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9 Using seasonal genomic changes to understand historical adaptation to new environments:

10 parallel selection on stickleback in highly-variable estuaries

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29 **Abstract**

30 Parallel evolution is considered strong evidence for natural selection. However, few studies
31 have investigated the process of parallel selection as it plays out in real time. The common
32 approach is to study historical signatures of selection in populations already well adapted to
33 different environments. Here, to document selection under natural conditions, we study six
34 populations of threespine stickleback (*Gasterosteus aculeatus*) inhabiting bar-built estuaries
35 that undergo seasonal cycles of environmental changes. Estuaries are periodically isolated from
36 the ocean due to sandbar formation during dry summer months, with concurrent
37 environmental shifts that resemble the long-term changes associated with postglacial
38 colonization of freshwater habitats by marine populations. We used pooled whole-genome
39 sequencing (Pool-WGS) to track seasonal allele frequency changes in six of these populations
40 and search for signatures of natural selection. We found consistent changes in allele frequency
41 across estuaries, suggesting a potential role for parallel selection. Functional enrichment among
42 candidate genes included transmembrane ion transport and calcium binding, which are
43 important for osmoregulation and ion balance. The genomic changes that occur in threespine
44 stickleback from bar-built estuaries could provide a glimpse into the early stages of adaptation
45 that have occurred in many historical marine to freshwater transitions.

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47 **Introduction**

48 Knowledge of the genomic targets of natural selection is central to understanding the
49 mechanisms responsible for adaptive evolution at the molecular level. Generating this
50 knowledge often involves comparing patterns of genomic differentiation (e.g., F_{ST}) between
51 populations adapted to distinct ecological conditions (e.g. Hoekstra et al. 2006; llardo et al.
52 2018). In such studies, natural selection is considered a strong candidate for the mechanism
53 driving phenotypic diversification when multiple closely related but independently-evolved
54 populations use the same genetic pathways to reach a shared adaptive solution to an
55 environmental challenge. Here, we refer to this phenomenon as parallel evolution (Elmer and
56 Meyer 2011). In vertebrates, studies of parallel evolution have provided insights into the
57 genetic mechanisms underlying adaptation to freshwater (Colosimo et al. 2005; Barrett et al.
58 2008; Schluter et al. 2010; Hohenlohe et al. 2010; Kitano et al. 2010; Lescak et al. 2015; Rudman
59 et al. 2019; Fang et al. 2020), novel pathogens (Alves et al. 2019), low oxygen availability
60 (McCracken et al. 2009; Foll et al. 2014; Wang et al. 2014; Graham and McCracken 2019; Lim et
61 al. 2019), crypsis (Comeault et al. 2016; Jones et al. 2018; Barrett et al. 2019), nutrient-limited
62 environments (Riddle et al. 2018), and dissolved ion (H^+) profiles (Haenel et al. 2019). However,
63 nearly all studies of parallel evolution are retrospective in the sense that they investigate
64 reasonably well adapted populations long after selection for successful habitat transition
65 occurred.

66 Retrospective approaches thus have difficulty detecting the specific genetic changes
67 that were under natural selection during the initial habitat shift amongst the noise from local
68 effects and stochastic processes that accumulates afterward (Elmer and Meyer 2011). A
69 valuable addition to the inferential toolbox, then, is to study natural selection that takes place
70 *during* parallel habitat shifts. Most studies adopting this selection-based approach have used
71 artificial perturbations of genotypes or environments, which have revealed genomic targets of
72 strong selection (e.g. Soria-Carrasco et al. 2014; Nosil et al. 2018; Barrett et al. 2019). However,
73 the manipulations involved in such tests leaves uncertain the extent to which similar selection

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74 would attend natural habitat shifts. One solution is to take advantage of serendipitous events,
75 like studying populations before and after extreme weather events (Grant et al. 2017), like
76 hurricanes (Donihue et al. 2018), heat waves (Coleman et al. 2020; Gurgel et al. 2020) and cold
77 snaps (Campbell-Staton et al. 2017; Card et al. 2018). However, the location and timing of
78 extreme weather events are unpredictable by nature, limiting the study of how natural
79 populations respond to such events. The optimal situation, then, might be to study selection as
80 it occurs in natural populations experiencing large, yet predictable, shifts between alternative
81 environments, like those that occur during seasonal changes (e.g. Behrman et al. 2018;
82 Tourneur et al. 2020). Here, we search for the signature of natural selection in a set of
83 populations that experience parallel seasonal changes in local conditions that likely resemble
84 the early phase of a classic habitat transition. Specifically, we study allele frequency changes in
85 stickleback populations in environments that alternate between marine-like (brackish) and
86 freshwater conditions.

87

88 *Study system*

89 The threespine stickleback (*Gasterosteus aculeatus*, 'stickleback' hereafter) is a classic model
90 system for studying parallel evolution (Boughman et al. 2005; Colosimo et al. 2005; Jones et al.
91 2012a, 2012b; Deagle et al. 2013; Hendry et al. 2009, 2013; Lescak et al. 2015; Paccard et al.
92 2018; Haenel et al. 2019; Smith et al. 2020). Over the past approximately 12,000 years, marine
93 stickleback have repeatedly colonized and become adapted to freshwater environments, often
94 through parallel phenotypic changes (Reimchen 1983; Colosimo et al. 2005) linked to predator
95 defence (Reimchen 2000; Marchinko 2009; Miller et al. 2019; Wasserman et al. 2020) and ion
96 regulation (Gibbons et al. 2016, 2017; Hasan et al. 2017). The genomic basis of such adaptation
97 is partly known. For instance, researchers have identified genes of large effect underlying
98 differences in the number of bony armour plates (e.g. *Eda* gene, e.g. Colosimo et al. 2005),
99 pelvic spines (e.g. *Pitx1* gene, Chan et al. 2010), and the ability to osmoregulate (e.g. *Kcnh4*
100 gene, Jones et al. 2012a; *Atp1a1a* gene, McCairns and Bernatchez 2010; Jones et al. 2012b;

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101 Hasan et al. 2017). These genes have been found primarily through a retrospective approach of
102 studying signatures of selection millennia after the initial colonization.

103 Stickleback populations in bar-built estuaries along the coast of California represent a
104 natural system for studying parallel selection over seasonal timescales. These populations
105 experience repeated bouts of strong and abrupt temporal changes driven by wet winters and
106 dry summers. With heavy winter rains, increased water flow breaches the wall of sediment (i.e.,
107 'sandbar') that, during the summer, typically isolates estuaries from the ocean. When rains
108 subside, sandbars are re-built by wave action and sand deposition in the spring or summer,
109 isolating estuaries from the ocean and creating coastal lagoons (Behrens et al. 2009; Behrens et
110 al. 2013; Rich and Keller 2013). The changes in precipitation that lead to the build-up of
111 sandbars and the subsequent breaching can result in drastic environmental shifts in, for
112 example, predator abundance (Becker and Reining 2008), salinity (Williams 2014), and habitat
113 structure (Heady et al. 2015). For example, in between breaching events, a shift takes place
114 from lotic (i.e. moving) brackish water to lentic (i.e. pond-like) freshwater (Heady et al. 2015;
115 Des Roches et al. 2020). Salinity also becomes stratified on the water column (Williams 2014),
116 with freshwater forming the top layer. During the time that the estuary is closed, the surface
117 freshwater layer progressively increases in thickness (see Figure 2.17 of Williams 2014).
118 Following a breaching event there is a mixing of freshwater and saltwater, resulting in drastic
119 increases and decreases in salinity in the top and bottom of the water column, respectively (see
120 Figures 2.19, 2.24 - 2.27 of Williams 2014). These seasonal habitat shifts may be analogous to
121 the environmental changes experienced by stickleback populations during postglacial marine-
122 to-freshwater colonization events and are replicated both spatially (in different estuaries) and
123 temporally (with seasonal changes in precipitation).

124 To study natural selection in action, we sampled stickleback from six bar-built estuaries
125 at two time points between breaching events, when the estuaries were isolated from the
126 ocean. Using a whole-genome SNP dataset, we characterized the extent of allele frequency
127 change between the sampling times, which should reflect, at least in part, natural selection
128 taking place during that interval. Then, to evaluate evidence of parallel natural selection, we ask

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129 several questions. (1) What genomic regions show relatively large changes in allele frequency
130 across time in multiple estuaries? (2) For these regions, do the changes in allele frequency
131 occur in parallel across estuaries? (3) Do genes putatively under parallel selection show
132 enrichment of genetic functions consistent with the changes in the environment? By obtaining
133 this information over a seasonal timescale in multiple estuaries, we hope to gain insight into
134 the genetic changes driven by selection that may have occurred when postglacial stickleback
135 populations first colonized freshwater environments from the ocean.

136

137 **Methods**

138 *Field sampling and DNA extraction*

139 In 2016, we sampled stickleback from six bar-built estuaries (Figure 1), three from small coastal
140 watersheds (< 7 km^2; Old Dairy, Lombardi, and Younger) and three that are relatively large
141 watersheds (> 22 km^2; Laguna, Scott, and Waddell; see Table S1 for full estuary names and size
142 metrics). We sampled in the spring at the end of the breaching season upon completion of the
143 sandbar (after winter rain), and again in the fall before the breaching season (before the winter
144 rain, Figure S1). Thus, we are testing for selection during the part of the year that estuaries are
145 isolated from the ocean, which provides a single-season analogue of the marine to freshwater
146 transition that is a classic theme in stickleback research (e.g. Colosimo et al. 2005; Bassham et
147 al. 2018; reviewed by Hohenlohe and Magalhaes 2019). At each time point in each estuary, we
148 collected 40 adult stickleback (> 30 mm in length) by means of minnow traps and beach seines.
149 Although sampling time is less than one generation (~6 months), some stickleback may have
150 given birth shortly after our first sample, with progeny growing large enough to be sampled as
151 adults in our second sample. Selection during our sampling period therefore reflects both
152 differential mortality and reproduction. Fish were euthanized with tricaine methanosulphonate
153 (MS-222) and tissue samples (pectoral fin) were stored in 95% ethanol prior to DNA extraction.
154 Collections were made in accordance with California Scientific Collector's Permit SC-12752.
155 Animal handling protocols were approved by the University of California, Santa Cruz IACUC
156 under protocols Palke-1306 and Palke-1310. We extracted DNA following a standard phenol-

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157 chloroform procedure. Briefly, tissue samples were placed in digestion buffer containing
158 proteinase K and incubated at 55 °C. We then isolated DNA using an isoamyl-phenol-chloroform
159 solution, followed with ethanol precipitation.

160 *Sequencing*

161 We quantified all samples using a Picogreen® ds DNA assay (Thermo Fisher Scientific, Waltham,
162 USA) on an Infinite® 200 Nanoquant (Tecan Group Ltd. Männedorf, Switzerland). Samples were
163 normalized to a dsDNA concentration of 15ng/µl, re-quantified, and pooled according to
164 sampling location and time of sampling. Thus, we created 12 pools of 40 individuals each (i.e.
165 six estuaries sampled at two time points). Whole-genome libraries of each pool were prepared
166 at the McGill University and Genome Quebec Innovation Center, Montreal, Canada, and
167 sequenced across five lanes of Illumina HiSeq2500 with paired-end, 125bp reads.

168 *Bioinformatics*

169 We filtered raw reads based on quality (--quality-threshold 20) and length (--min-length 50)
170 with the *trim-fastq.pl* script of *Popoolation* (Kofler et al. 2011a). The resulting reads were
171 mapped to the stickleback reference genome (BROADS S1) using *BWA mem* v. 0.7.13 (Li and
172 Durbin 2009). We then used *SAMtools* (Li et al. 2009) to convert SAM files to BAM format and
173 remove reads with mapping quality below 20 (samtools view -q 20). We then generated a
174 mpileup file (samtools mpilep -B) and filtered for a minimum depth of coverage of 5X. We
175 converted the mpileup file to the synchronized (sync) format using *Popoolation2* (Kofler et al.
176 2011b) for downstream analysis.

177 *Analysis of repeated genomic differentiation*

178 In this study we use F_{ST} to measure changes in allele frequency within a lineage across time
179 points (rather than differentiation between two lineages, Burri 2017). First, within each estuary,
180 we calculated F_{ST} (Hartl and Clark 1997) at the SNP level to identify variants showing relatively
181 large changes in allele frequency (i.e. outliers) between the two time points. We then
182 quantified the extent of overlap in these outliers among estuaries. To obtain genome-wide F_{ST}
183 estimates, we applied the *fst-sliding.pl* script of *Popoolation2* for each of the six estuaries post-

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184 breaching (i.e. brackish conditions) versus pre-breaching (freshwater) (--min-count 2, --min-
185 coverage 5, --max-coverage 100, --min-covered-fraction 0, --window-size 1, --step-size 1, --pool-
186 size 40:40:40:40:40:40:40:40:40:40:40:40, --suppress-noninformative). We only analyzed
187 genomic regions assembled at the chromosome level (i.e. scaffolds excluded). We included data
188 from chromosome 19 (allosome) as we did not find evidence of any artefact on this
189 chromosome or large differences in the coverage (mean = 23.92, SD: 6.48, range 5-74) relative
190 to the genome-wide average (see Results). We identified F_{ST} outliers as SNPS that fell in the top
191 5% of the F_{ST} distribution. These loci were excluded from calculations of genome-wide F_{ST} and
192 allele frequency change distributions to obtain estimates for putatively neutral SNPs (e.g.
193 Batista et al. 2016). To discover candidates potentially under selection, we focused on SNPs that
194 showed large allele frequency changes in multiple estuaries. Because drift and sampling
195 variance will affect loci at random across the genome within any particular estuary, it is unlikely
196 that consistent genetic changes across three or more different estuaries will be due to
197 stochastic processes. We used a custom bash script to quantify F_{ST} outlier overlap across
198 estuaries and identify SNPs that qualify as outliers in at least three out of the six estuaries.
199 However, because evidence of repeated changes in allele frequency in the same SNP (as shown
200 by F_{ST}) does not necessarily mean that these changes were parallel (i.e. in estuary *X* an allele
201 shows a large *increase* in frequency, while in estuary *Y* the same allele experiences a large
202 *decrease* in frequency), we also tested for parallelism in allele frequency change.

203 *Parallel changes in allele frequencies in response to seasonality*

204 We identified SNPs showing consistent directional changes in allele frequency across our
205 estuaries using the program *PoolFreqDiff* (Wiberg et al. 2017). *PoolFreqDiff* uses a generalized
206 linear model with a quasibinomial error distribution (qGLM). Wiberg et al. (2017) showed that
207 the qGLM has a substantially lower false positive rate than the Cochran-Mantel-Haenszel test, a
208 method commonly used in pool-seq studies to identify consistent changes in allele frequency
209 across replicates. We used the same flags (e.g. minimum read count and coverage settings) in
210 the *PoolFreqDiff* program as in our F_{ST} analysis. The qGLM test implemented in *PoolFreqDiff* has
211 the potential to identify parallel selection reflected as subtle, but consistent, changes in allele

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212 frequency in multiple estuaries. Such small changes in allele frequency are unlikely to be
213 identified as F_{ST} outliers in individual estuaries. We used the 'no rescaling' option of
214 *PoolFreqDiff* (re-scaling allele counts relative to the effective sample size gave similar results).
215 We corrected for population structure using the empirical null-hypothesis approach (Caye et al.
216 2016; François et al. 2016). Visual inspection of the histogram of corrected P-values confirmed a
217 uniform distribution, indicating that confounders were controlled (Figure S2). Next, we
218 corrected for multiple hypothesis testing using the false discovery rate (FDR) procedure
219 implemented in the R package *qvalue* V2.14 (Storey et al. 2018). We analyzed three sets of
220 outliers to study potential targets of selection. First, to look for strong and parallel changes in
221 allele frequency, we categorized 'F_{ST}-qGLM outliers' as SNPs that are an F_{ST} outlier in at least
222 three estuaries and also significant (FDR = 1%) under the qGLM model. Second, we identified
223 outliers from each of the two distinct approaches (F_{ST} and qGLM) but with more conservative
224 thresholds than those used in the overlapping F_{ST}-qGLM outlier set. For 'F_{ST} candidates', we
225 identified F_{ST} outliers (SNPs in the top 5% of the F_{ST} distribution in a single estuary) that were
226 shared across at least four of six estuaries (as opposed to the three estuaries minimum
227 requirement in the F_{ST}-qGLM outlier set). Note that the frequency changes across these
228 estuaries may not be parallel and thus this outlier set accounts for potential causes of selection
229 that may differ in direction among estuaries. We also tested whether estuary size may influence
230 the likelihood of shared targets of selection (F_{ST} outliers; see supplemental information). Finally,
231 'qGLM outliers' are SNPs identified as highly significant using the quasibinomial GLM test for
232 parallel changes in allele frequency, here using an FDR = 0.01% as opposed to the less
233 conservative FDR = 1% used for the F_{ST}-qGLM outlier set. We obtained estimates of allele
234 frequency change across time points for F_{ST}-qGLM outliers and putatively neutral loci with
235 respect to F_{ST} (not in top 5% F_{ST} distribution) using the *snp-frequency-diff.pl* script of
236 *Popoolation2*.

237 *Identification of candidate genes and analysis of molecular function*

238 To identify genes putatively under parallel selection, we used a custom bash script to map
239 outliers (i.e. F_{ST}-qGLM outliers , F_{ST} candidates, and qGLM outliers) to protein-coding genes in

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240 the reference genome. We limited our search to a set of 14,252 protein-coding gene
241 annotations with attributes “ID=gene” and “biotype=protein_coding”. To gain insights into the
242 traits under selection, we analyzed candidate genes for enrichment of molecular functions. To
243 do this, we obtained gene names and gene ontology (GO) information from the stickleback
244 reference genome on *Ensembl* using the R package *biomaRt* (Smedley et al. 2009). We
245 compared the three lists of candidate genes with the reference set of 14,252 genes (‘gene
246 universe’) and tested for functional enrichment using the package *TopGO* 2.34.0 (Rahnenfuhrer
247 2018) and the Fisher’s exact test. To reduce false positives, we pruned the GO hierarchy by
248 requiring that each GO term have at least 10 annotated genes in our reference list (“nodeSize =
249 10”).

250 **Results**

251 *Data processing and F_{ST} estimates in response to seasonal sandbar formation*

252 Our sequencing efforts led to an average of 23,914,973,875 bases sequenced per pool (SD:
253 1,760,685,042). After filtering data, we obtained 101,911,501 bases for variable sites, providing
254 F_{ST} estimates for 4,024,542 SNPs distributed across 21 stickleback chromosomes. The average
255 minimum coverage per SNP was 25.32 (SD = 6.96, range: 5 – 84, Figure S3) among pools.
256 Overall, allele frequency changed relatively little within estuaries, showing a mean ‘neutral’ F_{ST}
257 of 0.0253 across time points (Waddell = 0.0224; Lombardi = 0.0230; Old Dairy = 0.0216;
258 Younger = 0.0243; Scott = 0.0236; Laguna = 0.0369).

259 *Consistent changes in allele frequency and the signature of parallel selection*

260 To identify candidates under temporally varying parallel selection, we looked for an overlap
261 among estuaries of SNPs that fall in the top 5% of the F_{ST} distribution. As expected, most SNPs
262 found in the top 5% of the distribution in an estuary only reach this threshold in a single estuary
263 (Figure 2). Yet, we find 22,111 SNPs in the top 5% in three or more estuaries. The majority of
264 these SNPs, 19,390 SNPs (87.7%), are confined to exactly three estuaries, with 2,721 ‘F_{ST}
265 candidates’ found in four or more estuaries. At a FDR of 1%, we identified 37,687 SNPs using
266 *PoolFreqDiff*, 705 of which overlapped with the SNPs that were F_{ST} outliers in at least three

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267 estuaries ('F_{ST}-qGLM outliers'; Figure 3 and Figure 4). We also identified 2,411 SNPs with a FDR
268 of 0.01% ('qGLM outliers').

269 *Candidate genes and analysis of molecular function*

270 We mapped outlier SNPs to genes and found 710 genic F_{ST} candidates in 579 different genes
271 (Table S2), 704 qGLM outliers in 569 genes (Table S3), and 190 F_{ST}-qGLM outliers in 169
272 different genes (Table S4). All three sets of outliers have candidate genes associated with ion
273 transfer, including *Wnk4* (Table S2 and Table S3) and *Nalcn* (Table S3 and Table S4). We find
274 consistent changes in allele frequency in ATPase genes that code for proteins that transport, for
275 example, sodium and potassium (e.g. *Atp1b1a*, Table S2) and phospholipids (*Atp8b5a*, Table S2
276 and Table S4). Potassium transport channels are also found among our candidate genes (e.g.
277 *Kcnma1a*, Table S3 and Table S4; *Kcnn1a*, Table S2) as well as genes involved in calcium binding
278 or transport, like the calcitonin receptor (*Calcr*, Table S2 and Table S3), calmodulin (*Calm1b*,
279 Table S2) and the calcium channel *Cacna1d* (Table S4). Yet others code for various mitogen-
280 activated protein kinases (e.g. *Map3k12*, Table S3; *Mapkbp1*, Table S4). We also found an F_{ST}
281 outlier mapping to *Ccny* (Table S2).

282 *GO analysis*

283 For an overall assessment of the gene functions that are most represented among our three
284 sets of candidate genes, we tested for enrichment of molecular function. We find that the
285 candidate genes from all three outlier sets have in their top ten most significant GO terms
286 molecular functions related to ion channel activity. For example, F_{ST} candidates show enriched
287 GO functions related to calcium ion binding (Table S5). Among the most enriched molecular
288 functions among qGLM candidate genes are metal ion transmembrane transporter activity and
289 calcium ion transmembrane transporter activity (Table S6). Similarly, F_{ST}-qGLM outlier genes
290 are enriched for ion transmembrane transporter activity and ligand-gated ion channel activity
291 (Table S7). We also found extracellular matrix structural constituent (Table S5) among the
292 significant molecular functions (see Discussion).

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294 **Discussion**

295 To document natural selection, we studied stickleback populations from estuaries experiencing
296 seasonal fluctuations in environmental conditions. We found evidence of parallel selection on
297 genes linked to ion transport and salinity adaptation. Consistent with a change in the ionic
298 environment, we found that the most statistically significant functions were related to ionic
299 homeostasis. Our findings suggest that intermittent connectivity with the ocean results in
300 episodic shifts in selection regime, a change that may resemble the initial phase of freshwater
301 colonization that occurred during the marine-freshwater transitions of postglacial stickleback
302 populations. More generally, our study adds to a growing literature that collectively shows that
303 natural selection can drive genetic change over very short timespans.

304

305 *Parallel selection over a seasonal timescale*

306 We found evidence of natural selection in the form of consistent changes in allele frequency in
307 stickleback populations from different bar-built estuaries. Our results showed changes in allele
308 frequency on genes with functions related to ion balance. This suggests that seasonal
309 fluctuations of environmental conditions shift the selection regime within bar-built estuaries.
310 The time scale involved in the overall subtle but consistent changes in allele frequency detected
311 here conforms with studies that show selection for freshwater adaptation can be detected
312 within a single year rather to decades (Bell 2001; Kristjánsson et al. 2002; Bell et al. 2004;
313 Lescak et al. 2015; Marques et al 2018). These results are also consistent with the idea that
314 adaptation to a particular environment, like freshwater, likely happens in the first few
315 generations after colonization (e.g. reviewed by Reimchen et al. 2013). Importantly, our results
316 set a new bar for how quickly selection can result in genetic adaptation during freshwater
317 colonization by stickleback.

318

319 *Seasonal allele frequency changes in genes involved in ion balance*

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320 Over the last 10,000 years, post-glacial stickleback populations have adapted to the different
321 osmotic conditions found in freshwater (Spence et al. 2012). In this study, we found that
322 seasonal isolation from the ocean led to repeated changes in allele frequency in many genes
323 linked to ion balance. For example, genes such as *Nalcl* and *Wnk4* show signs of potential
324 selection as shared outliers across multiple estuaries. *Nalcn* is a salt-sensing gene that was
325 recently found to be rapidly evolving in saline-alkaline lake-dwelling fish (Tong and Li 2020).
326 Similarly, *Wnk4* codes for an intracellular chloride sensor (Chen et al. 2019) implicated in
327 salinity-tolerance in stickleback (Wang et al. 2014). Additionally, we found evidence of parallel
328 selection on the gene *Ccny*. A recent epigenomic study linked *Ccny* to salinity adaptation,
329 showing that *Ccny* is differentially methylated in stickleback populations along a gradient of
330 decreasing salinity (Heckwolf et al. 2020). We also found evidence of selection on genes from
331 the *Mapk* family. These genes are differentially expressed in fish in response to many
332 environmental stressors (Mateus et al. 2017), including osmotic stress (Tse et al. 2011; Tian et
333 al. 2019; reviewed by Kültz 2012).

334 We also found evidence of selection on genes for calcium balance, for example, the
335 calcium sensing gene calmodulin (*calm1b*, Chin and Means 2000). Another gene we found to be
336 putatively under seasonal parallel selection is the calcitonin receptor (*Calcr*), part of a family of
337 genes known to regulate calcium homeostasis (Naot et al. 2019). Indeed, a gene from the same
338 family, the calcitonin gene-related peptide type 1 receptor (*Calcrl*), has been implicated in
339 salinity tolerance or osmoregulatory adaptation in postglacial stickleback populations
340 (Kusakabe et al. 2017). These findings suggest a potential role for selection on these loci.
341 However, to avoid false conclusions based on selective assessment of particular genes we
342 analyzed the overall genetic functions of candidate genes.

343

344 *Temporal changes in bar-built estuaries likely select for ionic homeostasis*

345 We focused on the top ten most significant GO terms in each outlier set and found functions
346 related to ion transmembrane transport among candidate genes. Our findings are consistent
347 with previous studies on freshwater adaptation that have documented divergence in genes

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348 linked to ion channels (e.g. sodium/potassium channels) during parallel adaptation to
349 freshwater (DeFaveri et al. 2011; Jones et al. 2012a; Jones et al. 2012b; Gibbons et al. 2016;
350 Gibbons et al. 2017; Hasan et al. 2017; Rudman et al. 2019; Heckwolf et al. 2020). Freshwater
351 adaptation has occurred independently in a wide range of taxa through selection on genes
352 involved in osmoregulation. For instance, annelids (Horn et al. 2019), arthropods (Lee et al.
353 2011), and fish (Velotta et al. 2016), including sculpin (Dennenmoser et al. 2016) and
354 stickleback (Jones et al. 2012b; Kusakabe et al. 2016; Hasan et al. 2017), have all shown genetic
355 changes in ion channel genes following colonization of freshwater habitats. Our findings are
356 also in line with a recent study (Tong and Li 2020) on adaptation of fish to a saline-alkaline lake,
357 which showed that rapidly-evolving genes, those with an elevated rate of non-synonymous
358 substitutions, are overwhelmingly involved in ion transport. Our findings of gene functions
359 related to transmembrane ion transport could also be in part driven by changes in temperature
360 over the time period sampled (during the summer months). Increases in temperature may
361 disrupt osmoregulation. A study on estuary fish from California showed that experimentally
362 exposing fish to higher temperature results in differential expression of some of the same
363 genetic functions we found overrepresented among our candidate genes, including ion channel
364 activity and extracellular matrix structural constituent (Jeffries et al. 2016). Thus, changes in
365 temperature could amplify the osmoregulatory challenges experienced by populations
366 responding to changes in salinity.

367 Freshwater adaptation in stickleback also could involve genetic changes to maintain
368 calcium homeostasis (Gibbons et al. 2016). Calcium binding proteins play an important
369 physiological role in maintaining calcium balance in fish (Evans et al. 2005). Calcium must be
370 continuously absorbed from freshwater, which is hypoosmotic relative to fish plasma (Liem et
371 al. 2001; reviewed by Evans et al. 2005). Fittingly, not only did we find consistent changes in
372 allele frequency in relevant genes, but we also found an overall enrichment of gene functions
373 related to calcium ion binding. This provides additional evidence that genes for ion regulation
374 are targets of selection during freshwater transitions that last from months to millennia.

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376 **Conclusion**

377 We found evidence of natural selection for osmoregulatory adaptation, likely brought into
378 operation by seasonal changes in the ionic environment within estuaries. Repeated changes in
379 allele frequency across estuaries suggests parallel selection is occurring, highlighting the power
380 of this system for studying adaptive evolution over very short timescales. Our results are
381 consistent with the idea that cyclical isolation and exposure to the ocean results in seasonally
382 oscillating selection, although time-series data over multiple instances of sandbar formation is
383 needed for confirmation. The threespine stickleback found in bar-built estuaries along coastal
384 California thus provide the rare opportunity to study parallel selection in real-time, *in natura*.

385

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389 Climate.

390

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686

687 **Data availability statement**

688 Raw sequence reads are available at the National Center for Biotechnology Information
689 Sequence Read Archive under the accession number PRJNA704280. Quality-filtered allele
690 frequency data for each estuary per time point (synchronized file) are available in Dryad
691 (doi:10.5061/dryad.fbg79cntg) and the scripts used in this study at
692 https://github.com/garfring/Stickleback_Scripts.

693

694 **Contributions**

695 AP, EPP, APH, and RDHB conceived the study. AGE, AP, APH, and RDHB designed the
696 methodological approach. BAW collected samples. AP performed the molecular work. AGE and
697 AP performed the bioinformatics. AGE analyzed the data and created figures with assistance
698 from TJT. AGE wrote the manuscript with intellectual input from all authors.

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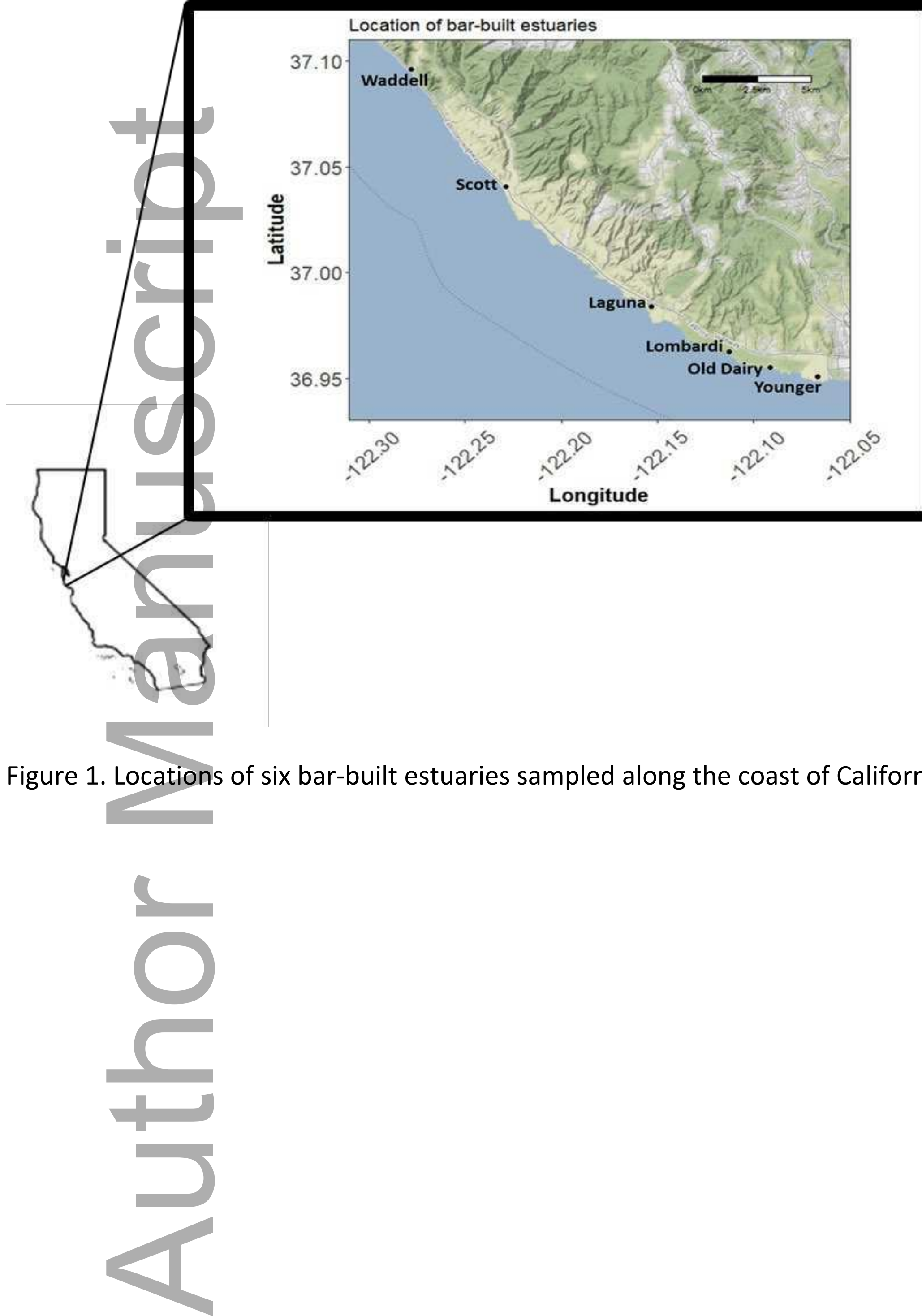

Figure 1. Locations of six bar-built estuaries sampled along the coast of California USA.

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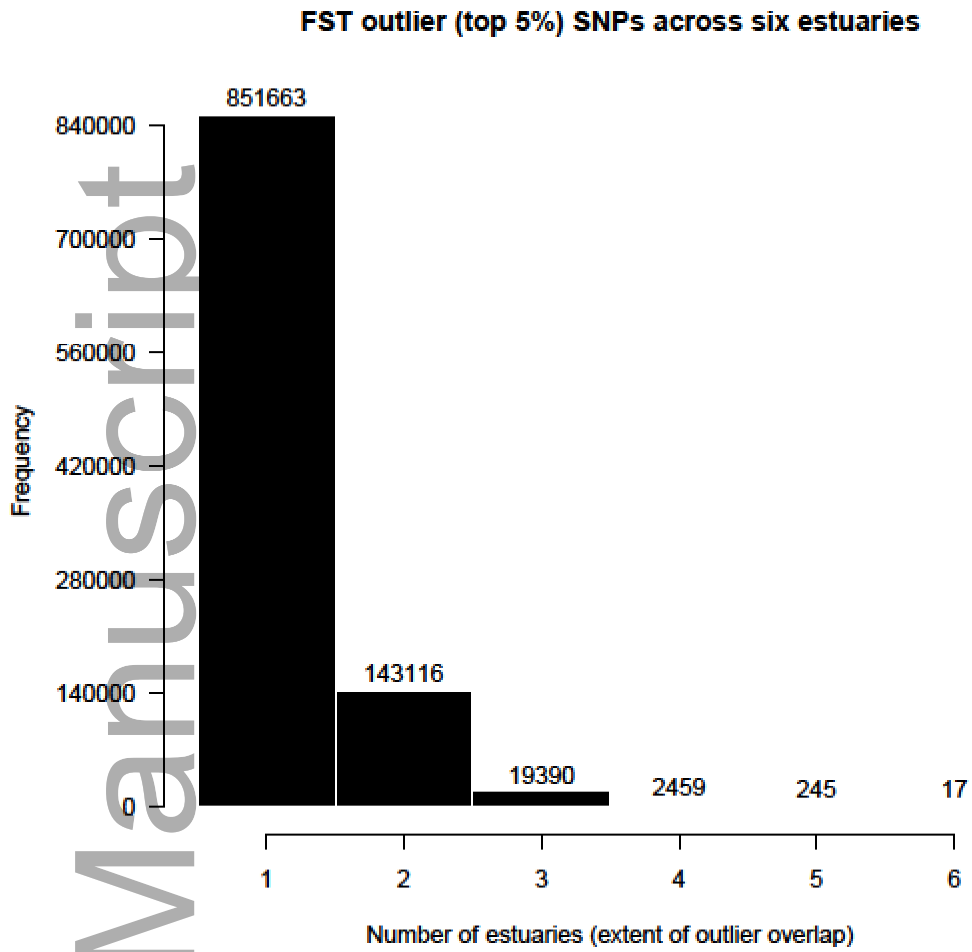

Figure 2. Extent of overlap of F_{ST} outlier loci across six estuaries.

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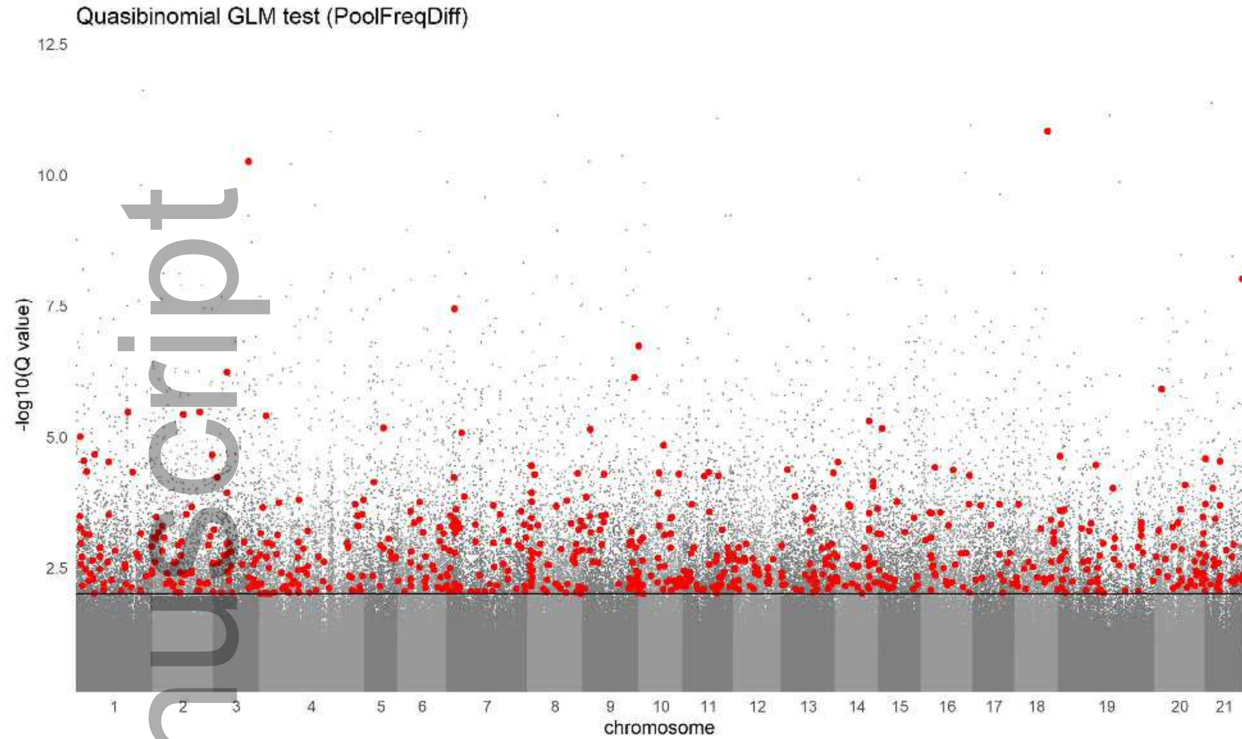

Figure 3. Result of qGLM test (*PoolFreqDiff*) for parallel changes in allele frequency. Each dot represents a single SNP (MT chromosome and unplaced scaffolds excluded). The 705 loci identified by F_{ST} analysis as candidates in at least three estuaries and by the qGLM test as significant (FDR = 1%) are shown in red ('F_{ST}-qGLM outliers'). Black line demarks the 1% false discovery rate.

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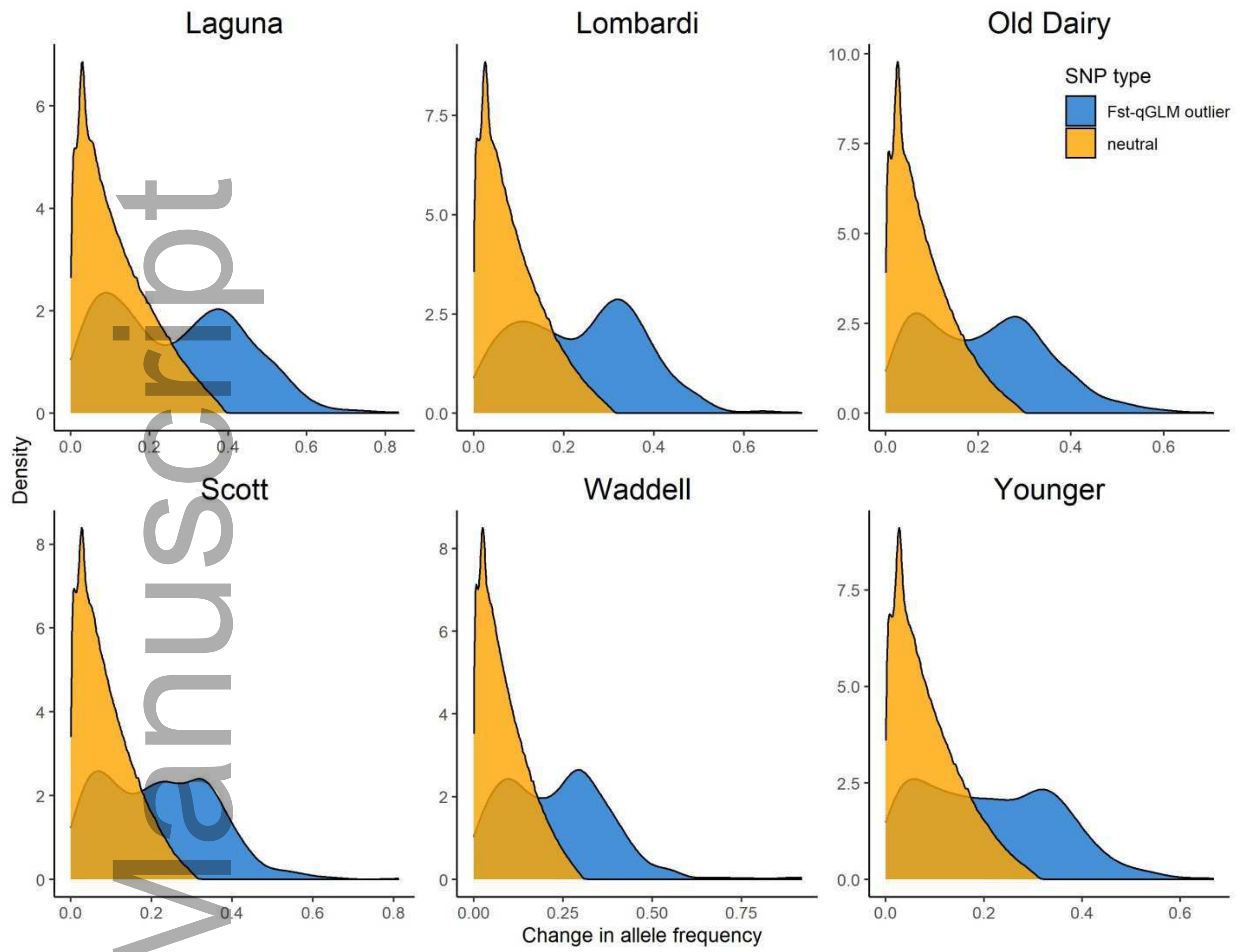

Figure 4. Distribution of allele frequency change in genome-wide SNPs and F_{ST}-qGLM outliers.

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