TITLE

The Evolution of Ecological Specialization Across the Range of a Broadly Distributed Marine Species

Submitted to *Evolution*

i. Title: The Evolution of Ecological Specialization Across the Range of a Broadly Distributed Marine Species

ii. Running title: Ecological Specialization in the Sea

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1111/evo.13930](https://doi.org/10.1111/evo.13930).

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vi. Author contributions: ABW and AW conceived and designed the analysis; AW collected the data; ABW and AW performed the analysis; ABW wrote the manuscript, with assistance from AW, IA and JMSG.

vii. Acknowledgements: We thank Jasmin Winkler and Kai Stölting for their support in project planning and logistics, and Stefan Zehnder, Jessica Mantel and the members of the Wilson group for their assistance with animal care. Field support for pipefish collections in the Lagoon of Venice was provided by Mariella Rasotto, Matteo Pizzolon and Francesco Pascoli of the University of Padova. Special thanks to Michael Collyer, Dean Adams, and Chris Klingenberg for their support with morphometric analyses, and to Michael Collyer and two anonymous referees for their comments on our manuscript. This research was supported with funding from the Swiss Academy of Sciences, the Swiss National Science Foundation, the University of Zurich, Brooklyn College and the City University of New York. Animal husbandry and experimental procedures were approved by the Veterinäramt Zürich (Permit 161/2006).


**ABSTRACT**

Ecological specialization is an important engine of evolutionary change and adaptive radiation, but empirical evidence of local adaptation in marine environments is rare, a pattern...
that has been attributed to the high dispersal ability of marine taxa and limited geographic barriers to gene flow. The broad-nosed pipefish, *Syngnathus typhle*, is one of the most broadly distributed syngnathid species, and shows pronounced variation in cranial morphology across its range, a factor that may contribute to its success in colonizing new environments. We quantified variation in cranial morphology across the species range using geometric morphometrics, and tested for evidence of trophic specialization by comparing individual-level dietary composition with the community of prey available at each site. While the diets of juvenile pipefish from each site were qualitatively similar, ontogenetic shifts in dietary composition resulted in adult populations with distinctive diets consistent with their divergent cranial morphology. Morphological differences found in nature are maintained under common garden conditions, indicating that trophic specialization in *S. typhle* is a heritable trait subject to selection. Our data highlight the potential for ecological specialization in response to spatially variable selection pressures in broadly distributed marine species.

**KEYWORDS**

Adaptation, dietary analysis, functional morphology, geometric morphometrics, *Syngnathus typhle*, trophic ecology

**INTRODUCTION**

Ecological specialization is an important driver of evolutionary change, and key to adaptive radiation (Streelman and Danley 2003; Gavrilets and Losos 2009; Nosil 2012). Specialization allows organisms to capitalize on unique resources (i.e. food, space, or mates), promoting local coexistence via niche
partitioning (Bellwood et al. 2006; Herder and Freyhof 2006; Belmaker et al. 2012). While specialization is frequently inferred based on differential resource use, understanding the evolutionary origins of the morphological, physiological, and behavioral mechanisms facilitating specialization is key to understanding its adaptive significance (Ferry-Graham et al. 2002). Functional morphology studies the relationship between organismal structure and function, and provides a mechanistic framework for understanding adaptive change.

Trophic partitioning is perhaps the best studied form of ecological specialization (e.g., Smith and Skulason 1996; Rübert et al. 1999; Schluter 2000; von Rintelen et al. 2004), and has been implicated in the well-known adaptive radiations of African cichlid fishes (Burress 2015) and Galapagos finches (Grant and Grant 2011). Trophic specialists are thought to trade off improved feeding efficiency on a preferred food resource against the cost of reduced niche breadth, and may benefit when competition for resources is high (Futuyma and Moreno 1988; Ferry-Graham et al. 2002).

While adaptive radiations highlight the importance of specialization in ecological speciation, there is a growing appreciation that geographically widespread species may exhibit resource specialization at the population level (Fox and Morrow 1981; Shipley et al. 2009; Loxdale et al. 2011). Marine fishes often show complex adaptations for resource acquisition and processing (e.g., Wainwright et al. 2004, 2015; Cooper et al. 2017), creating the potential for trophic partitioning and ecological specialization in spatially heterogeneous environments. Molecular data suggest that ecological specialization has likely contributed to the high diversity of marine groups such as coral reef fishes (Wainwright et al. 2004; e.g., Rocha et al. 2005), but empirical evidence of dietary specialization remains rare (Barnett et al. 2006; Bellwood et al. 2006), making it difficult to link pattern and process in marine systems (Puebla 2009).

Suction feeding is an important mechanism of resource acquisition in aquatic environments, and has led to a variety of functional innovations for enhanced feeding efficiency in ray-finned fishes.
Syngnathid fishes (seahorses, pipefish and seadragons) are specialized suction feeders that use a sit-and-wait predation strategy to target zooplankton, crustaceans, and small fishes in nearshore marine environments. The unique morphology of syngnathids has attracted considerable interest (Consi et al. 2001; Hoffman et al. 2006; Van Wassenbergh et al. 2011b), and the feeding kinematics of the group have been explored in detail (e.g., de Lussanet and Muller 2007; Leysen et al. 2011). High resolution video imaging indicates that increases in syngnathid snout length create explosive capture velocities and the ability to target prey at greater distances, enhancing the capture of highly mobile prey (de Lussanet and Muller 2007; Van Wassenbergh et al. 2011a). However, mathematical modelling indicates that optimal snout length in this group is inversely correlated with snout height, creating a trade-off between capture efficiency and the maximum prey size that can be consumed (de Lussanet and Muller 2007). An interspecific analysis of trophic ecology in syngnathid fishes is consistent with this hypothesis, and indicates that long-snouted species consume significantly more mobile and smaller prey than do species with short snouts (Kendrick and Hyndes 2005). Increases in snout to head length ratios have also been associated with increases in brain size in this group, a relationship that may reflect the importance of enhanced visual information processing when feeding on highly mobile prey (Tsuboi et al. 2017).

The broad-nosed pipefish (Syngnathus typhle) is one of the most broadly distributed syngnathid species, and shows pronounced variation in cranial morphology across its range (Kendrick and Hyndes 2005; Leysen et al. 2011). S. typhle is distributed from the Baltic Sea in northern Europe and along the Atlantic and Mediterranean coasts through into the Black Sea (Fig. 1), and inhabits a variety of nearshore environments and conditions (Wilson and Eigenmann Veraguth 2010), offering opportunities for trophic specialization to capitalize on locally abundant food resources. As offspring develop within the male brood pouch and are released as free-living juveniles (Rispoli and Wilson 2008), dispersal in S. typhle is thought to be limited, but the species is genetically diverse, and exhibits a phylogeographic history consistent with the post-glacial colonization of northern Europe.
Here, we investigate the role of ecological specialization in the evolution of morphologically-divergent populations of *S. typhle*. We first use geometric morphometrics to quantify intraspecific variation in the snout morphology of *S. typhle* from across the species range, and then study associations between prey availability and dietary composition across ontogeny in populations showing pronounced morphological divergence. Finally, we use a common garden experiment to test whether observed morphological differences among populations reflect short-term ecological responses to locally abundant prey, or fixed genetic differences associated with trophic specialization (e.g., Meyer 1987; Warner 1997; Mittelbach et al. 1999). We predict that the presence of long, low-snouted phenotypes, and high, short-snouted phenotypes in natural populations reflects trophic specialization to feed on small, mobile prey, and large, low motility prey, respectively (e.g., Kendrick and Hyndes 2005), and that the relative prevalence of these phenotypes at the population-level reflects local prey availability, consistent with trophic divergence in a spatially heterogeneous marine environment.

**MATERIALS AND METHODS**

*Specimen Collection*

Species-level variation in the cranial morphology of *S. typhle* was initially quantified using randomly-selected specimens from twelve populations collected from across the species range (Fig. 1). Detailed information on specimen collections is provided in Wilson and Eigenmann Veraguth (2010), which analyzed genetic diversity and population structure across the species range.

Intrapopulation variation in cranial morphology and dietary composition was quantified for 21 juveniles and an equal number of reproductively mature adults (11 females and 10 males)
collected from the Gullmar Fjord, Sweden (KLU), Ria Formosa, Portugal (RIA) and Venice Lagoon, Italy (VEN) (Table 1). Pipefish specimens were collected at depths of 0.5-2m by beach seine or beam trawl during June and August 2001 (RIA) and 2008 (KLU and VEN). Animals were euthanized with an overdose of MS-222 (Tricaine), reproductive status was identified by the assessment of gonad morphology, and the digestive tract was dissected for dietary analysis. RIA animals included a subset of specimens collected as part of a larger study of commercially important fish species in the Ria Formosa (Erzini et al. 2002). A detailed analysis of the dietary composition of these specimens has been published elsewhere (Oliveira et al. 2007).

**Morphological Analyses**

Specimens were digitally photographed in standard orientation with closed gape for morphometric analyses using a Nikon D300 camera with 50mm f/1.8D lens, and total length (TL), head length (HL) and snout length (SnL) were measured to the nearest 0.5 mm using a sizing board. Gape size of each specimen was calculated as the average of the height and width of the open mouth measured with digital calipers, following the method of Kendrick & Hyndes (2005).

Morphological landmarks (LM) were selected to capture the major components of cranial variation (Fig. 2b; Table S1). A total of 13 homologous landmarks and four semi-landmarks were recorded for each specimen using TpsDig v2.25 (Rohlf 2016). Semi-landmarks (LM4-7) demarcated the dorsal snout surface, and were equally spaced between LM2 and LM8. Independent scoring of landmarks in the twelve-population sample by AW and ABW showed high repeatability of data (Procrustes ANOVA: R=0.927; Arnqvist and Martensson 1998), indicating minimal measurement error of cranial landmarks relative to sample variation.
A Generalized Procrustes fit of landmark data controlling for specimen size, position and orientation was performed in R (R Development Core Team 2016) using the geomorph v3.0.3 package (Adams and Otárola-Castillo 2013), restricting semi-landmarks to slide along tangent vectors to the outline curve using the Procrustes distance criterion (Ivan Perez et al. 2006). Canonical variate analyses (CVA) were carried out using the Morpho v2.7 package (Schlager 2016), and 95% confidence ellipses of population samples were generated using Car v3.0-6 (Fox and Weisberg 2011). Warped outlines summarizing morphological variation along major CVA axes were produced using the shape.predictor function in geomorph, using an outline trace of *S. typhle* prepared in TpsDig.

Global and pairwise tests of variation in cranial morphology were conducted for populations and developmental stages, against the null hypothesis of a lack of morphological variation among groups, using Procrustes ANOVAs, as implemented in the advanced.ProcD.lm function of geomorph, using randomized residual permutation (RRPP; n=10,000 permutations; Collyer et al. 2015). Sexual dimorphism in the cranial shape of KLU, RIA and VEN adults was separately tested using the same procedure to test for statistical differences between male and female morphology at each site. All analyses used Sequential (Type I) Sums of Squares. Sequential Bonferroni correction (Rice 1989) was used to correct for multiple testing.

Allometric trajectories of cranial morphology were compared by linear regression of Procrustes regression scores on the natural logarithm of centroid size (Adams et al. 2013; Klingenberg 2016), with population identity included as an additional factor. A global test of slope homogeneity was performed using ProcD.allometry, and pairwise comparisons of slope angles (in degrees) between populations were conducted using the advanced.ProcD.lm function in geomorph to test for allometric differences across populations. Statistical
significance was estimated using RRPP resampling (n=10,000 permutations). Allometric regressions were plotted separately for each population, along with the common allometric component (CAC), which reflects the expected allometric trajectory in the absence of population structure (Mitteroecker et al. 2004).

Dietary Analysis

Prey items were identified using a stereomicroscope (Wild Heerbrugg M3). An identification key has been prepared for prey items recovered in *S. typhle* from VEN and KLU (Wegmann 2009). Prey items were grouped into seven major categories (Amphipods, Copepods, Decapods, Fish, Isopods, Mysids, and Ostracods), following the methodology of Oliveira et al. (2007). Prey that were represented by only a single individual (a decapod postlarva, a polychaete, and a mollusk), and unidentifiable items/detritus were excluded from further analyses. Prey items were digitally photographed, and the length and width of intact prey were recorded. Average prey volume per group and population was estimated from length and width data using the volume proxy equation provided in Steffe et al. (1989). As prey width data were unavailable for the RIA population, average prey size for adult and juvenile pipefish at this site was estimated as the mean of the values for KLU and VEN. Similarly, as the isopod prey of VEN juveniles were not intact, the prey size for this group was taken from the intact prey collected from VEN adults.

The relative representation of prey categories for each group was calculated using both simple abundance and the Index of Relative Importance (IRI), a composite measure incorporating numerical abundance, prey volume and frequency of occurrence (Pinkas et al. 1971). The proportion of juveniles and adults recovered with empty stomachs in each population were compared using a 3-sample test for equality of proportions using the prop.test function in R.
Prey items were ranked by relative mobility using literature data (amphipods: (Kendrick and Hyndes 2005); copepods: Mauchline (1998); decapod shrimp: Arnott et al. (1998); fish: Rollo & Higgs (2008); isopods: Alexander (1988); mysids: Mauchline (1980); ostracods: Davenport (1990)). According to these estimates, amphipods are the slowest of the targeted prey, while copepods, ostracods, mysids, decapod shrimps, isopods and fish are ranked from lowest to highest mobility, respectively.

The relative abundance of zooplankton at each site was derived from survey data from the Gullmar Fjord (KLU), Ria Formosa (RIA), and Venice Lagoon (VEN) (Marques 2006; Riccardi 2010; Tiselius et al. 2015). Plankton surveys sampled nearshore surface waters (0-20m), the typical depth range of S. typhle (Froese and Pauly 2010), using standard 80-200μM mesh nets, recording the taxonomic diversity and numerical composition of nearshore plankton communities at each site. As the methodology of sampling differed somewhat between studies (surface tow for Marques et al. 2006; on-deck pump for Riccardi et al. 2010; vertical tow for Tiselius et al. 2015), the absence of particular prey groups from zooplankton records should not be taken as evidence that these prey are entirely absent from these sites, but rather as an indication that they are numerically rare relative to other prey groups.

Zooplankton community composition across sites was compared against the null hypothesis of no spatial structure using a permutation approach based on Bray-Curtis dissimilarities. Given differences in zooplankton sampling methods across sites, abundance data were first rarefied to a common sample size using rrarefy, and rarefied data were randomized by site, maintaining fixed row and column totals (N=10,000 permutations). Standardised z-scores based on the simulated data were used to test if observed dissimilarities were greater than that expected by chance. All analyses were carried out using vegan v2.5-6 (Oksanen et al. 2016).
Comparisons of zooplankton relative abundance and pipefish dietary composition at each site were carried out using a similar approach, excluding prey items not recovered in the pipefish diet. Data were again rarefied for each pairwise comparison, and adult and juvenile dietary compositions were compared to the local zooplankton community using oecosimu. Separate pairwise tests compared the dietary composition of juveniles and adults among sites to test for dietary differences between developmental stages. Comparisons of pipefish dietary composition were also carried out using % IRI, to correct for differences in prey size and frequency of occurrence across each population.

Two-block partial least squares analyses were used to test for an association between cranial morphology and dietary composition at each site using the two.b.PLS function in Geomorph. Dietary data were square-root transformed and mean-centered prior to analysis, and individuals with empty stomachs were excluded. The correlation between the primary PLS axes of shape and diet was tested by permutation (N=10,000 replicates). Shape outlines were prepared to show shape deformation along the line of best-fit, estimated using major-axis regression for each population. As cranial morphology showed evidence of allometric variation (see below), additional PLS analyses were carried out using adjusted Procrustes coordinate data for each population, combining fitted data from an intercept-only model with ontogeny-centered residuals, removing the effect of development.

**Common Garden Experiment**

To investigate the genetic basis of cranial variation in *S. typhle*, a common garden experiment was carried out using offspring from pregnant males from KLU (N=3) and VEN (N=5). Pregnant males from KLU were wild-caught, while 2 of the 5 VEN males were mated with VEN females after their arrival in the lab. Broods were raised separately under standard culture conditions (salinity 33-34 ppt, temperature 18-19°C and pH ~8.0), approximating summer breeding conditions at the KLU and VEN sites (Rispoli and Wilson 2008), and were
fed an identical diet consisting of fresh *Artemia* spp. nauplii for the first 10 weeks of development (OK Nature Cysts, INVE Aquaculture), and a combination of live *Artemia* and frozen adult *Artemia* spp. (Gamma Slice, Tropical Marine Centre) until the conclusion of the experiment.

Common-garden animals were raised until they reached the size of field-collected juveniles, which occurred after 129 ±10.0 days for the VEN offspring and 139 ± 4.0 days for KLU animals. Animals were anesthetized for photographing in a 100 mg/L solution of Aqui-S (10% eugenol; Aquatactics Inc.). A total of 4-5 juveniles per brood were analyzed for VEN, and 5-8 offspring per brood for KLU, to match the sample size of 21 individuals for the wild-caught samples.

**Qst – Fst Comparison: Inferring Adaptive Evolution**

Comparisons of the geographical partitioning of phenotypic and genotypic variation across populations can be used to test for adaptive evolution in natural populations (Gilbert and Whitlock 2015). We used the breeding design of our common garden experiment to estimate quantitative genetic variation (Qst) along the primary axes of cranial variation from our canonical variate analysis (Figure 3c), and compared the distribution of phenotypic variation to genetic variation of nine neutral microsatellite loci sampled in a previous study of population genetic structure in *S. typhle* (Wilson and Eigenmann Veraguth 2010). Estimates of Qst and Fst were calculated following the methodology of Gilbert and Whitlock (2015) using the QstFstComp v0.2 package in R (Gilbert and Whitlock 2014). Note that the unique reproductive system of syngnathid fishes creates the opportunity for both maternal and paternal effects that may influence estimates of genetic variation in quantitative traits (Sagebakken et al. 2017). *S. typhle* is polygynous in natural populations (Rispoli and Wilson 2008), and we provide Qst estimates based on half sib (*r*=0.25) and full sib (*r*=0.50) mating designs for comparative purposes. Confidence intervals of both Qst and Fst were estimated by parametric bootstrapping (N=10,000 permutations) under the null hypothesis of neutrality of phenotypic and
genetic variation. A comparison of the relative magnitude of Qst and Fst estimates (permutation test; N=10,000 permutations) provided a statistical test of adaptive evolution (i.e., Qst>Fst).

RESULTS

Intraspecific Variation in Cranial Morphology

Two individuals from each of 12 populations were analyzed in order to visualize species-level variation in the cranial morphology of *S. typhle* (Fig. 2a). Sampled animals ranged from 10.80-27.70 cm TL and differed significantly in relative snout length (SnL:HL range: 0.588-0.660; Table 1), exceeding the level of intraspecific variation observed in a previous study of twelve syngnathid species by Kendrick & Hyndes (2005). Similarly, gape sizes of individuals included in the species sample (range: 1.43-3.75 mm) exceeded that documented by Kendrick & Hyndes (2005), and were positively correlated with body length (*F*$_{1,11}$=8.82; *p*=0.013) after controlling for source population.

Canonical variates 1 (CV1) and 2 (CV2) of Procrustes-transformed landmark data explain more than 80% of standardized between-group variance in Procrustes coordinate data (Fig. 3a), and partition morphological variation in snout length (CV1: 52.6% of standardized between-group variance) and gape angle (CV2: 28.4%). Populations are clearly discriminated in trophic morphospace, and show strong geographic affinities, with northern populations (ASK, KLU) displaying an intermediate phenotype including a moderate snout length and a slightly angled gape, Atlantic coast populations (GRO, PED, RIA) with the exception of ROS showing a high and short snout with angled gape, and eastern populations (ERD, ODE, TIL, VEN) exhibiting a hyperextended snout and strongly angled gape, almost perpendicular.
to the anterior-posterior axis (Fig. 2a). LIV, MUR and ROS display the highest snouts and are distinct in having a protracted mandible, producing a distinctive blunt-ended snout (Fig. 2a).

**Interpopulation Analysis of Adults and Juveniles**

Three populations were selected for the comparative analysis of cranial morphology and dietary composition, representing the high (RIA), hyperextended (VEN), and intermediate snout (KLU) phenotypes (Fig. 2). Morphological variation in the population sample mirrors that observed in the intraspecific analysis, revealing significant differences in SnL:HL ratios among populations ($F_{2,120}=35.3; p<0.001$) and across developmental stages ($F_{1,120}=31.1; p<0.001$), with a significant population * ontogeny interaction ($F_{2,120}=7.2; p=0.001$). Among adults, VEN specimens have the longest snouts (SnL:HL ± SD: 0.639 ± 0.01), while KLU and RIA have similar SnL:HL ratios (KLU: 0.621 ± 0.02; RIA: 0.628 ± 0.01) (Table 1). Size-standardized gape (Gape size:HL) is greatest in RIA adults (0.089 ± 0.01mm), intermediate in KLU animals (0.080 ± 0.01mm) and smallest in the VEN population (0.062 ± 0.01mm), consistent with a trade-off between snout length and gape size in this species.

These morphological differences dominate geometric morphometric analyses, with RIA pipefish showing a high and angled snout, KLU animals exhibiting an intermediate phenotype, and VEN individuals showing a low and hyperextended snout (Fig. 3b). CV1 explains 62.0% of the standardized between-group variance, and shape changes along this axis parallel those found along CV1 in the broader species-level analysis (Fig. 3a). Variation in gape angle and snout height again dominate CV2 (28.7% of standardized between-group variance), ranging from the concave snout and slightly angled gape of the KLU population to the flat snout and moderately angled gape displayed by RIA pipefish (Fig. 2a).
Comparisons of adult males and females across sampling sites failed to detect a significant signal of sexual dimorphism in cranial morphology (Population: $F_{2,62}=39.34$, $Z=15.00$, $p<0.0001$, Sex: $F_{1,62}=0.92$, $Z=0.87$, $p=0.46$, Population * Sex: $F_{2,62}=0.97$, $Z=0.97$, $p=0.42$). Subsequent analyses were conducted on the combined adult sample for each site.

Tests of shape differences across the interpopulation sample revealed significant differences across ontogeny and among populations, and the pattern of ontogenetic change differed across populations, as evidenced by a significant population * ontogeny interaction (Population: $F_{2,125}=67.29$, $Z=25.72$, $p<0.0001$, Ontogeny: $F_{1,125}=12.79$, $Z=9.92$, $p<0.0001$, Population * Ontogeny: $F_{2,125}=4.77$, $Z=4.37$, $p<0.0001$). Pairwise comparisons using Procrustes distances indicated highly significant differentiation between all populations ($p<0.0002$ for all comparisons), but within-population differentiation between juveniles and adults was only significant for the RIA population after controlling for multiple testing (sequential Bonferroni correction: $p<0.0001$; Fig. 3b).

An analysis of shape allometry revealed only modest differences in allometric trajectories across populations (Fig. 4; Homogeneity of slopes test: $F_{2,125}=2.26$, $Z=2.03$, $p=0.02$), indicating that morphological differences among populations are already present in juvenile animals. While KLU and RIA exhibited significantly different allometric slopes ($p=0.006$), all other pairwise comparisons were not significant.
Dietary Composition

A total of 1,649 prey items were identified in 56 juvenile pipefish from across the three sites, the bulk of which were calanoid copepods (1,233/1,649: 74.8%). The proportion of individuals with empty stomachs differed significantly among sites ($\chi^2=6.11$, df=2, p=0.047), and 5 of 21 RIA juveniles (23.8%) carried no identifiable stomach contents (Table 2).

Adult diets at all three sites were dominated by larger prey, with a total of 388 prey items recovered in the adult sample (n=46 individuals) (Table 2). Similar to the pattern found in juvenile pipefish, RIA had the highest proportion of adults with empty stomachs (11/21: 52.4%), while 20 of 21 VEN adults (95.2%) carried at least one identifiable prey item. The proportion of individuals with empty stomachs differed significantly across populations ($\chi^2=12.25$, df=2, p=0.002).

The composition of local zooplankton communities differed significantly across the three survey sites (Fig. 5; average Bray-Curtis dissimilarity: 0.20, z-score: 41.3, p<0.0001), but communities at all three locations were dominated by copepods (54.5-82.2%), along with a diversity of groups not included in the pipefish diet (e.g., Mollusca, Cladocera, Tunicata, Ctenophora, Annelida, Cirripedia), comprising up to 43% of available zooplankton at each site (Fig. 5).

Despite qualitative similarities in zooplankton composition across sites, animals exhibited distinctive dietary compositions in each population, and clear ontogenetic shifts in dietary composition with growth (Fig. 5). Both juvenile and adult diets were significantly different from the dominant zooplankton found at each site (pairwise Bray-Curtis dissimilarity: 0.058-1.000, z-scores: 5.26-39.80, p<0.001 for all comparisons). The diet of KLU juveniles most closely resembled the local zooplankton community (Fig. 5; Bray-Curtis dissimilarity: 0.058), but remained significantly different even after correction for multiple testing.
Juvenile pipefish from KLU and RIA fed predominantly on copepods (94.2% and 87.8% of total diet by abundance, respectively), while the diet of VEN juveniles was dominated by amphipods (57.8%), along with a large (i.e. >10%) proportion of mysids (23.2%) and copepods (15.8%) (Fig. 5). Dietary composition shifted towards larger items in adults, and adult populations from the three sites were highly distinctive (pairwise Bray-Curtis dissimilarities: 0.643-0.786, z-scores: 3.49-10.98, p<0.002 for all comparisons). Copepods made up 47.2% of the diet of KLU adults in terms of numerical abundance, but the remainder of the diet for this population was dominated by fish (26.4%) and decapods (12.5%), the two largest prey categories (Table 2). The diet of RIA adults shifted dramatically toward larger prey, with decapods and fish comprising 57.1% and 35.7% of total diet, respectively. Most notably, low-snouted adult pipefish from VEN had a diet dominated by amphipods (57.9%), with smaller contributions from mysids (13.6%) and isopods (10.3%), and was qualitatively similar to the juvenile diet at this site (Bray-Curtis dissimilarity: 0.212). Nonetheless, adult diets differed significantly from juveniles at every site (pairwise Bray-Curtis dissimilarities: 0.212-1.000, z-scores: 6.90-8.55, p<0.0001 for all comparisons).

While copepods dominated the diets of KLU and RIA juveniles, the dietary value of this group is likely to be limited given their small size. Dietary composition was therefore also quantified using the Index of Relative Importance, which takes into account prey abundance, frequency of occurrence, and size (Pinkas et al. 1971). Relative prey composition shifted towards larger prey categories after the incorporation of prey size and frequency of occurrence (Fig. S1), but all developmental stages and populations remained highly distinct (Bray Curtis distances: 0.193-0.976, z-scores: 13.34-72.08, p<0.0001 for each pairwise comparison).
Intrapopulation variation in resource use is widespread, and may play an important role in niche partitioning and the early stages of adaptive speciation (Bolnick et al. 2003; Araújo et al. 2011). Two-block partial least squares analyses were used to test for an association between cranial morphology and dietary composition at each site (Fig. 6). While all three plots showed significant scatter (r=0.535-0.657, p>0.1058 for all comparisons), they revealed clear separation in dietary composition with cranial shape (Fig. 6). Small prey (copepods, ostracods, amphipods and mysids) exhibited negative loadings on PLS1 in both KLU and RIA, while large prey (decapods and fish) had positive loadings, indicating that individuals feeding on larger prey items at these sites have a distinctive trophic morphology. The pattern for the VEN population was distinct, with copepods, isopods and decapods showing negative loadings on PLS1, while amphipods, mysids and fish loaded positively on the PLS1 axis. Juveniles and adults were clearly separated along PLS1 in the RIA population (consistent with the significant separation of these two groups in shape space; Fig. 3b), and two-block PLS indicated a highly significant association between cranial morphology and diet in this population after controlling for ontogeny (r=0.843, p=0.001). Developmental stages overlapped in KLU and VEN, indicating that associations between cranial morphology and diet in these populations do not simply reflect underlying ontogenetic change.

Morphological Comparison of Wild-Caught and Laboratory-Reared S. typhle

Laboratory-reared juveniles from KLU and VEN juveniles raised on a common Artemia diet remain morphologically distinct (Fig. 3c), indicating that differences in cranial morphology among populations have an underlying genetic basis. Lab animals overlap with their population of origin in

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relative snout height (CV1), but are clearly separated along CV2, which explains 41% of standardized between-group variance in Procrustes coordinate scores in this comparison (Fig. 3c), and partitions morphological variation in gape angle. Both laboratory-reared populations have distinctive blunt-ended snouts (compare with LIV, MUR and ROS populations in Fig. 3a), but are clearly distinguishable by snout height, with the KLU laboratory population retaining a high, flat snout, and the VEN population showing the hyperextended low snout characteristic of the wild population. Lab and field populations were statistically different for all interpopulation comparisons (NPMANOVA, 10,000 permutations, p<0.002- for all comparisons after sequential Bonferroni correction).

While Qst estimates of cranial divergence in common garden animals are high along CV1 (Fig. 3c; Table 3), confidence limits are broad, and a statistical comparison of phenotypic and genetic variance between KLU and VEN failed to reject the null hypothesis of neutral evolution (P>0.075 for both half- and full-sib analyses; Table 3). We discuss the potential limitations of this analysis below.

DISCUSSION

Ecological specialization has played a central role in the majority of adaptive radiations (Streelman and Danley 2003), but is thought to be rare in marine species, given the limited geographic barriers to gene flow (Sotka 2012). While empirical examples of local adaptation in marine systems are limited, phylogenetic reconstructions of some of the most species-rich groups of marine fishes suggest that ecological specialization has been key to their diversification (Wainwright et al. 2004; Rocha et al. 2005), a phenomenon that has been dubbed the “marine speciation paradox“ (e.g., Bierne et al. 2003): How can local adaptation and specialization evolve in the face of high levels of gene flow?

Our data suggest that the extensive variation in snout morphology found in S. typhle is the product of dietary specialization at the population level. Similar to the classical example of resource partitioning in Darwin’s finches (Geospiza fortis), in which beak size reflects the size and hardness of seeds
consumed (Grant et al. 1976; Price 1987; De León et al. 2012), local populations of *S. typhle* have specialized to feed on distinctive subsets of available zooplankton, and juvenile and adult morphotypes reflect the dominant prey categories consumed at each site. Pipefish populations exhibit distinctive prey compositions despite the fact that zooplankton communities at all three localities are qualitatively similar, indicating that dietary composition does not simply reflect relative zooplankton abundance, but is consistent with selective foraging on the most energetically valuable prey available for a given gape size (e.g., Stephens and Krebs 1986). Morphological differences found in natural populations are maintained under common garden conditions, indicating that cranial morphology in *S. typhle* is a heritable trait subject to spatially variable selection.

Based on de Lussanet and Muller’s (2007) analysis of the functional morphology of feeding in *Syngnathus*, we predicted that the diets of high-snouted KLU and RIA individuals would be dominated by large prey items, while gape-limited VEN pipefish would feed on smaller prey. Consistent with this hypothesis, adult pipefish from RIA fed predominantly on the two largest prey categories (decapods and fish), and while copepods comprised a significant component of the KLU diet based on numerical abundance (Fig. 5), they contributed a negligible proportion of the total diet after controlling for prey size and frequency of occurrence, which indicated that fish and decapods dominated the diet of KLU adults (Fig. S1). The diet of VEN adults, in contrast, was dominated by amphipods, one of the smallest prey categories. Juvenile diets in all populations were dominated by the smallest prey items (copepods for KLU and RIA; amphipods for VEN), consistent with the important role of gape size in determining dietary composition in this species.

Although snout height was positively correlated with the dominant prey size consumed at each site, snout length did not strongly predict prey mobility, as expected based on the model of trophic
optimization proposed by de Lussanet & Muller (2007), and analyses of interspecific variation in
syngnathid fishes (Kendrick and Hyndes 2005). Short-snouted KLU and RIA adults consumed some
of the most mobile prey items (decapods and fish), while the diet of long-snouted VEN pipefish was
dominated by low mobility amphipods. This observation does not appear to be a sampling artifact, as
a longitudinal study of the dietary composition of VEN pipefish found that amphipods dominate the
diets of S. typhle throughout the summer sampling period (Franzo et al. 2004).

While the interspecific analysis of Kendrick & Hyndes (2005) revealed a strong positive association
between snout length and prey mobility in syngnathids, a closer investigation of the underlying data
suggests a possible explanation for the lack of association between these variables in S. typhle. S.
typhle has the longest relative snout length of the syngnathids examined by Kendrick and Hyndes
(2005), and its minimum snout length:head length ratio exceeds that of eight of the fourteen species
included in their analysis, suggesting that even short-snouted S. typhle may retain the ability to
capture mobile prey.

The results of our common garden experiment indicate that observed differences in snout morphology
among populations have a significant genetic component. Laboratory populations of pipefish raised
on a common Artemia diet remained morphologically distinct, and showed a cranial morphology
consistent with that observed in wild-caught animals (Fig. 3c). At the same time, cranial morphology
is also influenced by environmental conditions, as laboratory-specimens can be distinguished from
their wild counterparts in having a distinctive gape angle and dorsal snout surface (CV2 in Fig. 3c),
suggesting the potential for short-term responses to local conditions via phenotypic plasticity. While
the rearing temperature of our common garden experiment was selected to simulate average summer
breeding temperatures and developmental rates of KLU and VEN were approximately equal, skeletal
development is highly temperature-dependent in fish (Hubbs 1922), and it would be interesting to
explore the extent to which changes in ambient temperature influence cranial variation.
While quantitative genetic variation in cranial morphology is pronounced between KLU and VEN, neutral genetic divergence between these populations is high (Fst=0.325), limiting our ability to infer adaptive evolution in the sample based on comparisons of Qst and Fst. As genetic divergence between many pipefish populations showing pronounced morphological divergence (Fig. 3) is modest (Fst range: 0.004-0.458; Wilson and Eigenmann Veraguth 2010), quantifying the genetic component of phenotypic divergence between more closely related populations could offer an opportunity to explore adaptive evolution at an earlier stage in the divergence process.

The analysis of quantitative genetic variation based on our common garden experiment should also be considered preliminary, given the modest size of the experiment and unique aspects of the reproductive biology of syngnathid fishes, where both maternal and paternal effects can influence offspring phenotype (Sagebakken et al. 2017). As parental effects can both inflate and depress estimates of Qst, an ideal experiment would be based on a half-sib breeding design investigating variation in the F2 generation (Gilbert and Whitlock 2015). An expanded common garden experiment involving the analysis of several hundred F2 offspring from multiple half-sib families in each of several populations (e.g., MUR, VEN and RIA; Fig. 3a) would offer a robust test of adaptive evolution in this system, and would ideally be coupled by experimental comparisons of feeding performance in pipefish differing in trophic morphology.

Given the lack of evidence of a functional tradeoff between snout height and length in S. typhle, what factors could be responsible for the distinctive hyperextended snouts found in Venice pipefish? An answer may be found through the analysis of interspecific competition, which can influence resource availability, driving dietary specialization and trophic divergence (Connell 1983; Schluter 1994; Belmaker et al. 2012). Fish community diversity is significantly higher at southern sites, and Venice S. typhle occur together with four congeners (Pihl et al. 2006; Ribeiro et al. 2008; Franzoi et al. 2010), each of which exhibits differences
in foraging behavior and trophic morphology associated with resource specialization (Franzoi et al. 1993, 2004; Teixeira and Musick 1995). The hyperextended snout of S. typhle at these sites is an outstanding feature of the species (Hablützel and Wilson 2011), and may allow individuals to access novel resources in the face of strong competition. Experimental tests of feeding performance in S. typhle and its congeners, coupled with comparative analyses of dietary preferences, would provide a test of this hypothesis.

CONCLUSIONS

Our data indicate that geographically widespread marine species such as S. typhle may exhibit resource specialization at the population level despite a lack of geographic barriers to gene flow, generating the potential for ecological divergence and local adaptation. While a comparison of phenotypic and genetic divergence between common garden populations failed to reject the null hypothesis of neutral evolution, the genetic contribution to morphological variation was high, suggesting that cranial features associated with feeding performance could be subject to spatially-variable selection pressures. We discovered functional associations between morphological variation and dietary composition both within and across populations, an observation that parallels that found in classical models of resource specialization (Lavin and McPhail 1985; Price 1987). Extending functional studies such as this to a variety of temperate and tropical systems will help to clarify the extent to which ecological specialization has influenced broader patterns of marine diversity.
LITERATURE CITED


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Wegmann, A. 2009. Geographic variation in the trophic morphology of a syngnathid pipefish. University of Zurich, Zurich, Switzerland.


**Figure Legends:**

**Fig. 1.** Geographic distribution of sampled populations of *S. typhle*, highlighting the three focal localities (KLU, RIA, VEN).
Fig. 2. (a) Intraspecific variation of cranial morphology across populations of *S. typhle* (scale bar: 1cm) (see Fig. 1 for population locations); (b) 13 homologous landmarks and 4 semi-landmarks (LM4-7) were used to quantify head morphology using geometric morphometrics (skeletal diagram modified after Rauther (1925)).
Fig. 3. Canonical variate analysis showing major axes of variation in trophic morphology across (a) *S. typhle* species range (see Fig.1 for population locations); (b) adults and juveniles in three focal populations; and (c) wild-caught animals and captive-bred individuals raised in a common environment. 95% confidence ellipses delineate intrasample variation.
Fig. 4. Allometric trajectories of juvenile and adult populations from KLU (blue), RIA (green) and VEN (red), along with the common allometric component, showing the predicted allometric relationship in the absence of population structure (black). Shape outlines show major changes in cranial morphology estimated from CAC regression (Mitteroecker et al. 2004).
Fig. 5. Relative representation of free-living zooplankton communities and dietary composition of adults and juveniles at each of the three study sites.
Fig. 6. Primary axes of a two-block partial least squares analysis of pipefish shape and dietary composition for each site. Dashed line indicates the line of best fit between PLS1 axes for shape and diet. Prey items positioned along dietary axis according to relative loadings on PLS1. Juveniles (light points) and adults (dark points) separately shaded for clarity.
Table 1. Descriptive statistics for sampled populations

<table>
<thead>
<tr>
<th>Pop</th>
<th>Maturity</th>
<th>#</th>
<th>Total Length ± SD (cm)</th>
<th>Head Length ± SD (mm)</th>
<th>Snout Length ± SD (mm)</th>
<th>SnoutLength: Head Length</th>
<th>Gape Size ± SD (mm)</th>
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<tr>
<td>ASK</td>
<td>Adu 2</td>
<td>16.90 ± 3.25</td>
<td>29.78 ± 7.21</td>
<td>18.43 ± 4.48</td>
<td>0.619 ± 0.00</td>
<td>2.35 ± 0.64</td>
<td></td>
</tr>
<tr>
<td>KLU</td>
<td>Adu 2</td>
<td>16.10 ± 3.39</td>
<td>28.35 ± 4.91</td>
<td>17.48 ± 2.88</td>
<td>0.617 ± 0.01</td>
<td>2.50 ± 0.78</td>
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<td>ROS</td>
<td>Adu 2</td>
<td>25.20 ± 0.42</td>
<td>42.61 ± 0.63</td>
<td>27.00 ± 0.28</td>
<td>0.633 ± 0.00</td>
<td>3.53 ± 0.32</td>
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<td>PED</td>
<td>Adu 2</td>
<td>17.05 ± 3.75</td>
<td>28.59 ± 7.45</td>
<td>17.43 ± 5.26</td>
<td>0.606 ± 0.03</td>
<td>2.23 ± 0.25</td>
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<tr>
<td>GRO</td>
<td>Juv 2</td>
<td>11.20 ± 0.57</td>
<td>20.89 ± 1.03</td>
<td>12.44 ± 0.62</td>
<td>0.595 ± 0.00</td>
<td>1.93 ± 0.04</td>
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<td>RIA</td>
<td>Adu 2</td>
<td>18.60 ± 0.42</td>
<td>31.88 ± 0.06</td>
<td>19.81 ± 0.49</td>
<td>0.621 ± 0.01</td>
<td>3.01 ± 0.23</td>
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<tr>
<td>MUR</td>
<td>Adu 2</td>
<td>24.70 ± 0.99</td>
<td>42.70 ± 0.25</td>
<td>27.26 ± 1.20</td>
<td>0.638 ± 0.02</td>
<td>3.10 ± 0.18</td>
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<tr>
<td>LIV</td>
<td>Adu 2</td>
<td>25.85 ± 2.62</td>
<td>46.84 ± 1.34</td>
<td>30.17 ± 0.91</td>
<td>0.644 ± 0.00</td>
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<td>VEN</td>
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<td>13.95 ± 1.63</td>
<td>26.97 ± 3.15</td>
<td>17.51 ± 2.49</td>
<td>0.648 ± 0.02</td>
<td>1.57 ± 0.19</td>
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<td>ERD</td>
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<td>41.01 ± 0.87</td>
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<td>ODE</td>
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<td>21.80 ± 0.57</td>
<td>41.15 ± 2.54</td>
<td>25.86 ± 1.50</td>
<td>0.629 ± 0.00</td>
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<td>TIL</td>
<td>Adu 2</td>
<td>18.25 ± 1.20</td>
<td>32.99 ± 0.83</td>
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<td>0.638 ± 0.02</td>
<td>2.64 ± 0.41</td>
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<td><strong>Range (min–max)</strong>*</td>
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<td>24</td>
<td>10.80–27.70</td>
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<td>12.00–30.81</td>
<td>0.588–0.660</td>
<td>1.43–3.75</td>
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<table>
<thead>
<tr>
<th>Pop</th>
<th>Maturity</th>
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<th>Total Length ± SD (cm)</th>
<th>Head Length ± SD (mm)</th>
<th>Snout Length ± SD (mm)</th>
<th>SnoutLength: Head Length</th>
<th>Gape Size ± SD (mm)</th>
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<td><strong>Wild-caught specimens</strong></td>
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<td>KLU</td>
<td>Juv 21</td>
<td>13.05 ± 1.14</td>
<td>22.91 ± 2.05</td>
<td>14.05 ± 1.43</td>
<td>0.613 ± 0.01</td>
<td>1.54 ± 0.12</td>
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<tr>
<td>KLU</td>
<td>Adu 21</td>
<td>18.20 ± 3.15</td>
<td>31.58 ± 5.39</td>
<td>19.63 ± 3.58</td>
<td>0.621 ± 0.02</td>
<td>2.56 ± 0.71</td>
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<td>RIA</td>
<td>Juv 21</td>
<td>11.33 ± 2.03</td>
<td>20.12 ± 3.84</td>
<td>12.19 ± 2.53</td>
<td>0.604 ± 0.02</td>
<td>1.37 ± 0.32</td>
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</tr>
<tr>
<td>RIA</td>
<td>Adu 21</td>
<td>20.08 ± 3.32</td>
<td>34.64 ± 5.12</td>
<td>21.78 ± 3.34</td>
<td>0.628 ± 0.01</td>
<td>3.10 ± 0.65</td>
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<td>VEN</td>
<td>Juv 21</td>
<td>10.73 ± 1.41</td>
<td>20.43 ± 2.50</td>
<td>12.96 ± 1.67</td>
<td>0.634 ± 0.01</td>
<td>1.15 ± 0.18</td>
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<td>VEN</td>
<td>Adu 21</td>
<td>14.21 ± 3.11</td>
<td>26.42 ± 4.73</td>
<td>16.88 ± 3.06</td>
<td>0.639 ± 0.01</td>
<td>1.64 ± 0.38</td>
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<td><strong>Common-garden experiment</strong></td>
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<tr>
<td>KLU</td>
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<td>11.25 ± 1.66</td>
<td>20.74 ± 2.95</td>
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<td>11.99 ± 1.78</td>
<td>23.92 ± 3.36</td>
<td>15.27 ± 2.63</td>
<td>0.636 ± 0.03</td>
<td>n.a.</td>
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</tr>
</tbody>
</table>

Table 2: Stomach contents of juvenile and adult *S. typhle* listed by prey group, along with average prey size (length (L) x width (W) in mm). Prey sizes for RIA estimated as mean of VEN and KLU measures. Isopod length and width for VEN juveniles based on measured size for adults.
Table 3: Comparison of genetic (\textit{Fst}) and phenotypic (\textit{Qst}) variation between KLU and VEN. \textit{Fst} estimates derived from the analysis of nine microsatellite loci (Wilson and Eigenmann Vergath 2010), and \textit{Qst} estimates inferred from patterns of cranial variation in captive-reared animals measured along the two primary axes of the canonical variate analysis (CV1 and CV2; Fig. 3b). \textit{Qst} estimates calculated for half sib- and full sib-mating designs provided for comparative purposes. 95\% confidence limits of parameter estimates estimated by parametric bootstrapping (N=10,000 permutations).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate (95% CI)</th>
<th>\textit{Qst-Fst}</th>
<th>P-value (\textit{Qst} &gt; \textit{Fst})</th>
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</thead>
<tbody>
<tr>
<td><strong>Half Sibs (r=0.25)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Fst}</td>
<td>0.325 (0.188, 0.431)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Qst-CV1}</td>
<td>0.848 (-0.046, 1.089)</td>
<td>0.523 (-0.495, 0.958)</td>
<td>0.093</td>
</tr>
<tr>
<td>\textit{CV2}</td>
<td>0.074 (-0.651, 0.660)</td>
<td>-0.252 (-2.303, 1.911)</td>
<td>0.599</td>
</tr>
<tr>
<td><strong>Full Sibs (r=0.50)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Fst}</td>
<td>0.325 (0.188, 0.431)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textit{Qst-CV1}</td>
<td>0.918 (-0.090, 1.055)</td>
<td>0.593 (-0.593, 0.970)</td>
<td>0.075</td>
</tr>
<tr>
<td>\textit{CV2}</td>
<td>0.137 (-1.255, 1.253)</td>
<td>-0.188 (-2.345, 2.240)</td>
<td>0.499</td>
</tr>
</tbody>
</table>

Table S1. Description of cranial landmarks:

<table>
<thead>
<tr>
<th>Landmark ID</th>
<th>Landmark/Semi-landmark</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM1(^2)</td>
<td>Landmark(^1)</td>
<td>Anterior point of the upper snout (premaxilla)(^2)</td>
</tr>
<tr>
<td>LM2(^2)</td>
<td>Landmark(^1)</td>
<td>Highest point of the anterior part of the upper snout at the premaxilla(^2)</td>
</tr>
<tr>
<td>LM3(^2)</td>
<td>Landmark(^1)</td>
<td>Most posterior point of the maxilla, at maximal curvature(^2)</td>
</tr>
<tr>
<td>LM4(^2)</td>
<td>Semi-landmark(^1)</td>
<td>Semilandmarks spaced equally along dorsal snout between LM2 and LM8(^2)</td>
</tr>
<tr>
<td>LM5(^2)</td>
<td>Semi-landmark(^1)</td>
<td>Semilandmarks spaced equally along dorsal snout between LM2 and LM8(^2)</td>
</tr>
<tr>
<td>LM6(^2)</td>
<td>Semi-landmark(^1)</td>
<td>Semilandmarks spaced equally along dorsal snout between LM2 and LM8(^2)</td>
</tr>
<tr>
<td>LM7(^2)</td>
<td>Semi-landmark(^1)</td>
<td>Semilandmarks spaced equally along dorsal snout between LM2 and LM8(^2)</td>
</tr>
<tr>
<td>LM8(^2)</td>
<td>Landmark(^1)</td>
<td>Midpoint between the nostrils, at the dorsal surface, where the mesethmoidal bone meets the frontal bone(^2)</td>
</tr>
<tr>
<td>LM9(^2)</td>
<td>Landmark(^1)</td>
<td>Anterior end of anterior nostril(^2)</td>
</tr>
<tr>
<td>LM10(^2)</td>
<td>Landmark(^1)</td>
<td>Posterior ventral corner, at maximum curvature, of the lateral ethmoidal bone(^2)</td>
</tr>
<tr>
<td>LM11(^2)</td>
<td>Landmark(^1)</td>
<td>Center of the eye (triangulation of three landmarks around orbit of eye)(^2)</td>
</tr>
<tr>
<td>LM12(^2)</td>
<td>Landmark(^1)</td>
<td>Dorsal surface of the head at the midpoint of the eye (LM11)(^2)</td>
</tr>
<tr>
<td>LM13(^2)</td>
<td>Landmark(^1)</td>
<td>Anterior point of the operculum(^2)</td>
</tr>
<tr>
<td>LM14(^2)</td>
<td>Landmark(^1)</td>
<td>Ventral posterior edge of the preopercular bone where it intersects the operculum(^2)</td>
</tr>
<tr>
<td>LM15(^2)</td>
<td>Landmark(^1)</td>
<td>Ventral surface at the height of the anterior nostril (LM9)(^2)</td>
</tr>
<tr>
<td>LM16(^2)</td>
<td>Landmark(^1)</td>
<td>The most ventral point at the intersection of the mandible and quadrate bone(^2)</td>
</tr>
<tr>
<td>LM17(^2)</td>
<td>Landmark(^1)</td>
<td>Anterior point of the lower snout (mandible)(^2)</td>
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