Adaptive morphological shifts to novel habitats in marine sculpin fishes

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Abstract

Sculpin fishes of the North American Pacific Coast provide an ideal opportunity to examine whether adaptive morphological character shifts have facilitated occupation of novel habitat types because of their well-described phylogeny and ecology. In this group, the basal-rooted species primarily occupy the subtidal habitat, whereas the species in the most distal clades are found in the intertidal. We tested multiple evolutionary models to determine whether changes in body size and changes in number of scales are adaptive for habitat use in sculpins. Based on a statistically robust, highly resolved molecular phylogeny of 26 species of sculpins, in combination with morphometric and habitat affinity data, our analyses show that an adaptive model based on habitat use best explains changes in body size and number of scales. The habitat model was statistically supported over models of neutral evolution, stabilizing selection across all habitats, and three clade-based models. We suggest that loss of scales and reduction of body size in the intertidal may facilitate cutaneous breathing in air when tidepools become hypoxic during low tides. This study demonstrates how the combined use of phylogenetic, ecological and statistical approaches helps to identify traits that are likely adaptive to novel habitats.

Introduction

Studies of adaptation, especially with respect to habitat use, have been a cornerstone of evolutionary biology as they provide insights into the relationship between form, function and speciation. Although adaptation within highly diverse clades has been well studied in terrestrial (e.g. Caribbean *Anolis* lizards, Galapagos *Geospiza* finches) and freshwater systems (e.g. post-glacial lake *Gasterosteus* sticklebacks, African rift-lake Cichlidae, Lake Baikal Cottoidei sculpins), marine systems have been less well studied, resulting in a strong publication bias towards terrestrial and freshwater examples (Schluter, 2000; Losos & Mahler, 2010; Glor, 2011; Jones *et al.*, 2012). In addition, few studies within marine systems have explicitly addressed the question of whether the phenotype–environment relationship is due to adaptation or alternative evolutionary processes,

hindering our ability to assess the generality of the drivers that both promote and maintain diversity across terrestrial, freshwater and marine systems.

The intertidal environment provides an excellent opportunity in which to study adaptation in a marine setting, as it is influenced by the daily ebb and flow of tides resulting in dramatic fluctuations in environmental factors such as temperature and dissolved oxygen levels (Truchot & Duhamel-Jouve, 1980; Burggren & Roberts, 1991; Richards, 2011). Conversely, the adjacent subtidal environment experiences little fluctuation in these environmental factors (Stillman & Somero, 1996; Mandic *et al.*, 2009a). This environmental gradient with depth appears to affect species distributions, with more temperature and hypoxia-tolerant species located in the intertidal and less tolerant species found in subtidal environments (e.g. Stillman & Somero, 1996, 2000; Richards, 2011).

Daily extreme hypoxia during low tides can potentially drive strong natural selection for hypoxia tolerance (Martin & Bridges, 1999; Richards, 2011). Consequently, cutaneous breathing has evolved in a

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large number of intertidal fishes (including, but not limited to sculpins) to supplement oxygen intake by gills (Yoshiyama & Cech, 1994; Martin & Bridges, 1999; Nelson, 2006). Many marine intertidal fishes have the ability to respire in air (via lungs, gills, buccal cavity or cutaneous respiration) with the consumption of oxygen at the same rate in air as in water (Bridges, 1988) and release of carbon dioxide to air at the same rate as it is formed by metabolism (Martin, 1993). Marine air-breathing fishes generally have no unique organs solely for air-breathing, and instead rely on the same surfaces for respiration in air as they do in water (primarily the gills and the skin; Martin & Bridges, 1999). Since gills typically collapse out of water, reducing the surface area available for respiration (Helfman *et al.*, 2009), cutaneous respiration is often the primary alternative respiratory surface for air-breathing fishes (Martin & Bridges, 1999).

Two morphological factors that are likely important for cutaneous respiration are scale number and body size. Scales, where present on the body, prohibit cutaneous breathing by inhibiting the diffusion of oxygen across the skin membrane (Martin & Bridges, 1999). Further, repeated, phylogenetically independent loss of scales (Nelson, 2006) suggests selection operated on this trait in variable and low oxygen environments (Martin & Bridges, 1999). Body size may also be an important factor under selection to optimize cutaneous respiration. The surface area to volume ratio is important for cutaneous respiration, as a high surface area to volume ratio is beneficial for efficient oxygen diffusion (Graham *et al.*, 1987). The volume of an organism is tightly coupled to its metabolic demand, but in skin breathing organisms, respiration is limited by surface area (Graham *et al.*, 1987). Thus, a small body size would lead to more efficient cutaneous respiration, whereas a large body size would be detrimental. However, in subtidal and transitional habitats, oxygen is rarely limiting (Helfman *et al.*, 2009) and predatory pressure is generally stronger than it is in the intertidal (Helfman *et al.*, 2009; Love, 2011), so it may be advantageous to be larger and have greater scale covering in these habitats.

Sculpins (Teleostei: Cottidae) of the North American Pacific Coast provide an ideal opportunity to examine whether morphological trait shifts are associated with habitat use because basal-rooted species live primarily in the subtidal habitat, whereas species in the most distal clades are found in the intertidal where they have diversified extensively (see Figs 1 and 2; and Ramon & Knope, 2008; Mandic *et al.*, 2009a; Richards, 2011; Knope, 2013). In addition, Martin (1996) found evidence that intertidal sculpins have tidepool emergence behaviours and the ability to perform cutaneous respiration for surviving hypoxia by air-breathing out of water that similar sculpin species from deeper water lacked, suggesting that phenotypes beneficial to the intertidal

zone are due to adaptation instead of exaptation. Sculpins of the North American Pacific Coast are generally restricted to the narrow continental shelf and near-shore environment between Baja California and the Aleutian Islands, Alaska (Miller & Lea, 1972; Howe & Richardson, 1978; Knope, 2013). In particular, we chose to examine the putative subfamily Oligocottinae (Bolin, 1947; Knope, 2004; Ramon & Knope, 2008), a monophyletic clade comprised of 16 species, and ten representative species from closely related clades, with habitat affinities ranging from the subtidal to the intertidal.

Here, we utilize data from previously published molecular phylogenies (Ramon & Knope, 2008; Mandic *et al.*, 2009a), morphometric data (this study; Bolin, 1944) and habitat affinity data (Bolin, 1944; Miller & Lea, 1972; Mecklenburg *et al.* 2002; Ramon & Knope, 2008; Mandic *et al.*, 2009a) to test the hypotheses that changes in body size and number of scales are adaptive to life in the following habitats: the subtidal (below the tidal change), the transitional zone (the interface between intertidal and subtidal habitats) and the strictly intertidal. To this end, we use Hansen's (1997) Ornstein-Uhlenbeck model with Butler & King's (2004) Ornstein-Uhlenbeck Comparative Hypotheses (*OUCH*) approach to compare alternative evolutionary models that may be responsible for the diversity of scale covering and body sizes in marine sculpins.

Materials and methods

Phylogeny

Mitochondrial cytochrome *b* (cyt *b*) sequences in Ramon & Knope (2008) and Mandic *et al.* (2009a) were used for this study (GenBank Accession numbers EF521313EF521387; EU836693–EU836704 respectively). This locus has been used extensively in fish phylogenetics and its molecular evolution is well studied (e.g. Johns & Avise, 1998; Crow *et al.*, 2004). The cyt *b* locus is protein coding and exhibited no insertions or deletions. An alignment of 802 basepairs was constructed in Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI, USA).

To conduct the maximum likelihood (ML) phylogenetic analysis, Modeltest 3.7 (Posada & Crandall, 1998) was used to determine the appropriate model of sequence evolution for cyt *b*. Aikake Information Criterion (AIC) was used to determine which of 88 models of nucleotide evolution best fit the data (Burnham & Anderson, 2002). The Hasegawa, Kishino and Yano model (Hasegawa *et al.*, 1985), with rate variation among sites and invariant sites (HKY85 + Γ + I), was selected as the best-fit model. The HKY85 model assumes an equal substitution rate at all sites and uses empirical data for base frequencies. The transition rate is not assumed to equal the transversion rate, and the substitution rate is estimated (Hasegawa *et al.*, 1985).
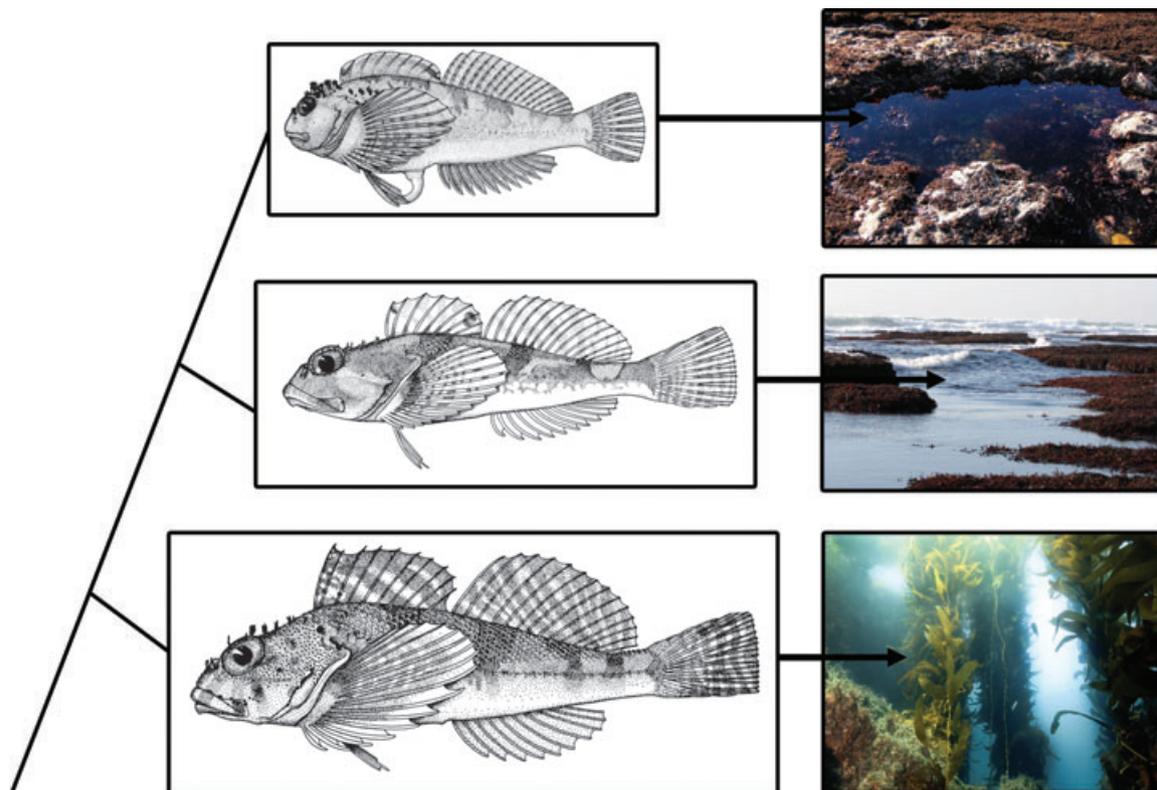
Fig. 1 A simplified conceptual diagram of sculpin phylogeny in relation to habitats where species are found. Image on bottom (*Ruscarius creaseri* Hubbs) representing the basal-rooted species found predominantly in the ancestral subtidal habitat. Image in middle (*Artedius notospilotus* Girard) representing the clade with species that can be found predominantly in the transitional zone. Image on top (*Clinocottus recalvus* Greeley) representing the clade with species found predominantly in the intertidal. Scientific illustrations from Bolin (1944). Photo credits (from top to bottom): M.L. Knope, M.L. Knope and K. Jameson.

The HKY85 + Γ + I model of sequence evolution was then used to find the best ML tree within the parameter search space using a heuristic tree search with stepwise addition and TBR branch swapping. *Jordania zonope* was chosen as the outgroup taxon based on published phylogenies (Bolin, 1947; Ramon & Knope, 2008), and trees were rooted for the heuristic search. Starting trees were obtained via stepwise addition with addition sequences 'as-is'.

We calculated the branch lengths on this topology in a maximum likelihood framework in PAUP* version 4.0b10 (Swofford, 2002). To test the sequences for a constant rate of substitution across lineages, a likelihood ratio test (LRT) was employed by calculating the log likelihood score of the best tree with the molecular clock enforced and comparing it with the log likelihood score previously obtained without enforcing the molecular clock (Jukes & Cantor, 1969; Goldman, 1993). In this case, the molecular clock is the null hypothesis. Enforcing the molecular clock constrains branch lengths by forcing terminal ends to be contemporaneous and sequence substitution rates to be equal across all lineages: $\chi^2 = 2 \times [(-\ln L_{clock}) - (-\ln L_{unconstrained})]$. The χ^2 distribution was calculated with $n-2$ d.f., where n is equal to the number of taxa sampled (Felsenstein, 1981).

Morphological data

Total number of scales above the lateral line and standard length for each individual were measured from specimens at the California Academy of Sciences Ichthyology Museum or taken from Bolin (1944). Mean standard length in millimetres (tip of maxilla to end of caudal peduncle) of each species was measured as a proxy for body size (following Bolin, 1944; Cailliet *et al.*, 1986; Moyle & Cech, 2003). A total of 1160 individuals were measured for body size (mean number of individuals per species = 45). The total number of scales above the lateral line posterior of the operculum (on right side, if looking anterior to posterior) were counted for 302 individuals (mean number of individuals per species = 12). Species means and ranges for standard length and number of scales are given in Table 1. Samples were limited to adult individuals only (post-juvenile morphology), and samples were collected in multiple locations using identical mesh size in
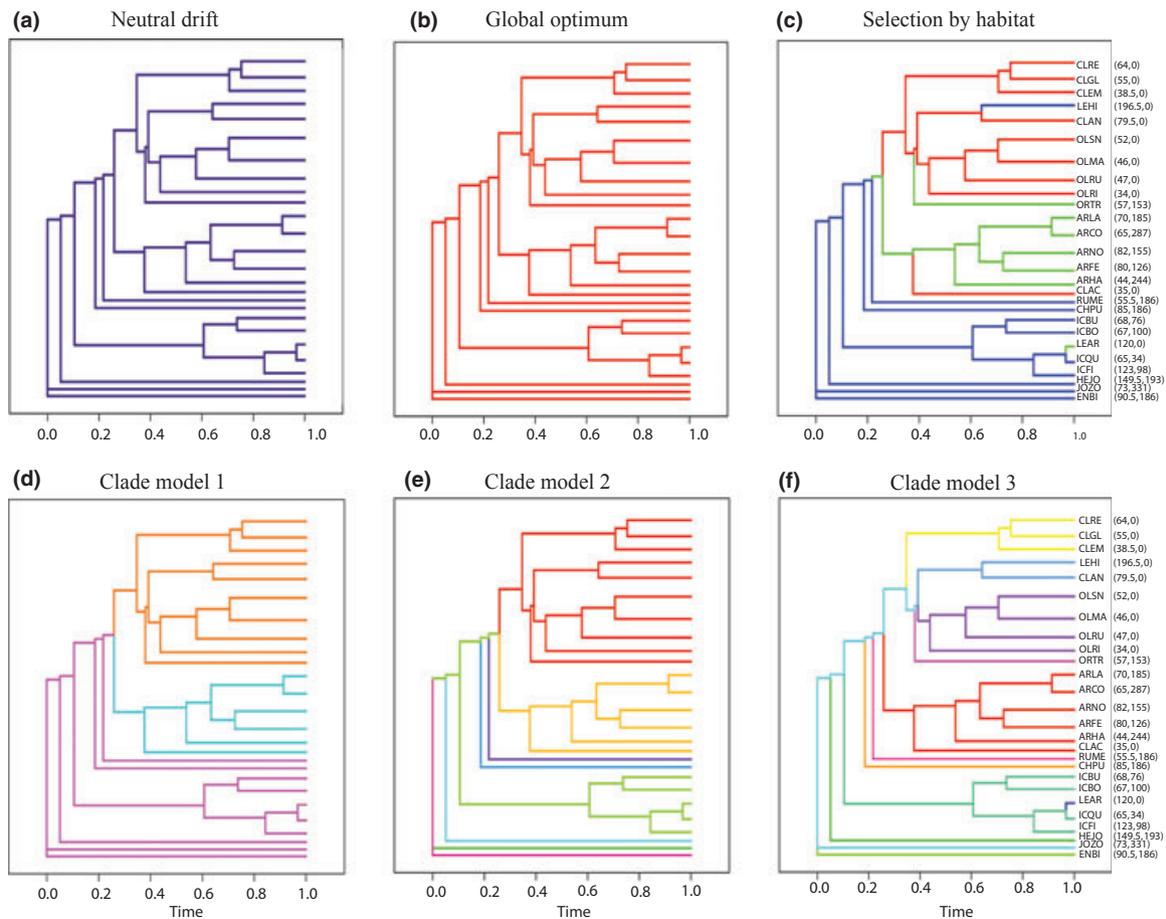
Fig. 2 Evolutionary models (hypotheses). From left to right, top to bottom: (a) Brownian motion or neutral drift model; (b) O-U global optimum model; (c) habitat model; (d) clade model 1; (e) clade model 2, and (f) clade model 3. Taxon abbreviation: first two letters denotes genus name, followed by first two letters of specific epithet (for full names please see Table 1). Each regime is 'painted' onto the branches of the phylogeny. In the Brownian motion model, there is only a single colour (purple) 'painted' onto the phylogeny to represent that the body size and scale number of all species are expected to be evolving in a neutral fashion. In the O-U global optimum model, there is a single colour (red) 'painted' onto the phylogeny to represent that the body size and scale number of all species are expected to be evolving towards the same optimal value. In the habitat model, there are three separate colours (red = intertidal, green = transitional and blue = subtidal) 'painted' onto the phylogeny to represent the three distinct selective regimes based on habitat affinity and that body size and scale number are expected to be evolving towards separate optima in each habitat type. In the three clade-based models, the clade (or grade) a species is a part of is 'painted' a separate colour to represent that each clade experiences a unique set of selective pressures on the evolution of body size and scale number. Values to the right of each species in parentheses are mean body size, followed by mean number of scales.

sampling nets. In addition, the mean number of individuals per species ($n = 45$) has been considered sufficient to accurately represent the species mean values in these fishes and serves as the basis for their formal species descriptions (Bolin, 1944). All data were natural log transformed prior to analysis, to improve fit to normality.

Evolutionary hypotheses (models)

Numerous methods exist for addressing phylogenetic nonindependence of species (e.g. Felsenstein, 1985;

Harvey & Pagel, 1991; Losos & Miles, 1994; Garland *et al.*, 2005; Losos, 2011). However, many of these methods assume Brownian motion to model the evolution of a character that is thought to be under selection (Harvey & Purvis, 1991; Harvey & Rambaut, 2000). For this reason, we used the *OUCH* method (Hansen, 1997; Butler & King, 2004), which allowed us to test alternative evolutionary hypotheses that include random drift and natural selection on the phenotypes of interest (body size and scale number, in this case). Both the evolution of body size and scale number were modelled using Hansen's (1997) model, as implemented in *OUCH*

Table 1 Species name, four letter abbreviation (first two letters of genus, followed by first two letters of specific epithet), mean standard length in millimetres (followed by range), mean number of scales (followed by range) and habitat affinity. Species are sorted by habitat affinity and listed alphabetically within each habitat type.

Species	Abbr.	Standard length	Scales	Habitat
Chitonotus pugetensis	CHPU	85 (54.3–143.2)	186 (185–187)	Subtidal
Enophrys bison	ENBI	90.5 (20.6–231)	32 (30–35)	Subtidal
Hemilepidotus jordani	HEJO	149.5 (83–215)	193 (175–210)	Subtidal
Icelinus borealis	ICBO	67 (52–80)	100 (88–106)	Subtidal
Icelinus burchami	ICBU	68 (36.6–107.5)	76 (72–81)	Subtidal
Icelinus filamentosus	ICFI	123 (51.1–2.1)	98 (90–104)	Subtidal
Icelinus quadriseriatus	ICQU	65 (51.8–73.4)	34 (30–36)	Subtidal
Jordania zonope	JOZO	73 (49.2–90.8)	331 (283–358)	Subtidal
Leiocottus hirundo	LEHI	196.5 (150–205)	0 (0–0)	Subtidal
Ruscarius meanyi	RUME	55.5 (40–85)	182 (170–190)	Subtidal
Clinocottus acuticeps	CLAC	35 (21.2–53.1)	0 (0–0)	Intertidal
Clinocottus analis	CLAN	79.5 (30.6–139.3)	0 (0–0)	Intertidal
Clinocottus embryum	CLEM	38.5 (18.4–60.7)	0 (0–0)	Intertidal
Clinocottus globiceps	CLGL	55 (25.2–153.8)	0 (0–0)	Intertidal
Clinocottus recalvus	CLRE	64 (21–108.5)	0 (0–0)	Intertidal
Oligocottus maculosus	OLMA	46 (23.8–73.2)	0 (0–0)	Intertidal
Oligocottus rimensis	OLRI	34 (21.8–45.2)	0 (0–0)	Intertidal
Oligocottus rubellio	OLRU	47 (23.3–60.2)	0 (0–0)	Intertidal
Oligocottus snyderi	OLSN	52 (25.5–75.6)	0 (0–0)	Intertidal
Artedius corallinus	ARCO	65 (42–91.5)	287 (275–300)	Transitional
Artedius fenestralis	ARFE	80 (51.3–103)	126 (126–127)	Transitional
Artedius harringtoni	ARHA	44 (17.9–81.3)	244 (204–280)	Transitional
Artedius lateralis	ARLA	70 (16.1–124.1)	185 (161–221)	Transitional
Artedius notospilotus	ARNO	82 (39.2–134.5)	155 (145–165)	Transitional
Leptocottus armatus	LEAR	120 (22.3–185.9)	0 (0–0)	Transitional
Orthonopius triacus	ORTR	57 (28.7–77)	153 (144–160)	Transitional

(Butler & King, 2004). Hansen's (1997) model is an Ornstein-Uhlenbeck (OU) process with distinct changes of selective regimes along the phylogeny. A selective regime is the overall combination of environmental and organismal traits associated with a particular lifestyle or behaviour (Baum & Larson, 1991). The OU model (eqn 1) expresses the change in a phenotypic trait, dX (t), over time, t, based on stabilizing selection (α: the strength of selection) and random drift (σ: strength of drift) while allowing the optimal trait value, $\theta(t)$, to vary across selective regimes. Thus, the Hansen model assumes that the trait under study remains close to a fitness optimum due to selection.

$$dX(t) = \alpha[\theta(t) - X(t)]dt + \sigma d\beta(t) \quad (1)$$

The model further assumes that the strength of selection can be interpreted similarly across selective regimes, and, as with other phylogenetic comparative methods, assumes the phylogeny accurately reflects the evolutionary history of the species being analysed. The Butler & King (2004) method incorporates Hansen's model and assigns regimes (which can also include neutral drift and clade-based selection regimes) to branches along the phylogeny to produce distinct models (hypotheses) of evolution. These models are then fit to the data and the

model fits are compared using maximum likelihood-based model selection criteria, allowing alternative evolutionary hypotheses to be explicitly tested (Butler & King, 2004). The optimal trait value for each selective regime is estimated as the weighted mean of the species in a given regime (weighted by the amount of time spent in the selective regime).

We tested six evolutionary hypotheses for the evolution of body size and number of scales by assigning (*sensu* Butler & King, 2004) regimes to individual branches of our phylogeny (Fig. 2). Ancestral states were assigned based on linear parsimony. The first two models are a neutral Brownian motion model and a single optimum model. The Brownian motion, or a random drift model, is the simplest model and assumes the character in question (either body size or number of scales) is evolving in a random fashion across the phylogeny (Fig. 2; neutral drift model). The single optimum model assumes stabilizing selection across the phylogeny with a single optimum trait value for all species (Fig. 2; global optimum model), and predicts that all sculpins share the same adaptive regime and the species traits are evolving towards a single optimal value. This situation would occur if the functional demands of the morphologies of all sculpin species were identical, irrespective of habitat affinity.

The remaining four models are adaptive. The first of these models is an adaptive model based on habitat affinities (Fig. 2; multiple optima habitat model). This model contains three separate trait value optima based on the habitat in which each species occurs and suggests that body size and scale number vary across habitats due to selection. Sculpin species were classified as strictly subtidal, transitional (for those species that can be found at the interface between the intertidal and subtidal and may extend into the subtidal habitats) or strictly intertidal based on published accounts of habitat use (Bolin, 1944; Miller & Lea, 1972; Mecklenburg *et al.* 2002; Ramon & Knope, 2008; Mandic *et al.*, 2009a).

The final three models are clade-specific selection models. These models assume sculpin morphologies are the result of all species in a clade sharing a common, distinct set of selective pressures. The first of these clade models splits the phylogeny into two major clades with derived traits (with *Oligocottus-Clinocottus* including *Leiocottus hirundo* Girard as a clade, and the *Artedius* species and *Clinocottus acuticeps* Gilbert, which consistently groups with *Artedius* [this study; Ramon & Knope, 2008; Knope, 2013], as the other derived clade) and all remaining basal-rooted species as a single grade (Fig. 2; clade model 1). This clade model would occur if the two major derived clades experience different selective regimes. The second clade model is essentially the same as clade model 1, except that the basal species are coded by genera rather than as a single grade (Fig. 2; clade model 2). The final clade model is based on genera, except for *Clinocottus analis* Girard plus *L. hirundo* as a clade as these two species appear most closely related to one another and *Clinocottus*, as it is currently described, is not a natural group (this study; Howe & Richardson, 1978; Ramon & Knope, 2008; Knope, 2013) (Fig. 2; clade model 3). This model would occur if selective pressures differ across genera, but are constant within genera (with *C. analis*/*L. hirundo* also treated as a genus).

Statistical analyses

We fit the six evolutionary models to the body size and scale data assuming the cyt *b* phylogeny and branch lengths. The models of evolution were fit using a ML approach as employed by the *OUCH* package (Butler & King, 2004) in the *R* statistical computing environment (R Development Core Team 2011). The fit of each of the models to the data (ML scores) were compared using both the Akaike Information criterion corrected for small sample size (AIC.c) and the more conservative Schwartz information criterion (SIC) following Butler & King (2004). Under both AIC.c and SIC, a difference of two units is considered a statistically significant difference (Burnham & Anderson, 2002). Model selection frequencies and 95% confi-

dence intervals for model parameters were calculated using 2000 bootstrap replicates (Burnham & Anderson, 2002). Once the optima for each model were calculated, these parameters were back-transformed into standard length (mm) and number of scales to facilitate interpretation.

Results

Phylogeny

The tree topology recovered in the cyt *b* phylogeny (for shared species) is not statistically significantly different (Shimodaira-Hasegawa test; $P > 0.05$) from the phylogeny reconstructed from two other molecular markers (mitochondrial NADH1 and nuclear ribosomal S7) and from the consensus tree based on all three of these molecular markers (Ramon & Knope, 2008) and is in general agreement with phylogenies based on morphology (Bolin, 1947; Begle, 1989; Strauss, 1993). Only the cyt *b* data were utilized in this analysis because sequence data for all species in the phylogeny are not available for all three loci and because concatenating data sets conflates the ability to accurately assess relative branch lengths in the phylogeny. A χ^2 test fails to reject the null hypothesis that enforcing the molecular clock does not significantly add length to the tree ($P > 0.05$), indicating that branch lengths are proportional to time.

Morphology

The intertidal, transitional and subtidal sculpin species exhibit significant differences in body size ($F_{2,23} = 5.1$, $P = 0.014$; Tukey's HSD intertidal vs. transitional = n. s., subtidal vs. transitional = n.s., subtidal vs. intertidal $P < 0.05$) and number of scales ($F_{2,23} = 11.59$, $P < 0.000$; Tukey's HSD intertidal vs. transitional $P < 0.05$, subtidal vs. transitional = n.s., subtidal vs. intertidal $P < 0.05$). On average, the smallest species are found in the intertidal, species of intermediate size can be found in the transitional zone, and the largest species are found in the subtidal, but considerable variation exists (especially, among subtidal sculpins; Table 1). Also, intertidal sculpins have the fewest number of scales (all are completely without scales), and those species in the transition zone have (on average) the greatest number, while subtidal species have an intermediate number (Table 1). However, one subtidal sculpin, *Leiocottus hirundo*, which is only found in the warm waters of the Southern California Bight and immediately adjacent areas, has no scales. The mean number of scales is strongly associated with per cent cover of scales over the skin (adjusted $R^2 = 0.802$, $P < 0.000$). However, mean body size is not a good predictor of mean scale number (adjusted $R^2 = 0.027$, $P = 0.205$).

Model performance

Bootstrap model selection frequencies for AIC.c and SIC scores were highly similar and therefore only model selection frequencies for AIC.c scores are presented here (but see Tables 2 and 3 for SIC scores). According to both the AIC.c and SIC scores, the habitat model best explains the evolution of scale number in these species of sculpins (Table 2). In fact, all other models performed substantially worse (higher AIC.c and SIC scores), implying strong support for the habitat model. The superior performance of the habitat model for the evolution of scales is further supported by bootstrap model selection frequencies as the habitat model was selected as the best-fit model in 100% of the 2000 bootstrap replicates. The habitat model predicts that fish in the intertidal zone should have no scales, the greatest number of scales should occur on fish in the transitional zone, and an intermediate number of scales on subtidal species and this pattern matches the actual data (Table 4). However, the habitat model under-predicts the actual number of scales for the transitional and subtidal species (Table 4).

The habitat model also best explains the evolution of body size, with the lowest (best-fit) AIC.c and SIC scores (Table 3). In addition, bootstrap frequencies showed strong support for the habitat model as it was selected as the best-fit model 82% of the time. All other

Table 2 Model selection criteria for scales. ΔAIC.c and ΔSIC scores followed by per cent of 2000 bootstraps where model selected as the best fit. Greater values of ΔAIC.c and ΔSIC represent an inferior fit between an evolutionary model and the actual data.

Model	ΔAIC.c	ΔSIC
Habitat	0.00 (100%)	0.00 (100%)
Single optima	26.92 (0%)	26.32 (0%)
Brownian motion	32.42 (0%)	31.12 (0%)
Clade 1	31.16 (0%)	7.30 (0%)
Clade 2	10.82 (0%)	10.82 (0%)
Clade 3	29.98 (0%)	24.60 (0%)

Table 3 Model selection criteria for standard length. ΔAIC.c and ΔSIC scores followed by per cent of 2000 bootstraps where model selected as the best fit. Greater values of ΔAIC.c and ΔSIC represent an inferior fit between an evolutionary model and the actual data.

Model	ΔAIC.c	ΔSIC
Habitat	0.00 (82%)	0.00 (85%)
Single optima	8.17 (2%)	7.56 (3%)
Brownian motion	16.11 (1%)	14.82 (2%)
Clade 1	37.82 (0%)	13.96 (1%)
Clade 2	8.34 (6%)	8.34 (9%)
Clade 3	25.83 (9%)	20.46 (0%)

Table 4 The θ parameter (character modelled) estimates of the best fitting scale model as compared with the actual data for scales (50% quantile followed by the 2.5% and 97.5% quantiles, respectively, in parentheses). Actual data represent the average of all species mean values for each habitat type.

Habitat type	θ estimate	Actual data
Intertidal	0.1 (0.02, 0.34)	0
Transitional	96.33 (20.8, 473.6)	164.2
Subtidal	44.91 (14.3, 154.2)	123.2

Table 5 The θ parameter estimates (character modelled) of the best fitting body size model as compared to the actual data for standard length (50% quantile followed by the 2.5% and 97.5% quantiles, respectively, in parentheses). Actual data represent the average of all species mean values for each habitat type.

Habitat type	θ estimate	Actual data
Intertidal	48.4 (38.7, 59.8)	50.1
Transitional	68.56 (52.4, 88.7)	74.0
Subtidal	90.45 (74.6, 109.9)	97.3

models performed relatively poorly, with limited support for clade model 2 (6%) and clade model 3 (9%). The habitat model predicts that the smallest species should be found in the intertidal, species of intermediate size found in the transitional zone and the largest species found in the subtidal, closely matching the actual data (Table 5). All data and code are deposited in the Dryad repository: doi:10.5061/dryad.46635.

Discussion

Scale number

Our results provide strong evidence that habitat use governs the evolution of scale cover in sculpins (Table 2). Although sculpins in the transitional and subtidal zones (except *L. hirundo*) have numerous scales, all intertidal sculpins in this study have no scales, suggesting strong selection for loss of scales entirely in the intertidal. We suggest that this loss of scales may function to facilitate skin breathing during hypoxia in these fishes (Martin & Bridges, 1999). Water generally contains less than 1% oxygen by volume (Helfman *et al.*, 2009), and since gas solubility in liquids is a function of temperature (Charles Law; Gay-Lussac, 1802), warmer water (as often occurs in tide pools) contains less oxygen than cool water. In addition, the gills of fishes generally collapse when fish emerge from water to perform aerial respiration (Helfman *et al.*, 2009). Therefore, hypoxic tidepools likely drive strong selection for cutaneous respiration, which loss of scales facilitates (Martin & Bridges, 1999). In addition, relaxation of other selective forces, such as predation pressure (Love, 1996, 2011; Helfman *et al.*, 2009), may have also facilitated scale loss in the intertidal.

Interestingly, sculpins found in the transition zone have the greatest mean number of scales (Table 4). There may be selection for increased scale covering in the transition zone due to increased wave stress (to protect the body against rocks, etc.) and predatory attacks (Love, 1996, 2011; Helfman *et al.*, 2009). Also, there may be relaxed selection on scale cover in subtidal sculpins due to the relative constancy of their environment, and relatively large body size, which may deter some predators. However, the function of scale cover regarding wave stress and predation is still largely unstudied, so these hypotheses remain speculative.

Body size

Our results suggest that habitat use also drives the evolution of body size in sculpins (Table 3). Evolution of small body size in the intertidal may be beneficial for many reasons and selection for body size may be governed by factors such as predation pressure, prey size, pool size, thermal tolerance and desiccation resistance (Stearns, 1992; Roff, 2002; Bird, 2011). Although the above factors may all influence body size evolution, surviving hypoxia in the intertidal is likely a relatively strong selective pressure. A small body size may facilitate cutaneous breathing by increasing the surface area to volume ratio of the fish, facilitating diffusion of oxygen across the cutaneous membrane (Graham *et al.*, 1987). Furthermore, because body size is closely linked with metabolic rate, a small body would reduce metabolic demands, a potential benefit in a hypoxic situation. If we use a metabolic scaling equation for all fishes (Eqn 2 below; Withers, 1992) where M = body mass, we estimate that *Clinocottus globiceps* Girard, an intertidal species that weighs on average 8.1 g (Yoshiyama & Cech, 1994) uses roughly 30.25 J hr^{-1}. Conversely, *Chitonotus pugetensis* Steindachner, a subtidal species weighing roughly 21 g (estimated from Martin, 1996), would use 69.95 J hr^{-1}, or more than twice as much energy per hour. Such a difference in metabolic rate could be the difference between life and death during extended periods of time in a hypoxic tide pool.

$$\text{Metabolic rate (J hr}^{-1}) = 4.8M^{0.88} \qquad (2)$$

In addition, the possibility that small body size was an exaptation that allowed small-bodied subtidal sculpins to move into the intertidal zone, while larger bodied sculpins could not, cannot be excluded. However, Martin (1996) rejected the hypothesis that sculpins' ability to survive in the often-hypoxic intertidal environment was due to a pre-existing low metabolic rate.

Sculpins and hypoxia

Does the small body size and loss of scales in intertidal sculpins result in a performance advantage in hypoxic conditions? Ecophysiological studies of cutaneous breathing in sculpins across a depth gradient show that species of intertidal cottids are capable of skin breathing while subtidal species are not (Wright & Raymond, 1978; Martin, 1991, 1993, 1995, 1996), and loss of scales is required for the acquisition of this trait (Martin & Bridges, 1999). All of the intertidal cottids studied thus far are known to escape hypoxic tidepools and respire in air with little or no metabolic effects (as measured by whole body lactate; Martin, 1991, 1993, 1995, 1996; Sloman *et al.*, 2008; or by critical O$_2$ tensions; Mandic *et al.*, 2009a,b; Richards, 2011). For example, *Oligocottus maculosus* Girard can perform aerial respiration with no discernable adverse metabolic effects for up to 72 hrs (Sloman *et al.*, 2008). Further, *C. analis* performs approximately 30% of its respiration through the skin during emergence (Martin & Bridges, 1999).

Although our results suggest that scale number and body size are adaptive to habitat use and may facilitate survival in hypoxic conditions in the intertidal zone, there are several additional lines of evidence indicating that surviving hypoxic conditions may be a primary mechanism of selection. In addition to shifts in body size and scale covering, intertidal sculpins appear to have a wide array of other morphological, behavioural and physiological adaptations (Richards, 2011). For example, Mandic *et al.* (2009a) found that intertidal sculpins have higher hypoxia tolerance than subtidal sculpins and that of the factors they examined, routine O$_2$ consumption rate, mass-specific gill surface area and whole blood haemoglobin-O$_2$-binding affinity account for most of the variation in hypoxia tolerance. Martin (1996) also showed that intertidal sculpins have specific behavioural attributes that subtidal sculpins do not, including emergence from tidepools, allowing them to survive hypoxia. In addition, Martin (1996) found a high rate of mortality in three species of subtidal sculpins (*Icelinus borealis* Gilbert, *Jordania zonope* Starks and *Chitonotus pugetensis* Steindachner) during forced emergence in air, as compared with the intertidal sculpins *O. maculosus* and *Ascelichthys rhodorus* Jordan & Gilbert. Thus, surviving hypoxic conditions appears to be a strong selective pressure on sculpins that may have acted on a large suite of morphological and physiological traits.

In addition, intertidal sculpins likely have adaptations that are largely independent of coping with daily periodic hypoxia. For example, three exclusively intertidal species (*Clinocottus globiceps* Gilbert, *C. recalvus* Greeley and *C. embryum* Jordan & Starks) have unique head and jaw morphology not observed in subtidal sculpins (Bolin, 1944; Miller & Lea, 1972), which may be adaptive for acquisition of prey unique to the intertidal environment (Boyle & Horn, 2006). Thus, the shift into the intertidal zone may have facilitated further ecological adaptations and diversifications. However, it is important to note that these physiological, behavioural and

morphological attributes may be correlated with the evolution of body size and loss of scales.

Did adaptation to novel habitat types lead to diversification?

A prominent idea in evolutionary biology is that adaptation to novel habitats can lead to diversification (Schluter, 2000), which may be the case in sculpins. The following three lines of evidence suggest that these morphological adaptations may have facilitated further diversification of the sculpin clade. First, basal-rooted species of marine sculpins along the North American Pacific Coast are found predominantly in the subtidal and the shift to the intertidal resulted in diversification (Ramon & Knope, 2008; Mandic *et al.*, 2009a; Knope, 2013), giving rise to at least the seven transitional and nine intertidal species examined in this study (Fig. 2c). Second, our results demonstrate that species of sculpins found in the intertidal and transitional zones acquired body size and scale covering adaptations to these novel habitat types. Third, because scales inhibit cutaneous breathing (Martin & Bridges, 1999) and subtidal sculpins (all having scales, except the exclusively warm water inhabiting *L. hirundo*) are not able to survive hypoxia (Martin, 1996), it follows that the loss of scales (and perhaps the reduction of body size) is an adaptation necessary for occupation of the intertidal and for diversification within this habitat.

We hypothesize that adaptation to the novel intertidal habitat gave rise to 'ecological opportunity' resulting in further diversification of the already hyperdiverse sculpin clade that originated in the subtidal (Bolin, 1947; Ramon & Knope, 2008; Knope, 2013). The 'ecological opportunity' hypothesis predicts that when interspecific competition or predation is reduced, populations will exhibit increases in phenotypic variance, upon which diversifying selection can act, resulting in speciation (Schluter, 2000; Nosil & Reimchen, 2005). The intertidal habitat is well known as a refuge from predation pressure by larger predators, and many noncottid fish species occupy the intertidal as juveniles before an ontogenetic shift to deeper water as adults (Love, 1996, 2011; Helfman *et al.*, 2009). In addition, as demersal ambush predators themselves (Boyle & Horn, 2006), intertidal sculpins may have experienced less competition for prey resources, compared with closely related subtidal sculpins, during the initial phases of diversification in the novel intertidal habitats. However, while our analyses indicate morphological adaptations to these habitats, additional analyses would be required to answer these questions regarding 'ecological opportunity' and diversification.

Summary

Although many diversification events are presumed to be adaptive, few have been convincingly demonstrated to be so, and these generally come only from well-studied terrestrial and freshwater systems (Schluter, 2000; Losos & Mahler, 2010). Our results suggest that adaptive morphological character shifts are associated with differential habitat use in sculpins. Such morphological adaptation may facilitate the occupation of novel habitat types in marine systems, including the intertidal environment. Future work should increase the phylogenetic breadth of sculpins investigated and test adaptive and alternative hypotheses for other morphological, physiological and behavioural characters associated with the habitat shifts. In addition, cutaneous breathing may be a 'key innovation' that allowed for occupation of the novel intertidal habitat in sculpins and future work that explicitly tests this hypothesis would be valuable. More broadly, additional marine studies are needed to further evaluate generalities in the factors that facilitate diversification across terrestrial, freshwater and marine systems.

Acknowledgments

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