

Research

Ecosystem size shapes antipredator trait evolution in estuarine threespine stickleback

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Ecosystem size is known to influence both community structure and ecosystem processes. Less is known about the evolutionary consequences of ecosystem size. A few studies have shown that ecosystem size shapes the evolution of trophic diversity by shaping habitat heterogeneity, but the effects of ecosystem size on antipredator trait evolution have not been explored. Ecosystem size may impact antipredator trait evolution by shaping predator presence (larger ecosystems have longer food chains) and habitat complexity (larger ecosystems may have more diverse habitat structure). We tested these effects using threespine stickleback from bar-built estuaries along the Central Coast of California. These stickleback populations are polymorphic for *Ectodysplasin-A* (*Eda*), a gene that controls bony lateral plates used as antipredator defense. We inferred *Eda* genotypes from lateral plate phenotypes and show that the frequency of the complete (C) allele, which is associated with greater number of lateral plates, increases as a function of ecosystem size. Predator presence and habitat complexity are both correlated to ecosystem size. The strongest proximate predictor of *Eda* allele frequencies was the presence of predatory fishes (steelhead trout and sculpin). Counter to expectations, habitat complexity did not have a strong modifying effect on *Eda* allele frequencies. Our results point to the importance of ecosystem size for determining predator presence as being the primary pathway to evolutionary effects. Ecosystem size has received much attention in ecology. Our work shows that it may be an important determinant of adaptive evolution in wild populations.

Keywords: antipredator traits, bar-built estuaries, ecosystem size, *Ectodysplasin A* gene, *Gasterosteus aculeatus*, predation

Introduction

Ecosystem size is a fundamental characteristic of natural habitats that has widespread ecological effects. The physical size of an ecosystem plays an important role in structuring the community (Spencer and Warren 1996, Post et al. 2000, Sabo et al. 2010)

and affects ecological functions such as rate of primary production and decomposition (Wardle et al. 2003, Ward and McCann 2017). Larger ecosystems often have more habitat complexity and structural diversity, thereby providing more open niche space and ecological opportunity (Barbour and Brown 1974, Brönmark 1985). Increases in the diversity of available niche space with ecosystem size may help drive the positive relationship between species number and ecosystem area (Gavrilets and Losos 2009). However, we still understand relatively little about how ecosystem size affects trait evolution and through which ecological pathways it acts.

While the ecological effects of ecosystem size are diverse and well-studied, evolutionary effects have received less attention. In some adaptive radiations, ecosystem size has been shown to be positively correlated with speciation rate, where increased ecosystem size represents increased habitat heterogeneity or ‘ecological opportunity’ (Losos and Schluter 2000, Parent and Crespi 2006, Seehausen 2006, Kisel and Barraclough 2010). Here we instead focus on the effects of ecosystem size on trait evolution within species. With such far-reaching ecological effects, ecosystem size could influence natural selection on traits through a wide variety of proximate mechanisms. For example, several studies have shown that lake size influences habitat heterogeneity and therefore resource diversity and distribution, which in turn influences genetic, morphological and ecological diversity in postglacial fishes (Nosil and Reimchen 2005, Lucek et al. 2016, Recknagel et al. 2017, Doenz et al. 2019, Bolnick and Ballare 2020). Taken together, these examples suggest that ecosystem size influences resource diversity, which in turn influences intraspecific competition and subsequent trophic diversification.

In addition to resource diversity, another ubiquitous source of natural selection on populations that may be related to ecosystem size is predation risk. As with resource diversity, food chain length also tends to increase with ecosystem size, and therefore the smallest ecosystems often lack top predators (Tonn and Magnuson 1982, Schoener 1989, Post et al. 2000, Takimoto et al. 2008). Ecosystem size may limit top predator presence for a number of reasons. Small ecosystems may have insufficient resources to support top predators (Elton 1927, Yodzis 1984). Top predators may have diverse habitat requirements (Lawrence et al. 2018) or might be limited by disturbances like flooding, drought or other physical and chemical extremes (Tonn and Magnuson 1982, Sabo et al. 2010). If predator presence is correlated with ecosystem size, then local adaptation of prey to different predation regimes may be a proximate effect that is ultimately driven by variation in ecosystem size (Nosil and Reimchen 2005). Yet the relative importance of predator presence and habitat complexity as proximate mechanisms shaping the evolutionary effects of ecosystem size remains unexplored (Table 1).

Here we test the effect of ecosystem size on the evolution of antipredator traits in estuarine threespine stickleback *Gasterosteus aculeatus* populations along the Central Coast of California, USA. The majority of these estuaries are only intermittently connected to the ocean by surface water due to the seasonality of rainfall and oceanographic deposition of sand along the shore (Heady et al. 2014). Such bar-built estuaries, also called intermittently closed/open lakes and lagoons (ICOLs), are found in wave dominated coastlines across the world (Mcsweeney et al. 2017). The top aquatic predators in California bar-built estuaries include predatory sculpins

Table 1. Previous studies that report significant effects of ecosystem size on trait evolution. Comparison of mechanisms proposed and tested.

Ecosystem size mechanisms proposed	Alternative mechanisms proposed	Mechanisms measured	Significant responses	Taxa	References
Habitat (resource) heterogeneity		habitat (resource) heterogeneity	variation in individual diet specialization	stickleback	Bolnick and Ballare 2020
Habitat (resource) heterogeneity			number of trophically and genetically distinct morphotypes	Arctic charr	Doenz et al. 2019
Habitat (resource) heterogeneity			trophic trait, defensive trait, neutral genetic divergence	stickleback	Lucek et al. 2016
Habitat (resource) heterogeneity			trophic trait variation	Arctic charr	Recknagel et al. 2017
Habitat (resource) heterogeneity			trophic trait min, mean, max	Arctic charr	Recknagel et al. 2017
Habitat heterogeneity	productivity	productivity	trophic diversification	whitefish	Siwertsson et al. 2010
Habitat heterogeneity			trophic trait variation	stickleback	Nosil and Reimchen 2005
Predator presence		predator presence	defensive trait polymorphism	stickleback	Moodie and Reimchen 1976
Predator presence		predator presence	defensive trait mean	stickleback	Reimchen 1994
Predator presence			defensive trait variation	stickleback	Nosil and Reimchen 2005
Unspecified		presence of other fish species	trophic trait mean	stickleback	Moodie and Reimchen 1976

and salmonids that eat a mix of invertebrates and small fishes including threespine stickleback. Salmonids (*Oncorhynchus* spp.) and sculpins (*Cottus* spp.) require adequate perennial freshwater habitat upstream for spawning, and salmonid population viability is predicted to be correlated with the amount of freshwater habitat upstream (Moyle 2002, Williams et al. 2016).

Threespine stickleback vary widely in predator defense traits, including the number and arrangement of a row of bony armor plates along the flank which begin behind the head and end in a keel on the caudal peduncle and protect stickleback against predatory fishes (Reimchen 1994, Barrett 2010). There is extensive inter- and intra-population variation in plate number and arrangement reflective of variation in predation pressure (Hagen and Gilbertson 1972, Bell et al. 1993, Reimchen et al. 2013). Experimental studies confirm that higher plate numbers allow increased survival during encounters with predatory fishes including salmonids (Reimchen 1991, 1992, 2000). Variation at the *Ectodysplasin-A* (*Eda*) locus explains 75–80% of the variation in plate number (Colosimo et al. 2004, Kitano et al. 2008, Des Roches et al. 2020). Individuals with two copies of the low allele (L) tend to have few plates (<10), those with two copies of the complete allele (C) tend to have a continuous row of plates (>30 in some populations), and heterozygotes are more variable but generally have an intermediate phenotype or look like homozygous completes (Colosimo et al. 2005, Miller et al. 2015). Marine or anadromous fish are usually homozygous for the complete allele, whereas many derived freshwater resident populations are homozygous for the low allele (Colosimo et al. 2005).

Stickleback plate number is also correlated with factors other than predator presence. In California, stickleback populations transition from primarily completely plated anadromous populations in the north to exclusively low-plated, freshwater resident populations in the south, a shift that tracks changes in temperature, precipitation and habitat (Baumgartner and Bell 1984, Des Roches et al. 2020). Bar-built estuary stickleback populations along the Central Coast of California are located in a transition zone between anadromous and resident populations and are polymorphic for plate number and underlying *Eda* genotype (Baumgartner and Bell 1984, Des Roches et al. 2020). However, site-to-site differences in *Eda* allele frequencies can be large (Paccard et al. 2018). Thus, latitudinal gradients might not explain more local differences in stickleback plate number among neighboring estuaries in the Central Coast transition zone. Our focal sites are south of the range of anadromous threespine stickleback and thus, while polymorphic for *Eda* and plate phenotype, these stickleback populations are made up of resident freshwater fish and are unlikely to represent a hybrid zone between anadromous and resident types (Howe 1973, Paccard et al. 2018).

One factor that might modify the effect of predators on stickleback plates is habitat complexity and the availability of cover (Leinonen et al. 2011). Low plate counts might be

avored over complete plates in complex, vegetated habitats such as the estuary if the relative risks of predation between genotypes differs in vegetated habitats and open-water habitats. There are a number of reasons why relative predation risk might differ as a function of habitat complexity, including differences in predator type or density, predator preference or prey escape probability (Reimchen et al. 2013). Experimental evidence indicates that natural selection by pike favors completely plated fish in open habitat, but favors low plated fish in habitats with more refuge (Leinonen et al. 2011). Low plates might be favored if hiding in refuge is an effective antipredator strategy, but large numbers of plates reduce the flexure and fast-start speeds necessary to quickly retreat to cover (Reimchen 1983, Taylor and McPhail 1986, Bergstrom 2002). Selection against low plates from fish predators may be relaxed if those fish predators prefer open water habitats and are less dense in the vegetated habitat. A study across the whole state of California found that higher frequencies of low plate morphs in estuaries that had lower proportions of flowing riverine wetlands and more lotic habitat (Des Roches et al. 2020).

In this study we test for the effect of ecosystem size on prey traits and compare the roles of predator presence and habitat complexity to explain that effect. We hypothesize that ecosystem size determines the presence of predatory fish, which is the major determinant of stickleback plate evolution. However, we further predict that habitat complexity modifies the role of predators on stickleback plates by favoring different antipredator traits in different environments.

Material and methods

Ecosystem size

We studied 20 estuaries along the coasts of Santa Cruz and San Mateo counties, California, USA (Table 2). We measured ecosystem size in several complementary ways as is common in studies of riverine ecosystems (Post et al. 2007). We measured the total stream length (km) of the river network draining into each estuary using ArcGIS ver. 10.2 (ESRI 2013). Then we measured estuary area, since water levels (and therefore estuary area) in bar-built estuaries fluctuate dramatically during the annual cycle of wet and dry seasons, breaches and impoundments (Fig. 1), (Webb et al. 1991, Behrens et al. 2013, Williams and Stacey 2016, Orescanin and Scooler 2018). To do so we used GIS data from the National Wetlands Inventory, which consists of polygons classifying wetlands using Cowardin's classification scheme (Cowardin et al. 1979, US Fish and Wildlife Service 1993, Federal Geographic Data Committee 2013). This dataset consists of polygons of wetland and open water habitats that were developed from expert interpretation of high-altitude aerial photographs (US Fish and Wildlife Service 1993). We calculated the Channel area by adding up the area of all wetland polygons of either estuarine deepwater or tidal

Table 2. Site characteristics and stickleback sample sizes for 20 bar-built estuaries in Santa Cruz and San Mateo Counties, California, sorted in order from north to south along the coast. Stickleback samples with fewer than 10 fish are not reported here or included in our analyses. In 2016 and 2017, fish were only sampled at six sites.

Site	Latitude	Longitude	Channel area (km ²)	Permanently flooded area (km ²)	Total wetland area (km ²)	Total stream length (km)	Proportion vegetated area	Steelhead	Sculpin	Spring 2014		Spring 2015		Spring 2016		Spring 2017	
										Fall	Spring	Fall	Spring	Fall	Spring	Fall	Spring
San Gregorio Creek	37.32	-122.40	0.0352	0.0352	0.0770	122.826	0.543	present	present	18	30						
Pomponio Creek	37.30	-122.41	0.0102	0.0102	0.0157	13.411	0.349	present	present	17	30	30	30				
Pescadero Creek	37.27	-122.41	0.1511	0.3160	1.0686	206.965	0.704	present	present	30	32	10					
Bean Hollow	37.23	-122.41	0.0086	0.0086	0.0278	13.023	0.691	absent	absent	30	30	59	30				
Yankee Jim Gulch	37.19	-122.40	0.0004	0.0004	0.0004	2.648	0.000	absent	absent	30		30	30				
Gazos Creek	37.17	-122.36	0.0053	0.0053	0.0053	26.368	0.000	present	present	30	30	12					
Waddell Creek	37.10	-122.28	0.0204	0.0204	0.1691	56.463	0.879	present	present	30	30	30	30	18	30		
Scott Creek	37.04	-122.23	0.0064	0.0128	0.1252	67.360	0.897	present	present	60	60	121	91	45	26	30	35
Laguna Creek	36.98	-122.15	0.0203	0.0219	0.0536	21.743	0.592	present	present	30	30	30	30	11	31	29	11
Baldwin Creek	36.99	-122.11	0.0041	0.0120	0.0627	11.982	0.809	present	present			30					
Lombardi Creek	36.96	-122.11	0.0012	0.0012	0.0280	6.389	0.958	absent	absent	30	37	60	59	30	30	23	30
Old Dairy Creek	36.96	-122.09	4.0E-05	4.0E-05	0.0133	11.032	0.997	absent	absent	30	60	60	25	30	30	21	30
Younger Lagoon	36.95	-122.07	0.0108	0.0108	0.0209	1.919	0.484	absent	absent	60	88	60	59	31	34	54	30
Moore Creek	36.95	-122.06	0.0032	0.0032	0.0415	4.388	0.924	absent	absent	30		30					
San Lorenzo River	36.97	-122.01	0.0874	0.0874	0.0992	322.680	0.119	present	present	30	30						
Corcoran Lagoon	36.96	-121.98	0.0003	0.0614	0.0770	9.932	0.202	absent	absent	30	42	37	30				
Moran Lake	36.96	-121.98	0.0098	0.0100	0.0100	0.805	0.000	absent	absent	30		30					
Soquel Creek	36.97	-121.95	0.0273	0.0273	0.0373	99.094	0.269	present	present	30	39	14	55				
Aptos Creek	36.97	-121.91	0.0077	0.0077	0.0112	55.120	0.310	present	present	30		29	17				
Pajaro River	36.85	-121.81	0.2483	0.2597	1.2855	3280.012	0.798	present	present	12		30					

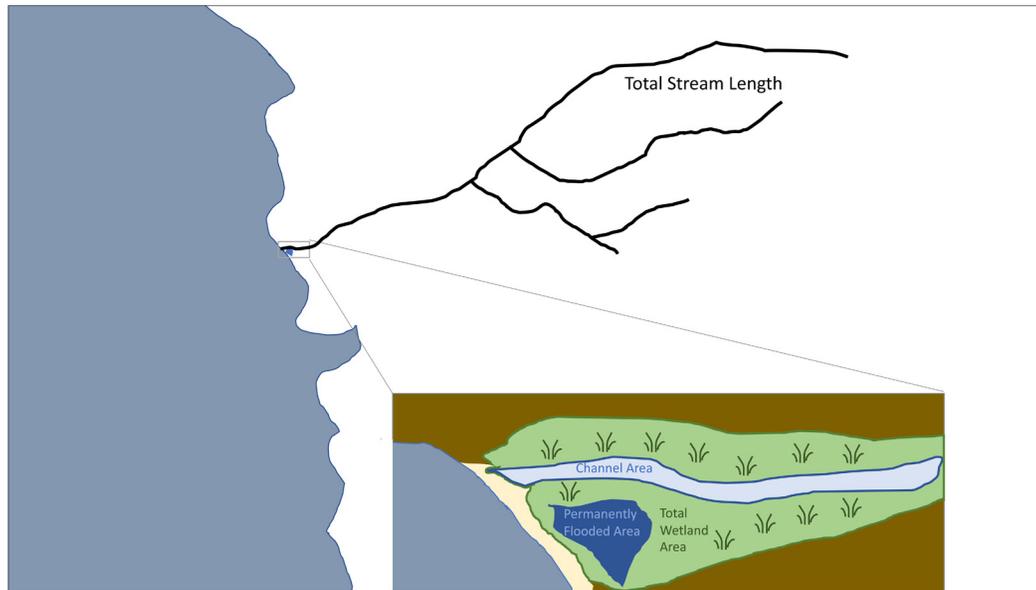


Figure 1. Schematic diagram of estuary wetland habitat types. We used the Cowardin et al. (1979) wetland classification system to create three nested metrics of estuary size. Channel area (light blue fill and outline in inset) is comprised of all wetland polygons of ‘Estuarine deepwater’ and ‘Tidal riverine’ habitats that were part of the main river channel, as opposed to side-channels, ponds and lakes. Permanently flooded area (dark blue fill and outline in inset) includes all polygons in the channel area plus any estuarine, riverine or palustrine deepwater polygons within the floodplain below the upstream extent of tidal riverine habitat. Total wetland area (light green fill and dark green outline in inset) includes all polygons in the permanently flooded area plus any estuarine, riverine or palustrine wetland polygons that were immediately adjacent to the channel and permanently flooded areas. Thus, channel area is nested within permanently flooded area, which is nested within total wetland area. Finally, we measured the total stream length (black lines, main panel) of all stream segments in the river network. In the inset, brown represents upland habitat that does not flood and yellow represents the sandbar that closes the stream off from the ocean seasonally.

riverine habitats that were part of the main river channel, as opposed to side-channels, ponds and lakes. We calculated the Permanently flooded area by adding the Channel area plus any estuarine, riverine or palustrine deepwater polygons within the floodplain below the upstream extent of tidal riverine habitat. We calculated the Total wetland area by adding the Permanently flooded area plus any estuarine, riverine or palustrine wetland polygons that were immediately adjacent to the Channel and Permanently flooded areas. All areas were measured in square kilometers.

The proximate mechanism for the habitat complexity hypothesis is the increased availability of structured habitats. Unlike the deepwater polygons in the channel area and permanently flooded area metrics, the additional habitats included in the total wetland area metric are mostly emergent marsh and scrub/shrub wetlands that seasonally dry. When emergent marsh and scrub/shrub habitats are inundated, vegetation remains above the water level. These seasonally flooded habitats are therefore highly structured. While there is some emergent vegetation included in Channel area, Permanently flooded area and Total stream length, it is likely only on the margins and doesn’t make up the majority of those wetland polygons. Therefore, from these ecosystem size metrics we also calculated a simple index of the availability of complex habitat for prey to use to avoid predators: proportion vegetated area (PVA) = $1 - \text{permanently flooded area} / \text{total wetland area}$.

Predator presence

We determined the presence of juvenile steelhead *Oncorhynchus mykiss* based on published accounts since these larger, faster predators often evade the type of sampling gear we used to target stickleback (Becker and Reining 2008). Coho salmon *Oncorhynchus kisutch* are present but locally rare and only occur at a subset of the sites with steelhead, so we did not consider them further (Williams et al. 2016). We recorded the presence of sculpin during our stickleback surveys. Sculpin were not identified to species, but three different species are present in the area: marine Pacific staghorn sculpin *Leptocottus armatus*, freshwater prickly sculpin *Cottus asper* and freshwater coastrange sculpin *Cottus aleuticus*. At some sites we encountered sculpins frequently and in large numbers. At other sites we did not encounter any sculpin or caught sculpin only infrequently (<5% of traps or seines) in small numbers. In the latter type of site, a follow-up study revealed that captured sculpin were most often juvenile marine Pacific staghorn sculpin caught during spring sampling following recent estuary breaching and were not found again in the following fall sample (B. A. Wasserman unpubl.). It is likely that sites at which we have not caught sculpin are also occasionally visited by marine accidentals in this way. Rather than distinguishing between sites where we caught sculpin and those where we did not, we think the more ecologically appropriate distinction is

between two types of sites: those with resident sculpin of any species (present) and those sites with no sculpin or only accidental Pacific staghorn sculpin (absent). Sites where we caught sculpin in more than 5% of traps or seines were defined as having sculpin present. Sites where we caught sculpin in less than 5% of traps or seines were defined as having sculpin absent. Since resident sculpin and steelhead distributions overlap almost entirely (Table 2), we could not disentangle their independent effects, and we did not use them as separate predictors in the same model. We chose to use the slightly more widespread sculpin (which occurred at one additional site that did not have steelhead) as a predictor of overall predatory fish presence, though results from analyses using steelhead were qualitatively similar.

Prey traits and genotype inference

We collected stickleback using minnow traps and beach seines semiannually just after sandbar formation in the spring (usually April–June, but sometimes as late as August) and just before sandbar breaching following sufficient rain in the fall (usually November–December, but occasionally as early as September and as late as January). We attempted to collect fish from all 20 sites in 2014 and 2015, and we continued sampling at six sites during 2016 and 2017 (Table 2). Fish were euthanized with an overdose of MS-222, frozen in the field, and then stored in a freezer until they could be processed. We collected fish longer than 30 mm and targeted a sample size of 30 fish per sample. Fish shorter than 30 mm were not used because they may not have fully developed plates (Bell 1981). Our analyses only use samples that included at least ten fish.

We counted the left lateral plates of each fish under a dissecting microscope. The spring 2014 fish were part of a previous study in which a subset of 287 was genotyped for *Eda* (Paccard et al. 2018). Since our populations are polymorphic for *Eda*, plate count distributions represent mixtures of distributions based on a latent categorical variable: *Eda* genotype (Supplementary material Appendix 1 Fig. A1). We therefore quantified the relationship between plate count and *Eda* genotype using the fish with known genotype in order to infer the *Eda* genotype of all 2952 fish.

We used a Gaussian mixture model to infer the most likely *Eda* genotype for each individual based on their plate count using the R package mixtools version 1.1.0 (Benaglia et al. 2009). We fit a model with three latent states (*Eda* genotypes) using an expectation–maximization algorithm and initialized the plate count distribution of each latent state with the sample mean and standard deviations of lateral plate counts for the corresponding genotype based on data from the individuals with known genotype (Dempster et al. 1977). We assigned all individuals in the study their inferred genotype based on maximum likelihood. We calculated the inferred allele frequency of each sample from the inferred genotypes of individual fish in that sample and used these inferred allele frequencies as a response variable in our analyses.

Data analysis

We used confirmatory path analysis (Shipley 2000) to model the effects of ecosystem size on predator presence and PVA and the effects of predator presence and PVA on inferred C allele frequency. All metrics of ecosystem size were log-transformed to meet assumptions of normality. We transformed the inferred C allele frequency using the empirical logistic transformation to improve heteroscedasticity of model residuals, where $\text{logit}(C) = \log((C + \epsilon)/(1 - C + \epsilon))$, and where ϵ is equal to the minimum non-zero value of inferred C allele frequency (Warton and Hui 2011). We conducted the analysis using the R statistical environment ver. 4.0.0 (<www.r-project.org>) using the packages lme4 ver. 1.1-23 (Bates et al. 2015) and piecewiseSEM ver. 2.1.0 (Lefcheck 2016) which accommodates complex model structures such as random effects and generalized linear models in the structural equation modeling framework (Shipley 2009, Lefcheck 2016). The effect of ecosystem size on predatory fish presence was modeled with logistic regression. The effect of ecosystem size on PVA was modeled using a linear regression. We then modeled the effect of predator presence and PVA on inferred C allele frequency using linear mixed models with a random effect of site. In piecewiseSEM we specified that there was no causal relationship, but allowed for the possibility of correlated error, between predator presence and PVA (Lefcheck 2016).

We used two different methods to test whether the effect of ecosystem size on inferred C allele frequency acted primarily through the predator presence or habitat-mediated pathways. First, we used Shipley’s test of directed separation to determine whether a simpler path analysis, which dropped the effect of PVA on inferred C allele frequency, was adequate to explain the data. In Shipley’s test of directed separation the included causal links are a sufficient description of the data if the calculated value of Fisher’s C could have easily occurred by chance ($p > 0.05$) (Shipley 2000). Therefore, if the simpler model has a probability of $p > 0.05$, it is considered a sufficient explanation and the more complex model is rejected. As a second way of evaluating through which causal pathway ecosystem size influenced inferred C allele frequency, we performed this path analysis separately for each of the four metrics of ecosystem size and compared the results (Post et al. 2007).

Results

Predatory fish were more likely to be found in larger ecosystems regardless of the ecosystem size metric used (Fig. 2). The standardized regression coefficient (β_s) for the effect of ecosystem size on predator presence was strongest for total stream length ($\beta_s = 0.8914$, $p < 0.0001$) and channel area ($\beta_s = 0.7185$, $p < 0.0001$), intermediate for permanently flooded area ($\beta_s = 0.6004$, $p = 0.0003$), and weakest for total wetland area ($\beta_s = 0.3044$, $p = 0.0271$) (Fig. 3).

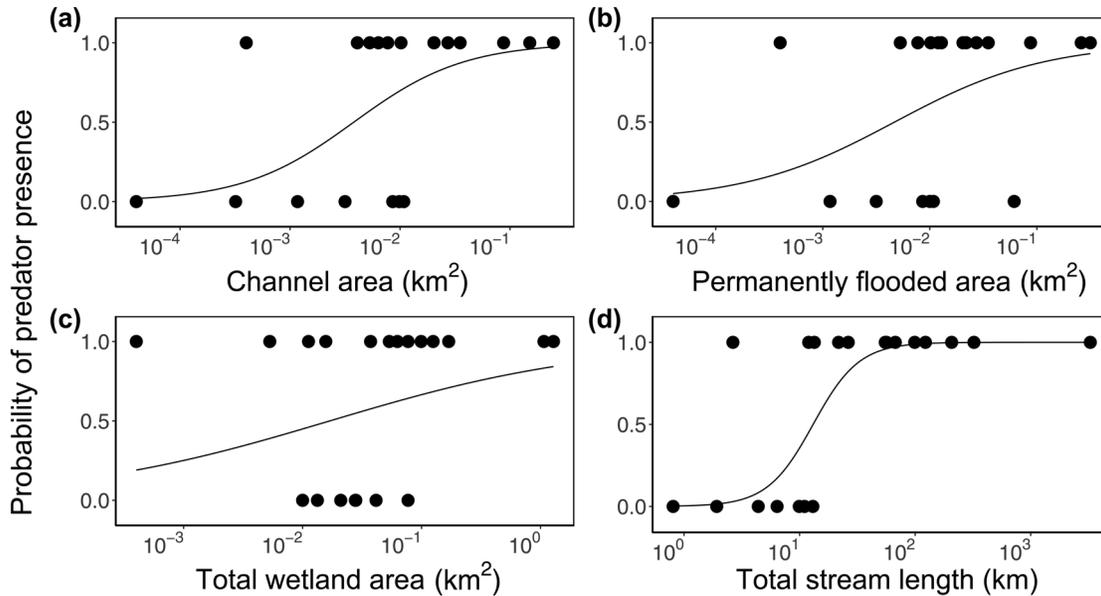


Figure 2. Predatory fish presence as a function of ecosystem size. Points show the raw data as a binary: either present (1) or absent (0), and lines show the fits of the logistic regressions. Ecosystem size measured as (a) channel area (km²), (b) permanently flooded area (km²), (c) total wetland area (km²) and (d) total stream length (km).

The degree to which estuary area measurements were correlated to PVA varied as expected (Fig. 4). The standardized regression coefficient (β_s) for the effect of ecosystem size on PVA was largest for log total wetland ($\beta_s = 0.4261$, $p = 0.0001$), intermediate for permanently flooded area ($\beta_s = -0.3280$, $p = 0.0023$) and channel area ($\beta_s = -0.2243$, $p = 0.0402$), and not significant for total stream length ($\beta_s = 0.1308$, $p = 0.2356$) (Fig. 3). Unexpectedly, PVA decreased with channel area and permanently flooded area (Fig. 3).

The mixture model classified fish with plate counts of 3–8 as LL homozygotes, those from 9 to 21 as CL heterozygotes, and those from 22 to 28 as CC homozygotes (Supplementary material Appendix 1 Fig. A1). The overall misclassification rate for the fish with known genotypes was 19.9%. LL and CC fish were correctly matched to their known genotype 93% and 96% of the time, respectively (Supplementary material Appendix 1 Table A1). CL fish were harder to classify: they were only correctly classified 46% of the time (Supplementary material Appendix 1 Table A1). Both the overall and the genotype-specific classification rates are in line with other estimates of the causal effects of *Eda* on plate counts (Colosimo et al. 2004, Paccard et al. 2018). Taken together, these misclassification rates mean that our inferred genotypes likely underestimated the number of CL fish but overestimated the number of CC fish. They also underestimated the number of LL fish, but only slightly. There were differences in the genotype frequencies by site, but no clear seasonal pattern across time (Supplementary material Appendix 1 Fig. A2).

Inferred C allele frequency was higher in sites with predatory fish than in sites without them ($\beta_s = 0.7067$, $p = 0.0006$, Fig. 5) but the effect of PVA on inferred C allele frequency was small ($\beta_s = -0.0355$, $p = 0.8218$) (Fig. 3). Shipley's test

of directed separation indicated that the simpler model, with only the predator presence pathway, was a sufficient explanation of the data for channel area (Fisher's $C = 3.085$, $df = 4$, $p = 0.544$), for permanently flooded area (Fisher's $C = 6.41$, $df = 4$, $p = 0.171$), and for total stream length (Fisher's $C = 6.107$, $df = 4$, $p = 0.191$), but not for total wetland area (Fisher's $C = 11.698$, $df = 4$, $p = 0.02$). Indeed, the full model with both paths was not a sufficient explanation of the data for total wetland area (Fisher's $C = 9.688$, $df = 2$, $p = 0.008$). So, we re-ran that model and included the only other possible path, a direct effect of total wetland area on inferred C allele frequency. In this saturated model of the effects of total wetland area on inferred C allele frequency, the effect of predator presence was even stronger ($\beta_s = 0.8522$, $p < 0.0001$); the effect of PVA on inferred C allele frequency was still not significant, though it was now positive ($\beta_s = 0.2247$, $p = 0.1694$); and the direct effect of total wetland area on inferred C allele frequency was negative ($\beta_s = -0.4257$, $p = 0.0079$) (Fig. 6). The magnitude of the predator presence pathway (calculated by multiplying $\beta_{s\text{TWA} \rightarrow \text{PP}} \times \beta_{\text{PP} \rightarrow \text{C}} = 0.2594$) was smaller than the magnitude of the direct pathway ($\beta_{s\text{TWA} \rightarrow \text{C}} = -0.4257$).

Discussion

Predatory fish can have an important evolutionary effect on their prey, and yet they are often absent from the smallest ecosystems (Tonn and Magnuson 1982, Stanley et al. 1994, Sabo et al. 2010). Our results show that the presence of top predators is correlated with ecosystem size in bar-built estuaries in central California. Further, threespine stickleback populations sympatric with predatory fish are more armored and have higher frequencies of the *Eda* C allele than those

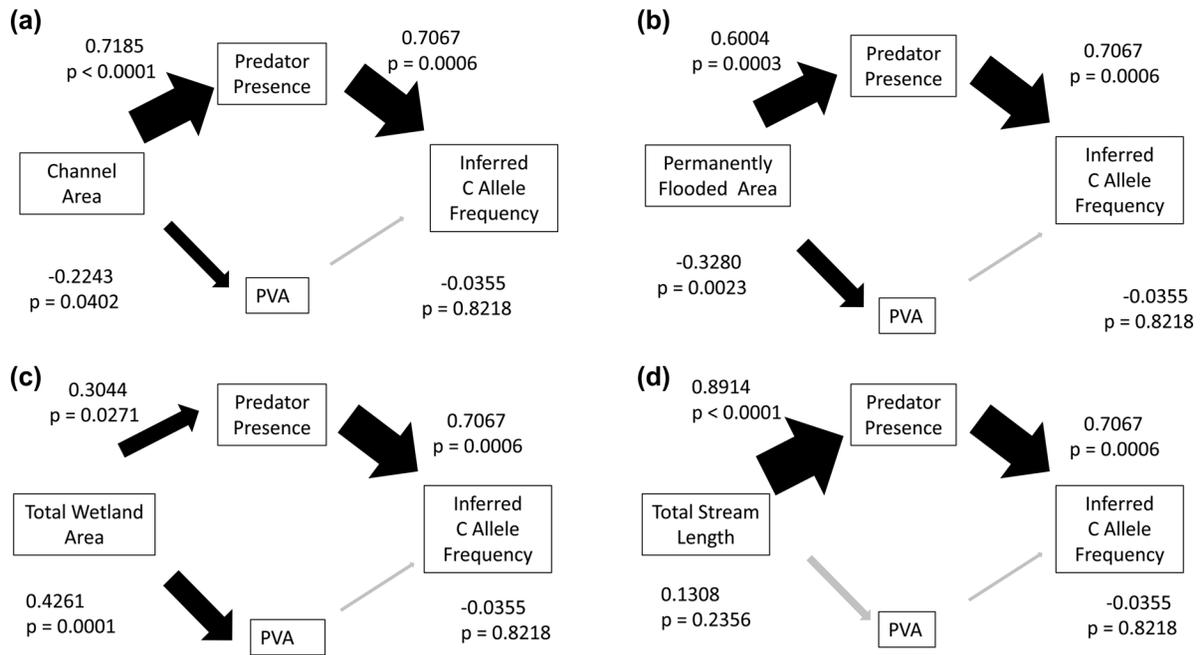


Figure 3. The results of the path analyses for empirical-logistic transformed C allele frequency, predatory fish presence, proportion vegetated area (PVA) and log-transformed ecosystem size. The widths of the arrows are scaled to the standardized coefficients which are also reported with the corresponding p-values next to each arrow. Significant relationships are shown in black, while non-significant relationships are shown in gray. Ecosystem size measured as (a) channel area (km²), (b) permanently flooded area (km²), (c) total wetland area (km²), (d) total stream length (km).

that occur in the absence of predators. Therefore, in our study system, there is an effect of ecosystem size on the evolution of prey traits which occurs primarily through predator presence. Meanwhile, habitat complexity did not have a significant effect on inferred *Eda* C allele frequency. Indeed, PVA

wasn't even consistently related to ecosystem size across different metrics.

A growing number of studies describe a positive effect of ecosystem size on predator presence and food chain length, especially in freshwater ecosystems (Tonn and Magnuson

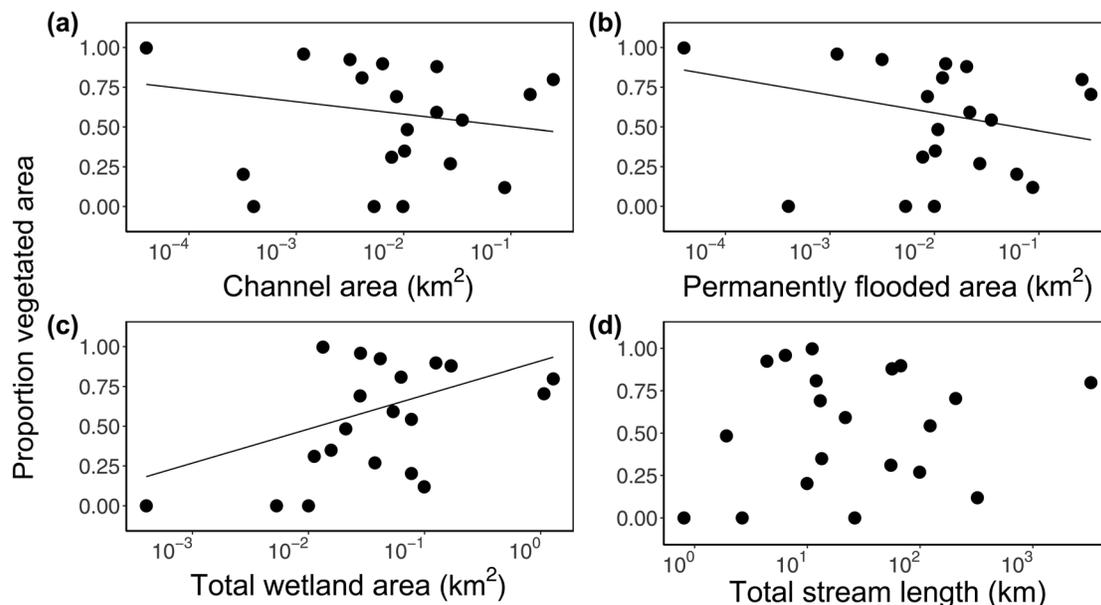


Figure 4. Proportion vegetated area (PVA) as a function of ecosystem size. Points show the raw data. Only significant regression lines are shown. Ecosystem size measured as (a) channel area (km²), (b) permanently flooded area (km²), (c) total wetland area (km²) and (d) total stream length (km).

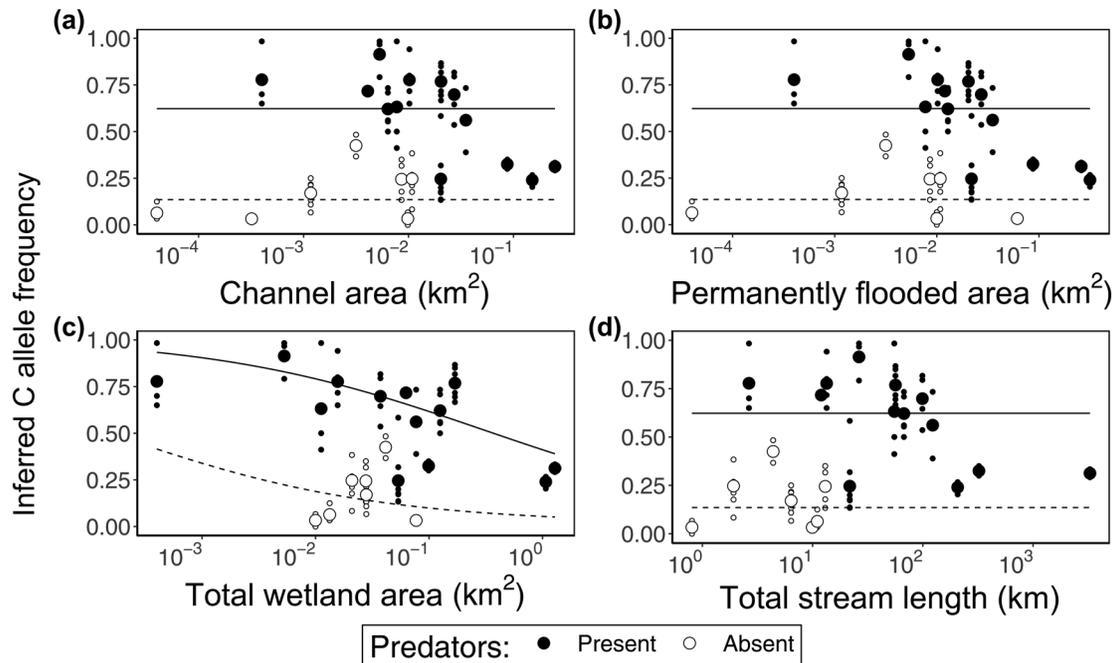


Figure 5. Inferred C allele frequency as a function of ecosystem size and predatory fish presence with ecosystem size measured as (a) channel area (km²), (b) permanently flooded area (km²), (c) total wetland area (km²), (d) total stream length (km). Small points represent the C allele frequency of an individual temporal sample; large points represent the mean of all samples from a given site. Solid regression lines represent the predicted value of inferred C allele frequency for estuaries of a given size with predators present, and dashed lines the predicted values of estuaries of a given size with predators absent. We fit the model in each panel that is best supported by Shipley's test of directed separation. Therefore panels (a) channel area, (b) permanently flooded area and (d) total stream length show model fits with only the indirect effect of ecosystem size (via predator presence) on inferred C allele frequency, whereas panel (c) total wetland area, shows the full model fit with a direct effect and an indirect effect (via predator presence) of ecosystem size on inferred C allele frequency.

1982, Post et al. 2000, Sabo et al. 2010). We too found that increasing ecosystem size is correlated with an increased chance of predator presence (Fig. 2, 3). The ecosystem size metric most strongly correlated with predator presence was Total Stream Length, as we predicted (Fig. 2, 3). Salmonids and sculpins in the genus *Cottus* require adequate amounts of freshwater habitat for breeding in order to maintain viable populations (Moyle 2002, Williams et al. 2016). Total stream

length would appear to account for this requirement well. Channel area and permanently flooded area also reflect this habitat requirement, whereas the total wetland area metric includes a great deal of marginal habitat that is not necessary for the breeding of these predators.

The presence of these predators is correlated with increased inferred frequency of C alleles and associated armor phenotypes in bar-built estuary stickleback populations (Fig. 3). This concurs with previous studies of these populations (Paccard et al. 2018). Stickleback armor traits have been shown to evolve in response to a wide range of predator selection regimes (Bell et al. 1993, Reimchen and Nosil 2002, Barrett et al. 2008, Spence et al. 2013). While we do not have direct evidence of selective predation by steelhead and sculpins in these estuaries, it has been shown elsewhere. Freshwater stickleback populations in British Columbia that live in sympatry with sculpins have two more plates, on average, than those that are allopatric to sculpin, and they have higher survival in mesocosms with sculpins present (Ingram et al. 2012, Miller et al. 2015, but see Maccoll and Chapman 2011). Stickleback from predominately low-predation populations in lakes in Washington state survived predation attempts by rainbow trout (the same species as our steelhead, *Oncorhynchus mykiss*) better with the modal 7-plated phenotype than with either fewer or more plates (Hagen and Gilbertson 1973). Other trout species have also been

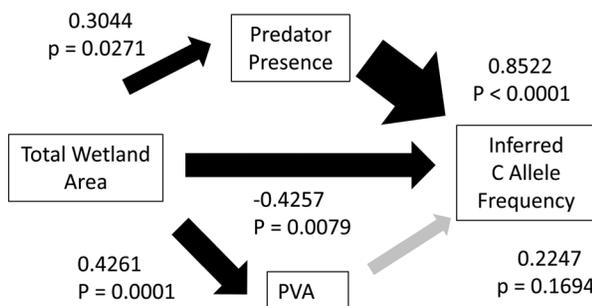


Figure 6. The results of the saturated path analyses for empirical-logistic transformed C allele frequency, predatory fish presence, proportion vegetated area (PVA) and log-transformed ecosystem size. The widths of the arrows are scaled to the standardized coefficients which are also reported with the corresponding p-values next to each arrow. Significant relationships are shown in black, while non-significant relationships are shown in gray.

shown to cause higher mortality of stickleback with fewer plates (Reimchen 1991, 1992, 2000). It is therefore likely that predation by one or more of the predators in our system is causing natural selection on *Eda*. This selection may derive either from predators intentionally targeting prey based on plate phenotypes or because more heavily plated stickleback are more likely to survive unsuccessful predator attacks (Reimchen 1991).

The correlation of stickleback armor to predator presence represents a proximate driver of armor evolution, but ecosystem size appears to be one of the ultimate drivers. Our path analyses show that the strongest effect of ecosystem size on stickleback armor was the predator–presence mechanism (Fig. 3). For three out of four ecosystem size metrics, the predator–presence pathway was a sufficient explanation of C allele frequencies, as demonstrated with Shipley’s test of directed separation. Only in the path analysis utilizing the fourth metric of ecosystem size, Total wetland area, was predator presence insufficient to explain the effect of ecosystem size on prey traits (Fig. 3). However, to our surprise, PVA still was not significant, and Shipley’s test of directed separation instead revealed that a direct effect of total wetland area was worth including (Fig. 6). The largest direct effect on allele frequency in this model was the effect of predator presence. However, since total wetland area so poorly predicted predator presence, the negative effect of total wetland area was the stronger pathway (Fig. 6).

We did not find evidence that habitat complexity (as measured by PVA) was inversely correlated to the number of lateral plates in stickleback. This is in contrast to a recent survey of stickleback throughout California, which found climate-driven habitat change to be an important driver of platedness (Des Roches et al. 2020). Our sites are all at similar latitude and so do not vary widely in climate. Despite not finding evidence for an effect of PVA on inferred C allele frequency, perhaps other ecological changes associated with increased total wetland area explain the decrease in C allele frequency. For example, total wetland area may influence predator density or the relative importance of predators with different selectivities, such as grappling invertebrate predators, which preferentially consume stickleback with complete plates (Marchinko 2009), as opposed to the predatory fishes studied here which preferentially consume stickleback with low plates (Reimchen 2000). This could be due to differences in the relative abundance of the two types of predators, differences in stickleback space use as a function of total wetland area if the two predators are primarily active in different habitats, or a combination of the two. Stickleback armor polymorphisms have previously been shown to reflect a balance between alternative forms of predation (Reimchen 1997, Reimchen and Nosil 2002).

Ecosystem size can also influence non-adaptive evolutionary processes such as genetic drift and gene flow. Genetic drift is unlikely to have created the correlation between mean C allele frequency and ecosystem size. If genetic drift strongly affects C allele frequencies, it should affect the variance of C allele frequency as a function of effective population size (which should

increase monotonically with ecosystem size) but not the mean C allele frequency as we show here. Gene flow between sites is substantial; analysis of microsatellite markers suggests that there is not much divergence between our focal populations (Paccard et al. 2018). If estuary size predicts the amount of time an estuary stays connected to the ocean because higher winter flows keep larger river mouths open longer, then it is possible that ecosystem size affects the opportunity for gene flow (Paccard et al. 2018). However, an analysis of neutral microsatellite loci reveals that individuals which are homozygous for the complete allele are well mixed into the local population rather than being associated with marine fish; therefore, gene flow from marine stickleback is not likely to be meaningfully altering C allele frequencies (Paccard et al. 2018).

When, more generally, might we expect adaptive evolutionary responses to variation in ecosystem size? We might expect evolutionary effects when abiotic and biotic correlates of ecosystem size alter the selective landscape. Broadly, we expect ecosystem size may influence trait evolution when it alters the presence (Nosil and Reimchen 2005, this study), the diversity (Recknagel et al. 2017) or the relative importance (Bolnick and Ballare 2020) of selective agents. Those sources of natural selection on the focal species might include resources, natural enemies, abiotic stressors or the relative importance of the three (Hiltunen et al. 2014, Lawrence and Barraclough 2016).

In this study, we measured multiple mechanisms to determine how ecosystem size affects antipredator trait distributions in prey. Our results suggest that ecosystem size can affect the evolutionary consequences of predator–prey interactions as well as those of competitive interactions (Nosil and Reimchen 2005, Lucek et al. 2016, Recknagel et al. 2017, Doenz et al. 2019, Bolnick and Ballare 2020). As in the competition examples, ecosystem size acts indirectly on trait evolution by altering the community structure. In the case of competition, resource diversity is correlated to ecosystem size and therefore impacts competitor trait evolution. Here ecosystem size affects prey traits primarily by determining predator presence. Many of the ecological consequences of ecosystem size are due to indirect effects on community structure or material and energy processing (Spencer and Warren 1996, Wardle et al. 2003). Future work investigating the selective impacts of these indirect effects of ecosystem size could give us a greater understanding of their potential for affecting adaptive evolution.

Data availability statement

Data are available from the Dryad Digital Repository: <<https://doi.org/10.7291/D1Z08B>> (Wasserman et al. 2020).

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Author contributions – BAW, TMA, RDHB, APH and EPP conceived the study. BAW, TMA and EPP designed the study. BAW, TMA, SD collected the data. AP and RDHB contributed the molecular genetic data. BAW analyzed the data with help from SD and EPP. BAW wrote the first draft of the manuscript. All authors contributed critically to editing and gave final approval for publication.

Conflicts of interest – We declare we have no competing interests.

Permits – Animal handling protocols were approved by the Univ. of California, Santa Cruz IACUC under protocols Palke-1306 and Palke-1310. Collections were made pursuant to California Scientific Collector's Permit SC-12752.

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Supplementary material (available online as Appendix oik-07482 at <www.oikosjournal.org/appendix/oik-07482>). Appendix 1.