We get by with a little help from our friends: diversity begets diversity through shared adaptive genetic variation

Emilie J. Richards^{1,2} and Christopher H. Martin^{1,2}

¹Department of Integrative Biology, University of California, Berkeley

²Museum of Vertebrate Zoology, University of California, Berkeley

Correspondence: *chmartin@berkeley.edu*

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Abstract

Adaptive radiations involve astounding bursts of phenotypic, ecological, and species diversity. However, the microevolutionary processes that underlie the origins of these bursts are still poorly understood. We report the discovery of a cryptic ‘wide-mouth’ intermediate scale-eating ecomorph in a sympatric radiation of *Cyprinodon* pupfishes which provides crucial information about the evolutionary and ecological transition from a widespread algae-eating generalist to a novel microendemic scale-eating specialist. We first show that this ecomorph occurs in sympatry with generalist *C. variegatus* and scale-eating specialist *C. desquamator* across several hypersaline lakes on San Salvador Island, Bahamas, but is genetically differentiated, morphologically distinct when reared in a common garden, and sometimes consumes scales. We then compared the timing of selective sweeps on shared and unique adaptive variants in both scale-eating species to characterize the evolutionary path to scale-eating. We predicted that adaptation to the intermediate *C. sp.* ‘wide-mouth’ scale-eating niche aided in the rapid divergence of the more specialized scale-eater *C. desquamator*. Therefore, selection for shared adaptive variants should occur first in ‘wide-mouth’. Contrary to our prediction, four of the six sets of shared adaptive alleles in both scale-eating species swept significantly earlier in *C. desquamator*. Adaptive introgression from *desquamator* into the ‘wide-mouth’ ancestor may have resulted in parallel evolution of their dietary niche. Conversely, no adaptive alleles for scale-eating were reused in a third sympatric molluscivore specialist *C. brontotheriodes*, despite sharing 9% of hard selective sweeps. Our work provides a microevolutionary framework for investigating how ‘diversity begets diversity’ during adaptive radiation.

Author Summary

Adaptive radiations present a paradox to our understanding of the mechanisms that drive speciation. The hallmark of adaptive radiation is a rapid, sustained burst of ecological, phenotypic, and species diversity yet many theoretical models predict that diversification should slow down with each event. Alternatively, radiations could be self-propagating and the diversity generated within the first stages of radiation could beget further diversity. The microevolutionary processes underlying this hypothesis are poorly understood. We propose that the exchange of adaptive alleles from one population could help other populations colonize new fitness peaks and promote further diversification across the fitness landscape. Here we explore the role of shared adaptive alleles in a recent radiation of trophic specialist pupfishes and characterize a cryptic new scale-eating species (*C. sp.* ‘wide-mouth’) through morphological, dietary, and genomic analyses. We discovered shared selective sweeps among all three specialists in this radiation despite their divergent morphologies and ecologies. We also found that adaptive introgression from the more specialized scale-eater *C. desquamator* may have provided access to a similar niche in the recently discovered intermediate scale-eater. Our findings of shared adaptive alleles and selective sweeps across multiple specialists within a radiation provide support for the hypothesis that diversity begets diversity and insight into the underlying microevolutionary process.

Introduction

Rapid bursts of diversification and repeated bouts of speciation like those seen in adaptive radiations contradict many of our existing mechanistic models for understanding speciation (reviewed in [1]). For example, many speciation models predict that diversification should slow with time as available niche space becomes increasingly subdivided and disruptive selection becomes weaker with each recurrent speciation event (e.g. [2–5]). Diversification on complex adaptive landscapes with multiple empty fitness peaks corresponding to different niches provides an alternative mechanism to niche subdivision and might better explain how a rapid burst of radiation can occur [6–9]. There is increasing empirical support for the complexity of fitness landscapes in nature [10–16]. However, these landscapes present a new problem: How do populations manage to escape local optima, cross fitness valleys, and access new fitness peaks [10–12,17–20]?

Colonizing new fitness peaks on the adaptive landscape often requires transitions in behaviors, morphological traits, or a combination of the two that allow organisms to adapt to new ecological niches [21]. Some of the most spectacular ecological transitions occur during adaptive radiations, such as blood-drinking [22] or plant carnivory [23,24]. Adaptation to novel fitness peaks is a hallmark of adaptive radiation, yet it is still poorly understood how such seemingly discontinuous transitions occur. An early hypothesis for how these transitions occurred was through ‘macromutational’ leaps that resulted in ‘hopeful monsters’ [25,26]. Although these ideas were eventually discounted by the insights of the Modern Synthesis [27–30], recent empirical and theoretical research on hybrid speciation has revived an appreciation for the rapid evolution of novelty through transgressive phenotypes resulting from hybridization. For example, hybridization between two species of sunflowers led to new hybrid species with suites of transgressive traits that allowed them to colonize novel sand dune and salt marsh

habitats [31,32]. However, the transgressive effects of hybridization are so myriad [33,34] that conditions for hybridization triggering the evolution of new species or new ecological niches are still poorly understood and contentious in diploid taxa [35,36].

Recent conceptual frameworks for understanding adaptation to novel fitness peaks suggest that ecological transitions are more likely to occur in stages [37–39]. The initial emergence of a novel trait is likely to require further adaptive refinement to become successfully incorporated into the functional ecology of an organism. One of the best examples is the evolution of the novel niche of aerobic citrate metabolism in *E. coli*, in which evolution of *citT* promotor capture in one intermediate lineage actualized the phenotype of aerobic citrate metabolism while further mutations at *dctA* in subsequent lineages refined the efficiency of citrate utilization that allowed it to fully occupy this niche [39–42]. This important set of studies suggests that novel ecological transitions are highly contingent on a complex series of mutations that potentiate, actualize, and refine the adaptations to colonize new fitness peaks [43].

This idea that adaptive alleles from one lineage can set the stage for further diversification of more lineages also underlies some general hypotheses about adaptive radiations, such as the hybrid swarm and syngameon hypotheses – in which radiations are driven by the exchange of genetic variation either from distinct lineages outside the radiation or within the radiation itself [44–46]. There is a wealth of evidence for extensive histories of hybridization in radiations (e.g. [47–53]), that suggests radiations are fueled by diversity that was generated across many lineages. Well documented cases of exchanges of adaptive variation aiding further diversification within a radiation include the exchange of beak shape alleles across Darwin’s finches [54] and wing pattern alleles across *Heliconius* species [49,55–57] that allow multiple species to converge on similar Mullerian mimicry patterns. However, we are only just beginning

to explore how the introduction of genetic diversity from such groups gives recipient lineages access to new fitness peaks and generates the wealth of ecological, morphological, and species diversity of adaptive radiations [1,58,59]. This is necessary to fully understand the extent to which diversity can self-perpetuate during the process of radiation [60].

An adaptive radiation of trophic specialist pupfishes on San Salvador Island in the Bahamas is an excellent system for understanding how the rapid evolution of major ecological transitions occur in nature. This radiation contains a wide-spread generalist pupfish species (*Cyprinodon variegatus*) that occurs in sympatry with two previously described trophic specialists that are endemic to many of the hypersaline lakes on the island: a molluscivore (*C. brontotheroides*) with a novel nasal protrusion which is an oral-sheller of gastropods (St. John et al. 2020) and a scale-eating specialist (*C. desquamator*) with two-fold larger oral jaws [61]. The evolutionary novelties in this system originated recently; the hypersaline lakes on San Salvador Island were dry during the last glacial maximum 6-20 kya years ago [62–64]. Intriguingly, we recently discovered a fourth species of pupfish living in sympatry with the two specialists and generalist on San Salvador Island. This species exhibits intermediate jaw morphology between *C. desquamator* and *C. variegatus* (Fig 1). Furthermore, in a previous whole genome resequencing study, individuals with this intermediate jaw morphology unexpectedly formed a monophyletic group across lake populations distinct from *C. desquamator* using the unsupervised machine-learning approach Saguaro [51,65]. Here we refer to this new ecomorph as the ‘wide-mouth’ because its mouth is wider than any other species in the radiation. The multi-peak fitness landscape driving this radiation suggests that *C. desquamator* is isolated by a large fitness valley from *C. variegatus* and *C. brontotheroides* [10,66] and this intermediate ‘wide-mouth’ may provide clues how this valley was traversed.

Here we first investigate the position of the ‘wide-mouth’ on the ecological spectrum from generalist to scale-eating specialist using a combination of morphological, behavioral, dietary, and genomic data. We then estimated the demographic history of the ‘wide-mouth’ and explore the timing of selection on shared and unique genetic variation involved in adaptation to scale-eating to better understand this ecological transition. Additionally, we explore the extent to which all trophic specialist pupfishes on this island share adaptive genetic variation, despite occupying divergent ecological niches. Our results show that diversification of one species in a radiation can promote further diversification by sharing adaptive genetic variation useful for specialization.

Fig. 1: Distribution of generalist, *C. brontotheriodes*, *C. desquamator*, and *C. sp. 'wide-mouth'* populations in hypersaline lakes on San Salvador Island. Pie charts indicate the presence of sympatric *Cyprinodon* species in each lake and are color-coded with representative pictures of generalist *C. variegatus* (gold), recently discovered *C. sp. 'wide-mouth'* ecomorph (red-orange) with intermediate jaws, scale-eater *C. desquamator* (teal) with the largest oral jaws, and molluscivore *C. brontotheroides* (purple) with characteristic nasal protrusion. Labeled lakes contain all known *C. sp. 'wide-mouth'* populations sampled for this study. Satellite image from Google Earth.

157

158 **Results**

159 *Wide-mouth ecomorph is ecologically intermediate and morphologically distinct*

160 We found the ‘wide-mouth’ ecomorph to be morphologically distinct from *C. desquamator* and
 161 *C. variegatus* across a suite of craniofacial traits (Fig. 2A-B). The lower jaw length of ‘wide-
 162 mouth’ was intermediate between *C. desquamator* and *C. variegatus* (Fig. 2C), while the buccal
 163 width and adductor mandibulae height were 8% larger in ‘wide-mouth’ than *C. desquamator*
 164 (Fig. 2E-F). These morphological differences were consistent across lakes that contained all
 165 three species (Osprey and Oyster) and within Great Lake which contained only ‘wide-mouth’
 166 and generalists (Fig S1). Trait differences in the ‘wide-mouth’ were heritable in a common
 167 garden laboratory environment after one generation: ‘wide-mouth’ displayed 4% larger buccal
 168 width and 2% larger adductor mandibulae insertion height in the lab environment than *C.*
 169 *desquamator* (Fig S2).

Fig 2. The *C. sp. 'wide-mouth'* ecomorph has distinct morphology within the San Salvador Island adaptive radiation across multiple traits. A) First two principal components of morphological diversity for 8 size-corrected traits and 95% confidence ellipses by species (*C. variegatus* : gold; *C. sp. 'wide-mouth'*: red-orange; *C. desquamator*: teal; *C. brontotheriodes* not shown). Shapes of points represent different lakes. PC1 is mainly described by lower jaw length and PC2 by adductor mandibulae insertion height, buccal width, and neurocranium width. B) Depictions of the three external measurements that best distinguished *C. sp. 'wide-mouth'* from both *C. desquamator* and *C. variegatus*, measured using digital calipers. C-E) The relationship between standard length (mm) of individuals and their C) lower jaw length, D) buccal cavity

width, and E) adductor mandibulae insertion height (AM insertion) across individuals of the three species in Osprey Lake. 95% confidence bands for linear models in gray.

‘Wide-mouth’ occupies a distinct intermediate scale-eating niche

We found that ‘wide-mouth’ ingest scales, but at a significantly lower frequency than *C. desquamator* (Wilcoxon Rank Sum test, $P = 0.004$; Fig. 3A). We did not detect any scales in *C. variegatus* guts (Fig 3A). A previous gut content analysis of more individuals also found nearly zero occurrence of scales in the *C. variegatus* diet [67]. All three sympatric species from Osprey Lake collected on the same day from the same site differed in $\delta^{15}\text{N}$ levels (ANOVA, $P > 1.29 \times 10^{-7}$; Fig. 3B); ‘wide-mouth’ $\delta^{15}\text{N}$ was intermediate between *C. variegatus* and *C. desquamator* (Tukey HSD; $P=0.04$ & 0.03 respectively). Similarly, high-speed scale-eating strikes on euthanized fish prey indicate an intermediate lower jaw angle in the ‘wide-mouth’ relative to generalist and scale-eating strikes (Fig. 3C; also see St. John et al. 2019 for analysis of scale-eating strikes relative to generalist).

Fig. 3: The *C. sp. 'wide-mouth'* individuals ingest scales. A) Dot plot of individual scale counts from gut content analysis of the hindgut of Osprey pupfish populations (10 individuals per species). B) Individual variation in relative trophic position ($\delta^{15}\text{N}$ stable isotope ratio) and

dietary carbon source ($\delta^{13}\text{C}$ stable isotope ratio) with 95% confidence ellipses for the three species. C) Still images from Phantom VEO high-speed videos (1,100 frames per second) of scale-eating strikes in *C. desquamator* and *C. sp.* ‘wide-mouth’. Note the distinct lower jaw angle with the suspensorium at the point of impact with the prey.

‘Wide-mouth’ did not result from hybridization between *variegatus* and *desquamator*

We tested whether ‘wide-mouth’ was the product of recent hybridization. First, extensive multi-year sampling efforts show that ‘wide-mouth’ populations are not found in every lake where both *C. variegatus* and *C. desquamator* are present (e.g. Crescent Pond; Fig. 1). However, we explored this possibility in more depth by resequencing 24 genomes of ‘wide-mouth’ across lake populations and comparing these to 86 resequenced genomes of *C. variegatus*, *C. brontotheriodes*, and *C. desquamator* from a previous study [68].

Several lines of genomic evidence support the ‘wide-mouth’ ecomorph as a distinct species. First, ‘wide-mouth’ individuals do not occupy an intermediate position between *C. desquamator* and *C. variegatus* along either of the first two principal components that represent the major axes of genetic variation in the radiation. Instead, ‘wide-mouth’ occupy a distinct cluster relative to the other three species (Fig. 4A). This is further corroborated by the model-based approach of ADMIXTURE, in which ‘wide-mouth’ individuals share more ancestry with each other than with either *C. desquamator* or *C. variegatus* under the two most likely K values based on cross-validation error estimates (K=6 and 7; Fig. 4B). This pattern holds when we decrease or increase the K value used in the analysis to model more or less population structure within groups (Fig. S3). Lastly, we used formal tests for introgression and admixed populations, f_3 and f_4 -statistics, to assess the evidence that ‘wide-mouth’ are the byproduct of recent

admixture. The significant positive f_3 -statistics and the non-significant f_4 -statistics support our inference that the ‘wide-mouth’ is not a recent admixed population of any pairwise combinations of the *C. brontotheriodes*, *C. variegatus*, or *C. desquamator* populations (Table S1).

‘Wide-mouth’ and *C. desquamator* populations are sister species which diverged shortly after the onset of adaptive radiation on San Salvador Island

To better understand the history of divergence and gene flow among the four species on San Salvador Island, we used *fastsimcoal2* [69] to run coalescent simulations and compare 28 different demographic models of divergence and gene flow on San Salvador Island (Table S2). These models represented all possible topologies among the four species and explored zero, early, and current gene flow scenarios. We evaluated their fit against our empirical site frequency spectrum (SFS) calculated from populations of the four species that occur in sympatry in Osprey Pond.

In the best supported model, ‘wide-mouth’ was sister to *desquamator* with current gene flow and a divergence time estimate of 11,658 years ago (95 CI: 8,257-20,113 years; Fig 4C-D) (Table 1 and S2). This model indicates that the ancestor to ‘wide-mouth’ and *C. desquamator* populations first diverged from *C. variegatus* 15,390 years ago (95% CI: 10,722-23,927 years; Fig 4D). This estimate of the origin of the radiation overlaps well with geological age estimates based on the filling of hypersaline lakes on San Salvador Island after the end of the last glacial maximum period (~6-19K years ago; [62–64]). *C. variegatus* and *C. brontotheriodes* populations in Osprey Lake diverged much more recently than the two scale-eating populations 462 years ago (95% CI: 411-1,121 years; Fig 4D).

Table 1. Support for the five best-fitting demographic models for the evolution of the *C. sp.* ‘wide-mouth’ from the site frequency spectrum. The likelihood and AIC scores for the top five best-fitting models estimated in *fastsimcoal2* for the Osprey populations of *C. variegatus* (var), *C. brontotheroides* (bro), *C. desquamator* (des), and *C. sp.* ‘wide-mouth’ (wid) are presented here with a complete list of all models tested reported in Table S2. Change in likelihood (ΔLnL) represents the difference in likelihood from the expected simulated SFS in the demographic model tested. Change in AIC (ΔAIC) represents the difference in AIC scores from the model with the smallest ΔAIC . All models presented here represent different divergence scenarios with recent gene flow allowed, which were better supported than models with no gene flow or early gene flow. Visual representations of the top five models are depicted in Figure S5.

Divergence Model	LnL	ΔLnL	AIC	ΔAIC
(1) ((des,wid),(bro,var)) + (des,wid) diverges first + recent gene flow	-11701	1077	54133	0
(2) ((des,bro),var),wid)) + recent gene flow	-11780	1112	54296	35
(3) ((des,bro),wid),var)) + recent gene flow	-11806	1138	54418	61
(4) ((bro,wid),des),var)) + recent gene flow	-11808	1140	54423	63
(5) ((des,wid),(bro,var)) + (bro,var) diverges first + recent gene flow	-11846	1178	54602	101

Fig 4. *C. sp. 'wide-mouth'* population did not result from recent hybridization. A) Principal components analysis of the four focal groups on San Salvador Island based on an LD-pruned subset of genetic variants (78,840 SNPs). B) Ancestry proportions across individuals of the four focal groups. Proportions were inferred from ADMIXTURE analyses with 2 values of K with the highest likelihood on the same LD-pruned dataset in A. C) Best supported demographic model in which *C. desquamator* diverged from the *C. sp. 'wide-mouth'* based on an LD-pruned dataset with no missing information across all Osprey Lake individuals (67,400 SNPs). Divergence time is shown with the maximum likelihood point estimate from the run with the best fit and the 95% confidence interval for that parameter estimate based on 100 bootstrap replicates. D) Maximum

likelihood point estimate and 95% confidence intervals for migration rate parameters involved in the best fitting model depicted in units of the number of migrants per generation.

Shared signatures of selection across the three specialists in the radiation

Next, we investigated whether the evolution of new trophic specialists on San Salvador Island may have been aided by reuse of the same underlying genetic variation. We characterized regions of the genome under selection in the Osprey Lake populations of the four species using the sliding window SFS-based approach SweeD (Pavlidis et al. 2013) and neutral simulation thresholds based on demographic changes in effective population size over time (Fig S4; Table S3). A higher percentage of the genome was under positive selection in all specialist populations compared to the *C. variegatus* population (Fig 5A). This pattern might be expected given the divergent selection pressures the specialists face to adapt to different trophic niches. Of all the specialists, *C. brontotheriodes* exhibited both the most selective sweeps and the longest selective sweeps (Fig 5A). This suggests that *C. brontotheriodes* have undergone selection most recently among all the species in Osprey Lake and is supported by the recent divergence time between the *C. brontotheriodes* and *C. variegatus* populations (Fig 4D).

Additionally, we assessed patterns of genetic divergence across the four species in Osprey Lake. Allowing for some level of gene flow between species, we calculated the number of fixed and nearly-fixed SNPs ($F_{st} \geq 0.95$) between all pairwise combinations of populations in Osprey Lake. All specialists, including the two scale-eating species, were more genetically diverged from each other than to *C. variegatus* species based on the number of SNPs fixed or nearly-fixed between them (Fig 5B). This pattern of stronger genetic divergence between

specialists also held across fixed SNPs, the top 1% of F_{st} , and genome-wide average F_{st} (Table S4). Next, we identified a set of candidate adaptive alleles for each specialist by filtering for SNPs that were fixed or nearly fixed ($F_{st} \geq 0.95$) relative to *C. variegatus* and found within a hard selective sweep in the focal specialist. In this set of candidate adaptive alleles, *C. brontotheriodes* had the fewest adaptive alleles while *C. desquamator* had the most (Fig 5B).

Figure 5. Patterns of selection and genetic divergence in specialist genomes. A) Histogram of selective sweep length distributions across all four San Salvador Island species. Rug plot below each histogram representing the counts of selective sweeps binned into different lengths is included to highlight the presence of several larger selective sweeps in the tails of the

distribution. B) The total number of fixed or nearly-fixed SNPs ($F_{st} \geq 0.95$) between each group in Osprey Pond. C) Ridgeline plots for length distributions of selective sweeps shared between different combinations of specialists (*C. brontotheriodes*: purple, *C. desquamator*: teal, *C. sp.* ‘wide-mouth’: red-orange). Full results of the GO enrichment analysis of the 57 genes contained in the shared selective sweep regions across all specialists. D) The number of adaptive alleles (nearly-fixed SNPs [$F_{st} \geq 0.95$] relative to *C. variegatus* and under selection [CLR thresholds in Table S3]) in each population of specialists in Osprey Lake. Venn diagram highlights those adaptive alleles that are unique to each specialist and shared with other specialists.

Next, we looked for shared patterns of genetic divergence and selection across the specialist genomes. Despite divergent trophic specializations and morphology, we found evidence of 44 shared selective sweeps across all three specialist populations that were not shared with *C. variegatus* populations (Fig 5C). These shared regions under selection in all specialists were significantly enriched for genes annotated for metabolic processes (Fig 5C), suggesting shared selection for a metabolism associated with a more protein-rich diet across trophic specialists (also see [70]). We also found evidence of shared selection across all pairwise combinations of two specialists. The shared selective sweeps were shorter than any of the selective sweeps unique to each of the specialists, suggesting that selection for these shared regions was not the most recent or strongest in any of the specialists (Fig 5C). We also found no shared fixed or nearly fixed ($F_{st} \geq 0.95$) adaptive alleles across all three specialists, although shared alleles did occur at lower frequencies ($F_{st} < 0.62$; Fig S6).

Shared and unique adaptive alleles in ‘wide-mouth’ and *C. desquamator*

We discovered that the two scale-eating populations shared a set of the same adaptive alleles not found in *C. brontotheriodes* or *C. variegatus* (Fig 5D). This consisted of 15 alleles in 6 shared hard selective sweeps in *C. desquamator* and ‘wide-mouth’: 10 SNPs were in unannotated regions, two were in the introns of the gene *daam2*, and three were in regulatory regions of the genes *usp50*, *atp8a1*, and *znf214* (one variant each).

Shared adaptive alleles in the gene *daam2*, a wnt signaling regulator, are intriguing because knockdown of this gene causes abnormal snout morphology, osteoporosis, and changes in insulin and alkaline phosphate levels in mice [71], and abnormal cranial and skeletal development in zebrafish (Kida et al. 2007). Craniofacial morphology is one of the rapidly diversifying traits in this system, suggesting that divergence in *daam2* may play a role in the shared craniofacial divergence of the two scale-eating species. Similarly, *usp50* functions in protein metabolism and deubiquitination [72] and may play a role in shared metabolic adaptations to the higher-protein content of a scale-eating or snail-eating diet (also note microbiome divergence of *C. desquamator* with increased prevalence of collagenase-digesting bacteria when reared in a common garden:[73]). *atp8a1* is an ion transporter [74], *znf214* is a transcription binding factor, and the ten unannotated variants were not associated with lower jaw size variation in a previous genome-wide association study of the radiation (Richards et al. 2021).

Despite the divergent craniofacial features of the ‘wide-mouth’, none of the adaptive alleles unique to the ‘wide-mouth’ appear to be in or near genes annotated for craniofacial phenotypes in model organisms. In *C. desquamator*, three of the 13 sets of unique adaptive alleles are in or near genes annotated for craniofacial phenotypes: a *de novo* non-synonymous

coding substitution in the gene *twist1*, several putative regulatory variants near the gene *gnaq*, and 8 variants in and near the gene *bri3bp*, which is located inside a QTL region for cranial height in pupfish (St. John et al. 2021). In *C. brontotheriodes*, there is also a candidate craniofacial adaptive allele: a non-synonymous coding substitution in the gene *kat6b*, which is associated with abnormal craniofacial morphologies, including shorter mandibles in mice [75,76] and Ohdo Syndrome and bulbous noses in humans [77].

This pattern of unique alleles relevant to craniofacial phenotypes in *C. brontotheriodes* and *C. desquamator*, but not ‘wide-mouth’, holds if we lower the threshold to the top 1 percentile of F_{st} between specialists and generalist. ‘Roof of mouth development’, ‘bone development’ and ‘skeletal system development’ are in the top 20 most significantly enriched GO terms for *desquamator* candidate adaptive alleles, with 6 significantly enriched terms relevant to cranial and skeletal development (out of 165 enriched terms total). Similarly, ‘embryonic skeletal system development’ and ‘skeletal system development’ were significantly enriched terms for *brontotheriodes* candidate adaptive SNP alleles (out of 8 enriched terms total). However, ‘wide-mouth’ adaptive alleles at this lower F_{st} threshold were not significantly enriched for any GO terms related to craniofacial or skeletal morphology (n=52 terms; Fig S7).

Selection for adaptive alleles shared in both scale-eating populations preceded selection for adaptive alleles unique to C. desquamator

Additionally, we estimated the timing of selection on unique and shared adaptive alleles in ‘wide-mouth’ and *desquamator* to explore the evolution of scale-eating in this system. Previous studies indicate that the *C. desquamator* scale-eater phenotype is separated from *C. variegatus* generalist niche by a deep fitness valley on the adaptive landscape (Martin and Gould 2020;

Martin and Wainwright 2013), suggesting a difficult ecological transition. Adaptation to an intermediate scale-eating niche such by the ‘wide-mouth’ lineage may have provided an easier first step and the adaptive alleles involved may have set the stage for the more specialized scale-eater *C. desquamator* to evolve. To test this, we estimated the age at which the adaptive alleles first started sweeping in each population using the coalescent-based approach starTMRCA [78].

Across the 30 sets of adaptive alleles with selective sweep ages, we found an overall difference in timing of selection between shared and unique adaptive alleles in the two scale-eater populations (ANOVA P -value = 0.00478). In *desquamator*, shared adaptive alleles were under selection before any unique adaptive alleles (Tukey HSD P -value = 0.003217; Fig. 6). We found a similar trend in ‘wide-mouth’: shared adaptive alleles were generally under selection before those unique to the species (Fig. 6). However, this difference was not significant due to one set of unique adaptive alleles near the gene *slitrk5* which was the oldest estimated sweep (ANOVA, Tukey HSD; P = 0.8367).

In contrast to our predictions, selection on 4 of the 6 shared sets of adaptive alleles occurred significantly earlier in *desquamator* than ‘wide-mouth’. Only a single set of these adaptive alleles had an older median age estimate in ‘wide-mouth’ than *desquamator*, although the 95% HPD intervals surrounding these point estimates overlapped between the two populations (Fig. 6). The other adaptive allele set that had overlapping 95% HPD intervals between the two populations were a set of unannotated de novo mutations.

Fig 6. Timing of selection on adaptive alleles in trophic specialists nested within the species demographic history. The mean and 95% HPD estimates for the timing of selection on sets of fixed or nearly fixed SNPs (named by the gene they are in or within 20-kb of) for *C. desquamator*, *C. sp. 'wide-mouth'*, and *C. brontotheroides* populations found in sympatry in Osprey Lake (sweeps in *C. variegatus* not shown). The age of each beneficial allele is color coded by the species it swept in and the inferred demographic history is displayed in the background for comparison. Gene names highlighted in bold are associated with oral jaw size in model organisms.

Shared adaptive variation may have resulted from introgression between desquamator and 'wide-mouth' scale-eaters

Finally, we explored several selection scenarios surrounding the shared adaptive alleles between 'wide-mouth' and *desquamator*. The most parsimonious answer for why these two populations share adaptive alleles is that these alleles swept in their common ancestor and were retained in both post divergence. However, the timing of selection on these alleles is much more recent in both populations than the inferred divergence time (Fig 6), suggesting that these alleles were not selected upon in their most recent common ancestor. Second, the timing of selection on most of these shared adaptive alleles is significantly different between the populations (Fig 6), suggesting that these alleles were not selected upon in a single event in the shared ancestor. This left us to explore two other scenarios that could explain the shared adaptive alleles: 1) these alleles were segregating in both populations after they initially diverged from each other and independently swept to fixation or 2) these adaptive alleles introgressed from one population to another after being selected for in one population.

To distinguish between these two scenarios, we assessed the regions surrounding these shared adaptive alleles for signatures of introgression. Introgression between recently diverged sister species is often more challenging to detect in comparison to introgression with a more distantly related outgroup because of the high genetic similarity between sister species. However, introgression between sister species can be detected from population level polymorphism data by looking for regions of the genome with lower absolute genetic divergence than expected given the genome-wide average [79–82]. We thus calculated D_{xy} in sliding windows across the genomes of the Osprey Lake populations and compared the distribution of D_{xy} values in the regions surrounding the shared adaptive alleles to the distribution of D_{xy} values

across the genome. The average D_{xy} of the shared adaptive alleles was two times lower than average genome-wide values between *desquamator* and ‘wide-mouth’ (Fig 7A). It was also slightly lower (1.2X) than the average D_{xy} for all selective sweep regions in the ‘wide-mouth’ genomes, suggesting it is not due to selection on these regions in general (Table S5). This lower D_{xy} was predominantly due to four out of the six regions containing shared adaptive alleles having ~2-5 times lower D_{xy} values than the genome-wide average. The other two regions had comparable (*znf214*) and higher (unannotated region on scaffold 43) D_{xy} values than the genome-wide average (Fig 7B). The lower genetic distance between *C. desquamator* and the ‘wide-mouth’ in these adaptive regions suggests they may have introgressed between the two groups rather than independent selection on ancestral polymorphisms since they diverged approximately 11,000 years ago.

Fig. 7. Absolute genetic divergence between ‘wide-mouth’ and *desquamator*. A) Distribution of D_{xy} values across 20-kb regions for the whole genome and regions with adaptive alleles shared between *desquamator* and ‘wide-mouth’. B) D_{xy} values across the 1 million base pairs surrounding the six regions of shared genetic variation between *desquamator* and ‘wide-mouth’. Vertical solid black lines indicate the position of the shared adaptive alleles between ‘wide-mouth’ and *desquamator*, horizontal dashed gray lines represent the genome-wide average D_{xy} between *desquamator* and ‘wide-mouth’. The smoothed lines represent D_{xy} between *C. desquamator* and *C. sp. ‘wide-mouth’* (teal), *C. brontotheriodes* (purple), and *C. variegatus* (gold) across these portions of the scaffold. The D_{xy} values were summarized with smoothed splines using the `smooth.spline()` function in R for ease of visualization across the different comparisons. Gray boxes represent the nearest gene to the adaptive alleles and unannotated

adaptive alleles were those in which no gene was located within 20-kb of the SNP (an approximate LD estimate from a previous study; [68]).

Discussion

Discovery of a new cryptic scale-eater evolved through reuse of shared adaptive alleles

The hallmark of adaptive radiation is a rapid burst of diversification which is predicted by theory to slow down over time as niche width driving further divergent selection diminishes (Martin and Richards 2019). An alternative possibility is that radiations can be self-propagating and that the diversity generated within the first stages of radiation helps beget further diversity [60]. This could happen through exploitation of new trophic levels created by new species (Seehausen et al. 2019) or physical alterations of the environment by new species (niche construction) that may create additional opportunities for speciation (reviewed in [1,58]). The diversity begets diversity hypothesis can be visualized as the exploration of a complex multi-peaked fitness landscape; as species in the radiation colonize new peaks, this provides access to additional neighboring fitness peaks to fuel rapid radiation.

At the microevolutionary level, shared adaptive variants can also help other populations colonize new or ecologically similar areas of the fitness landscape. However, the genetic basis and microevolutionary processes underlying major ecological transitions are still poorly understood in nature. Our discovery of a cryptic new scale-eating species through morphological, dietary, and genomic analyses revealed shared selective sweeps among all three trophic specialists in a radiation of pupfishes despite the divergent morphological and ecological adaptations between them. However, only in the two scale-eating populations, *C. desquamator*

and *C. sp.* ‘wide-mouth’, did we find shared nearly-fixed or fixed adaptive alleles. These species are sister species, suggesting a single origin for scale-eating. Nonetheless, these shared adaptive alleles were not selected upon in the ancestral population and swept first in the *C. desquamator* population. This contradicts our initial prediction that the evolution of the intermediate ‘wide-mouth’ scale-eater would aid the evolution of the more specialized scale-eater *C. desquamator*. Instead, adaptive alleles from *C. desquamator* may have introgressed and then selected upon in the ‘wide-mouth’ population. Additionally, unlike *C. brontotheriodes* and *C. desquamator*, the cryptic ‘wide-mouth’ appears to have little unique genetic variation relevant to craniofacial morphology, which is a major axis of diversification in the other trophic specialists [83]. An intriguing implication of these findings is that adaptive variants within one specialist may aid the evolution of another into a similar niche and promote further ecological divergence within radiations, in line with the diversity begets diversity hypothesis [60].

An adaptive walk underlies the major ecological transition from generalist to scale-eating specialist

One of the foundational models of adaptation is that it proceeds in ‘adaptive walks’ towards fitness optima that involve the sequential fixation of adaptive alleles that move a population in the phenotypic direction of the local optimum [84–88]. The distinct timing of selection across different adaptive alleles suggests that the ecological transition from generalist to novel scale-eating specialist involved such an adaptive walk rather than a sudden burst of concurrent selection events after some major environmental shift. These distinct, multiple bouts of selection could be caused by mutation-limited (i.e. waiting for new beneficial alleles; [89–91]) or mutation-order processes (i.e. epistatic interactions; [92–94]).

We found that all of the shared adaptive alleles between the scale-eating populations occurred at low frequency in generalist populations on other Caribbean islands in a previous study ([68]; Table S7). For example, three copies of the shared *usp50* adaptive allele were found outside of San Salvador Island (Table S7). This indicates that initial bouts of selection occurred on available standing genetic variation and that staggered timing of hard selective sweeps in each trophic specialist most likely reflects mutation-order processes in which selection on a beneficial allele was contingent on prior fixation of other adaptive alleles in each specialists' genetic background. However, several adaptive alleles originated from introgression or de novo mutations found only on San Salvador Island (Table S7), so part of the adaptive walk may have also been mutation-limited.

Cryptic scale-eating species reveals features of the adaptive walk towards scale-eating specialization

Although the two scale-eating species shared a set of adaptive alleles and a recent common ancestor, 5 of 6 shared adaptive regions swept at significantly different times between the two species. This difference in timing may result from several different scenarios: 1) independent adaptive walks to the same scale-eating niche, 2) independent adaptive walks to different scale-eating niches or 3) the adaptive walk of one population depends on the adaptive walk of the other population. We explore each of these scenarios in turn.

First, the difference in timing of selection on the same shared adaptive alleles could indicate independent adaptive walks to the same scale-eating peak that occurred at different times and/or by slightly different routes. *C. desquamator* and 'wide-mouth' populations have

predominantly abutting ranges, with only a small amount of geographic overlap on San Salvador Island in four lakes, Osprey, Oyster, Little Lake, and Mermaid's Pond (Fig 1). If this current distribution is representative of their historical ranges and the two lineages began diverging about 11,000 years ago, it is possible that the adaptive walks took place in different lakes and were largely independent of one another. The differences in timing on the shared adaptive alleles and the presence of unique alleles observed in each population are reminiscent of mutation-order speciation [93,95]. In mutation-order speciation the same alleles are favored in populations that are adapting to similar environments, yet by chance and/or epistatic interactions with different genetic backgrounds, similar adaptive alleles fix in just one population. At least one set of alleles near the gene *slitrk5* are unique to the 'wide-mouth' scale-eater and fixed well before selection occurred on the shared adaptive variants between the two scale-eater populations (Fig 5). Epistatic interactions of these *slitrk5* alleles may have prevented the 'wide-mouth' from fixing additional segregating alleles that are uniquely fixed in *C. desquamator*.

Second, the difference in timing of selection may have occurred because the two scale-eating populations are adapting to two different scale-eating niches. The intermediate diet, distinctly sized morphological traits, and smaller body size in the 'wide-mouth' scale-eater compared to *C. desquamator* may indicate the 'wide-mouth' scale-eater is adapting to a different scale-eating niche. While the two scale-eating populations do share some overlap in adaptive alleles and selective sweeps, the majority of selective sweeps are unique to each species (Fig 5 & 6), including neurogenesis, brain, and nervous system development (*slitrk5*, *sema4g*, and *smarcel1*), whereas unique adaptive alleles in *desquamator* include craniofacial development annotations (*olfm1*, *gnaq*, *twist1*) as well. This difference in gene annotations can also be seen at the broader level of regions of the genome under selection (Fig S7). Therefore, the differences in

timing at shared adaptive alleles might reflect differences in relative strength of selection due to different selective regimes experienced by the two scale-eating populations. While starTMRCA is fairly robust in its timing estimates to different strengths of the selection [78], we cannot rule out that differences in relative timing of selection might partially reflect differences in strength of selection occurring in the two scale-eating populations.

Third, another scenario that could have led to the older timing of selection across shared adaptive alleles in *desquamator* than ‘wide mouth’ is one in which the adaptive walk of one population depended on adaptive alleles from another. The significantly older timing of selection in *desquamator* on shared adaptive alleles and the low absolute genetic divergence between these two scale-eating population in these regions (Fig 6) suggests that these alleles may have introgressed between the two populations. Given that these shared adaptive alleles appear as standing genetic variation across Caribbean populations, albeit at very low frequency in our sampling, it is likely that the shared alleles were present in the ancestor and were segregating in both populations after they diverged. There are several reasons why adaptive introgression may have been necessary for adaptive divergence of ‘wide-mouth’ despite the alleles initially segregating in the ancestral population. In line with mutation-order processes (e.g. Mani and Clarke 1990; Schluter 2009; Good et al. 2017), these alleles might not have been adaptive until the right genetic background was present in the ‘wide-mouth’. Introgression from *C. desquamator* in which the allele was already swept to high frequency could have raised the frequency of these alleles in ‘wide-mouth’ and increased the likelihood of fixation for the beneficial alleles (reviewed in [96]). This is consistent with previously proposed hypotheses for how diversity begets further diversity in adaptive radiations (Whittaker 1977; Stroud and Losos 2016; Martin and Richards 2019).

562

563 *Shared aspects of genomic landscape of selection among all specialists additionally supports*
 564 *specialization promoting further specialization*

565 Intriguingly we also find evidence of selective sweeps shared across all three specialists despite
 566 their divergent adaptations and lack of a shared specialist ancestor. However, there are no
 567 commonly shared adaptive alleles fixed against *C. variegatus* in these shared selective sweep
 568 regions. Hard selective sweeps indicate a selection scenario in which a single beneficial allele of
 569 large effect on a trait is swept to high frequency (reviewed in [97]). The lack of highly divergent
 570 alleles in these regions might indicate polygenic selection events underlie the shared signatures
 571 across specialists. The broad-scale transition from dietary generalist to dietary specialist may
 572 involve polygenic selection with small shifts in the frequency of many alleles. Despite very
 573 different expectations about the genetic basis of adaptation, polygenic selection events may have
 574 been detected as a hard selective sweep in this study as the two selection types can be
 575 challenging to distinguish based solely on patterns of genetic variation [98–101]. Recently
 576 developed frameworks provide additional criteria to help distinguish between the two selective
 577 regimes [99] but are beyond the scope of this current study.

578 These shared selective sweeps across all specialists are not the longest selective sweeps
 579 in any of the specialist genomes (> 50-kb; Fig 5C), indicating they are not the most recent or
 580 strongest selection events. However, these shared selective sweeps appear to be relevant to
 581 dietary specialization as these regions are enriched for genes annotated for metabolic processes
 582 such as short chain fatty acid and propionate metabolism (Fig 5). Propionates and other short
 583 chain fatty acids are common microbiome metabolites [102]. A recent microbiome study in lab-
 584 reared populations found that *C. desquamator* microbiomes appear enriched for

Burkholderiaceae bacteria that can digest collagen, further supporting an adaptive role for microbiomes in the radiation [73].

We also see pairwise shared selective sweeps between all combinations of two specialists. In contrast to the sweeps shared between all specialists, we see stronger genetic divergence among species in these regions and a proportion of the selective sweeps shared between two specialists do contain fixed or nearly-fixed alleles. However, the specialists that share selective sweeps in these regions are fixed for different adaptive alleles, consistent with previously observed patterns of parallel differential gene expression in specialists despite divergent genotypes [70]. These alleles were possibly under balancing selection in the ancestor (most of these alleles are segregating at intermediate frequency in *C. variegatus* currently) and the alternate alleles were driven to fixation between the specialists during their respective divergences. While the species have discrete phenotypes across several morphological traits important to divergence in this system, these traits often lie on a continuous axis (i.e. shorter oral jaw lengths in *C. brontotheriodes*, intermediate jaw lengths in *C. variegatus*, and longer oral jaw lengths in ‘wide-mouth’ and *C. desquamator*). Some of the alleles that are alternatively fixed between specialists at the same position may have incomplete dominance effects on such traits.

In line with adaptation to divergent trophic niches across the species, we do still see many unique signatures of selection among the specialists. The *C. variegatus* population had the fewest selective sweep signatures. All three specialists had more selective sweeps potentially resulting from strong directional selection for adapting to new fitness peaks. However, while we might expect more selective sweeps in *C. desquamator* genomes given the deeper valley on the fitness landscape isolating the scale-eater phenotypes [10,12], *C. brontotheriodes* had the most selective sweeps detected in their genome. They also have the longest sweeps detected (e.g. 100-kb),

suggesting that selection may have occurred most recently in the *C. brontotheriodes*. This matches with the most recent divergence event being between generalist and *C. brontotheriodes* in Osprey Lake. An intriguing implication of this result is the potentially long wait time for speciation of *C. brontotheriodes* species compared *C. desquamator* despite the shallower fitness valley isolating *brontotheriodes* from generalists. However, we cannot yet rule out that the recent divergence time estimated in Osprey Lake reflects a more recent arrival of *C. brontotheriodes* to that lake after initially diverging long ago in another lake not incorporated in our demographic model.

Methods

Sampling

The *C. sp.* ‘wide-mouth’ individuals were collected on San Salvador Island in the Bahamas using hand and seine nets between 2017 and 2018. ‘Wide-mouth’ individuals were collected from 5 lakes in which taxa occurs in sympatry with just *C. variegatus* (Great Lake, and Stout’s Lake) or with generalist and *C. desquamator* (Oyster Pond, Osprey Lake, and Mermaid’s Pond,; Fig. 1). In Oyster Pond and Little Lake, *C. desquamator* is extremely rare (only a single Oyster Pond individual grouped genetically and morphologically with *C. desquamator*), so Osprey Lake appears to be the only lake where all three species and the recently discovered ‘wide-mouth’ coexist at appreciable frequencies in sympatry. ‘Wide-mouth’ individuals have also been collected in Little Lake in the past (Bruce Turner, pers. obs. 1990s).

Fishes were euthanized in an overdose of buffered MS-222 (Finquel, Inc.) following approved protocols from the University of North Carolina at Chapel Hill Animal Care and Use

Committee (#18-061.0), and the University of California, Berkeley Animal Care and Use Committee (AUP-2015-01-7053) and preserved in 95-100% ethanol.

Ecological and morphological characterization of 'wide-mouth' scale-eater

Multiple individuals from 3 lake populations (Osprey Lake, Great Lake, and Oyster Pond) in which we had measurable specimens of the species (n=84) were measured for 9 traits using dial calipers. Traits were selected for specific connections to foraging performance [103,104] and that differed across the three groups in a previous study when externally measured [10]. For each specimen we measured (1) standard length from the anterior tip of the premaxilla to the posterior margin on the midline of the caudal peduncle, (2) lower jaw length from the medial point of the dentary on the lower jaw to the distal point of rotation on the quadrate-articular joint, (3) the width of the buccal from the distance between the proximal sides of the buccal cavity in dorsal view, (4) the height of the adductor mandibulae insertion from the vertex between the vertical and horizontal arms of the preopercle to the dorsal margin of the hyomandibula, (5) the distance from the insertion of the first ray of the dorsal fin to the insertion of the first ray of the anal fin, (6) the height of the caudal peduncle from the dorsal and ventral procurrent rays on the caudal fin, (7) neurocranium width from the narrowest distance between the eyes from the dorsal view, (8) body width from just behind the posterior margins of each operculum in dorsal view, and (9) diameter of the orbit. Measurements were made by a single observer (EJR). Repeatability of measurements was assessed by remeasuring a random subset of 10 individuals per species twice. Measurements on the full dataset were not taken until repeatability was above 85% per trait (r^2 in a linear regression of both sets of measurements). To remove the effects of body size on trait size, we used the residuals from a linear regression of the log-transformed trait on log

transformed standard length. We also compared these morphological measurements between lab-reared F2 and wild caught specimens to rule out strong plasticity in traits of interest.

For a subset of individuals collected from each population (*C. variegatus*, *C. desquamator*, and ‘wide-mouth’) in Osprey Lake, diet was characterized from stomach content analyses ($n=10$ each) and stable isotope analyses ($n=75$; 42 *C. variegatus*, 16 *C. desquamator*, 17 ‘wide-mouth’ individuals). These are two complementary approaches that reflect short-term and long-term snapshots of the dietary differences among these groups. For stomach content analysis, a single observer (EJR) dissected out a 1 cm portion of the gut from the posterior hindgut region and counted the number of scales found. For stable isotope analyses, muscle tissue samples were taken from the caudal peduncle region of each individual immediately after euthanasia in an overdose of MS-222. Muscle samples were individually labeled and dried at 60° C for at least 24 hours. Foil-wrapped tissue samples were then sent to the UC Davis Stable isotope facility and analyzed on a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer to characterize the natural abundances of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ among individuals.

Genomic library preparation

We sequenced 24 ‘wide-mouth’ individuals following protocols used in a previous study [105] in which we sequenced genomes from *Cyprinodon variegatus*, *C. desquamator*, and *C. brontotheroides*. Raw reads were mapped from these 24 individuals to a de-novo assembly of *Cyprinodon brontotheroides* reference genome (v 1.0; total sequence length = 1,162,855,435 bp; number of scaffolds = 15,698, scaffold N50 = 32 Mbp) with bwa-mem (v 0.7.12; [106]). Duplicate reads were identified using MarkDuplicates and BAM indices were created using

BuildBamIndex in the Picard software package ([http://picard.sourceforge.net\(v.2.0.1\)](http://picard.sourceforge.net(v.2.0.1))). We followed the best practices guide recommended in the Genome Analysis Toolkit (v 3.5;[107]) to call and refine our single nucleotide polymorphism (SNP) variant dataset using the program HaplotypeCaller. Variants in ‘wide-mouth’ individuals were called jointly with the 202 individuals sequenced from a previous study [105]. We filtered SNPs based on the recommended hard filter criteria (i.e. QD < 2.0; FS > 60; MQRankSum < -12.5; ReadPosRankSum < -8;[107,108]) because we lacked high-quality known variants for these non-model species. After selecting for only individuals from San Salvador Island, variants were additionally filtered to remove SNPs with a minor allele frequency below 0.05, genotype quality below 20, or containing more than 10% missing data across all individuals at the site using vcftools (v.0.1.15;[109]). Variants in poorly mapped regions were then removed using a mask file generated from the program SNPable (<http://bit.ly/snpable>; k-mer length =50, and ‘stringency’=0.5). Our final dataset after filtering contained 6.4 million variants with 7.9x median coverage per individual.

Testing for signatures of recent hybrids

We first tested whether these ‘wide-mouth’ individuals represented recent (e.g. F1/F2) hybrids of *C. variegatus* and *C. desquamator* in the wild. These two young species do reproduce with each other in the lab and rare hybrid spawning events have been observed in the wild (unpublished data ME St. John). As a first visual assessment, we conducted principal component analysis on *C. desquamator*, generalist, and ‘wide-mouth’ individuals to look for the genome-wide pattern expected in PCAs in which recent hybrids between two populations are included. To perform the PCA, the genetic dataset was first pruned for SNPs in strong linkage disequilibrium using the LD

pruning function (--indep-pairwise 50 5 0.5) in plink (v1.9;[110]), leaving 2.6 million variants.

We then ran a principal component analysis using the eigenvectors output by plink's pca function (--pca). The first two principal components were plotted in R (R Core Team 2018 v3.5.0).

As a complementary assessment, we used an admixture model-based approach to estimate the proportion of shared ancestry among individuals in our dataset using ADMIXTURE (v.1.3.0)[111]. The number of populations (K) was decided upon using ADMIXTURE's cross-validation method (--cv) across 1-10 population values of K. K = 4 was then chosen using the broken-stick method. Ancestry proportions estimated by ADMIXTURE were plotted in R for the K value with the highest likelihood and the two K values surrounding to explore whether the strong signatures of population structure in Crescent Pond *C. desquamator* individuals was masking hybridization signatures in any of the 'wide-mouth' populations.

Lastly, to discriminate among alternative evolutionary scenarios for the origin of 'wide-mouth' and to estimate divergence time among all four species, we used demographic modeling based on the folded minor allele frequency spectrum (mSFS). The observed mSFS was computed using the SFStools script from D. Marques available on github (<https://github.com/marqueda/SFS-scripts>). For these demographic model comparisons, we used only individuals from Osprey Lake (*C. desquamator*: n=10; *C. variegatus* : n=12; *C. sp.* 'wide-mouth': n=11; *C. brontotheriodes*: n=13) to avoid additional complex population structure across ponds. We contrasted 28 demographic models of different topologies and gene flow scenarios across the four groups (Fig S5; Table 1 and S2). For each model, the fit to the observed multidimensional mSFS was maximized using the composite-likelihood method fastsimcoal (v2.6.0.3;[69] with 100,000 coalescent simulations, 40 expectation-maximization cycles, and

pooling all entries with less than 20 SNPs). For parameter estimates, we used a wide search range with log-uniform distributions with the range of some priors informed by previous estimates of effective population size from MSMC and the age of the last glacial maximum (~20kya) prior to which the lakes would have been dry on SSI. These ranges were not upper bounded by these specified priors and so the simulations were free to explore parameter space that exceeded the priors.

For each demographic model, we ran 100 independent *fastsimcoal2* runs to determine the parameter estimates with the maximum likelihood. The best fitting demographic model was identified using Akaike information criteria (AIC). To get confidence intervals for the parameter estimates from the best fitting model, we simulated 100 mSFS based on the maximum likelihood parameter estimates from our best fitting model and ran 50 independent runs with this model on each simulated SFS. The parameter point estimates from the run with the highest likelihood (of the 50 independent runs) from each simulated SFS were then used to compute 95 percentile confidence intervals as a measure of uncertainty in the parameter estimates from the observed SFS.

Testing for shared adaptive alleles

Patterns of directional selection

Various demographic histories can shift the distribution of low- and high-frequency derived variants to falsely resemble signatures of hard selective sweeps. In a previous study, we used MSMC analyses to infer histories of population size changes in *C. variegatus*, *C. desquamator* and *C. brontotheriodes* (Richards et al. 2021). In this study, we repeated the same analysis for the ‘wide-mouth’. In order to account for demography of the ‘wide-mouth’ population in

downstream analyses, we used the MSMC (v. 1.0.1;[112] to infer historical effective population size (N_e) changes in the ‘wide-mouth’. We ran MSMC on unphased GATK-called genotypes separately for a single individual ‘wide-mouth’ from Osprey Lake with 16x mean coverage across its genome (Fig S4). As recommended in the MSMC documentation, we masked sites with less than half or more than double the mean coverage for that individual or with a genotype quality below 20. We also excluded sites with <10 reads as recommended by Nadachowska-Brzyska et al. [113]. To scale the output of MSMC to real time and effective population sizes, we used a one-year generation time [114] and the estimated spontaneous mutation rate of 1.56×10^{-8} estimated from high coverage sequencing of two independent pedigreed parent-offspring crosses of San Salvador Island species from a previous study (Richards et al. 2021).

Across all four populations in Osprey Lake, we looked for regions that appeared to be under strong divergent selection in the form of a hard selective sweep from the site frequency spectrum using SweeD (v.3.3.4;[115]). In this calculation of the composite likelihood ratio (CLR) of a sweep, we incorporated our empirical estimate of the decrease in population size for each focal population estimated from MSMC analyses in 50-kb windows across scaffolds that were at least 100-kb in length (99 scaffolds; 85.6% of the genome). We also calculated CLR ratios across 100,000 scaffolds consisting of neutrally evolving sequences simulated with ms-move [116], controlling for the impact of the inferred population size decreases over time for each population from MSMC runs mentioned above (Fig. S4; Table S3). The CLR ratios for the simulated datasets were then used to assess outlier CLR ratios from the empirical dataset. Regions with CLR ratios above the 95th percentile value of CLR from the neutral simulated dataset were considered candidate hard selective sweep regions (Table S3). We compared

selective sweeps across *C. variegatus*, *C. desquamator*, *C. brontotheriodes*, and ‘wide-mouth’ to look for shared and unique selective sweeps among the four groups.

Characterization of candidate adaptive alleles

We searched for candidate adaptive alleles underlying divergent traits among the species by overlapping selective sweeps regions with regions of high genetic divergence based on fixed or nearly fixed SNPs between groups. We chose to look at nearly fixed SNPs over only fixed variation to accommodate the ongoing gene flow occurring between these young species. We took two approaches to finding fixed or nearly-fixed variants: 1) a fixed threshold of $F_{st} \geq 0.95$ across all comparisons and 2) and threshold of the 99.9th percentile of F_{st} , which varied among comparisons (range of F_{st} 0.73-0.83).

We made the following pairwise comparisons for F_{st} calculations including a) between *C. variegatus* and each of the specialists, b) each specialist against all other groups and c) shared between two or three specialists against *C. variegatus*. For nearly fixed variants with the $F_{st} \geq 0.95$ threshold, we looked for putative function of the candidate adaptive alleles by looking at gene annotations of any gene the variant was in or near (within 20-kb of the gene, which is within the 50-kb LD decay estimate). We used available gene annotations from model organisms of mice and zebrafish from MGI, ZFIN, and we checked other annotation databases and studies for verification of putative function, including Phenoscape Knowledgebase (<https://kb.phenoscape.org/#/home>), NCBI’s PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>), and the Gene Ontology database using AMIGO2 [117] .

For the 99.9th percentile F_{st} variants: We performed gene ontology (GO) enrichment analyses for genes near candidate adaptive variants using ShinyGo (v.0.51;[118]). In the *C.*

brontotheroides reference genome annotations (described in *de novo* genome assembly and annotation section), gene symbols largely match human gene symbols. Thus, the best matching species when we searched for enrichment across biological process ontologies curated mostly human gene functions.

Timing of selection on candidate adaptive alleles

Lastly, we also determined the relative age of candidate adaptive alleles by generating estimates of coalescent times using the R package starTMRCAs (v0.6-1; [78]). For each candidate adaptive allele that was unique to the three specialists and the 16 shared alleles between *C. desquamator* and ‘wide-mouth’, a 1-Mb window surrounding the variant was extracted into separate vcfs for individuals within each species. The program requires complete genotype data so we first filtered out any individuals with more than 10% missing data (1 *C. brontotheriodes* and 2 ‘wide-mouth’ individuals). With Tassel5 [119], we then used the LD KKN command to infer missing sites based on LD for remaining individuals with less than 10% missing data. Subsequently we removed the small number of sites with any missing data across individuals within each population.

These sets of variants were then analyzed in starTMRCAs with the mutation rate of 1.56×10^{-8} substitutions per base pair, and a recombination rate of 3.11×10^{-8} (from genome-wide recombination estimate for stickleback; [120]). Each analysis was run three times per focal adaptive allele and all runs were checked for convergence between and within runs. Most runs rapidly converged within the initial 6000 steps, but 5 runs did not converge after an additional 4000 steps and were discarded from further analysis.

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Author Contributions

Conceptualization: EJR,CHM; Data Collection: EJR,CHM; Statistical analyses: EJR; Resources: CHM; Visualization: EJR,CHM; Original draft: EJR; Revising: EJR, CHM

Declaration of Interests

The authors declare no competing interests.

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Keywords : fitness landscape ; adaptive radiation ; introgression ; standing genetic variation ; de novo mutation
Introduction
First conceptualized by Sewell Wright in 1932 , the adaptive landscape describes the complex relationship between genotype or phenotype and fitness (1). The landscape is a concept , metaphor , and an empirical measurement that exerts substantial influence over all evolutionary dynamics (2 – 6). Fitness landscapes were originally depicted as high-dimensional networks spanning genotypic space in which each genotype is associated with fitness (1). Simpson (7) later described the phenotypic evolution of populations through time on a rugged landscape , in which isolated clusters of fitness peaks represent ‘ adaptive zones ’ to which populations evolve from adjacent regions of low fitness (8). Lande and Arnold formalized the analysis of selection and estimation of phenotypic fitness landscapes (9 – 11), leading to empirical studies of fitness landscapes in numerous empirical systems (12 – 18). Fitness surfaces also provide a central component of speciation models and theory (19 – 21). Populations may speciate through a process of divergent selection (22), either on static landscapes or negative frequency-dependent disruptive selection (23 – 28). A fundamental concept in fitness landscape theory is that not all genotypic trajectories are accessible (5 , 29 , 30); only those paths that monotonically increase in fitness at each mutational step are evolutionarily accessible , an

- expectation stemming from Fisher ' s fundamental theorem (31). These accessible
genotypic trajectories can thus be considered potential adaptive walks under Fisher ' s
geometric model (31 , 32). Experimental studies of adaptive walks in nature are
increasingly common , including the evolution of toxin resistance in monarch butterflies (33),
metabolism and citrate exploitation in *E. coli* (34) (35), and host-shift. 2021;
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Supplemental Figures and Tables:

We get by with a little help from our friends: diversity begets diversity through shared adaptive genetic variation

Emilie J. Richards^{1,2} and Christopher H. Martin^{1,2}

¹Department of Integrative Biology, University of California, Berkeley

²Museum of Vertebrate Zoology, University of California, Berkeley

Correspondence: *chmartin@berkeley.edu*

1190 Keywords: ecological speciation, ecomorph, adaptive radiation, novelty, innovation, selective

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1198 Figure S1. Relationships between three divergent traits and standard length across ponds. The
 1199 relationship between standard length (mm) of individuals and their D) lower jaw length, E)
 1200 buccal cavity width, and F) adductor mandibulae muscle insertion height across individuals the
 1201 three species *C. desquamator* (teal), *C. variegatus* (gold), and *C. sp.* ‘wide-mouth’ (red-orange)
 1202 across different ponds (Osprey, Oyster, and Great Lake). Colored lines represent linear model of
 1203 these relationships for each species with their 95% confidence bands in gray.

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Figure S2. Comparison of the three focal divergent traits in lab and wild populations. A. 95% CI of the standardized sizes of lower jaw length (left), buccal width (center) and preopercular insertion height (right) for *C. variegatus* (gold), *C. sp. 'wide-mouth'* (red-orange) and *C. desquamator* (teal). Lines (wild: solid; lab: dashed) are the 95% CI interval and symbols (wild: circle; lab: triangle) represent the mean values of traits from each population. Lab raised individuals include F1 and F2 generations.

Figure S3. *C. sp. 'wide-mouth'* is not an admixed population. ADMIXTURE analysis of San Salvador Island populations using an LD-pruned subset of genetic variants from 109 individuals (78,840 variants). A) Cross validation error estimates from ADMIXTURE that indicate a K model of 6-7 were the best fit. B) Ancestry proportions across individuals of the three focal groups. Proportions were inferred from ADMIXTURE analyses with 2 values of K with the highest likelihood on the same LD-pruned dataset in A.

Fig S4. Changes in effective population size over time for Osprey populations inferred using MSMC [112] on high-coverage (18-36X) genomes from single individuals of each of the four species in Osprey Lake, San Salvador Island Bahamas: *C. variegatus* (gold), *C. brontotheroides* (purple), *C. desquamator* (teal), and *C. sp. 'wide-mouth'* (red-orange). Time was scaled using a mutation rate of 1.56×10^{-8} mutations/basepair/generation and a generation time of 1 year.

Fig S5. Top five best fitting demographic models for Osprey Lake. From a genetic dataset that was LD-pruned and had no missing information across individuals comprised of 67,400 SNPs. Listed in order of lowest AIC scores (Table 1 and S2) with best supported model in A. All top models were of recent bidirectional gene flow but varied in topology among the four species.

Fig S6. Genetic divergence among populations in Osprey Lake in regions of shared selection across two or more specialists. Each panel represents pairwise F_{st} comparison between two populations for every SNP found in regions under shared selection across two or more specialist species on San Salvador Island: *C. brontotheroides* (bro); *C. desquamator* (des), *C. sp.* 'wide-mouth' (wid). Regions shared across all three specialists (bro+des+wid) do not contain highly divergent SNPs between any of the species in the radiation. Highly divergent SNPs ($F_{st} > 0.75$) are only found in regions shared between one or two specialists.

Fig S7. Top 20 enriched GO categories for divergent alleles in each of the three specialists. GO enrichment analyses were performed on genes in or near (within 20-kb) of a SNP that was under a hard selective sweep and strongly diverged from generalist species (top 1% of *Fst* values across genome) for a) 'wide-mouth' b) *C. desquamator*, and c) *C. brontotheriodes*. GO categories that were significantly enriched for relevant terms corresponding to craniofacial development, the major axes of morphological divergence in this radiation, are highlighted in red. All terms included were significant at an FDR < 0.05 and full list of terms in Data S1-3.

Table S1. No significant genome-wide signature of admixture among populations on San Salvador Islands. Results from three formal tests for introgression to assess whether ‘wide-mouth’ populations are hybrids between generalist *C. variegatus* and scale-eater *C. desquamator*: D-, f_3 - and f_4 -statistics across all possible combinations of Osprey Lake populations of generalist species. Significant f_3 -statistic had Z-scores > -2; significant f_4 and D-statistic Z-scores were smaller than -2 and greater than 2. The only significant signature of admixture (bolded Z-scores) comes from D- and f_4 -statistics based on relationships that violate the expected tree (((*C. desquamator*; ‘wide-mouth’), Generalist), Outgroup) and therefore should not be interpreted as evidence of ‘wide-mouth’ being an admixed population. *C. variegatus* population from Rum Cay, the nearest neighbor island in the Bahamas to San Salvador Island was used as an outgroup population for D- and f_4 -statistics.

f_3 -statistic						
A	B	C	--	f_3	stderr	Z
<i>C. variegatus</i>	<i>C. desquamator</i>	C. sp. ‘wide-mouth’		0.004626	0.00088	5.257
f_4 - statistic						
P1	P2	P3	P4	f_4	stderr	Z
C. sp ‘wide-mouth’	<i>C. desquamator</i>	<i>C. variegatus</i>	Rum Cay	-0.000196	0.000164	-1.2
C. sp ‘wide-mouth’	<i>C. variegatus</i>	<i>C. desquamator</i>	Rum Cay	0.003971	0.000268	14.836
C. sp ‘wide-mouth’	<i>C. variegatus</i>	<i>C. brontotheroides</i>	Rum Cay	0.000186	0.000201	0.927
D-statistic						
P1	P2	P3	Outgroup	D	stderr	Z
C.sp.‘wide-mouth’	<i>C. desquamator</i>	<i>C. variegatus</i>	Rum Cay	-0.0018	0.001498	-1.199
C. sp. ‘wide-mouth’	<i>C. variegatus</i>	<i>C. desquamator</i>	Rum Cay	0.035	0.002326	15.068
C. sp. ‘wide-mouth’	<i>C. variegatus</i>	<i>C. brontotheroides</i>	Rum Cay	0.0017	0.001792	0.931

Table S2. Support for the 28 demographic models for the evolution of the ‘wide-mouths’ from the site frequency spectrum. The likelihood and AIC scores all demographic models estimated in *fastsimcoal2* for the Osprey populations of *C. variegatus* var), *C. brontotheroides* (bro), *C. desquamator* (des), and *C. sp.* ‘wide-mouth’ (wid) are presented here with a complete list of all models tested reported in Table S2. Change in likelihood (ΔLnL) represents the difference in likelihood from that of a simulated SFS expected by the demographic model tested. Change in AIC (ΔAIC) represents the difference in AIC scores from that of the model with the smallest ΔLnL . All models presented here represent different divergence scenarios with recent gene flow allowed, which had better support from the data than models with no gene flow or early gene flow. Visual representations of the top five models are depicted in Figure S5.

Divergence Model	LnL	ΔLnL	AIC	ΔAIC
(1) ((des,wid),(bro,var)) + (des,wid) diverges first + recent gene flow	-11701	1077	54133	0
(2) ((des,bro),var)) ,wid)) + recent gene flow	-11780	1112	54296	35
(3) ((des,bro),wid),var)) + recent gene flow	-11806	1138	54418	61
(4) ((bro,wid),des),var)) + recent gene flow	-11808	1140	54423	63
(5) ((des,wid),(bro,var)) + (bro,var) diverges first + recent gene flow	-11846	1178	54602	101
(6) ((des,wid), bro),var)) + recent gene flow	-11855	1187	54642	110
(7) ((des,bro),var)) ,wid))+ early gene flow	-12016	1348	55364	271
(8) ((des,var),bro), wid)) + recent gene flow	-12024	1356	55426	279
(9) ((wid,bro),des),var)) + early gene flow	-12086	1418	55688	341
(10) ((wid,des),bro),var)) + early gene flow	-11855	1419	55692	342
(11) ((des,wid),(bro,var)) + early gene flow	-12139	1471	55929	394
(12) ((des,wid), bro),var)) + no gene flow	-12315	1648	56738	570
(13) ((wid,bro),des),var)) + no gene flow	-12326	1658	56787	581
(14) ((des,bro),var)) ,wid))+ no gene flow	-12334	1667	56826	589
(15) ((des,wid),(bro,var)) + recent sister gene flow only	-12394	1726	57106	649
(16) ((des,wid),(bro,var))+ no gene flow	-12396	1728	57109	651
(17) ((wid,var)),bro),des)) + recent gene flow	-12399	1731	57148	654
(18) ((bro,var)) ,wid),des)) + recent gene flow	-12401	1733	57157	656
(19) ((wid,bro),var)),des)) + recent gene flow	-12401	1734	57163	657
(20) ((des,wid),var),bro)) + recent gene flow	-12420	1752	57244	675
(21) ((wid,var)),des),bro)) + recent gene flow	-12425	1757	57265	680
(22) ((des,var),wid),bro)) + recent gene flow	-12425	1757	57267	680
(23) ((wid,var)),(bro,des)) + no gene flow	-23245	12577	107070	11500
(24) ((wid,var)),(bro,des)) + early gene flow	-23658	12990	108976	11913
(25) ((wid,var)),(bro,des)) + recent gene flow	-23659	12991	109003	11914

(26) ((des,var),(bro,wid)) + early gene flow	-34983	24315	161129	23238
(27) ((des,var),(bro,wid)) + no gene flow	-34983	24315	161125	23238
(28) ((des,var),(bro,wid)) + recent gene flow	-34983	24315	161149	23238

Table S3. Parameters for selective sweep analyses in SweeD.

The 95th percentile of composite likelihood ratio threshold based on neutral simulations under the demographic scenario of decreasing population size through time inferred with MSMC (Fig S4), and the population size change and haplotype number information parameters required by SweeD for each species.

Species	CLR threshold	SweeD Commands
<i>C. variegatus</i>	0.40320	-folded -strictPolymorphic -G 0.407 -eN 5.45 181.8 -s 24
<i>C. brontotheroides</i>	0.43473	-folded -strictPolymorphic -G 0.389 -eN 5.88 196 -s 32
<i>C. desquamator</i>	0.40537	-folded -strictPolymorphic -G 0.218 -eN 8.11 270 -s 16
<i>C. sp. 'wide-mouth'</i>	0.39262	-folded -strictPolymorphic -G 0.203 -eN 8.57 276 -s 22

Table S4. Genetic divergence among the four populations in Osprey Lake, San Salvador Island across different thresholds of F_{st} . Relative measure of genetic divergence was calculated in pairwise combinations of the different species as the number of fixed SNPs between them, the number of fixed or nearly fixed SNPs between them, the top 1% of F_{st} between them from the distribution of F_{st} between SNPs and the genome-wide average F_{st} across all SNPs in the genome.

F_{st} Comparison	Number of fixed SNPs ($F_{st} = 1$)	Number of nearly fixed SNPs ($F_{st} \geq 0.95$)	Top 1%	Genome-wide average
<i>C. sp. 'wide-mouth'</i> vs <i>C. desquamator</i>	5,212	5,915	0.84	0.131
<i>C. sp. 'wide-mouth'</i> vs <i>C. brontotheroides</i>	5238	14,602	0.85	0.15
<i>C. sp. 'wide-mouth'</i> vs <i>C. variegatus</i>	247	869	0.68	0.104
<i>C. desquamator</i> vs <i>C. brontotheroides</i>	6941	22,088	0.86	0.17
<i>C. desquamator</i> vs <i>C. variegatus</i>	414	1,964	0.71	0.116
<i>C. variegatus</i> vs <i>C. brontotheroides</i>	110	567	0.637	0.09

Table S5. Comparison of the average absolute genetic divergence (D_{xy}) between the four species in Osprey Lake ‘wide-mouth’, *C. variegatus*, *C. desquamator* and *C. brontotheriodes* and the shared adaptive alleles between scale-eater populations.

Comparison	Mean D_{xy}
<i>C. variegatus</i> vs <i>C. brontotheriodes</i>	0.167
<i>C. variegatus</i> vs <i>C. desquamator</i>	0.169
<i>C. variegatus</i> vs <i>C. sp. ‘wide-mouth’</i>	0.165
<i>C. brontotheriodes</i> vs <i>C. desquamator</i>	0.162
<i>C. brontotheriodes</i> vs <i>C. sp. ‘wide-mouth’</i>	0.156
<i>C. desquamator</i> vs <i>C. sp. ‘wide-mouth’</i>	0.157
Selective sweeps in <i>C. sp. ‘wide-mouth’</i>	0.123
Shared genetic variants with <i>C. desquamator</i>	0.0814
Unique genetic variants in <i>C. sp. ‘wide-mouth’</i>	0.24

Table S7. The distribution of shared adaptive alleles between the two scale-eating species across the Caribbean. Numbers represent the copies of the *C. desquamator* and ‘wide-mouth’ allele present in outgroup populations collected from San Salvador Island (SS), Rum Cay (RC), Cat Island (CT), Exumas Islands (EX), Long Island (LG), Mayaguana (MG), New Providence Island (NPI) in the Bahamas. Lagunas Bavaros (BAV) and Etang Saumautre (ETA) in Dominican Republic, Sarasota Estuary in Florida (FL), Curacao (CUR), Caicos Island (CAI) and Isla Margarita (MAR) in Venezuela.

<i>C. variegatus</i>												<i>C. laciniatus</i>	<i>C. higuey</i>	<i>C. bondi</i>	<i>C. dearborni</i>		
Allele	Gene	Distribution	SS	RC	CT	EX	LG	MG	FL	NC	NPI	BAV	ETA	CUR	CAI	MAR	
HiC_scaffold_6:907101	NA	SGV	0	0	1	0	0	0	0	12	0	0	0	0	0	0	
HiC_scaffold_6:907425	NA	SGV	1	0	0	0	0	0	0	7	0	0	0	0	0	0	
HiC_scaffold_6:907665	NA	SGV	0	0	0	0	0	0	0	7	0	0	0	0	0	0	
HiC_scaffold_6:908229	NA	SGV	1	0	1	0	0	0	0	6	3	0	0	0	0	0	
HiC_scaffold_6:908889	NA	SGV	1	0	0	0	0	0	0	6	0	0	0	0	0	0	
HiC_scaffold_6:923182	NA	SGV	0	5	0	1	0	0	1	0	0	0	0	0	0	0	
HiC_scaffold_6:923590	NA	SGV	0	5	0	2	0	0	0	0	0	0	0	0	0	0	
HiC_scaffold_6:924203	NA	de novo	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
HiC_scaffold_6:929621	NA	SGV	1	2	1	0	0	0	0	0	0	0	0	0	0	0	
HiC_scaffold_9:31763392	daam2	SGV	6	3	0	1	0	1	1	1	6	1	0	0	0	0	
HiC_scaffold_9:31763400	daam2	SGV	8	5	0	1	1	0	1	8	10	8	0	0	0	0	
HiC_scaffold_24:5390450	usp50	SGV	1	1	0	0	0	0	0	0	1	0	0	0	1	0	
HiC_scaffold_43:27385265	NA	SGV	3	1	0	2	1	1	0	5	1	0	1	2	0	11	
HiC_scaffold_43:27389200	NA	SGV	5	1	0	0	1	1	1	12	12	2	1	3	1	12	
HiC_scaffold_44:14963656	atp8a1	SGV	3	1	0	1	0	1	1	12	12	2	1	3	1	12	
HiC_scaffold_60:1798737	znf214	SGV	3	0	0	0	0	1	0	0	2	0	0	0	0	0	

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