [8,9] and between allopatric populations within a species [10,11], but the timescale over which these differences have evolved is unknown. Laboratory selection experiments in a range of taxa indicate that natural populations have copious standing genetic variation for learning ability and that learning performance can improve rapidly under direct selection [12–16]. However, selection on learning in nature may be indirect and mediated by the fitness consequences of the many behaviours it modifies. An environmental change that results in an increased advantage of learning in the context of one behaviour (e.g., foraging) might have no effect on, or even decrease, its benefits in a different context (e.g., predator avoidance), thus dampening net selection on learning.

Here we test if learning performance evolves rapidly over an annual timescale in a natural population of *D. melanogaster*. This multivoltine species is a promising system to study rapid evolution because each generation experiences different conditions as seasons progress. The alternating conditions between different selection regimes of winter and summer drive rapid cyclical evolution of *Drosophila melanogaster* life history, stress resistance and immunity traits [17–19]. One might predict that increased ability to learn should be favoured in the summer

72 because learning modulates behaviours that are key to fitness in the summer, such as foraging,
73 pathogen avoidance, and sexual and social interactions [20–25]. However, learning might also
74 contribute to behaviours that are important for overwintering survival (e.g., shelter identification,
75 foraging, pathogen avoidance). Finally, evolutionary dynamics of learning ability may be driven
76 by selection on genetically correlated traits. Such genetic correlations may reflect the high
77 energetic costs of the central nervous system [26,27], leading to trade-offs with other fitness
78 traits. Such trade-offs have been reported between learning and reproduction in *Pieris rapae* [28]
79 and *Drosophila* [29], as well as between learning and stress resistance in *Drosophila* [30,31].
80 Thus, seasonal evolution in learning ability could be driven by indirect effects of selection on
81 correlated traits – particularly on fecundity in the summer or stress tolerance in the winter.

82 We assessed aversive and appetitive learning in common-garden-raised flies derived from
83 spring and fall collections made at the same locality in the wild over three consecutive years. We
84 found that learning performance was consistently higher in the post-winter (i.e., spring)
85 collections compared to the post-summer collections in the fall. A reproductive cost of learning
86 ability was indicated by an inverse relationship with fecundity between seasonal populations and
87 at an individual level.

88 Next, we tested if natural variants of the pleiotropic RNA-binding protein *couch potato*
89 (*cpo*) contribute to the relationship between learning and fecundity. Fecundity and reproductive
90 dormancy differ among natural variants in *cpo* that change in frequency with latitude and season
91 [32–35]. We hypothesized that *cpo* may be involved in learning because it is highly expressed in
92 the nervous system including the centre of associative learning, the mushroom body [36–38]. We
93 found that learning performance and fecundity in flies carrying natural *cpo* genotypes that are
94 more common in spring versus fall paralleled the seasonal differences in learning within the

natural population. These results suggest that rapid fluctuating evolution of learning ability in wild *Drosophila* is at least in part driven by pleiotropic effects of *cpo* polymorphism.

2. Methods

(a) Seasonal populations derived from nature

The evolution of learning differences over seasonal time was assessed by comparing seasonal reconstructed outbred populations (ROP) from isofemale lines, hereafter referred to as spring and fall ROPs. Gravid females aspirated off decaying fruit in the spring (June) and fall (November) at Linvilla Orchards in Media, PA (39.9ºN, 75.4ºW), across three consecutive years (2012-2014) were used to establish isofemale lines that were maintained in standard laboratory conditions (25ºC, 12L:12D on cornmeal-molasses medium) on a four-week transfer cycle. Populations representing each collection were constructed after all collections were complete using 40 isofemale lines per collection. The populations were subsequently maintained in common garden culture for more than 10 non-overlapping generations. Systematic differences in traits between spring and fall ROPs assessed in the common garden can be attributed to genetic differences of the spring and fall allele frequencies.

(b) cpo recombinant outbred populations

Three intronic SNPs in *cpo* are associated with climatic adaptation: 3*R*:17964408, 3*R*:17965558, 3*R*:17967866 (*D. melanogaster* reference genome v.6). The temperate genotype (cpo^{TTA}) is more common at high latitudes and in the spring while the tropical genotype (cpo^{CGT}) is more common at low latitudes and in the fall [35]. The polymorphism at 3*R*:17967866 was

118 identified as a non-synonymous coding change in exon 5 with functional effects on reproductive
119 dormancy and other life history traits [33,37], however, its specific molecular function has been
120 re-evaluated as it not identified in the modENCODE classification of functional elements [39].
121 Learning performance of the genotypes was assessed using ROPs that were each fixed for one
122 genotype with a randomized genetic background [40]. ROPs for cpo^{TTA} and cpo^{CGT} were
123 constructed using nine independent, homozygous lines from the *Drosophila* Genetics Reference
124 Panel [35,41]. Ten gravid females from each line were permitted to lay eggs for 48h; after at
125 least 10 non-overlapping generations of recombination with the other lines containing the same
126 genotype each ROP was fixed for either cpo^{TTA} or cpo^{CGT}, and any linked loci, in a
127 heterogeneous, outbred background.
128
129 *(c) Aversive shock learning in the laboratory*
130 We quantified the performance of flies in an aversive olfactory learning assay that
131 assessed their ability to remember and avoid an odour previously associated with mechanical
132 shock [30]. Flies were reared in common laboratory conditions (25˚C, 12L:12D) at standardized
133 density of 100 eggs per vial, sorted into groups by sex under light CO_2 anaesthesia 24 hour prior
134 to the learning assessment, and then assayed at 3-5 days of age. Single-sex groups of 30 flies
135 were conditioned to associate an odorant (the conditioned stimulus, CS+) with a mechanical
136 shock. Three cycles of conditioning were conducted, each consisting of the sequence: 30 seconds
137 exposure to CS+ paired with mechanical shock (pulsating 1 second every 5 seconds), 60 seconds
138 break of humid air, 30 seconds exposure to another odorant (CS−) with no shock, and 60 seconds
139 humid air. Methylcyclohexanol (MCH, 800 uL/L) and 3-octanol (OCT, 600 uL/L) were used as
140 odorants; half of the fly groups were conditioned to avoid each odorant to account for innate

141 preference for either odorant (defining two conditioning directions). After a one-hour memory
142 retention interval we tested odorant preference of the conditioned flies by giving them 60
143 seconds to choose between the odorants in an elevator T-maze. The *cpo* genotypes were also
144 assessed for immediate response (5-minute retention) and long-term memory (24-hour retention).
145 To exclude impairment in odour perception or differences in innate preference we
146 performed two additional controls. Controls for absolute preference subjected flies to mechanical
147 shock cycle without odorant before presenting a choice between a single odorant and air in the T-
148 maze. Controls for relative preference tested innate odour preference in naïve flies by giving a
149 choice between the two odorants in the T-maze.
150 All statistical analyses were performed using the R software (R Core Team, version R
151 3.2.2). To analyse learning performance, we modelled proportion of flies selecting the odour not
152 previously associated with shock (CS−) using generalized mixed models with logit link and
153 binomial error distribution, fitted by maximum likelihood (package *lme4,* Bates et al. 2014). To
154 test for the significance of main effects and interactions, we used likelihood ratio test from the
155 package *afex* [43] and functional analysis of variance (ANOVA, Fox and Weisberg 2011). Flies
156 that chose no odour (i.e., remained in the centre of the T-maze) were excluded from analysis. For
157 illustration purposes, the proportion of flies learning was rescaled as 2 × proportion – 1 to make
158 it range from –1 to 1 with 0 indicating no learning. We used the following models where ROP
159 (season or *cpo* genotype), year, sex, and conditioning treatment are fixed effects and block is a
160 random effect grouping samples tested at the same time:
161
162 Natural populations:
163

Proportion = Year +ROP + Sex + Conditioning treatment + Year:Conditioning treatment + Sex:Conditioning treatment + Block

cpo genotypes:

Proportion = ROP + Sex + Conditioning treatment + Retention Interval + ROP:Conditioning treatment + ROP:Retention Interval + Block

(d) Appetitive learning in a natural environment

The appetitive conditioning learning assays tested the flies' ability to remember and prefer the flavour previously associated with food, with preference assessed as number of flies caught in traps of two alternative flavours. It was adapted from [15,24], but implemented in a natural setting in outdoor cages over two replicate days (August 30 and 31, 2016).

Flies were reared in standard laboratory (25˚C, 12L:12D) conditions at controlled density (approximately 200 eggs per bottle), and 3-day cohorts were sorted into groups of 50 by sex. Flies were marked with fluorescent powder using four colours (Blue 1162B, Red 1162R, Yellow 1162Y and White 1162W; Bioquip, Rancho Dominguez, CA) according to learning treatment and source population. A pinch of powder was added to an empty vial before adding the flies and gently rolling the vial until the flies were coated. Flies were then transferred to a non-nutritional agarose for 12 hours to recover from the marking process and to become starved, so they were more motivated to feed. The flies were subsequently exposed to either strawberry or apple flavoured food (two directions of conditioning) for an 8-hour training period, followed by a 4-hour retention period on a fresh agar substrate, before being released into the outdoor cages for

testing. The use of four marking colours permitted the simultaneous release in the same cage of flies from the two genotypes being tested (either spring and fall or cpo^{TTA} and cpo^{CGT}) conditioned with either flavour (400 flies per conditioning direction, gentoype season and sex). The cages (0.6 × 0.6 × 1.8 m), made of mesh and with plant bedding covering the ground, were placed underneath a peach tree. Four pairs of strawberry and apple unidirectional traps were dispersed around the cage. Flies trapped in each trap within 14 hours after release were sexed and attributed to season and conditioning direction based on colouring. All of the trapped flies showed traces of the powder; thus, marking was effective, and no flies infiltrated the experiment from outside.

Learning performance was quantified as proportion of flies selecting traps flavoured with the same flavour that they had been previously exposed to during the conditioning. The same statistical analyses were performed as on the aversive conditioning, with year, ROP (season or *cpo* genotype), sex, and conditioning treatment are fixed effects and trap and day are random effects.

Natural populations:

Proportion = Year + ROP + Sex + Conditioning treatment + Year:Conditioning treatment + Sex:Conditioning treatment + Trap + Day

cpo genotypes:

Proportion = ROP + Sex + Conditioning treatment + ROP:Sex + Trap + Day

210

211 *(e) Fecundity*

212 Fecundity of the reconstructed seasonal populations was measured by placing 25 virgin
213 females from each reconstructed seasonal population into culture bottles [40] with 25 males
214 from a standardized stock (BDSC-3605). Food plates were changed daily for ten days to count
215 the number of eggs and record any mortalities. Daily mean fecundity per female was calculated
216 across 10 replicate bottles per population, taking into account any mortality that occurred.
217 Therefore, population measurement of fecundity was assessed using mixed model ANOVAs,
218 with the fixed effects of season and year (seasonal ROP) and genotype (*cpo* ROP).

219 Within-population relationship between learning and reproduction was assessed in the
220 spring reconstructed seasonal populations from 2013 and 2014. Virgins were collected, aged in
221 vials of standard food for 3 days, and subject to the aversive olfactory conditioning and test
222 described above. The flies were thus divided in "learners" (those that chose CS-) and "non-
223 learners" (those that chose CS+). From each replicate test, five "learner" and five "non-learner"
224 females were placed onto food with 5 males from a tester stock; all available flies were used in
225 the few replicates with fewer than five flies. The flies were housed on food supplemented with
226 topical yeast to promote egg production before the assay and on grape-agar substrate during the
227 egg collection. Daily fecundity was counted for 3 days post learning assessment and the mean
228 fecundity per day per female was calculated based on the number of females in the vial. It was
229 then compared between "learners" and "non-learners" from the same replicate test with a paired
230 T-test.

231

232

233 **3. Results**

234 *(a) Rapid evolution of learning and fecundity in a natural population*

235 Learning ability in the wild *D. melanogaster* population evolved rapidly and repeatedly
236 across seasonal time. The spring collections learned better than the fall from the same year when
237 assessed using laboratory aversive conditioning (χ_1^2 = 4.68, p = 0.03; Figure 1a, Table 1). These
238 behavioural differences were not due to differences in ability to perceive the odorants, as there
239 was no effect of season in absolute preference between odorant and solvent in unconditioned
240 flies (χ_1^2= 0.03, p = 0.85 Figure 1b).

241 The spring collections also were better at learning an association between food and
242 flavour tested in outdoor mesocosms. The spring flies were more likely than the fall flies to be
243 trapped in traps flavoured with the same flavour as the food they previously encountered (χ_1^2=
244 5.6, p = 0.017, Figure 1c).

245 The seasonal pattern of fecundity was opposite to that for learning ability. At the
246 population level, the fall populations laid on average 30% more eggs per female per day than the
247 spring populations (Figure 1d; $F_{1,57}$ = 44.2, p < 0.0001). A phenotypic trade-off between learning
248 and reproduction was also apparent at the individual level within the spring populations. Females
249 within each population that chose the "wrong" odour (i.e., the odour associated with shock) in
250 the aversive learning assay subsequently laid an average of six more eggs (25% more) per day
251 compared to the females that made the "correct" choice, i.e., avoided the shock-associated odor
252 (t_{14}= −2.84, p = 0.013, Figure 1e).

253

254 *(b) Natural variation in cpo sequence affects learning and fecundity*

255 Recombinant outbred populations (ROPs) homozygous for the spring *cpo*^{TTA} genotype

had higher aversive learning than the fall ROP; this pattern persisted across all retention intervals between conditioning and testing (χ_1^2 = 27.12, p = 1.91×10^{-7}; Figure 2a, Table 1). In the absence of conditioning, the spring ROPs showed a weaker avoidance of the odorants compared to the fall with 1-hour between shock exposure and the test (χ_1^2=7.39, p=6.52 × 10^{-3}, Figure 2b), but not at 5-minutes or 24-hour retention intervals. The *cpo* ROPs did not differ in the relative preference of naïve flies choosing between the odorants (χ_1^2 = 1.11, p = 0.29, Figure 2c), although there was an interaction between genotype and odorant (χ_1^2 = 6.24, p = 0.012) with the spring ROP having a slight preference for MCH and the fall for OCT. However, replicate flies were conditioned in both directions to avoid either odour so this slight preference should not affect the quantification of learning ability. Thus, the difference in learning performance is not explainable by differences in odour perception. The spring ROPs also had a higher appetitive associative learning when assessed in the natural mesocosms (χ_1^2=4.22, p=0.039, Figure 2a). The spring cpo^{TTA} ROP was characterized by lower fecundity, with females laying 36% fewer eggs than females from the fall cpo^{CGT} ROP (Figure 2d, F_2=8.23, p=0.01).

4. Discussion

(a) Rapid evolution in natural populations

Our data show that learning and fecundity both evolve rapidly in a natural population of *D. melanogaster* over the scale of approximately 10-15 generations from spring to fall and approximately 1-2 generations between fall and spring. The differences can be attributed to annual cyclical genetic changes because environmental effects are removed by rearing and testing these populations in common laboratory conditions. The repeatability across years indicates that this is not a result of genetic drift but instead of deterministic evolutionary

279 processes. Genomic data strongly suggest that gene flow through migration is not generating the
280 observed seasonal cycles in allele frequency [45,46]. Therefore, the rapid and repeatable
281 seasonal changes in learning and reproduction are more likely to reflect seasonally fluctuating
282 selection. Learning is thus not only a mechanism of plasticity that allows organisms to respond
283 rapidly to environmental change; we demonstrate that learning ability itself can evolve rapidly in
284 nature.

285 The learning differences between spring and fall populations were consistently observed
286 in a laboratory aversive conditioning assay and in an appetitive conditioning assay performed in
287 outdoor mesocosms. In both assays, flies originating from the spring collections performed better
288 than those from the fall. Learning associations between odours and both aversive and appetitive
289 stimuli appear to be mediated by the same molecular mechanisms of synaptic plasticity in the
290 mushroom body of the brain [47]. The memory of appetitive and aversive associations is formed
291 at least partly in different subsets of mushroom body neurons, and acting on the appetitive and
292 aversive memories involves different neurotransmitters [47]. Here, the similar pattern in learning
293 performance in both assays implies that the evolutionary change affected a shared component of
294 the learning process. This is consistent with an experimental evolution study in which
295 *Drosophila* populations selected for appetitive learning also performed better in the aversive
296 shock-odour assay [48].

297 The higher learning in the spring population is counter to the prediction that learning
298 should improve during the summer when flies are active and perform many behaviours known to
299 depend on learning. However, cognitive abilities may evolve to match the demands posed by an
300 organism's biological and physical environment [49,50]. Energetically challenging environments
301 (e.g., cold winters or severe droughts) are hypothesized to favour cognitive performance at the

302 cost of other physiological systems receiving less resources [51]. For example, cache seed
303 recovery success in several bird species suggests that learning may be important for
304 overwintering survival in harsh environments [52–54]. Although flies do not cache food, it is
305 possible that learning is also important for other aspects of *D. melanogaster* overwintering
306 survival in hash climates, such as the ability to find a suitable overwintering site or temperature
307 dependent patterns of foraging [55]. However, it is also possible that seasonal evolution of
308 learning may be driven by selection on genetically correlated traits, such as fecundity.
309
310 *(b) Links between resilience, fecundity and learning*
311 We found that flies from fall collections show higher fecundity than those from the
312 spring. The opposite trend has previously been reported in wild-caught females: higher fecundity
313 during the first 24-hours after capture in flies collected in the spring compared to those collected
314 in the fall [18]. However, many factors that change with season (e.g., age and nutrition) also
315 affect fecundity; thus, the previously identified differences among wild-caught flies could have
316 reflected these environmental effects rather than genetic differences. In the present study these
317 environmental effects were excluded by rearing flies in a common garden, exposing for the first
318 time a genetic component of seasonal changes in reproductive output. Fecundity is likely to be
319 under strong selection during the summer months of rapid population expansion. Furthermore,
320 previous studies have demonstrated that fly lines collected in the spring are more resilient than
321 those from the fall, with higher propensity for reproductive dormancy [17], greater resistance to
322 thermal stress [18], starvation [18] and bacterial pathogens [56]. The winter conditions are
323 expected to favour such resilience traits, and at least some of these traits are known to trade-off
324 with fecundity [57,58]. Altogether, these results support the hypothesis of alternating selection

325 for resilience required for survival in the harsh winter conditions and for reproduction during the
326 summer expansion [59,60].

327 The seasonal changes learning performance could in principle have evolved for reasons
328 unrelated to those driving the seasonal fluctuations in resilience and fecundity. However, in
329 addition to the inverse relationship between learning performance and fecundity in spring versus
330 fall populations, we found a negative phenotypic correlation between these traits at the individual
331 level within populations. Females that made the "correct" odour choice in the aversive assay had
332 lower fecundity that females from the same populations that made the "incorrect" choice (i.e.,
333 chose the odour previously associated with shock). The inverse relationship between learning
334 and fecundity is consistent with previously reported trade-offs between these traits in a range of
335 taxa [28,29,61]. The trade-off supports the hypothesis that the decline in learning performance
336 from spring to fall in the natural *Drosophila* population is connected with selection for fecundity
337 during the summer months. If so, the concerted annual evolutionary changes in learning
338 performance, fecundity and resilience traits would be mediated by seasonal fluctuations in allele
339 frequencies at loci with pleiotropic effects on all those traits.

340

341 *(c) Candidate polymorphism at cpo*

342 Our results suggest *cpo* as one such pleiotropic candidate gene that mediates the annual
343 seasonal evolutionary cycle. Variants of *cpo* show latitudinal clines and seasonal fluctuations in
344 frequency [33,34,45,46,62,63] and affect many traits including activity, dormancy, fecundity and
345 lifespan [33,35,37]. We show that genetic variants of *cpo* also affect learning performance: the
346 ROP homozygous for the *cpo* genotype that is more frequent in the spring than in the fall and
347 increases in frequency with latitude [35] performed better in the aversive learning assay than the

348 ROP homozygous for the alternative fall genotype. Furthermore the spring ROP had a lower
349 fecundity than the fall ROP [35]. The *cpo* ROPs that represent annual frequency oscillations and
350 latitudinal patterns of three loci in *cpo* are able to reconstruct the seasonal antagonistic effects on
351 fecundity and learning performance that we observed in the wild-derived collections. This makes
352 *cpo* a strong candidate to contribute to the cyclic annual evolution of these traits in the natural
353 population of *D. melanogaster*.

354 Learning is a new phenotype associated with *cpo* even though the RNA-binding protein
355 is known to be expressed in the mushroom bodies and *cpo* mutants have behaviour phenotypes
356 [36,64]. *cpo* knockdown mutants have reduced odour avoidance [65], which we saw in flies
357 carrying the spring *cpo* genotype that had weaker avoidance of odours when paired with air
358 during the control. This leaves the possibility that the spring *cpo* genotype impairs perception of
359 odours; however, this should have led to impaired rather than improved learning performance.
360 Thus, the difference in learning between the genotypes is unlikely to be due to differences in
361 olfaction, also confirmed by the odour response controls we report above. It is more plausible
362 that the natural *cpo* genotypes we studied, or genetic variants linked to them, affect the neural
363 processes of memory formation, storage and/or recall, although the mechanism by this RNA-
364 binding protein acts on the function of neurons remain known.

365

366 *(d) Pleiotropy as a driving force in evolution of learning*

367 The discussion of learning evolution often assumes that inter- and intra-specific
368 differences in learning performance are mainly driven by ecological factors that affect the
369 benefits of learning, such as environmental predictability, reliability of cues, opportunities for
370 learning and costs of making mistakes [1,50,66,67]. However, our study points out that natural

371 genetic variants that affect learning genes are likely to have broad pleiotropic effects. Similarly, a
372 natural polymorphism in *D. melanogaster foraging* (*for*) gene is also pleiotropic: in addition to
373 learning [68,69] it affects many traits, including foraging behaviour [70], aggregation [71] and
374 competitive ability [69]. Highly pleiotropic effects of genes involved in learning also emerges
375 from the study of mutant phenotypes [72,73]. The overall force of selection acting on such
376 pleiotropic polymorphisms in natural populations reflects their aggregate impact on survival and
377 reproduction mediated by the diverse ecologically relevant traits they influence. This makes it
378 difficult to disentangle which traits are directly under selection for their fitness benefits and
379 which mainly evolve as a by-product of natural selection on correlated traits. It is unclear in our
380 results if changes in learning performance are driven by ecological factors that affect the
381 usefulness of learning or if they evolve as a pleiotropic effect of selection acting on the focal
382 polymorphisms for reasons unrelated to learning performance. Such pleiotropy may reflect
383 alternative coadapted strategies or it may be a non-adaptive mechanistic consequence of gene
384 action. Nonetheless, even if evolving mainly as correlated response to selection on other traits,
385 the learning differences that we identify between our spring and fall populations and between the
386 *cpo* genotypes may have ecologically relevant consequences for behaviour.
387
388
389 **Acknowledgements:**
390 This work was supported by NSF GRF DGE-0822 (ELB), the Rosemary Grant Award from the
391 Society for the Study of Evolution (ELB), the Teece Dissertation Research Fellowship from the
392 University of Pennsylvania (ELB), the Peachey Environmental Fund from the Department of

Biology, University of Pennsylvania (ELB), and NIH R01GM100366 (PS), as well as research funding from the University of Lausanne to TJK.

Author contributions:

ELB, TJK and PS designed the experiment. ELB performed the experiment and did the analyses. ELB, TJK and PS wrote the manuscript.

399 **References:**

400 1. Dukas R. 2004 Evolutionary Biology of Animal Cognition. *Annu. Rev. Ecol. Evol. Syst.* **35**, 347–
401 374. (doi:10.1146/annurev.ecolsys.35.112202.130152)
402 2. Paenke I, Sendhoff B, Kawecki TJ. 2007 Influence of Plasticity and Learning on Evolution under
403 Directional Selection. *Am. Nat.* **170**, E47–E58. (doi:10.1086/518952)
404 3. Raine NE, Chittka L. 2008 The correlation of learning speed and natural foraging success in
405 bumble-bees. *Proc. R. Soc. B Biol. Sci.* **275**, 803–808. (doi:10.1098/rspb.2007.1652)
406 4. Dawson EH, Chittka L. 2014 Bumblebees (*Bombus terrestris*) use social information as an
407 indicator of safety in dangerous environments. *Proc. R. Soc. B Biol. Sci.* **281**, 20133174.
408 (doi:10.1098/rspb.2013.3174)
409 5. Verzijden MN, ten Cate C, Servedio MR, Kozak GM, Boughman JW, Svensson EI. 2012 The
410 impact of learning on sexual selection and speciation. *Trends Ecol. Evol.* **27**, 511–519.
411 (doi:10.1016/J.TREE.2012.05.007)
412 6. Battesti M, Moreno C, Joly D, Mery F. 2012 Report Spread of Social Information and Dynamics
413 of Social Transmission within Drosophila Groups. *Curr. Biol.* **22**, 309–313.
414 (doi:10.1016/j.cub.2011.12.050)
415 7. Reichert MS, Quinn JL. 2017 Cognition in Contests: Mechanisms, Ecology, and Evolution.
416 *Trends Ecol. Evol.* **32**, 773–785. (doi:10.1016/j.tree.2017.07.003)
417 8. Odling-Smee LC, Boughman JW, Braithwaite VA. 2008 Sympatric species of threespine
418 stickleback differ in their performance in a spatial learning task. *Behav. Ecol. Sociobiol.* **62**, 1935–
419 1945. (doi:10.1007/s00265-008-0625-1)
420 9. Hoedjes KM, Smid HM. 2014 Natural variation in long-term memory formation among Nasonia
421 parasitic wasp species. *Behav. Processes* **105**, 40–45. (doi:10.1016/j.beproc.2014.02.014)
422 10. Froissart L, Giurfa M, Sauzet S, Desouhant E. 2017 Cognitive adaptation in asexual and sexual
423 wasps living in contrasted environments. *PLoS One* **12**. (doi:10.1371/journal.pone.0177581)
424 11. Croston R, Branch CL, Kozlovsky DY, Roth TC, LaDage LD, Freas CA, Pravosudov V V. 2015

Potential mechanisms driving population variation in spatial memory and the hippocampus in food-caching chickadees. In *Integrative and Comparative Biology*, pp. 354–371.(doi:10.1093/icb/icv029)

12. Tryon RC. 1940 Studies in individual differences in maze ability. VII. The specific components of maze ability, and a general theory of psychological components. *J. Comp. Psychol.* **30**, 283–335. (doi:10.1037/h0054238)
13. Mcguire TR, Hirschth J. 1977 Behavior-genetic analysis of Phormia regina: conditioning, reliable individual differences and selection. *Proc. Natl. Acad. Sci.* **74**, 5193–5197.
14. Brandes C, Frish B, Menzel R. 1988 Time course of memory formation differs in honeybee lines selected for good and poor learning. *Anim. Behav.* **36**, 981–985.
15. Mery F, Kawecki TJ. 2002 Experimental evolution of learning ability in fruit flies. *Proc. Natl. Acad. Sci.* **99**, 14274–14279. (doi:10.1073/pnas.222371199)
16. Zwoinska MK, Maklakov AA, Kawecki TJ, Hollis B. 2017 Experimental evolution of slowed cognitive aging in Drosophila melanogaster. *Evolution* **71**, 662–670. (doi:10.1111/evo.13156)
17. Schmidt PS, Conde DR. 2006 Environmental heterogenity and the maintenance of genetic variation for reproductive diapause in Drosophila melanogaster. *Evolution* **60**, 1602. (doi:10.1554/05-430.1)
18. Behrman EL, Watson SS, O'Brien KR, Heschel MS, Schmidt PS. 2015 Seasonal variation in life history traits in two Drosophila species. *J. Evol. Biol.* **28**, 1691–1704. (doi:10.1111/jeb.12690)
19. Behrman EL, Howick VM, Kapun M, Staubach F, Bergland AO, Petrov DA, Lazzaro BP, Schmidt PS. 2018 Rapid seasonal evolution in innate immunity of wild Drosophila melanogaster. *Proc. R. Soc. B Biol. Sci.* **285**. (doi:10.1098/rspb.2017.2599)
20. Dukas R. 1998 Evolutionary Ecology of Learning. In *Cognitive Ecology: The Evolutionary Ecology of Information Processing and Decision Making*, pp. 129–174.
21. Dukas R. 2005 Learning affects mate choice in female fruit flies. *Behav. Ecol.* **16**, 800–804. (doi:10.1093/beheco/ari057)

451 22. Sarin S, Dukas R. 2009 Social learning about egg-laying substrates in fruitflies. *Proc. R. Soc. B*
452 *Biol. Sci.* **276**, 4323–4328. (doi:10.1098/rspb.2009.1294)
453 23. Battesti M, Moreno C, Joly D, Mery F. 2012 Spread of Social Information and Dynamics of Social
454 Transmission within Drosophila Groups. *Curr. Biol.* **22**, 309–313.
455 (doi:10.1016/j.cub.2011.12.050)
456 24. Zrelec V, Zini M, Guarino S, Mermoud J, Oppliger J, Valtat A, Zeender V, Kawecki TJ. 2013
457 *Drosophila* rely on learning while foraging under semi-natural conditions. *Ecol. Evol.* **3**, 4139–
458 4148. (doi:10.1002/ece3.783)
459 25. Hollis B, Kawecki TJ. 2014 Male cognitive performance declines in the absence of sexual
460 selection. *Proc. R. Soc. B Biol. Sci.* **281**, 20132873. (doi:10.1098/rspb.2013.2873)
461 26. Laughlin SB, de Ruyter van Steveninck RR, Anderson JC. 1998 The metabolic cost of neural
462 information. *Nat. Neurosci.* **1**, 36–41. (doi:10.1038/236)
463 27. Laughlin S. 2001 Energy as a constraint on the coding and processing of sensory information.
464 *Curr. Opin. Neurobiol.* **11**, 475–480. (doi:10.1016/S0959-4388(00)00237-3)
465 28. Snell Rood EC, Davidowitz G, Papaj DR. 2011 Reproductive tradeoffs of learning in a butterfly.
466 *Behav. Ecol.* **22**, 291–302. (doi:10.1093/beheco/arq169)
467 29. Mery F, Kawecki TJ. 2004 An operating cost of learning in Drosophila melanogaster. *Anim.*
468 *Behav.* **68**, 589–598. (doi:10.1016/j.anbehav.2003.12.005)
469 30. Mery F, Kawecki TJ. 2005 Cost of Long-Term Memory in Drosophila. *Science* **308**, 1148.
470 (doi:10.1126/science.1111331)
471 31. Mery F, Kawecki TJ. 2003 A fitness cost of learning ability in Drosophila melanogaster. *Proc. R.*
472 *Soc. B Biol. Sci.* **270**, 2465–2469. (doi:10.1098/rspb.2003.2548)
473 32. Zhao X, Bergland AO, Behrman EL, Gregory BD, Petrov DA, Schmidt PS. 2015 Global
474 Transcriptional Profiling of Diapause and Climatic Adaptation in Drosophila melanogaster. *Mol.*
475 *Biol. Evol.* **33**, 707–720. (doi:10.1093/molbev/msv263)
476 33. Schmidt PS, Zhu C-T, Das J, Batavia M, Yang L, Eanes WF. 2008 An amino acid polymorphism

477 in the couch potato gene forms the basis for climatic adaptation in Drosophila melanogaster. *Proc.*
478 *Natl. Acad. Sci.* **105**, 16207–16211. (doi:10.1073/pnas.0805485105)
479 34. Cogni R, Kuczynski C, Koury S, Lavington E, Behrman EL, O'Brien KR, Schmidt PS, Eanes WF.
480 2013 The Intensity of Selection Acting on the Couch Potato Gene-Spatial-Temporal Variation in a
481 Diapause Cline. *Evolution* **68**, 538–548. (doi:10.1111/evo.12291)
482 35. Behrman EL, Bergland AO, Petrov DA, Schmidt PS. 2020 Intragenic epistasis in couch potato and
483 its effect on climatic adaptation in natural populations in Drosophila melanogaster. *BioRxiv*
484 36. Bellen HJ, Kooyer S, D'Evelyn D, Pearlman J. 1992 The Drosophila couch potato protein is
485 expressed in nuclei of peripheral neuronal precursors and shows homology to RNA-binding
486 proteins. *Genes Dev.* **6**, 2125–36.
487 37. Bellen HJ, Vaessin H, Bier E, Kolodkin A, D'Evelyn D, Kooyer S, Jant YN, Yuh Nung Jan. 1992
488 The Drosophila couch potato gene: An essential gene required for normal adult behavior. *Genetics*
489 **131**, 365–375.
490 38. Glasscock E, Tanouye MA. 2005 Drosophila couch potato Mutants Exhibit Complex Neurological
491 Abnormalities Including Epilepsy Phenotypes. *Genetics* **169**, 2137–2149.
492 (doi:10.1534/genetics.104.028357)
493 39. Celniker SE *et al.* 2009 Unlocking the secrets of the genome. *Nature* **459**, 927–930.
494 (doi:10.1038/459927a)
495 40. Paaby AB, Bergland AO, Behrman EL, Schmidt PS. 2014 A highly pleiotropic amino acid
496 polymorphism in the Drosophila insulin receptor contributes to life-history adaptation. *Evolution*
497 **68**, 3395–3409. (doi:10.1111/evo.12546)
498 41. Mackay TFC *et al.* 2012 The Drosophila melanogaster Genetic Reference Panel. *Nature* **482**, 173–
499 178. (doi:10.1038/nature10811)
500 42. Bates D, Mächler M, Bolker B, Walker S. 2014 Fitting Linear Mixed-Effects Models using lme4.
501 *J. Stat. Softw.* **67**, 1–48. (doi:10.18637/jss.v067.i01)
502 43. Singmann H, Bolker B, Westfall J, Aust F. 2016 afex: Analysis of Factorial Experiments.

503 44. Fox J, Weisberg S. 2011 An {R} Companion to Applied Regression. See
504 http://socserv.socsci.mcmaster.ca/jfox/Books/Companion (accessed on 18 December 2017).
505 45. Bergland AO, Behrman EL, O'Brien KR, Schmidt PS, Petrov DA. 2014 Genomic Evidence of
506 Rapid and Stable Adaptive Oscillations over Seasonal Time Scales in Drosophila. *PLoS Genet.* **10**,
507 e1004775. (doi:10.1371/journal.pgen.1004775)
508 46. Machado HE *et al.* 2019 Broad geographic sampling reveals predictable and pervasive seasonal
509 adaptation in Drosophila. *bioRxiv* , 337543. (doi:10.1101/337543)
510 47. Cognigni P, Felsenberg J, Waddell S. 2018 Do the right thing: neural network mechanisms of
511 memory formation, expression and update in Drosophila. *Curr. Opin. Neurobiol.*
512 (doi:10.1016/j.conb.2017.12.002)
513 48. Mery F, Pont J, Preat T, Kawecki TJ. 2007 Experimental evolution of olfactory memory in
514 Drosophila melanogaster. *Physiol. Biochem. Zool.* **80**, 399–405. (doi:10.1086/518014)
515 49. Healy SD, Jones CM. 2002 Animal learning and memory: an integration of cognition and ecology.
516 *Zoology* **105**, 321–327. (doi:10.1078/0944-2006-00071)
517 50. Smid HM, Vet LEM. 2016 The complexity of learning, memory and neural processes in an
518 evolutionary ecological context. *Curr. Opin. Insect Sci.* **15**, 61–69.
519 (doi:10.1016/j.cois.2016.03.008)
520 51. Maille A, Schradin C. 2016 Ecophysiology of cognition: How do environmentally induced
521 changes in physiology affect cognitive performance? *Biol. Rev.* **92**, 1101–1112.
522 (doi:10.1111/brv.12270)
523 52. Olson DJ, Kamil AC, Balda RP, Nims PJ. 2004 Performance of Four Seed-Caching Corvid
524 Species in Operant Tests of Nonspatial and Spatial Memory. *J. Comp. Psychol.* **109**, 1–9.
525 53. Bednekoff PA, Balda RP, Kamil AC, Hile AG. 1997 Long-term spatial memory in four seed-
526 caching corvid species. *Anim. Behav.* **53**, 335–341. (doi:10.1006/anbe.1996.0395)
527 54. Pravosudov V V, Clayton NS. 2002 A test of the adaptive specialization hypothesis: Population
528 differences in caching, memory, and the hippocampus in black-capped chickadees (Poecile

529 atricapilla). *Behav. Neurosci.* **116**, 515–522. (doi:10.1037/0735-7044.116.4.515)

530 55. Brankatschk M *et al.* 2018 A Temperature-Dependent Switch in Feeding Preference Improves
531 Drosophila Development and Survival in the Cold. *Dev. Cell* , 781–793.
532 (doi:10.1016/j.devcel.2018.05.028)

533 56. Behrman EL, Howick VM, Kapun M, Staubach F, Bergland AO, Petrov DA, Lazzaro BP, Schmidt
534 PS. 2018 Rapid seasonal evolution in innate immunity of wild Drosophila melanogaster. *Proc. R.*
535 *Soc. B Biol. Sci.* **285**. (doi:10.1098/rspb.2017.2599)

536 57. Stearns SC. 1992 The Evolution of Life Histories. *Oxford Univ. Press. London* **6**, 304–306.
537 (doi:10.1046/j.1420-9101.1993.6020304.x)

538 58. Roff DA. 2002 *Life history evolution*. Sinauer Associates.

539 59. Schmidt PS, Paaby AB, Heschel MS. 2005 Genetic variance for diapause expression and
540 associated life histories in Drosophila melanogaster. *Evolution* **59**, 2616–2625.
541 (doi:10.1111/j.0014-3820.2005.tb00974.x)

542 60. Schmidt PS, Paaby AB. 2008 Reproductive diapause and life-history clines in North American
543 populations of Drosophila melanogaster. *Evolution* **62**, 1204–1215. (doi:10.1111/j.1558-
544 5646.2008.00351.x)

545 61. Galea LAM, Kavaliers M, Ossenkopp KP, Innes D, Hargreaves EL. 1994 Sexually dimorphic
546 spatial learning varies seasonally in two populations of deer mice. *Brain Res.* **635**, 18–26.
547 (doi:10.1016/0006-8993(94)91419-2)

548 62. Fabian D, Kapun M, Nolte V, Kofler R, Schmidt PS, Schlötterer C, Flatt T. 2012 Genome-wide
549 patterns of latitudinal differentiation among populations of Drosophila melanogaster from North
550 America. *Mol. Ecol.* **21**, 4748–4769. (doi:10.1111/j.1365-294X.2012.05731.x)

551 63. Kolaczkowski B, Kern AD, Holloway AK, Begun DJ. 2011 Genomic Differentiation Between
552 Temperate and Tropical Australian Populations of Drosophila melanogaster. *Genetics* **187**, 245–
553 260. (doi:10.1534/genetics.110.123059)

554 64. Bellen HJ, Vaessin?' H, Bier E, Kolodkin A, D'evelyn D, Kooyer S, Jant YN. 1992 The

Drosophila couch potato Gene: An Essential Gene Required for Normal Adult Behavior.

65. Sambandan D, Yamamoto A, Fanara J-J, Mackay TFC, Anholt RRH. 2006 Dynamic genetic interactions determine odor-guided behavior in Drosophila melanogaster. *Genetics* **174**, 1349–63. (doi:10.1534/genetics.106.060574)

66. Johnston TD. 1982 Selective costs and benefits in the evolution of learning. *Adv. Study Behav.* **12**, 65–106.

67. Dunlap AS, Stephens DW. 2016 Reliability, uncertainty, and costs in the evolution of animal learning. *Curr. Opin. Behav. Sci.* **12**, 73–79. (doi:10.1016/J.COBEHA.2016.09.010)

68. Mery F, Belay AT, K-C So A, Sokolowski MB, Kawecki TJ, Robinson GE, So AKC. 2007 Natural polymorphism affecting learning and memory in Drosophila. *Proc. Natl. Acad. Sci.* **104**, 13051–13055. (doi:10.1073/pnas.0702923104)

69. Kaun KR, Hendel T, Gerber B, Sokolowski MB. 2007 Natural variation in Drosophila larval reward learning and memory due to a cGMP-dependent protein kinase. *Learn. Mem.* **14**, 342–349. (doi:10.1101/lm.505807)

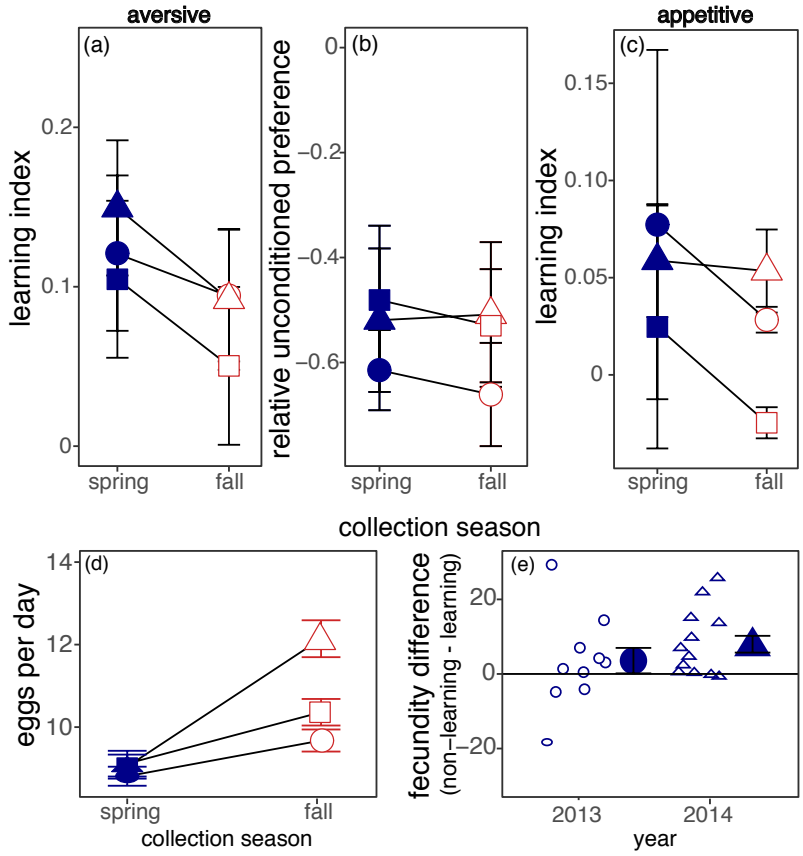
70. Fitzpatrick MJ, Sokolowski MB. 2004 In Search of Food: Exploring the Evolutionary Link Between cGMP-Dependent Protein Kinase (PKG) and Behaviour. *Integr. Comp. Biol.* **44**, 28–36. (doi:10.1093/icb/44.1.28)

71. Wang S, Sokolowski MB. 2017 Aggressive behaviours, food deprivation and the foraging gene. *R. Soc. Open Sci.* **4**, 170042. (doi:10.1098/rsos.170042)

72. Dubnau J, Chiang A-S, Tully T. 2002 Neural substrates of memory: From synapse to system. *J. Neurobiol.* **54**, 238–253. (doi:10.1002/neu.10170)

73. Butcher LM, Kennedy JK, Plomin R. 2006 Generalist genes and cognitive neuroscience. *Curr. Opin. Neurobiol.* **16**, 145–151. (doi:10.1016/j.conb.2006.03.004)

579



580

581 Figure 1. Rapid evolution of learning and reproduction in natural populations of *Drosophila*

582 *melanogaster*. (a) Higher learning (mean +/- S.E) in the spring populations compared to the fall

583 replicated across three years: 2012 (square), 2013 (circle), 2014 (triangle). (b) No difference in

584 absolute preference in either population when unconditioned flies are given the choice between

585 one odour and solvent. (c) Spring populations return to positive conditioning food at a higher rate

586 than fall populations. (d) Fewer eggs laid per day in the spring populations compared to the fall

587 averaged over ten days. (e) Individuals that do not learn in the aversive conditioning assay have

588 higher daily reproductive output over three days than those that learn. Difference between

589 individuals is shown in small outlined shapes and the mean difference +/- SE in the large, filled

590 shapes.

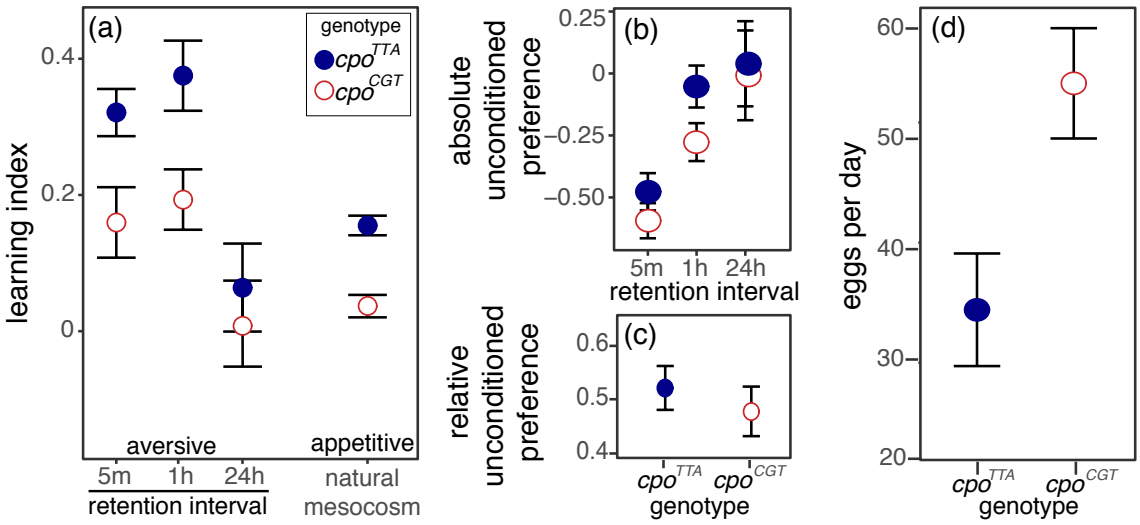


Figure 2. Natural variants in *couch potato* (*cpo*) involved in trade-off between learning and reproduction. (a) Flies containing the spring (cpo^{TTA}) genotype show better learning than flies that contain the fall (cpo^{CGT}) genotype, across all retention intervals between conditioning and test in the aversive shock assay, as well as in the appetitive conditioning of the natural mesocosm. (b) Unconditioned flies containing the spring genotype have a slightly higher absolute preference for odour instead of solvent compared to the flies containing the fall genotype. (c) However, there is no difference in relative preference for either of the experimental odours in unconditioned flies. (d) Flies containing the fall *cpo* genotype had higher daily reproductive output than flies containing the spring *cpo* genotype. In all panels the symbols are mean +/- SE.

602 Supplemental Material
603

Table 1. Analysis of Deviance (Likelihood ratio test)

	Aversive learning			Absolute unconditioned preference			Relative unconditioned preference			Appetitive learning		
	df	Chisq	p	df	Chisq	p	df	Chisq	p	df	Chisq	p
Seasonal populations												
Year	2	0.078		2	8.56					2	14.25	***
Season	1	4.68	*	1	0.24					1	5.6	*
Sex	1	0.0013		1	0.072					1	13.02	***
Odor	1	42.41	***	1	1.91	***				1	1391.7	***
Year:Odor	2	12.75	**	2	1.99					2	23.05	***
Sex:Odor	1	4.36	*	1	4.96					1	15.61	***
cpo genotypes												
Genotype	1	27.12	***	1	7.39	**	1	1.19		1	4.22	*
Sex	1	6.87	**	1	0.24		1	0.059		1	0.85	
Odor	1	166.99	***	1	12.18	***	1	1.18		1	454.23	***
Recovery Time	1	54.13	***	1	66.56	***						
Genotype:Odor	1	10.41	**	1	6.94	**	1	6.24	*			
Genotype:Time	1	4.62		1	53.41	***						
Genotype:Sex										1	2.98	0.084

Significance codes: * < 0.05, ** < 0.01, *** < 0.001

604