1   **A behavioral syndrome linking boldness and flexibility facilitates invasion success in**

2   **sticklebacks**

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4   Miles K. Bensky[1,3] and Alison M. Bell[1,2,4]

5   [1] Program in Ecology, Evolution, and Conservation Biology, School of Integrative Biology,

6   University of Illinois, 505 S. Goodwin Ave., Urbana, IL 61801

7   [2] Department of Evolution, Ecology and Behavior, Carl R. Woese Institute for Genomic Biology,

8   Neuroscience Program, University of Illinois, Urbana, Illinois 61801

9   [3] ORCID: 0000-0001-9776-2981

10   [4] ORCID: 0000-0001-8933-8494

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12   Corresponding author:

13   Miles Bensky, Program in Ecology, Evolution, and Conservation Biology, School of Integrative

14   Biology, University of Illinois, 505 S. Goodwin Ave., Urbana, IL 61801, e-mail:

15   bensky2@illinois.edu, telephone: (206) 419 - 2361

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ABSTRACT

Understanding the factors that allow a species to expand its range and adapt to changing habitats is essential for mitigating anthropogenic change. We evaluated how behavior and cognition facilitate colonization of new environments and evolve post establishment during natural biological invasions. Marine threespined sticklebacks are expert colonists with a penchant for invading freshwater environments and rapidly adapting to them. However, the role of behavior in facilitating rapid adaptation in this system has received little attention. By rearing replicate populations of sticklebacks under common garden conditions in the lab, we tested the hypothesis that boldness is favored in dispersers and that neophilia and flexibility are favored in recently-arrived immigrants. We found that dispersing populations comprised bold individuals, while sticklebacks from the invaded region were flexible in their behavior. Moreover, boldness and flexibility were negatively correlated with each another at the individual, family and population levels. Multiple lines of evidence suggest that there is a heritable component to boldness and flexibility, therefore their divergence is likely to be evolutionary in origin. If boldness is favored in invaders during the initial dispersal stage, while flexibility is favored in recent immigrants during the establishment stage, then the link between boldness and flexibility could generate positive correlations between successes during both the dispersal and establishment stages, and therefore play a key role in facilitating colonization success in this important model organism.

SIGNIFICANCE STATEMENT

For a species to expand its range, it needs to be good at dispersing and also capable of exploiting resources and adapting to different environments. Therefore, behavioral and cognitive traits such

47   as boldness, neophilia and behavioral flexibility could play key roles in facilitating invasion

48   success. Here, we show that dispersing sticklebacks are bold, while sticklebacks that have

49   recently established in a new region are flexible. Moreover, boldness and flexibility are

50   negatively correlated with one another. If boldness is favored in dispersers while flexibility is

51   favored in immigrants, then this behavioral syndrome could play a heretofore underappreciated

52   role in facilitating rapid adaptation in this important model organism.

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INTRODUCTION

Understanding the factors that allow a species to expand its range and adapt to changing habitats is increasingly important in the face of anthropogenic change. Natural biological invasions can reveal how and why certain organisms can excel in response to novel selection pressures (1). In addition to the importance of propagule pressure (2), stochasticity, and the opening of new niches at the edge of species boundaries, there is growing evidence that particular traits (e.g., r-selected life histories (3), habitat breadth (4), large brains/cognitive abilities (5)) might promote biological invasions.

Behavioral and cognitive traits are likely to play an important role in allowing animals to move into and become established in new environments (6-9). For example, behavioral plasticity allows animals to rapidly adjust their phenotype in response to changes in environmental cues (10-12). Cognitive processes – how animals perceive, process and retain information about their environment and then use that information to make decisions (13) – may play an especially important role during biological invasions because they influence the ability of animals to enter new habitats, exploit new niches, become established and spread (9, 14, 15). For example, recent immigrants have to be willing to approach and interact with novel stimuli in order to gain information about their new environment (i.e., neophilia (16)). Additionally, previously successful behavioral patterns may no longer be successful in new environments, so immigrants need to be able to stop persisting on ineffective responses and flexible enough to attempt new approaches (14).

Successfully colonizing a new environment can be broken down into discrete stages, e.g., dispersal, colonization, establishment and spread (6), and invasion success likely relies on different behavioral and cognitive traits in each stage (12). For example, dispersers need to be

78   bold, while immigrants can succeed in a new environment when they are willing to investigate

79   novel stimuli and when they are able to quickly inhibit old and ineffective behaviors, i.e., if they

80   are flexible (12). If boldness, neophilia and behavioral flexibility are no longer beneficial or even

81   costly once a population becomes established in a new environment, they may be lost (9, 17).

82   Numerous studies have documented differences in behavioral traits between invading and

83   established populations, e.g., (18-21), and a handful of studies have shown that they have a

84   heritable basis, e.g., (22, 23). If there is an underlying genetic basis to the behavioral and

85   cognitive traits that facilitate range expansions or biological invasions, then we might expect

86   those traits to evolve over the course of an invasion. According to this hypothesis, behavioral and

87   cognitive traits important for invasion should vary in a systematic way between dispersing

88   populations compared to newly-derived and well-established populations when they are reared in

89   a common garden. Moreover, if invasion success requires different behavioral and cognitive

90   traits in the different invasion stages, then mechanisms that package these traits together could be

91   key to the success of invasive species (12). For example, traits important for the dispersal and

92   establishment stages are coupled together in western bluebirds, and this facilitates the expansion

93   of their range (24).

94   Threespined sticklebacks (*Gasterosteus aculeatus*) are a model system for studying trait

95   evolution during biological invasions. Throughout their evolutionary history, marine sticklebacks

96   have repeatedly colonized freshwater environments, rapidly adapted to them and diversified (25).

97   Sticklebacks can also spread and have dramatic impacts on freshwater communities (26, 27).

98   Work on this system has primarily focused on a suite of morphological and physiological traits

99   that repeatedly evolves when marine sticklebacks invade freshwater habitats (28, 29), with

100   evidence that haplotypes containing a set of coadapted alleles are maintained at low frequency in

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101   the ocean and are repeatedly tapped during adaptation to freshwater (30). However, little is

102   known about the behavioral and cognitive mechanisms that may facilitate the invasion of

103   sticklebacks into new habitats and which may evolve once a population becomes established

104   (31).

105   We took advantage of the repeated invasion of freshwater habitats by sticklebacks by

106   comparing marine stickleback to sticklebacks from recently-derived and well-established

107   freshwater populations in Alaska, with replicate populations of each type. For each population,

108   we reared clutches of field-fertilized eggs in a controlled laboratory environment and scored

109   individuals for three informative and repeatable behavioral traits: boldness, behavioral flexibility,

110   and neophilia (32). We hypothesized that boldness is favored in dispersers, that neophilia and

111   behavioral flexibility are favored in newly-arrived immigrants, and that these traits may be

112   subject to relaxed selection and possibly lost once a population becomes established. The

113   replicate populations allow us to assess the generality of the patterns and comparing the newly-

114   established to well-established populations provides insight into whether different traits are

115   favored during initial establishment versus population persistence (33). Rearing animals under

116   common-garden conditions in the lab also allowed us to determine if behavioral variation within

117   and among populations likely reflect evolved, genetically based differences.
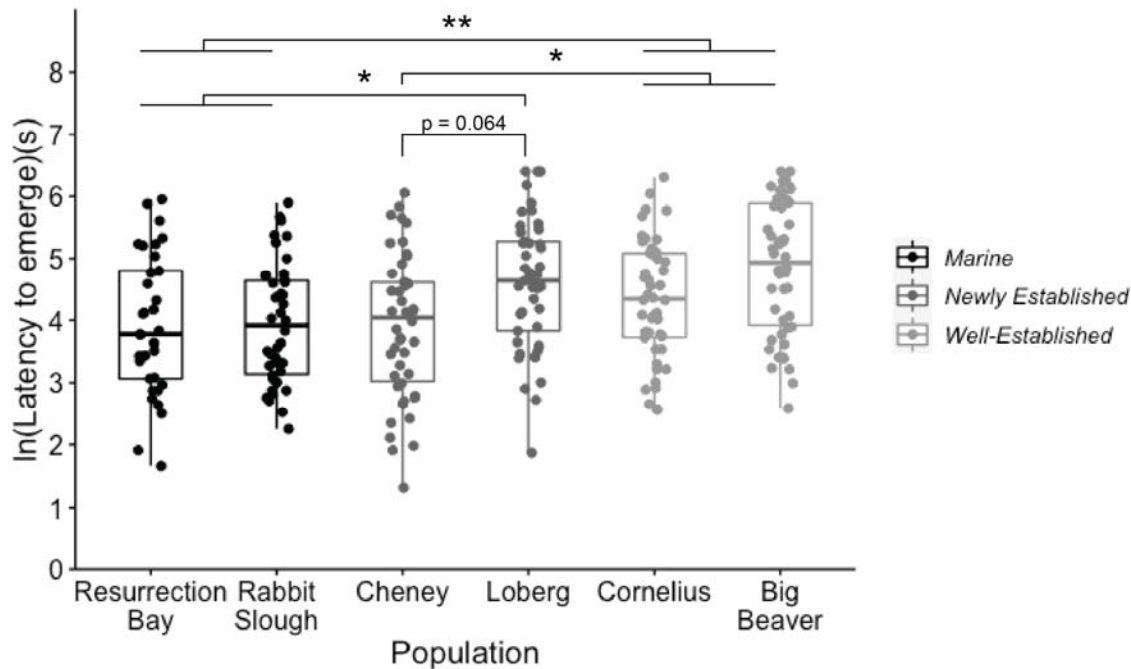
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119   **RESULTS**

120   ***Sticklebacks from dispersing populations were bold***

121   There was significant variation among populations in boldness (latency to emerge from a

122   refuge ($F_{5,59} = 3.30$, p = 0.01, Table S1, Figure 1)).

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Figure 1. Variation among populations in boldness (latency to emerge from a refuge). Smaller values indicate greater boldness. Figure shows boxplots with individual data points superimposed.

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Sticklebacks from dispersing populations (marine) were more bold than fish from well-established freshwater populations ($z = 2.745$, $n = 262$, $p = 0.006$). The two recently-derived populations provide insight into the rate at which boldness diverged from the ancestral marine behavioral type. Sticklebacks from Cheney Lake, a population that was established eight years prior to collection, resembled marine populations and differed from the well-established freshwater populations (Cheney vs. well-established: $z = -2.497$, $n = 262$, $p = 0.034$). In contrast, sticklebacks from Loberg Lake, a population that was established 28-34 years prior to collection, more closely resembled the well-established freshwater populations (Loberg Lake vs marine: $z = 2.429$, $n = 262$, $p = 0.040$). After correcting for multiple comparisons, there was a trend for

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140   sticklebacks from Cheney Lake to emerge faster compared to sticklebacks from Loberg Lake (z

141   = -2.249, n = 91, p = 0.064).

142   There was significant variation in boldness among families within populations (FamilyID

143   improved model fit (X^2 = 9.394, df = 1, p = 0.002; AIC_{with} = 774.50; $AIC_{without}$ = 781.89)).

144   Estimates of broad sense heritability of boldness in the six populations ranged from 0.1 to 0.75

145   (average = 0.48) and was significantly different from zero in the Big Beaver, Cornelius and

146   Resurrection Bay populations (Table S2).

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148   ***Sticklebacks from established populations were flexible***

149   There was significant variation among populations in behavioral flexibility (time at the

150   apex of a barrier ($F_{5,58}$ = 3.495, p = 0.008, Table S1, Figure 2)).

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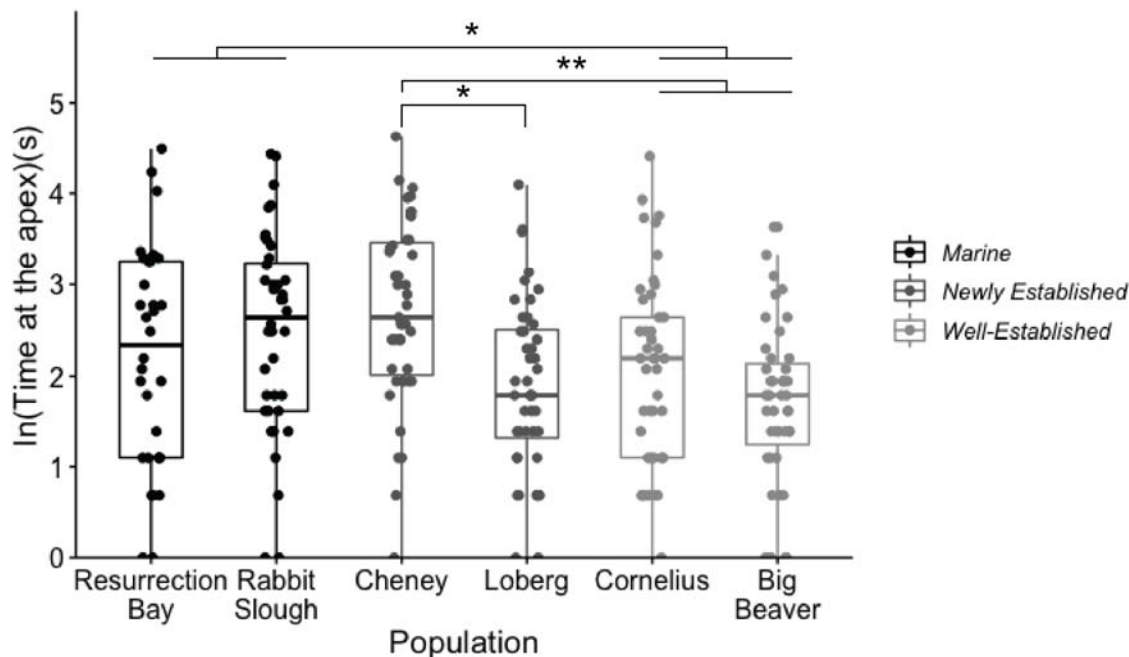
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153   **Figure 2**. Variation among populations in flexibility (time at the apex of the barrier). Smaller
154   values indicate greater flexibility, i.e., less time persisting on a previously-successful behavior
155   pattern. Figure shows boxplots with individual data points superimposed.

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157   Sticklebacks from the well-established populations were more flexible (spent less time at the

158   apex of a barrier), compared to sticklebacks from the dispersing marine populations (z = -2.175,

159   n = 158, p = 0.030). As was the case for boldness, the two recently-derived populations show

160   different patterns: flexibility in Cheney Lake – the most recently established freshwater

161   population – resembled flexibility in the dispersing populations, while flexibility in Loberg Lake

162   – the freshwater population that was established 28-34 years prior to collection – resembled

163   flexibility in the well-established freshwater populations (Cheney Lake vs Loberg Lake: z =

164   2.821, n = 87, p = 0.013, Cheney Lake vs well-established: z = 3.373, n = 131, p = 0.002).

165   There was significant variation among families within populations in flexibility (X^2 =

166   7.350, df = 1, p = 0.007; AIC_{with} = 723.74; $AIC_{without}$ = 729.09). Estimates of broad sense

167   heritability of flexibility in the six populations ranged from 0.22 to 0.86 (average = 0.45) and

168   was significantly different from zero in the Cornelius population (Table S2).

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170   ***Neophilia did not evolve systematically during the invasion***

171   Neophilia (time near and oriented to a novel object) did not vary among populations or

172   between the sexes (Table S1) and did not vary in a systematic manner among the different types

173   of populations (Figure 3).

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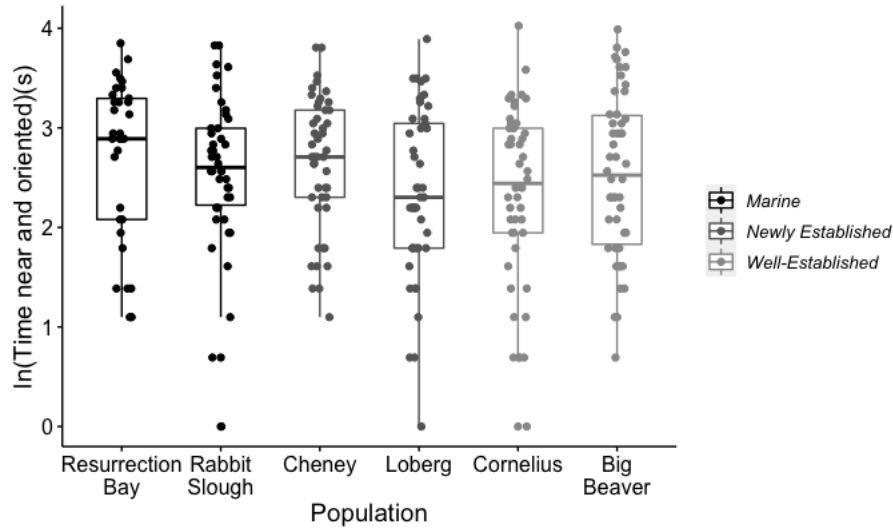
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Figure 3. Variation among populations in neophilia (time near and oriented to the novel object). Figure shows boxplots with individual data points superimposed.

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Larger fish were less neophilic (β = -0.033, t = -2.631, df = 247.79, p=0.009, Figure S2, Table S1). We did not detect variation among families within populations in neophilia, as FamilyID did not significantly improve model fit (X^2 = 2.369, df = 1, p = 0.124; AIC_{with} = 636.57; $AIC_{without}$ = 636.94). Estimates of broad sense heritability of neophilia in the six populations ranged from 0.09 to 0.87 (average = 0.34) and was significantly different from zero in the Cornelius population (Table S2).

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A boldness-flexibility syndrome facilitates invasion success

Given that sticklebacks from the dispersing populations were bolder and less flexible compared to sticklebacks from established populations, we tested how boldness and flexibility were correlated with one another. Consistent with the pattern described above, individuals that were more bold (quickly emerged from the refuge) were less flexible (spent more time at the barrier apex; r = -0.522, n = 245, p<0.001, Table S3), and this pattern was also evident at the family (r = -0.688, n = 62, p<0.001) and population (r = -0.91, n = 6, p=0.01) levels (Figure 4).
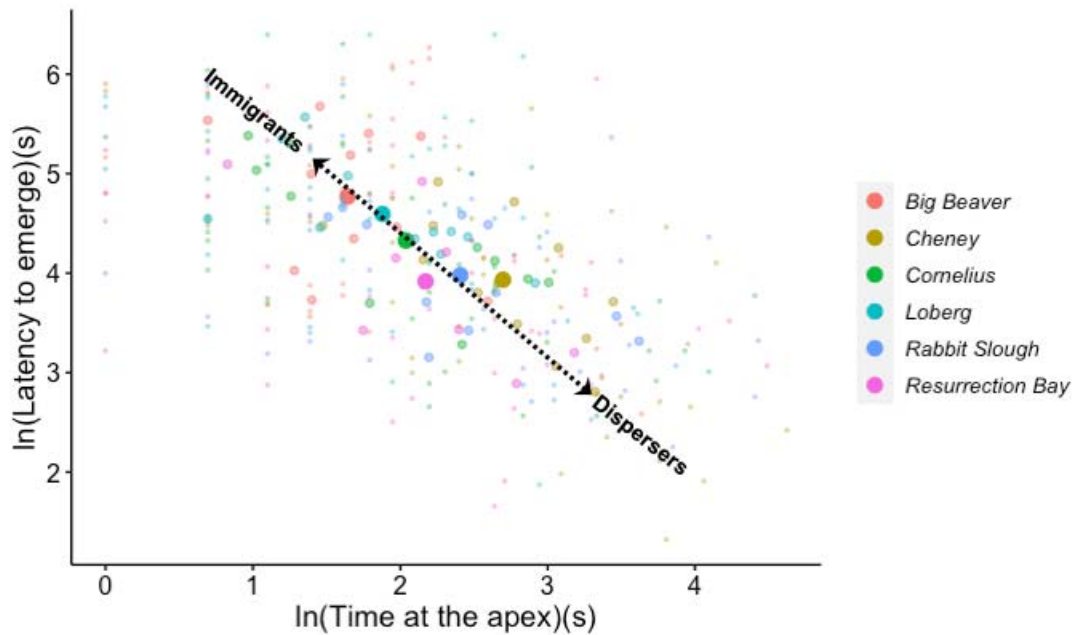
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Figure 4. Relationship between flexibility (time at the barrier apex) and boldness (latency to emerge) within and among populations. Dispersers are relatively bold (emerge quickly) while immigrants are relatively flexible (spend less time at the barrier apex). Large circles represent populations means, medium circles show family means, small circles show individual data points. Circles are color coded by population. The line and text drawn on the figure are for visual purposes only.

The average genetic correlation between boldness and flexibility within the six populations was r

= -0.69 (range: -0.59 to -0.77) and was significantly different from zero in the Cornelius

population (Table S3).

DISCUSSION

The possibility that behavioral and cognitive traits might facilitate and evolve during

natural biological invasions is intriguing but difficult to study directly. Here, we took advantage

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208   of a model system for invasions to test the hypothesis that boldness is favored in dispersers, that

209   neophilia and flexibility are favored in recently-arrived immigrants and that these traits are

210   subject to relaxed selection and possibly lost once a population becomes established in a new

211   environment. We found that boldness and flexibility evolve in a systematic way when marine

212   sticklebacks colonize freshwater habitats. Specifically, the dispersing populations were bold, and

213   well-established populations were flexible. These traits varied between the two recently

214   established populations relative to time since establishment. Moreover, boldness and flexibility

215   were negatively correlated with one another at the individual, family and population levels.

216   Differences in boldness and flexibility were evident in a common garden environment, there was

217   significant variation among families in both of these traits, and estimates of their heritability and

218   the genetic correlation between them were relatively high for behavioral traits (34). These lines

219   of evidence suggest that there is a heritable component to the traits and that their divergence is

220   likely to be evolutionary in origin.

221   If boldness is favored in invaders during the initial dispersal stage, while flexibility is

222   favored in recent immigrants during the establishment stage, then a tight link between boldness

223   and flexibility could generate positive correlations between successes during both the dispersal

224   and establishment stage, and therefore play a key role in facilitating invasion success in this

225   species (12). Selection favoring boldness in dispersers and flexibility in immigrants could cause

226   the population to move along a ridge of high fitness whenever sticklebacks diverge from the

227   ancestral marine behavioral type (Figure 4). An outstanding question is what maintains the

228   extensive phenotypic and genetic variation in boldness and flexibility within the populations

229   (Figures 1-4). Selection might not be strong enough to effectively purge the variation, and/or

230   there may be ongoing gene flow between marine and freshwater habitats which maintains

231 recessive alleles associated with behavioral flexibility at low frequency in marine populations

232 (transporter hypothesis (35)), as appears to operate at the *Eda* locus (36) and surrounding

233 genomic regions (30).

234 We found no support for the hypothesis that traits which promote dispersal and early

235 establishment in a new environment are lost once a population becomes well-established (9), i.e.,

236 no evidence that newly-derived populations were more bold, neophilic and flexible than well-

237 established populations. One possible explanation for this pattern is that *assortative mating*

238 within dispersing populations causes dispersing behavioral phenotypes to be maintained early in

239 the establishment process, even in the presence of selection favoring more flexible phenotypes

240 (37). The comparison between the two newly-derived populations (Cheney Lake and Loberg

241 Lake, respectively) is also insightful; the two newly derived populations either tended to

242 resemble the dispersing populations or the well-established populations. Cheney Lake was

243 founded more recently than Loberg Lake (9 versus 28-34 years prior to this study); if it takes

244 longer than 10 generations for behavioral and cognitive traits to diverge from the ancestral

245 marine behavioral type then *time since establishment* could be important. Alternatively, or in

246 addition, the phenotypic differences between sticklebacks from Loberg Lake and Cheney Lake

247 could reflect differences in the way that the two lakes were colonized; Loberg Lake was

248 naturally colonized, while sticklebacks were experimentally introduced to Cheney Lake. If

249 particularly flexible individuals were more likely to disperse into Loberg Lake, but a random

250 sample of behavioral types were artificially introduced into Cheney Lake (38-40), then *non-*

251 *random dispersal* could be contributing to the rapid evolution of increased flexibility in Loberg

252 Lake. Given evidence from the literature (39, 40) and from this study that bold individuals are

253 more likely to disperse, and that boldness and flexibility are tightly *negatively* correlated with

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254   one another, this explanation seems unlikely. Further studies tracking how behavioral and

255   cognitive traits change over time in the Cheney Lake population (and similar experimental lakes

256   (e.g., Scout Lake)) could help discriminate between the assortative mating, time since

257   establishment and non-random dispersal hypotheses.

258   We originally hypothesized that neophilia would be favored in recently-derived

259   populations because seeking and/or being willing to investigate novel stimuli may help newly-

260   arrived immigrants locate new habitats and discover novel resources, but we found no support

261   for this hypothesis. One possible explanation for the failure to find systematic differences in

262   neophilia among the populations is that neophilia may actually be disadvantageous in a new

263   environment because it can expose animals to dangerous stimuli they have never encountered

264   before. Another potential (nonexclusive) explanation based on our results is that neophilia may

265   not evolve as readily because it may be less heritable (effect of FamilyID was nonsignificant,

266   lower H^2 estimate). Instead, neophilia may be more influenced by age or experience: in this

267   study, smaller (and younger: $r = -0.159$, $t = -2.595$, $n = 262$, $p=0.01$) fish were more neophilic,

268   which is consistent with other studies which have shown that novelty-seeking decreases with age

269   (41).

270   Sticklebacks are a powerful model system for understanding how and why certain traits

271   repeatedly evolve whenever organisms invade new habitats. Accumulating evidence suggests

272   that sticklebacks have evolved mechanisms for rapidly adapting to new environments with

273   alleles conferring the freshwater-adapted phenotype maintained at low frequency in the ocean

274   (28). But the role of behavior and cognition in facilitating evolutionary processes in this system

275   has received less attention (31). Our results suggest that a behavioral mechanism – a behavioral

276   syndrome linking boldness and flexibility together – contributes to rapid adaptation in this

277   important model organism.

278

279   **MATERIALS AND METHODS**

280   Adult sticklebacks were collected via minnow traps from six populations ranging from

281   the Matanuska-Susitna Valley to the Kenai Peninsula of Alaska (Figure S1; Table S1) during

282   June 2017. Two populations (Rabbit Slough and Resurrection Bay) occur in the ancestral marine

283   environment, while the remaining four populations occur in freshwater. Two of the freshwater

284   populations (Big Beaver and Cornelius, hereafter referred to as "well-established") are derived

285   from natural colonization events that presumably occurred hundreds to thousands of years ago,

286   after the last glacial maximum, while the other two freshwater populations are recently derived

287   via natural recolonization (Loberg: 28-34 years prior to collection; (29)) or experimental seeding

288   (Cheney: 8 years prior to collection; (42)). As is typical for sticklebacks, the marine populations

289   are only weakly genetically differentiated from each other ($F_{ST} = 0.0076$; (43)) while the

290   freshwater populations are more strongly genetically differentiated (M. Bell, K Veeramah,

291   personal communication).

292   Eggs were fertilized in the field following previously established protocols (see (44)).

293   Two to three days post fertilization, the eggs were transferred to 50 mL canonical tubes and

294   shipped overnight in coolers filled with ice packs to the University of Illinois Urbana-Champaign

295   where they were raised in common garden conditions in the lab. Artificial incubation controls

296   for environmental paternal effects due to receiving paternal care therefore it is likely that

297   phenotypic differences among the lab-reared populations reflect heritable differences (although

298   environmental maternal effects could also contribute).

299   Clutches were reared in separate tanks (9.5L 32 x 21 x 19 cm) where the embryos were

300   incubated in a cup with a mesh bottom and placed over an air bubbler. Fish were kept at 60°F

301   with an even light cycle (12L:12D) for the entirety of the experiment. All families were kept on

302   one of two recirculating flow-through water racks, which consisted of a series of particular,

303   biological, and IV filters and had three different shelves (Aquaneering , San Diego, USA). 10%

304   of each tank's water was replaced each day. Family tank position was pseudo-randomly assigned

305   so that all populations were evenly distributed across both racks and the three levels of shelves.

306   Importantly, we elected to rear the fish and measure their behavior in freshwater (~5ppt), thereby

307   simulating the conditions that marine sticklebacks encounter when they move into freshwater.

308   Because the marine populations studied here are naturally anadromous (Rabbit Slough: (42);

309   Resurrection Bay: R. King, pers. comm.), i.e., they spawn in fresh/brackish waters, their early

310   offspring development typically occurs under low salinity.

311

312   ***Behavioral assays***

313   ***Summary of the behavioral assays.*** Neophilia was measured as response to a novel

314   object. Boldness was measured as latency to emerge from a refuge, a reliable and widely-used

315   behavioral assay in fishes (45, 46). Boldness was quantified at the individual level as average

316   latency to emerge across four independent trials. Behavioral flexibility was measured in a barrier

317   task: after pretraining individuals to expect a food reward upon emergence from a refuge,

318   individuals were confronted by a transparent barrier that they had to swim around in order to get

319   the food reward. Individuals that continue to follow the prepotent search pattern established

320   during training spend relatively more time at the point of the barrier closest to the food reward

321   ("barrier apex"), which we interpret as relatively low flexibility. In contrast, individuals that

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322   quickly break away from the previously-established behavior pattern spend relatively little time

323   spent at the apex of the barrier, which we interpret as relatively high flexibility. A previous study

324   in sticklebacks found that time at the apex of the barrier predicts reversal learning performance

325   (32), another common metric of behavioral flexibility.

326   ***Detailed experimental methods.*** 18 observation tanks (36L x 33W x 24H cm) were used

327   for behavioral testing. When the fish were approximately eight months of age (approximately 40

328   mm standard length), the testing phase of the experiment began. Behavioral assays were carried

329   out over the course of 5 months. Families, sexes and populations were measured in a

330   pseudorandomized order, such that male and female offspring from different families and from

331   different populations were measured in the same block. Individuals were randomly selected from

332   each family, and their weight and length were measured. During the testing phase of the

333   experiment, fish were only fed during the behavioral tests to help maintain motivation.

334

335   ***Acclimation phase***

336   In order to ensure that an individual had acclimated to their home tank and was motivated

337   to eat during the behavioral tests, the individual was presented with food via a petri dish at the

338   center of their home tank, and the individual had to eat the food within 10 minutes on three

339   consecutive days in order to proceed to the next step. On average, it took 5.1 days for fish to

340   meet this criterion (range = 3 to 17 days).

341

342   ***Novel object test (neophilia)***

343   Individuals' response to a novel object (toy lion; 10L x 7H cm; TERRA by Battat,

344   Montreal, Canada) was recorded the day after the fish met criterion in the acclimation phase. The

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345 toy lion was selected as a novel object because the fish had no prior experience with this object,

346 there was no presumed evolutionary history with the object's shape, and it was made up of

347 neutral colors.

348   The individual's behavior was measured for five minutes after their first approach to the

349 novel object (i.e., first time within one body length of the novel object and oriented directly

350 towards it). We interpret more time spent near and oriented towards the novel object as greater

351 time investigating the object (i.e., higher neophilia).

352

353 ***Latency to emerge (boldness)***

354   For each individual, we recorded their latency to emerge from a refuge on their first four

355 training trials for the barrier task (described below) and used the average of those measures as a

356 proxy for boldness (Movie S1). Latency to emerge was repeatable across the four trials within

357 each population (r=0.56-0.72, Table S4).

358

359 ***Barrier detour task (flexibility)***

360   Pretraining for the barrier detour task started on the same day the novel object test was

361 completed. The goals of pretraining were to train the individual to learn that there would

362 consistently be a food reward in the middle of the tank, establish food motivation in this context,

363 and create a prepotent response of leaving a shelter to directly approach and eat the food reward.

364   During pretraining, individuals were trained for one session per day, with each session

365 comprising four trials. To start the trial, the observer removed a cork from the side of the shelter

366 and the fish was given ten minutes to exit. Upon exiting the fish was allowed five minutes to eat

367 the worm.

368   After eating the worm, the fish was placed back into the shelter in preparation for the

369   next trial. If the fish did not emerge from the shelter within ten minutes after the cork was pulled

370   or eat within five minutes after emergence, the observer recorded the maximum times for these

371   behaviors, removed the food reward and gently poured the fish out of the shelter if necessary.

372   Latency to emerge from a refuge during the first pretraining session was used as a measure of

373   boldness (see above).

374   Training for the barrier task was criterion based. In order to move on to the barrier task

375   following pretraining, the individual had to emerge from the shelter within 10 minutes and

376   directly approach and eat the food reward within five seconds on three out of the four trials. The

377   one failed attempt could not be on the fourth trial; this requirement ensured that the fish would

378   be motivated throughout the four trials. Fish were given a maximum of four days to reach

379   criterion. Fifteen fish did not meet criterion (Big Beaver: 7, Cornelius: 1, Loberg: 1, Cheney: 3,

380   Rabbit Slough: 2, Resurrection Bay: 1).

381   Once an individual met criterion, the individual moved on to the barrier detour task the

382   following day. This task also comprised four trials. The first two trials were exactly the same as

383   the pretraining trials in order to reinforce the direct search pattern. On the third trial a transparent

384   semi-circular barrier was placed between the shelter and food reward. The opening into the

385   barrier area was positioned directly in front of the entrance to the shelter. After removing the

386   cork the individual was allowed 30 minutes to emerge from the shelter, navigate around the

387   barrier and eat the food reward (Movie S2). The observer recorded the duration of the first bout

388   (no break in contract longer than five seconds) at the apex of the barrier. In order to confirm that

389   the fish that spent little time at the barrier apex during the third trial were still motivated to eat,

390   the fish's behavior was observed for a fourth trial during which no barrier was present.

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391   Altogether, a total of n=262 individuals from n=8-11 families per population (n=2-7 full

392   sibs per family) completed the novel object test and boldness assay. A total of n=247 individuals

393   from n=8-11 families per population (n=1-4 full sibs per family) completed the barrier task. The

394   experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of

395   the University of Illinois Urbana-Champaign (IACUC protocol #15077).

396

397   ***Statistical analysis***

398   R 3.5.3 (http://www.r-project.org/) was used for statistical analyses. Positively-skewed

399   variables were log-transformed to improve normality, model residuals were also visually

400   inspected for deviations from normality.

401   We used linear mixed models (47)(package = "lme4"; function = "lmer") to examine the

402   behavioral data in each assay separately. We created models in which population and sex were

403   included as fixed factors, and body length was included as a covariate. FamilyID nested within

404   population was included as a random variable. The statistical significance of the effect of

405   FamilyID was assessed by AIC (48), i.e., by comparing models with and without the effect of

406   FamilyID (R Core Team 2016; package = "care"; function = "anova"). We infer that a trait has

407   an underlying heritable basis when the trait differs among populations (because they were reared

408   in a common garden), and/or when FamilyID improves model fit. To further explore the

409   heritable basis to these traits we also computed broad-sense heritabilities and genetic correlations

410   within each population following (49, 50). We used weakly informative inverse-gamma priors

411   for the 'residual' and 'genetic' effects (by setting the MCMCglmm parameters $V\square=\square1$,

412   nu$\square=\square0.002$). In order to test for genetic correlations, a 2x2 covariance matrix was specified

413   with the degree of belief parameter set to n=1.002. The raw positively-skewed duration data for

414　all the behavioral measures were used for this analysis, and thus all measures were rounded to

415　the nearest integer and a Poisson distribution was used. The posterior distribution was sampled

416　every 3000 times (thinning interval), following a burn-in period of 100□000 iterations with a

417　total run of 10□000□000 iterations. The relatively modest number of families and full sibs

418　within each family resulted in large confidence intervals around the estimates therefore we

419　interpret those results with some caution.

420　　　　To examine the structure of the phenotypic variation both among and within populations,

421　we examined correlations between traits at the individual, family and population level.

422　　　　See SI Methods for more details.

423

424　**ACKNOWLEDGEMENTS**

428

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557 **FIGURES**
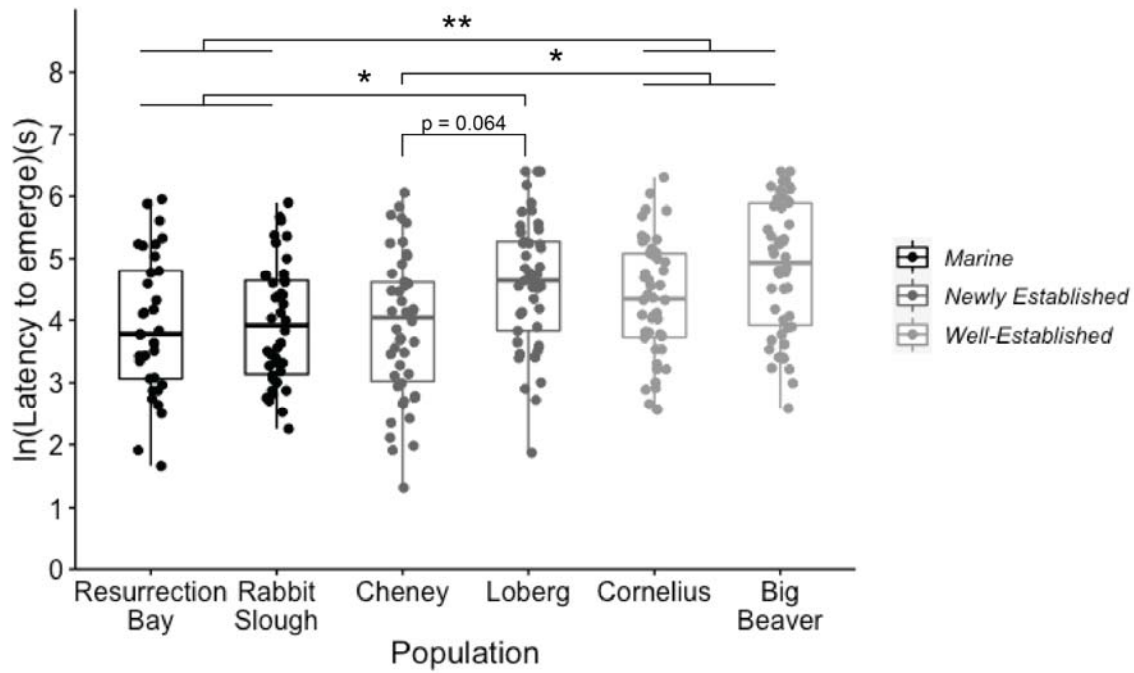
558

559 **Figure 1**. Variation among populations in boldness (latency to emerge from a refuge). Smaller

560 values indicate greater boldness. Figure shows boxplots with individual data points
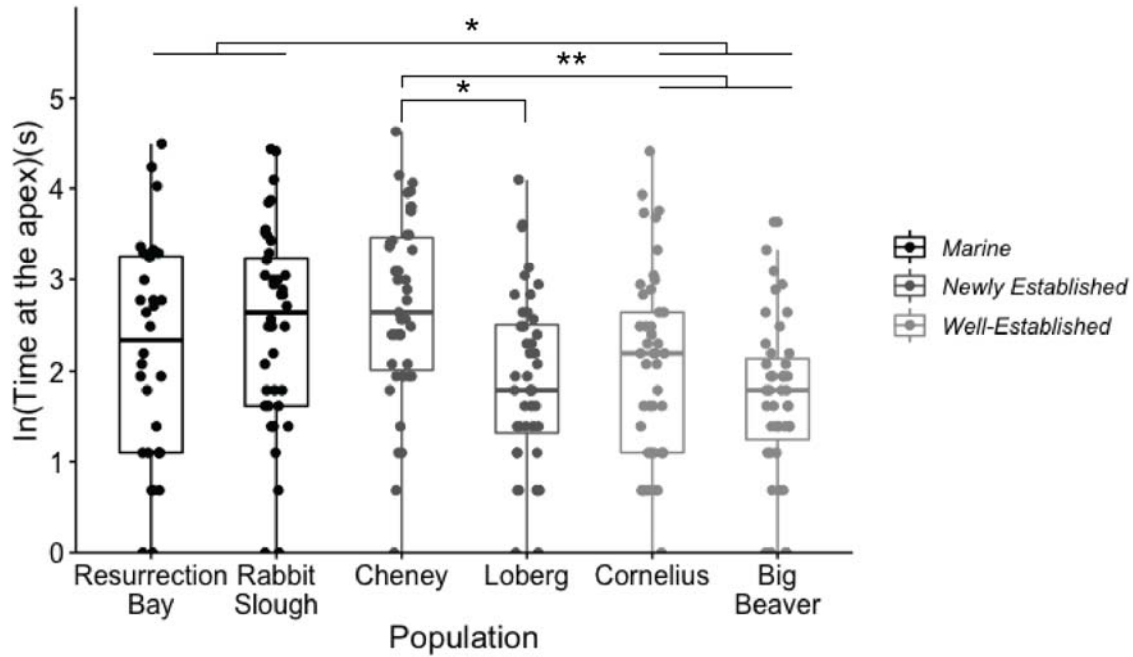
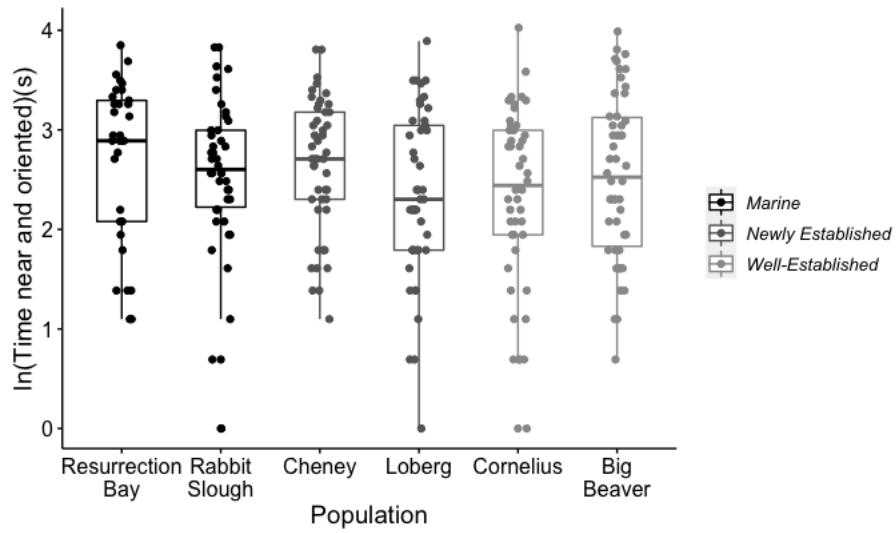561 superimposed.

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Figure 2. Variation among populations in flexibility (time at the apex of the barrier). Smaller

values indicate greater flexibility, i.e., less time persisting on a previously-successful behavior

pattern. Figure shows boxplots with individual data points superimposed.

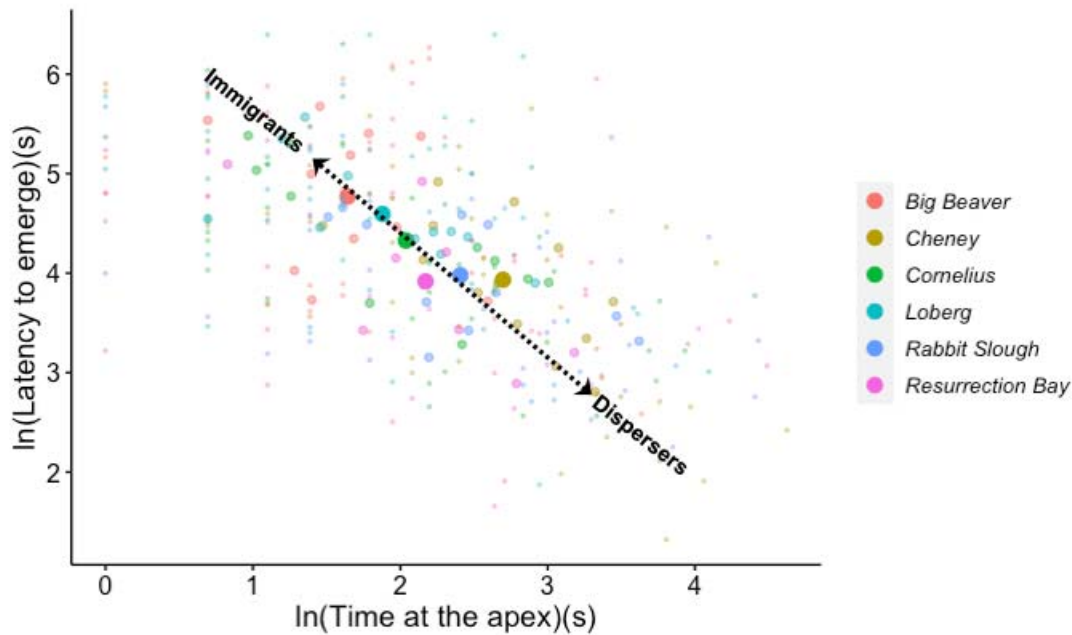573

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Figure 3. Variation among populations in neophilia (time near and oriented to the novel object).

Figure shows boxplots with individual data points superimposed.

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579

580 **Figure 4**. Relationship between flexibility (time at the barrier apex) and boldness (latency to

581 emerge) within and among populations. Dispersers are relatively bold (emerge quickly) while

582 immigrants are relatively flexible (spend less time at the barrier apex). Large circles represent

583 populations means, medium circles show family means, small circles show individual data

584 points. Circles are color coded by population. The line and text drawn on the figure are for visual

585 purposes only.

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589   **Contact and competing interest information for all authors.** The authors have no competing

590   interests.

591

592   **Data sharing plans (including all data, documentation, and code used in analysis).** The data

593   will be made available on Dryad.

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610   **Supplementary Information for**
611   A behavioral syndrome linking boldness and flexibility facilitates invasion success in sticklebacks
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614   Miles K. Bensky and Alison M. Bell

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616   Miles K. Bensky
617   Email: bensky2@illinois.edu
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619   **This PDF file includes:**
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621   Supplementary text
622   Figures S1 to S2
623   Tables S1 to S4
624   Legends for Movies S1 to S2
625   SI References
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627   **Other supplementary materials for this manuscript include the following:**
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629   Movies S1 to S2
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640 **Supplementary Information Text**

641

642 **Supplementary methods.** Embryos were generated via artificial fertilization and incubation. To collect
643 sperm, males from each population were euthanized using an overdose of buffered tricaine
644 methanesolfonate (MS-222), and their testes were immediately dissected and macerated. Eggs were
645 then gently extruded from gravid females from the same population into a petri dish and the macerated
646 testes were pipetted over the eggs to fertilize them. Distilled water with 6 ppt Instant Ocean ® was used
647 to repeatedly rinse the newly fertilized clutches before being stored in that solution in the petri dish. The
648 clutches were inspected daily for proper development; dead embryos and unfertilized eggs were
649 removed, and the water was changed.
650   Once the embryos arrived at the University of Illinois Urbana-Champaign, each clutch was
651 transferred into a plastic cup with a mesh bottom and clipped to the side of a tank (one clutch per tank;
652 (9.5L 32 x 21 x 19 cm)). A bubbler was placed under the cup to provide aeration. Tanks used for rearing
653 embryos were lined with gravel and had a refuge (artificial plant). Embryos were checked daily and dead
654 embryos were removed. Upon hatching (8-13 days post fertilization) fry were fed brine shrimp daily. Once
655 the eggs had hatched the mesh cup and bubbler were removed.
656   At approximately two months of age, the fish were gradually introduced to a mixed diet of frozen
657 bloodworms, frozen brine shrimp, and frozen Mysis shrimp and were fed ad lib once a day. To prevent
658 overcrowding, families were culled to a maximum of 30 fish per tank at two months, and to a maximum of
659 15 fish at approximately six months. Throughout the experiment each family was maintained in its own
660 tank. Therefore, tank effects could potentially contribute to differences among families. Tanks from each
661 population were evenly distributed around the fish room in an attempt to control for location effects.
662   Tissue for DNA was collected by swabbing the side of each fish with a sterile cotton swab to
663 collect DNA for non-invasively determining the sex of each fish with a genetic marker (1). Total genomic
664 DNA was extracted using the DNEasy® Blood and Tissue Kit (Qiagen, Venlo, Netherlands).
665   Tanks used for behavioral observations (36L x 33W x 24H cm) had lines drawn on the bottom to
666 separate it into equal thirds (i.e., left, center, right). Each observation tank was lined with gravel, but the
667 floor was cleared immediately around the lines so that they were still visible from a "top-down" view for the
668 novel object test. A plastic plant was placed into the middle third of each tank to provide a refuge. Opaque
669 dividers were inserted between the observation tanks during behavioral testing; otherwise individuals had
670 visual access to fish in neighboring tanks.
671   *Measuring neophilia as response to a novel object.* To set up the neophilia assay, the plastic
672 plant was removed, plastic dividers were placed on all sides of the tank, and a mirror was positioned at a
673 45-degree angle above the tank to provide a top-down view. A camcorder (JVC Everio HD Hard Dish
674 Camcorder Model No: GZ-HD40U) was used to record the trial via the mirror. A perforated tank divider
675 was used to block the fish from accessing the back part of the tank that was out of the camera's view.
676 One of the outside thirds of the tank was randomly selected, and a circular blind was used to corral the
677 individual into that area. The novel object was then placed in the opposite end. After five minutes, the
678 blind was removed. The observer recorded how much time was spent orienting towards the novel object
679 while within one body length of the object. Upon completion of the test, the home tank was reset to its
680 pretrial state.
681   *Measuring flexibility with the barrier task.* All barrier detour task-related trials occurred in a
682 separate testing tank. This tank had the same dimensions as the individual's home observation tank. No
683 gravel was present to help increase the salience of the food reward. To begin the session, the individual
684 was gently scooped with a white cup from their home tank and transferred to an opaque shelter that was
685 then placed into the back-center of the testing tank and the fish was left undisturbed for three minutes. A
686 60W mm circle was initially drawn on the center of the floor of the tank (~7 cm from the entrance of the
687 shelter), and a single blood worm was placed within that circle. Between trials fish were allowed to
688 reacclimate to the shelter for two minutes. After the fourth trial of the day the fish were returned to their
689 home tanks. If an individual did not reach criterion within four days, an additional individual from that
690 family was sampled later in the experiment if possible, in order to maximize the number of individuals
691 tested within each family. 15 individuals did not reach criterion. For the actual barrier trial fish were given
692 30 minutes to emerge and solve the task. Eleven fish did not solve the task and eat within 30 minutes. All
693 fish approached and ate the food on the fourth trial that followed the barrier trial, suggesting that those
694 that did not solve the task were still motivated by the food reward. Upon completion of the fourth trial, the
695 individual was returned to its home tank.

31

696   ***Compliance with ethical standards.*** Fish were caught in the field using baited minnow traps
697   under the approval of the State of Alaska's Department of Fish and Wildlife permit to M. Bensky (FRP P-
698   17-019 and FTP 17A-0020). In the lab, the fish were housed in groups before and after the experiment.
699   All experimental procedures were non-invasive. While the fish were undergoing training they were housed
700   individually, but were given visual access to neighboring fish when they were not participating in active
701   trials in order to enhance their welfare. Fish were transferred from their home tank to the training tanks by
702   gently scooping them in a cup to minimize stress.
703   ***Statistical analysis.*** When there was a significant effect of population, we first tested the
704   hypothesis that marine and well-established freshwater populations differ by creating a contrast matrix
705   that directly compared these two population types (2)(package = "multcomp"; function = "glht"). We then
706   performed post-hoc tests to compare the newly established freshwater populations. Contrast matrices
707   were constructed so that post-hoc p-values were adjusted for multiple comparisons. Post-hoc contrasts
708   focused on the two newly derived populations (Cheney Lake and Loberg Lake) because visual inspection
709   of the data suggested that they are different. The first post-hoc contrast was between Cheney Lake and
710   Loberg Lake. Then Cheney Lake, the newest freshwater population (8 years from collection), was
711   compared to the established freshwater populations, while Loberg Lake (28-34 years from collection) was
712   compared to the marine fish, and Cheney Lake and Loberg Lake were directly compared.
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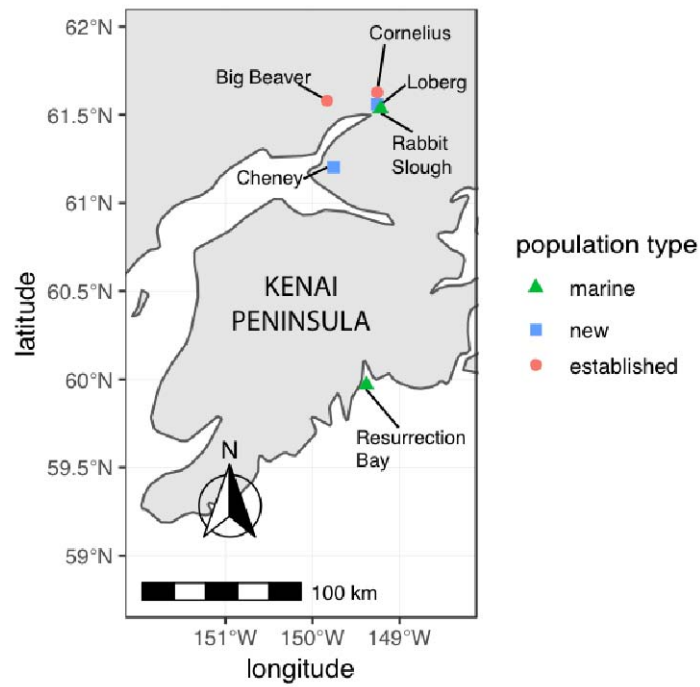
Fig. S1. Map of sampling sites.

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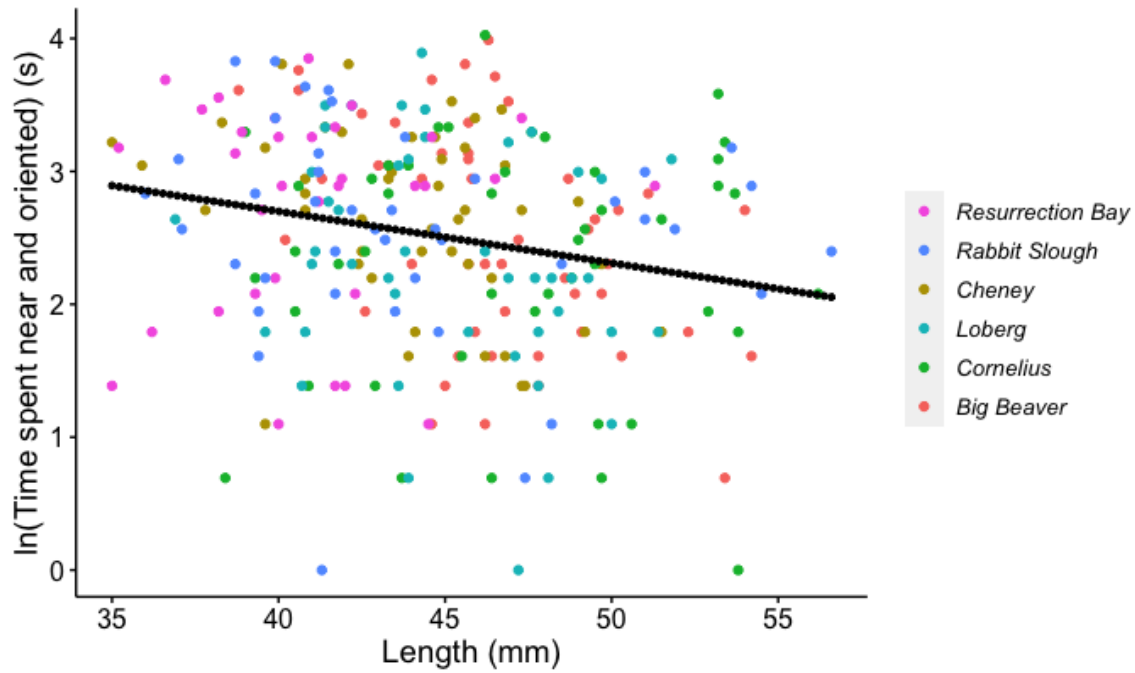
Fig. S2. Bigger fish were less neophilic (spent less time near and oriented to the novel object). Each data point represents a different individual, color coded by population.

723

724   **Table S1**. Linear mixed models testing the influence of population, length and sex on boldness (latency

725   to emerge), flexibility (time at the apex) and neophilia (time near and oriented to the novel object.

726   Significant terms are in bold.

727

Behavior	Factor	Sum Sq	Mean Sum Sq	Num DF	Den DF	F	p-value
Boldness (latency to emerge)							
	Population	**14.969**	**2.994**	**5**	**58.946**	**3.299**	**0.011**
	Length	0.037	0.037	1	253.774	0.041	0.840
	Sex	2.610	2.609	1	248.498	2.875	0.091
Flexibility (time at the apex)							
	Population	**15.459**	**3.092**	**5**	**58.009**	**3.495**	**0.008**
	Length	0.0001	0.0004	1	237.932	0.001	0.982
	Sex	1.008	1.008	1	235.742	1.140	0.287
Neophilia (time near and oriented to the novel object)							
	Population	1.474	0.295	5	59.568	0.512	0.766
	Length	**3.986**	**3.986**	**1**	**247.793**	**6.923**	**0.009**
	Sex	0.407	0.407	1	253.628	0.707	0.401

Table S2. **Broad-sense heritabilities and genetic correlations within each population.**

Heritabilities are on the diagonal, genetic correlations are on the off-diagonal, with 95% confidence intervals in parentheses. Bold indicates that the estimate is statistically different from zero. Greater boldness trait values correspond to lower boldness (i.e., slower to emerge), greater flexibility trait values correspond to less flexibility (i.e., more time persisting at the apex of the barrier).

Big Beaver. Sample size for correlations with flexibility is n=43, otherwise n=50

	Boldness	Flexibility	Neophilia
Boldness	**0.754 (0.153, 1.000)**	-0.588 (-0.880, 0.295)	-0.219 (-0.747, 0.443)
Flexibility	--	0.225 (0.000, 0.974)	-0.327 (-0.782, 0.431)
Neophilia	--	--	0.096 (0.000, 0.437)

Cheney Lake. Sample size for correlations with flexibility is n=43, otherwise n=46

	Boldness	Flexibility	Neophilia
Boldness	0.252 (0.000, 0.983)	-0.718 (-0.925, 0.134)	0.171 (-0.584, 0.667)
Flexibility	--	0.325 (0.000, 0.994)	0.0144 (-0.574, 0.647)
Neophilia	--	--	0.151 (0.000, 0.684)

Cornelius. Sample size for correlations with flexibility is n=45, otherwise n=46

	Boldness	Flexibility	Neophilia
Boldness	**0.737 (0.092, 1.000)**	**-0.777 (-0.931, -0.231)**	**-0.762 (-0.899, -0.206)**
Flexibility	--	**0.864 (0.378, 1.000)**	**0.599 (0.053, 0.895)**
Neophilia	--	--	**0.876 (0.458, 1.000)**

Loberg Lake. Sample size for correlations with flexibility is n= 44, otherwise n= 45

	Boldness	Flexibility	Neophilia
Boldness	0.103 (0.000, 0.511)	-0.619 (-0.903, 0.282)	-0.586 (-0.862, 0.381)
Flexibility	--	0.692 (0.014, 1.000)	0.585 (-0.258, 0.856)
Neophilia	--	--	0.378 (0.000, 0.993)

Rabbit Slough. Sample size for correlations with flexibility is n=40, otherwise n=42

	Boldness	Flexibility	Neophilia
Boldness	0.322 (0.000, 0.991)	-0.663 (-0.903, 0.298)	-0.206 (-0.691, 0.614)
Flexibility	--	0.329 (0.000, 0.996)	0.094 (-0.571, 0.758)
Neophilia	--	--	0.183 (0.000, 0.892)

Resurrection Bay. Sample size for correlations with flexibility is n=31, otherwise n=33

	Boldness	Flexibility	Neophilia
Boldness	**0.700 (0.012, 1.000)**	-0.756 (-0.936, 0.259)	-0.237 (-0.746, 0.614)
Flexibility	--	0.279 (0.000, 0.995)	-0.011 (-0.700, 0.753)
Neophilia	--	--	0.355 (0.000, 0.993)

Table S3. **Phenotypic correlations (Pearson) within each population.** Bold indicates P<0.05.

Populations combined. Sample size for correlations with flexibility is n=247, otherwise n=262

	Boldness	Flexibility	Neophilia
Boldness	--	**-0.522**	**-0.315**
Flexibility		--	**0.229**
Neophilia			--

Big Beaver. Sample size for correlations with flexibility is n=43, otherwise n=50

	Boldness	Flexibility	Neophilia
Boldness	--	**-0.297**	**-0.422**
Flexibility		--	0.038
Neophilia			--

Cheney Lake. Sample size for correlations with flexibility is n=43, otherwise n=46

	Boldness	Flexibility	Neophilia
Boldness	--	**-0.576**	-0.100
Flexibility		--	0.156
Neophilia			--

Cornelius. Sample size for correlations with flexibility is n=45, otherwise n=46

	Boldness	Flexibility	Neophilia
Boldness	--	**-0.618**	**-0.554**
Flexibility		--	**0.450**
Neophilia			--

Loberg Lake. Sample size for correlations with flexibility is n=44, otherwise n=45

	Boldness	Flexibility	Neophilia
Boldness	--	**-0.402**	**-0.422**
Flexibility		--	**0.293**
Neophilia			--

Rabbit Slough. Sample size for correlations with flexibility is n=40, otherwise n=42

	Boldness	Flexibility	Neophilia
Boldness	--	**-0.440**	-0.085

Flexibility		--	0.228
Neophilia			--

Resurrection Bay. Sample size for correlations with flexibility is n=31, otherwise n=33

	Boldness	Flexibility	Neophilia
Boldness	--	**-0.478**	-0.127
Flexibility		--	0.021
Neophilia			--

Table S4. **Repeatability of latency to emerge across four trials.** Repeatability estimate was calculated using Bayesian statistics with Markov Chain Monte Carlo simulations using MCMCglmm package (3) in R 3.5.3 (http://www.r-project.org/). A Poisson distribution was used because latency to emerge was positively skewed and used full integer time durations. We used non-informative proper priors with 1 000 000 iterations, thinning of 100 iterations, and burn-in of 100 000 iterations. Through these simulations, 95% confidence intervals were generated with significance of our estimate inferred according to whether the lower bound of the interval approached zero.

Population	n	Repeatability	Lower, Upper CI
Big Beaver	50	0.6985	(0.5620,0.7866)
Cornelius	46	0.6083	(0.4782,0.7367)
Loberg Lake	45	0.6565	(0.4950,0.7553)
Cheney Lake	46	0.6499	(0.5385,0.7824)
Rabbit Slough	42	0.5645	(0.3961,0.7019)
Resurrection Bay	33	0.7528	(0.6218,0.8605)

Movie S1 (separate file): Boldness assay (emergence from a refuge)

Movie S2 (separate file): Flexibility assay (response to a barrier)

SI References

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2. R. C. Team, *R: A language and environment for statistical computing* (R Foundation for Statistical Computing, Vienna, Austria, 2013).
3. J. D. Hadfield, MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. *2010* **33**, 22 (2010).