

Co-evolution of cleaning and feeding morphology in western Atlantic and eastern Pacific gobies

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Cleaning symbioses are mutualistic relationships where cleaners remove and consume ectoparasites from their clients. Cleaning behavior is rare in fishes and is a highly specialized feeding strategy only observed in around 200 species. Cleaner fishes vary in their degree of specialization, ranging from species that clean as juveniles or facultatively as adults, to nearly obligate or dedicated cleaners. Here, we investigate whether these different levels of trophic specialization correspond with similar changes in feeding morphology. Specifically, we model the evolution of cleaning behavior across the family Gobiidae, which contains the most speciose radiation of dedicated and facultative cleaner fishes. We compared the cranial morphology and dentition of cleaners and non-cleaners across the phylogeny of cleaning gobies and found that facultative cleaners independently evolved four times and have converged on an intermediate morphology relative to that of dedicated cleaners and non-cleaning generalists. This is consistent with their more flexible feeding habits. Cleaner gobies also possess a distinct tooth morphology, which suggests they are adapted for scraping parasites off their clients and show little similarity to other cleaner clades. We propose that evolutionary history and pre-adaptation underlie the morphological and ecological diversification of cleaner fishes.

KEY WORDS: Convergence, geometric morphometrics, gobiidae, macroevolution, pre-adaptation, specialization.

The link between ecology and morphology is a relationship well established in a myriad of lineages, with a keen emphasis on those that occupy narrow ecological niches (Losos 1990; Ferry-Graham et al. 2002; Summers et al. 2004; Gartner and Greene 2008; Tschapka et al. 2008). It is thought that highly specialized ecologies promote greater morphological specialization as well as strong morphological convergence (Ferry-Graham et al. 2002). Exemplar cases of this exist among vertebrates that exercise a narrow diet breadth, such as egg-eating snakes, nectar-feeding bats, shell-crushing sharks, and others. These taxa have evolved highly modified feeding apparatuses in response to the strong selective pressures exerted by their particular prey items (Summers et al. 2004; Gartner and Greene 2008; Tschapka et al. 2008). However, specialists often vary in their degree of spe-

cialization, and differ in how dependent they are on a specific resource (Mori and Vincent 2008). This is likely to have a substantial effect on their phenotypic evolution, yet it is not always clear how morphology might change with changes in ecological specialization.

Here, we investigate the evolutionary relationship between diet and morphology in fishes that clean and feed on the ectoparasites of other fishes. Across teleost fishes, cleaning is one of the most specialized trophic strategies and has been observed in only 36 families and just over 200 species (Vaughan et al. 2017). The family Labridae (wrasses, parrotfishes, and hogfishes) contains the greatest number of cleaner fishes, with 60 species (Baliga and Law 2016), while Gobiidae (gobies) contains the second most, with 17 cleaner species (Côté and Soares 2011). Cleaning

symbioses are mutually beneficial, complex interspecific relationships between cleaner and client (Limbaugh 1961; Feder 1966; Losey 1972). Successful cleaners are capable of amassing a large clientele and removing parasites efficiently. To attract clients, cleaners have evolved specialized behavioral and morphological characteristics including living near cleaning stations (small areas associated with cleaning behavior—often on coral reefs), using cleaner “dances,” and displaying bright coloration patterns (Feder 1966; Youngbluth 1968; Grutter 2004). While many studies have investigated and documented these traits associated with attracting clients, few have examined the feeding traits that facilitate efficient predation on parasites (Baliga and Metha 2015; Baliga and Law 2016; Baliga and Mehta 2018; Baliga et al. 2019). Even fewer studies have explored the feeding morphology of cleaner fishes beyond that of cleaner wrasses (Böhlke and Robins 1968).

Cleaner fishes can be assigned into three different categories based on their behavior and degree of specialization (Côté 2000). The most abundant type are juvenile cleaners that only clean early in their life history, but some facultative cleaners continue to clean occasionally across their ontogeny (Côté 2000). The rarest and most specialized cleaners are those that clean consistently throughout adulthood. The term “obligate” has often been used to describe these cleaners (Limbaugh 1961; Youngbluth 1968). However, despite cleaning much more frequently than facultative cleaners, few “obligate” species actually rely exclusively on cleaning, so we hereafter refer to them as “dedicated” cleaners instead (Vaughan et al. 2017). The nearly exclusive commitment to cleaning suggests that dedicated cleaners might be equipped with a highly modified feeding morphology for removing ectoparasites. We also suspect that facultative cleaners likely exhibit morphologies that reflect their less specialized ecology, compared to having the same morphologies as dedicated cleaners or alternatively, that of non-cleaning generalists. Studies on cleaner wrasses indicate that this may be the case; however, wrasses are mostly juvenile cleaners, with few dedicated and facultative cleaners (Baliga and Law 2016; Baliga and Mehta 2018). By contrast, Gobiidae contains the most species of dedicated and facultative cleaners, yet cleaner gobies remain poorly studied by comparison (Côté and Soares 2011). We propose that by examining cleaner gobies we can improve our understanding of the link between ecology and morphology with regards to the different degrees of specialization. We can also begin to answer macroevolutionary questions about the feeding morphology of cleaner fishes across lineages.

Gobies are among the most abundant cleaner species in the western Atlantic and eastern Pacific Oceans. Cleaner gobies fall within the genera of *Elacatinus* (neon gobies) and the closely related but polyphyletic genus *Tigriobius*, and exhibit many of the traits associated with attracting clients (Rüber et al. 2003). For

example, cleaning members of both genera occur near distinct microhabitats that serve as recognizable cleaning stations (Colin 1975; Taylor and Van Tassell 2002; Randall and Lobel 2009). Neon gobies also possess brightly colored lateral stripes for signaling conspecifics. A few dedicated cleaner gobies possess blue lateral stripes that are adapted for attracting clients and convergent with the blue stripes of dedicated cleaner wrasses (Cheney et al. 2009). Some species of neon gobies have variable coloration patterns, or color morphs, that are both genetically and geographically distinct (Taylor and Hellberg 2006; Colin 2010). While most of these color morphs have recently been elevated to species status, there are still several that have yet to be recognized as their own species (Colin 2010).

Empirical evidence suggests that cleaner gobies possess multiple feeding adaptations that differ from cleaner wrasses and other cleaner species (reviewed by Côté and Soares 2011). For example, some cleaner gobies have a modified inferior, or downturned, mouth position that is strongly associated with the blue lateral stripe and dedicated cleaning (Taylor and Hellberg 2005). Colin (2010) assigned species of *Elacatinus* into ecomorphs based on whether they possess a terminal, subterminal, or inferior mouth position following the definitions in Böhlke and Robins (1968). While cleaners were generally associated with inferior or subterminal mouths, Victor (2014) pointed out the difficulties associated with categorical assignments, suggesting a quantitative approach is preferred. Furthermore, while describing several species of *Elacatinus*, Böhlke and Robins (1968) also noted variable tooth morphologies between species and sexes that superficially corresponded with feeding ecology. However, no studies have explicitly compared the dentition of *Elacatinus* or *Tigriobius* in the context of cleaning behavior.

We examined the link between cleaning and morphology in gobies, focusing on the *Gobiosoma* subgroup of the Gobiosomatini tribe (Rüber et al. 2003; Van Tassell et al. 2011). This system, which includes *Elacatinus*, *Tigriobius*, and several non-cleaning genera, provides the opportunity to investigate how morphological specialization varies with different degrees of ecological specialization. The genera *Elacatinus* and *Tigriobius* possess species that display feeding behaviors ranging from non-cleaning to facultative and dedicated cleaning, yet the exact evolutionary origins of these behaviors and the morphologies that accompany them have not been formally investigated (Taylor and Hellberg 2005; Côté and Soares 2011). Using a comparative phylogenetic framework, we compared the cranial morphology, head shape, and dentition of these genera and assessed patterns of morphological convergence among cleaners. Here we addressed three questions. (1) How many times has cleaning evolved in gobies? (2) Does the degree of morphological specialization reflect the degree of cleaning behavior? (3) Are cleaner gobies converging on a specialized feeding morphology? Lastly, we also comment on the patterns of

morphological convergence in the feeding adaptations of cleaner fishes in general.

Methods

CLASSIFICATION OF CLEANER GOBIES

A literature review was conducted to classify each species in the phylogeny into a dietary group based on their feeding ecology or cleaning behavior. We categorized each species in our genetic dataset as a dedicated cleaner, facultative cleaner, or non-cleaner (Supporting Information Table S1). A caveat with this approach is that cleaners are unlikely to match these discrete groups perfectly, but they allow us to test general hypotheses surrounding the varying degrees of cleaning behavior. The non-cleaners were also further divided into generalists and specialists. Most of the non-cleaners are fairly generalist consumers but *Ginsburgellus novemlineatus* specializes on consuming the tube feet of sea urchins (Teytaud 1971). Both *E. atronasus* and *E. jarocho* (only the former was included in our phylogeny) are planktivores that were also considered to be specialists (Böhlke and Robins 1968; Taylor and Akins 2007). Additionally, the obligate sponge-dwelling *Risor ruber* has a highly modified feeding morphology and the species from this clade have two to four tusk-like canines that project outward from the upper and lower jaw. While it is unclear as to how these dentitions are used, this trait sets *R. ruber* apart from the rest of Gobiidae, as far as we know, so we also considered it to be a specialist (Tyler and Böhlke 1972).

Most of our cleaner assignments followed Côté and Soares (2011) with a few exceptions. Recent studies on *E. prochilos* and the yellow-blue color morph of *E. evelynae* (hereafter *E. evelynae* YB), suggest that these species may be facultative cleaners, rather than dedicated cleaners (Whiteman and Côté 2002; White et al. 2007). We agreed with the assignment for *E. prochilos*, due to the variation in cleaning behavior among sympatric coral and sponge-dwellers (Whiteman and Côté 2002). However, we opted to identify *E. evelynae* YB as a putative dedicated cleaner because the observed facultative cleaning behavior appears to be restricted to a single locality (White et al. 2007; Côté and Soares 2011). We also assigned *E. phthiophagus*, *E. pridisii*, and *E. randalli* as facultative cleaners because they are all similar to *E. prochilos*, in that they live on both coral and sponge and likely demonstrate differences in cleaning behavior based on microhabitat (Gasparini and Floeter 2001; Francini-Filho and Sazima 2008; Luiz Rocha and Raphael Macieira, pers. comm.). Furthermore, *E. xanthiprora* has traditionally been considered to be a non-cleaning sponge-dweller, but Victor and Krasovec (2018) observed facultative cleaning among individuals of this species from North Carolina and the Gulf of Mexico. Therefore, we considered this species a facultative cleaner. This resulted in six species,

with three additional color morphs, of dedicated cleaners and 11 species of facultative cleaners.

INFERRING THE EVOLUTIONARY HISTORY OF CLEANING IN GOBIES

There is currently no published goby phylogeny that incorporates all of the cleaner species and their respective color morphs. Therefore, we used published sequence data to infer the phylogenetic relationships of 36 species of gobies from the *Gobiosoma* subgroup of the tribe Gobiosomatini, and their respective color morphs for a total of 40 unique terminal taxa. Most of the sequences originated from Taylor and Hellberg (2005), which is the most comprehensive phylogeny of *Elacatinus* and *Tigriogobius* to date. We combined their sampled loci, which included two mitochondrial loci (*cyt b* and a segment spanning the two tRNAs, tRNA^{Glu}, and tRNA^{Pro}) and two nuclear genes (*rho* and *rag1*), with mitochondrial *coi* sequences from Weigt et al. (2012) and Victor (2014). A few supplemental sequences from other studies were also downloaded from GenBank (AF391348, GU224409, GU908158, HQ909467, KY781699, KY781700, KY781619, KY78161920, MF169003, and MF169006). The sequences were aligned in Geneious R10 (<http://www.geneious.com>); the full alignment was 4394 bp long. We used PartitionFinder2 (Lanfear et al. 2016) to select the best-fit substitution models and partitioning schemes using the “greedy” algorithm (Lanfear et al. 2012). The phylogeny was inferred using Bayesian phylogenetic inference in MrBayes 3.2 (Ronquist et al. 2012) using two parallel Metropolis-coupled Markov chain Monte Carlo (MCMC) runs, each consisting of four chains. The analysis was run for 10 million generations sampling trees every 1000 generations. We conservatively discarded the first 10% of trees as burn-in, and the remaining 9000 post burn-in trees from both runs were summarized into a single majority-rule consensus (MRC) tree.

We used BEAST 2 (Bouckaert et al. 2014) to estimate branch lengths and divergence times, with an uncorrelated, log normal, relaxed molecular-clock model. Because no fossil calibration points exist for our taxa of interest, we calibrated the molecular clock model using node ages derived from the most recent and comprehensive time-calibrated phylogeny of Gobiidae to date (Tornabene et al. 2016). That study primarily used molecular data from Agorreta et al. (2013) supplemented with additional species of Gobiosomatini, and two gobioid fossils and one geological calibration point. Based on the node age estimates and 95% highest posterior density intervals from that study, we set a prior on the most recent common ancestor of *Gobiosoma* and *Elacatinus* (root of our tree) as a normal distribution centered at a mean age of 28 million years with a 95% interval of 20–36.1 million years ($\sigma = 4.15$, offset = 28.0). A second calibration point was set at the most recent common ancestor of *Elacatinus* as a normal distribution with a mean of 17.3 million years and a 95% interval of 8.28–26.3 million years

($\sigma = 4.6$, offset = 17.3). To ensure the BEAST analysis would converge, we disabled the topology-estimating MCMC operators and ran the analysis using a fixed topology. The MRC tree from the MrBayes analysis was made ultrametric using the “chronopl” R function in the *ape* package (Paradis and Schilep 2018), to assign approximate node ages using a semiparametric method based on penalized likelihood (Sanderson 2002). This tree was then used as our starting tree topology for the BEAST analysis. We used the same partitioning scheme and substitution models that were used for MrBayes as well as a birth–death speciation/extinction prior. The analysis was run for 10 million generations and node ages and branch lengths were sampled every 1000 generations. After discarding first 10% of trees as burn-in, the remaining 9000 trees were summarized into a single maximum clade credibility (MCC) tree. We also retained a random sample of 100 post-burn-in trees to use for estimating the number of transitions in feeding behavior.

To infer the frequency of cleaning transitions across the evolutionary history of gobies, we used stochastic character mapping of discrete traits via SIMMAP (Bollback 2006). We used the “make.simmap” function in the *phytools* package in R (Revell 2012). All of the species and color morphs included in the phylogeny were also used in the SIMMAP analysis. We first performed a SIMMAP on the single MCC tree produced by the BEAST analysis, with 1000 simulations. For this analysis, we synonymized the non-cleaning generalists and specialists to reduce the number of character states. We ran the SIMMAP with a symmetrical rates model that prevented direct transitions from non-cleaning to dedicated cleaning. This restriction was considered to be more biologically relevant as it is unlikely that non-cleaners can instantaneously become dedicated cleaners without first transitioning through facultative cleaning. We also set a prior on the root node that defined it as a non-cleaner. To estimate the number of state transitions while accounting for uncertainty in our estimates of branch lengths and node ages, we employed a separate SIMMAP analysis with the three character states simulated on a subset of 100 post-burn-in trees from the BEAST analysis. Each SIMMAP analysis was performed with 100 simulations per tree (100 trees \times 100 simulations = 10,000 total simulations) and modeled with the same restricted symmetrical rates matrix and root node prior.

MORPHOLOGY VERSUS CLEANING: SPECIMEN ACQUISITION AND IMAGING

We compared the feeding morphology of 37 terminal taxa in our tree, and three additional species of *Elacatinus* for which genetic data were not available. All examined specimens were acquired from museum collections. Individuals were sexed based on the shape of their urogenital papilla, and at least one male and one

female were acquired for most species. A full list of examined lots can be found in the Supporting Information Table S2.

To visualize the skeletal anatomy of each specimen, we used a combination of clearing and staining, and micro-computed tomography (μ CT) scanning. Some of the museum specimens were previously cleared and stained. However, we cleared and double stained additional specimens for cartilage and bone following Dingerkus and Uhler (1977). To increase our morphological sample size, we μ CT scanned approximately 150 additional specimens due to the rarity of certain species and irreversible nature of clearing and staining. Scanning was conducted at the Karel F. Liem Bio-Imaging Center at the University of Washington owned Friday Harbor Laboratories, on a Bruker Skyscan 1173. Each fish was scanned at 65 kV, 123 μ A, 1150 or 1170 ms exposure time, and with a voxel size between 7.1 and 20.5 μ m. All scans were uploaded online and made publicly accessible on Morphosource.org (Media numbers: M20285, M20286, M43910-M43939, M43941-M43944, M43946, M43947, M44013-M44050, M44061-M44080, M44172-M44204, and M53025-M53028).

MORPHOLOGY VERSUS CLEANING: MORPHOMETRICS AND TOOTH MORPHOLOGY

To compare the cranial morphology and jaw mechanics of cleaners and non-cleaner gobies, we measured nine linear traits for 36 taxa ($n = 1$ –8 specimens per taxa). Lateral, dorsal, and ventral view images were taken for each cleared and stained specimen using a motorized Zeiss SteREO Discovery V20 stereomicroscope with an attached AxioCam digital camera and imaging workstation. The same was done for each μ CT specimen using the digital segmentation program Horos (The Horos Project 2015). We used ImageJ to measure the following traits to the nearest 0.01 mm: (1) head length from the tip of the snout to the posterior margin of the operculum, (2) head depth, measured at a vertical through the anterior tip of the sphenotic, (3) head width, or the maximum width of the frontals, (4) lower jaw length, (5) jaw closing in-lever from the jaw joint to the adductor muscle attachment point on the coronoid process, (6) jaw closing out-lever from the jaw joint to the tip of the anterior most tooth, (7) maxilla length, (8) premaxilla length, and (9) ascending process length (Fig. 1). The ratio of in-lever over out-lever was used to calculate mechanical advantage and quantify the trade-off between jaw closing speed and jaw closing force in the closing lever mechanism of the lower jaw. Standard length was also measured from the preserved specimens using a digital caliper to the nearest 0.01 mm.

We also classified and compared the tooth morphology of each specimen as either homodont or heterodont. Homodonty was defined as a row or rows, of same-sized, uniformly spaced teeth (Fig. 2). In contrast, heterodonty included any unevenly distributed teeth that varied in size (Fig. 2). We also recorded the presence or absence of enlarged recurved canines

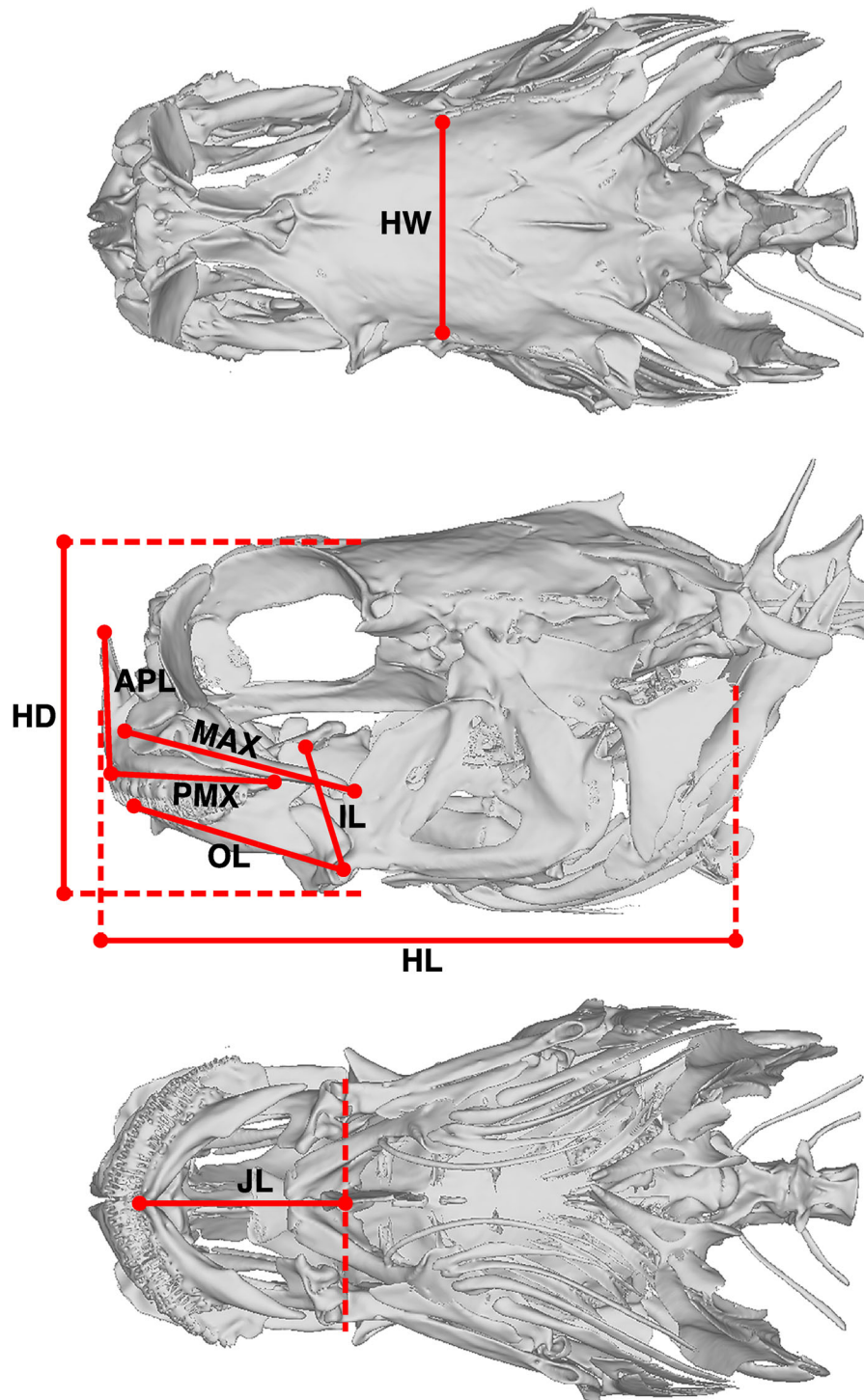


Figure 1. An example of the linear nine measurements taken from the cleared and stained specimens, and μ CT scans. Shown is a μ CT scan of the dedicated cleaner and neon goby, *Elacatinus oceanops*. Trait abbreviations are as follows: APL, ascending process length; HD, head depth; HL, head length; HW, head width; IL, closing in-lever; JL, jaw length; MAX, maxilla length; OL, closing out-lever; and PMX, premaxilla length.

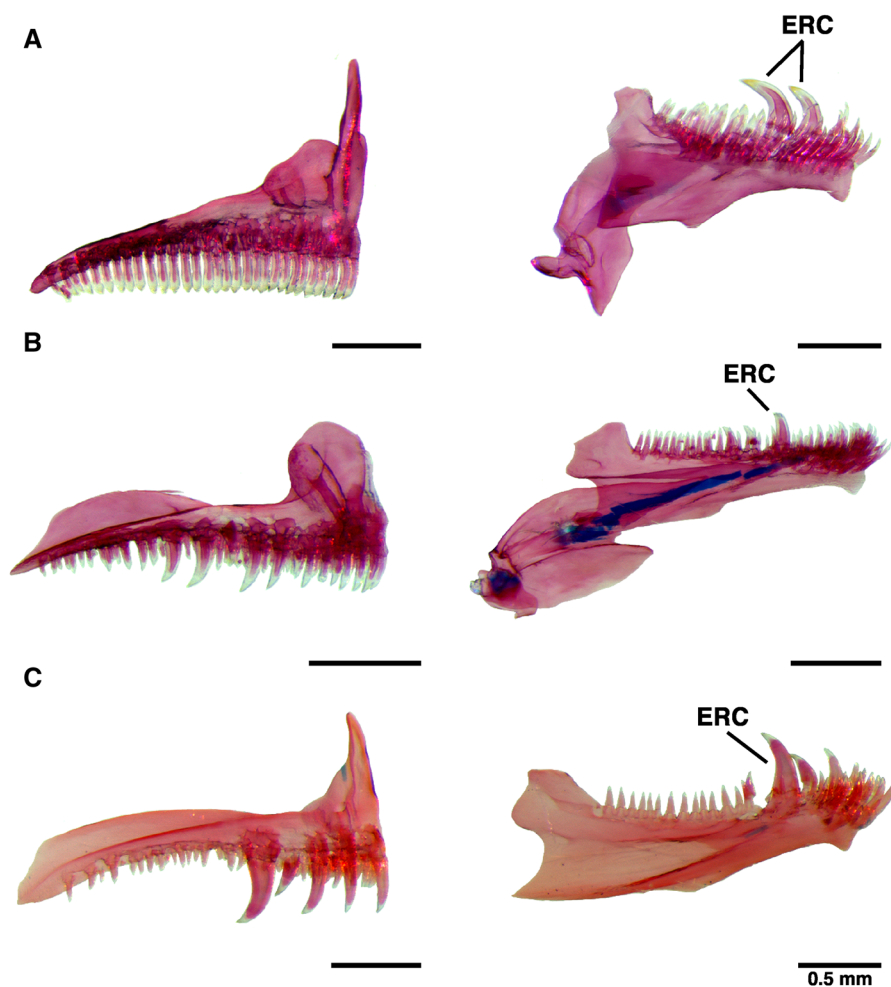


Figure 2. Lateral images of the premaxilla and dentary from a (A) male *Elacatinus evelynae* YB (USNM 198662), (B) male *Elacatinus colini* (USNM 360562), and (C) male *Tigrigobius janssi* (LACM 32524-46) showing type one (A) and type two (B and C). The arrows indicate the sexually dimorphic enlarged recurved canines (ERC) that were not considered when characterizing tooth morphology.

in the upper or lower jaw of each specimen (Fig. 2). However, these were determined to be a sexually dimorphic trait, present in males and absent in females, with no obvious associations with feeding ecology. As such, these canines were not considered when characterizing overall tooth morphology. Moreover, Bohlke and Robins (1968) observed sexually dimorphic and same-sex differences (beyond the enlarged canines) in overall tooth morphology for a few species of facultative cleaners and so we paid close attention to any intraspecific variation in dentition.

Additionally, we used 2D geometric morphometrics to quantify head shape and mouth position for the same 36 terminal taxa and additional four species. Left facing lateral images were taken from preserved alcohol specimens ($n = 1-6$ per species) in a glass squeeze-box using a Nikon D90 digital camera. Published type photos for *E. jarocho* and *E. phthiophagus* were also added into the data set (Taylor and Akins 2007; Sazima et al. 2008). Specimens were considered suitable for analysis if there was minimal

distortion and their jaw was fully closed. These criteria reduced our available sample size, so we were unable to acquire both male and female data for many species. We digitized nine fixed landmarks and 12 sliding semi-landmarks that captured overall head shape using the program *tpsDig2* (Rohlf 2006). In contrast to fixed landmarks, the semi-landmarks were placed equidistant from each other along two curves and allowed to slide along the curves to minimize bending energy. We used two sets of semi-landmarks, six sliding landmarks per curve, to capture the slope of the snout and the ventral side of the head, respectively (Fig. 3).

MORPHOLOGY VERSUS CLEANING: PHYLOGENETIC COMPARATIVE METHODS

The variation in cranial morphology was visualized in a morphospace with the phylogeny overlaid—a phylomorphospace (Sidlauskas 2008). Before this was done, we natural log-transformed each measurement and removed the effect of size

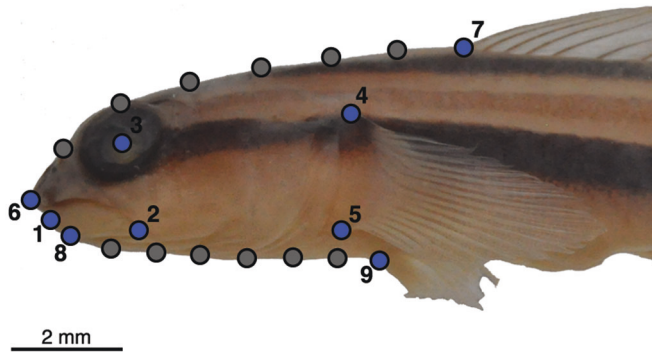


Figure 3. Lateral image of *Elacatinus illecebrosus* Y (UF 143030) with the landmarks used for geometric morphometrics. Fixed landmarks are in blue and sliding semi-landmarks are in grey. The fixed landmarks are as follows: (1) middle of the upper lip, (2) jaw joint, (3) center of the eye, (4) top of the pectoral fin base, (5) bottom of the pectoral fin base, (6) tip of the snout, (7) base of the first dorsal fin ray, (8) tip of the lower jaw, and (9) base of the pelvic fin. Two semi-landmark curves were used to capture the slope of the snout and the ventral side of the head, respectively.

by regressing each trait against standard length. The residuals of each linear regression were used as the new size-corrected measurements, with the exception of mechanical advantage, an already dimensionless and effectively size-corrected trait. Because comparative phylogenetic analyses require a single trait value per species, we then averaged the measurements by sex and then by species, to account for disproportionate sampling for males and females. The average species trait data were used to perform a phylogenetically corrected principal component analysis (pPCA) with a covariance matrix. This was done with the “*phyl.pca*” function from the *phytools* package (Revell 2012). For this analysis, we trimmed the single MCC BEAST tree to include only the examined taxa. However, two of the examined species were not present in the phylogeny (*E. serranilla* and *E. tenox*), and thus were not included in the pPCAs. We therefore conducted an additional non-phylogenetic principal component analysis that included these species (Supporting Information Fig. S1).

We also used a phylomorphospace to visualize the variation in head shape. We scaled, rotated, and aligned the geometric morphometric landmark data in a generalized Procrustes analysis using the “*gpgen*” function from the *geomorph* package (Adams et al. 2016). Procrustes coordinates for conspecifics were then averaged first by sex and then by species. We performed a pPCA (Revell 2012) with a covariance matrix on the average species Procrustes coordinates. The MCC BEAST tree was pruned again to include the additional taxa examined in this analysis. We also performed a non-phylogenetic PCA that included the examined taxa not in the phylogeny (*E. jarocho*; *E. seranilla*; *E. tenox*) (Supporting Information Fig. S2).

TESTING THE LINK BETWEEN CLEANING AND MORPHOLOGY

To test for the association between cleaning behavior and cranial morphology, we compared mean trait values between trophic groups using phylogenetic ANOVA. Because we were primarily interested in assessing how cleaners differ from trophic generalists, the non-cleaning specialists (*E. atronasus*, *G. novemlineatus*, *R. ruber*) were omitted from these analyses. We ran these analyses with subsequent post hoc pairwise tests using the “*phylANOVA*” function in the “*phytools*” R package (Revell 2012). Each test was performed with 1000 iterations and the Bonferroni method of correction for multiple comparisons.

To test the association between cleaning behavior and head shape, we used a phylogenetic Procrustes ANOVA and a post hoc pairwise test. The “*procD.pgls*” function in the *geomorph* package (Adams et al. 2016) was used to implement a randomized residual permutation procedure (RRPP) for significance testing with 1000 iterations with no additional corrections. The dietary groups (excluding the non-cleaning specialist) were used as the independent variables. We also performed a separate phylogenetic Procrustes ANOVA to quantify the proportion of head shape variation attributed to size, or in this case log(centroid size), as well as the interaction between size and feeding ecology.

To assess whether the evolution of cleaning behavior and tooth morphology are correlated, we used BayesTraits version 3 (Meade and Pagel 2017) to implement models of discrete binary character evolution. We ran MCMC analyses for independent and dependent models of evolution for cleaning (cleaning or non-cleaning) and tooth morphology (homodonty or heterodonty). The first model assumes that the two traits are evolving independently from one another; whereas, the latter assumes they are correlated. The significance of the dependent model was tested using Bayes factors, where values greater than 10 indicate strong evidence for correlated evolution. Because we observed same-sex intraspecific variation in dentition for males but not females, we ran these analyses twice to test for the correlation between cleaning and tooth type for each sex separately. In the rare instances where we observed intraspecific variation in tooth morphology among same-sex individuals, we opted to code tooth type as the most commonly observed state. Each analysis was performed on a set of 100 post-burn-in trees with 100 stepping stones and 10,000 iterations per stone.

ASSESSING MORPHOLOGICAL CONVERGENCE

To test whether cleaners have converged on similar morphologies, we applied the pattern-based measures of convergence as described by Stayton (2015). This method includes four metrics (C_1 – C_4) that quantify clustering in the morphospace, but we only used C_1 and C_2 to assess patterns of convergence. C_1 quantifies the degree of morphological convergence between taxa. However,

taxa that exhibit similar levels of convergence but vary in their ancestral starting points will have similar C_1 values. Therefore, the C_2 metric was used to quantify the magnitude of morphological convergence between taxa. For these tests, we specified sets of putatively convergent taxa and compared them to the non-cleaning generalists. We ran the tests three ways with the dedicated cleaners, facultative cleaners, and all cleaner species designated as convergent taxa. We used the “convratsig” function in the *convevol* R package (Stayton 2015) to apply the *convevol* tests separately to the PC scores for the first three axes of the pPCA with linear measurements, and to the PC scores for the first four axes of the head shape pPCA. The pPCA axes were retained using a visual Cattell scree test. To assess significance, “convratsig” compares the observed patterns of morphology to a distribution of 500 generations of trait data simulated across the phylogeny under Brownian motion.

Results

EVOLUTION OF CLEANING IN GOBIES

The stochastic character mappings performed on the single MCC BEAST tree and post-burn-in trees indicated that transitions from non-cleaning to facultative cleaning occurred four times. The transitions most likely occurred along the branches leading up to (1) the clade of cleaner *Tigrigobius* species, (2) *E. puncticulatus*, (3) *E. xanthiprora*, and (4) the *E. randalli* and *E. evelynae* clade (Fig. 4). A less likely second scenario inferred a fifth transition towards cleaning, with a subsequent loss of cleaning along the same branch, but the branch where these transitions occurred varied between simulations. In a third scenario, cleaning evolved in the ancestor of *Elacatinus*, with a subsequent loss of cleaning on the branch leading up to the *E. horsti* and *E. xanthiprora* clade, and a second cleaning transition in *Tigrigobius*. However, the first scenario was considered to be the most likely case scenario because it was observed most often across simulations (Supporting Information Fig. S3). Meanwhile, reversals from facultative cleaning back to non-cleaning were very rare in simulations. The SIMMAP analyses also suggested a scenario that involved a single transition from facultative cleaning to dedicated cleaning along the branch leading up to the ancestor of the *E. illecebrosus* and *E. evelynae* clade (Fig. 4). Dedicated cleaning was then lost in the facultative cleaner, *E. prochilos*, and the planktivore, *E. atronatus*.

THE LINK BETWEEN CLEANING AND MORPHOLOGY

The cranial variation between dedicated cleaners, facultative cleaners, and non-cleaners was best described by lower jaw length, maxilla length, premaxilla length, and head length. In general, the dedicated cleaners occupied areas in the phylomorphospace associated with smaller size-corrected trait values relative to the non-cleaners. The facultative cleaners exhibited intermediate trait val-

ues that fell in between the dedicated cleaners and non-cleaners. The first three PC axes of the pPCA performed on the cranial measurements accounted for 87.3% of the total variation (Fig. 5; Supporting Information Table S3). PC 1 explained 53.8% of the total variation and loaded strongly for jaw length, premaxilla length, head length, and maxilla length. PC 2 explained 19.6% of the total variation and loaded for head width, ascending process length, and head depth. PC 3 explained 13.7% of the total variation and primarily accounted for mechanical advantage, ascending process length, head width, and maxilla length.

The phylogenetic ANOVA revealed that cleaning behavior was associated with head length, head depth, jaw length, and mechanical advantage. The dedicated cleaners exhibited distinctly shorter heads ($t = 6.922$, $P = 0.021$) and shallower heads ($t = 7.893$, $P = 0.009$) than the non-cleaners. The dedicated cleaners also possessed significantly shorter jaws than the facultative cleaners ($t = 7.672$, $P = 0.003$) and the non-cleaners ($t = 11.794$, $P = 0.003$). By contrast, the dedicated cleaners possessed significantly greater mechanical advantage than the facultative cleaners ($t = 4.725$, $P = 0.018$) and non-cleaners ($t = 5.984$, $P = 0.048$). All remaining comparisons were not significant.

Nearly all cleaners had the homodont dentition, while non-cleaners exhibited the heterodont dentition (Fig. 6). One exception was the homodont dentition found in female *E. atronatus*, a planktivore. Moreover, the facultative cleaners *E. puncticulatus* and *E. xanthiprora* were the only cleaner species where neither the male nor female possessed homodont dentition. We consistently observed homodonty in all other female cleaners, but in some cases the cleaner males possessed a heterodont tooth morphology. Males of the facultative cleaner *E. randalli* only possessed heterodont morphologies, but in some species tooth morphology varied between conspecifics. Individual males of the cleaner species *E. figaro*, *E. lobeli*, *E. prochilos*, *T. digueti*, and *T. inornatus* possessed either heterodont or homodont dentition, which also corresponded with the presence or absence of enlarged recurved canine and a cranial morphology more similar to that of the generalist and dedicated cleaners, respectively. For male *E. lobeli*, *T. digueti*, and *T. inornatus*, homodonty was more commonly observed, but for *E. figaro* and *E. prochilos*, heterodonty was more commonly observed. For all other species, the heterodont tooth morphology was consistently observed in both the males and females. Furthermore, the homodont morphology varied somewhat between cleaner *Elacatinus* and cleaner *Tigrigobius*. In *Elacatinus*, the premaxilla has a single row of teeth, while the dentary has three to four distinct rows. By contrast, cleaner *Tigrigobius* possess four distinct rows in both the upper and lower jaw. The BayesTraits analyses indicated strong support for the correlated evolution between cleaning behavior and homodonty in both males (Bayes factor = 14.34) and females (Bayes factor = 15.83).

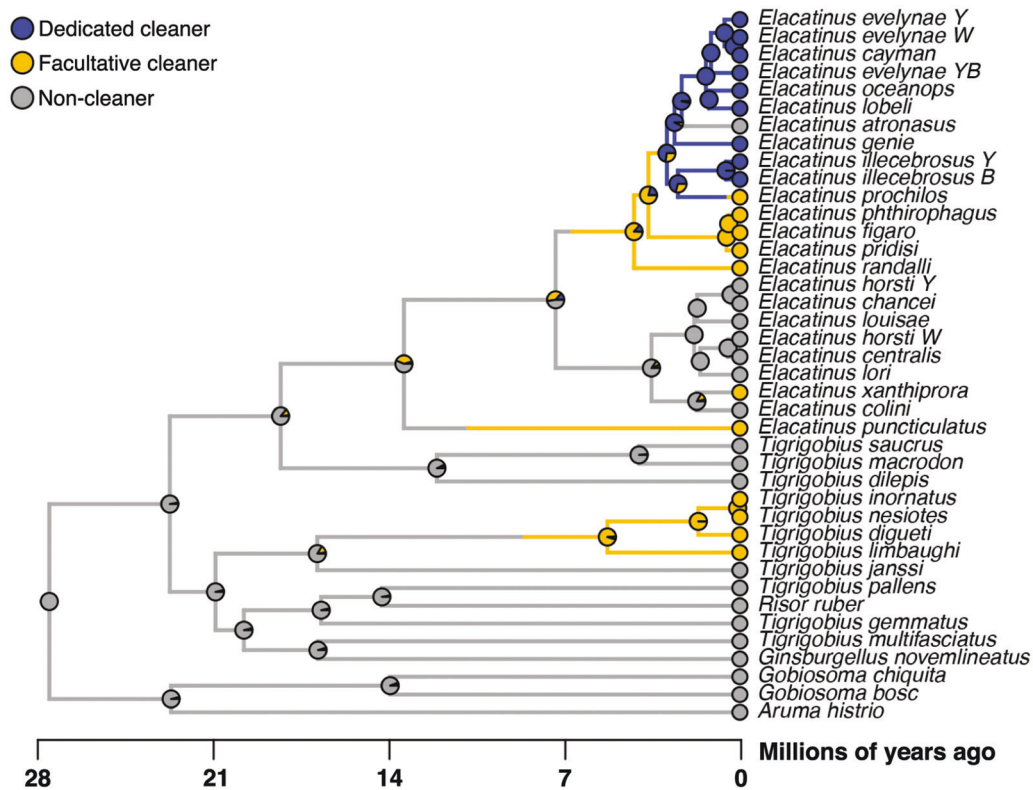


Figure 4. The MCC tree showing the evolutionary history of cleaning from a single SIMMAP simulation. The circles at each tip represent the trophic ecology of the extant taxa, while the pies represent the estimated posterior probabilities of each character state. Colors along the branches depict the simulated evolution of cleaning and the topological transitions between states.

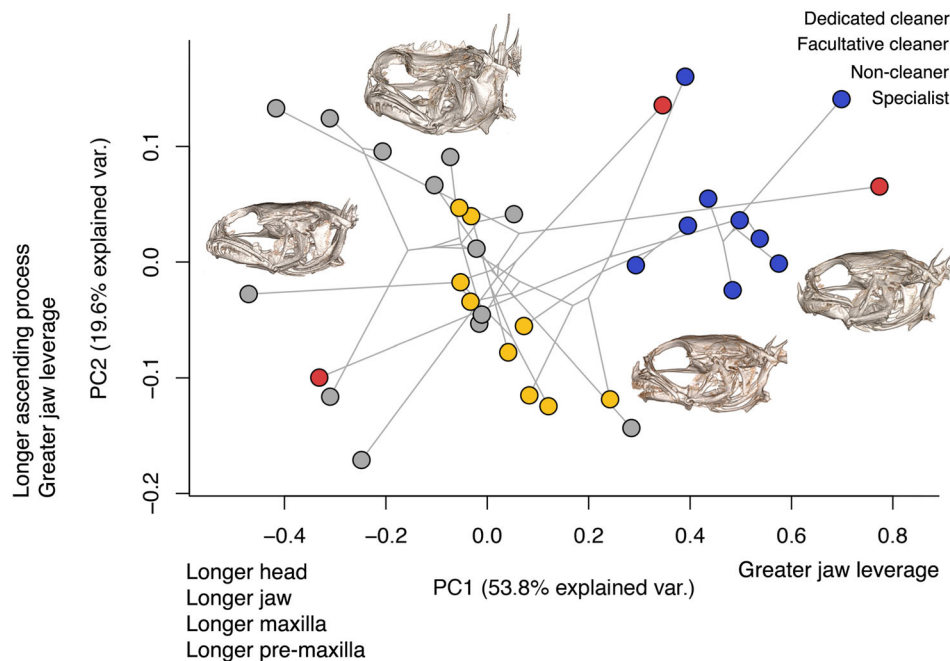


Figure 5. A phylomorphospace showing the variation in cranial morphology. The analysis was performed on the linear measurements taken from the μ CT scans and cleared and stained specimens. Each point represents the averaged data for a single species colored by its trophic group. Example skulls are also included to show the morphology variation on each end of PC 1 (left: *Tigrigobius janssi*, top: *Elacatinus horsti* Y, right: *Elacatinus evenlynnae* W, bottom: *Elacatinus figaro*).

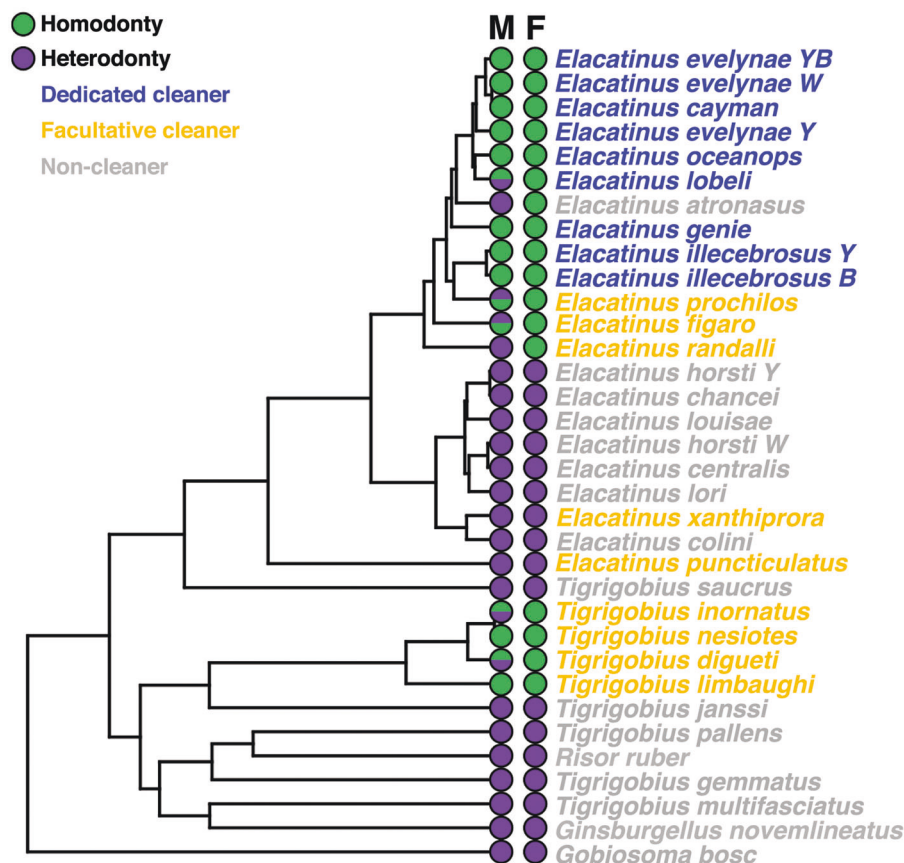


Figure 6. A phylogram showing the distribution of tooth types found in the males and females of each species. Green circles represent type one (homodonty) and purple circles represent type two (heterodonty). Multicolored circles indicate same-sex intraspecific variability. Tip labels are colored by diet groups. Note the strong correlation between cleaning and the type one tooth morphology.

Dedicated cleaners differed in head shape from the facultative cleaners by exhibiting a more inferior mouth, whereas a few facultative cleaners exhibited a subterminal, or less extreme downturned mouth. All other facultative cleaners possessed a more terminal mouth. The first four PC axes of the geometric morphometric pPCA accounted for 85.4% of the total head shape variation (Fig. 7). PC 1 explained 48.6% of the total shape variation and was associated with a stouter head in the negative direction, and a longer and shallower head in the positive direction. PC 2 explained 21.8% of the total shape variation and was associated with mouth position. A terminal (forward-facing) mouth was associated with the negative direction, while a more inferior (downturned) mouth was associated with the positive direction. PC 3 explained 8.4% of the total shape variation and was associated with a longer lower jaw in the negative direction and a shorter lower jaw in the positive direction. PC 4 explained 6.5% of the total shape variation and was associated with a smaller and more inferior mouth in the negative direction, and a larger more terminal mouth in the positive direction.

The phylogenetic Procrustes ANOVA did not detect a significant association between feeding ecology and head shape

($F = 0.866$, $P = 0.921$), which only accounted for 3.0% of the total shape variation. No pairwise comparisons were statistically significant. However, a separate Procrustes ANOVA found that size had a significant effect on head shape and accounted for 30.1% of the variation ($F = 16.705$, $P = 0.001$). The analysis also found a significant interaction between size and feeding ecology ($F = 4.54$, $P = 0.002$), which accounted for 16.4% of the variation.

MORPHOLOGICAL CONVERGENCE OF CLEANER GOBIES

The convergence tests performed on the cranial measurements detected low levels of convergence, or phenotypic similarity, for dedicated cleaners ($C_1 = 12.7\%$, $P = 0.471$), but a significant amount of change attributed to convergent evolution ($C_2 = 0.031$, $P = 0.004$). Similar results were found for all cleaners in the cranial morphospace ($C_1 = 18.9\%$, $P = 0.244$; $C_2 = 0.082$, $P = 0.048$). However, the analysis did detect high levels of morphological convergence among facultative cleaners ($C_1 = 36.1\%$, $P = 0.001$), and a significant amount of convergent evolution ($C_2 = 0.125$, $P = 0.018$). In contrast, the convergence tests performed on the head shape data detected low levels of phenotypic

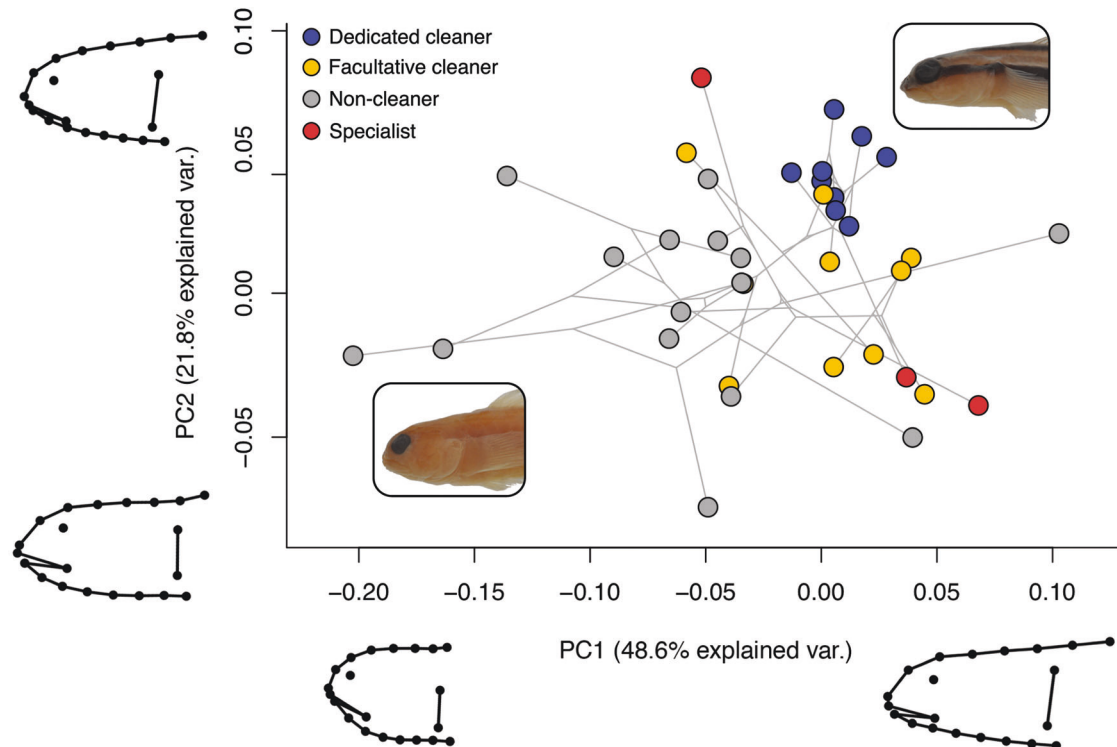


Figure 7. A phylomorphospace showing the variation in head shape. The pPCA was performed on shapes coordinates produced by a generalized Procrustes superimposition analysis. Each point represents the averaged data for a single species colored by its trophic group. The average head shape on either ends of each PC axis is shown with the reference point plots. Example head shapes are also included to show difference between inferior (top: *Elacatinus illecebrosus* Y) and terminal (bottom: *Elacatinus horsti* Y) mouth positions.

similarity and convergent evolution for all sets of potentially convergent taxa. The dedicated cleaners showed an average of 9.95% ($P = 0.383$) convergence and a C_2 value of 0.004 ($P = 0.124$). The facultative cleaners showed an average of 22.1% ($P = 0.064$) convergence and a C_2 value of 0.019 ($P = 0.070$). All cleaners showed an average of 17.6% ($P = 0.146$) convergence and a C_2 value of 0.015 ($P = 0.072$).

Discussion

CO-EVOLUTION OF CLEANING AND MORPHOLOGY IN GOBIES

We found strong evidence for four independent origins of cleaning within Gobiidae and three independent origins within *Elacatinus* alone. This contradicts a previous empirical hypothesis that states that cleaning may have evolved once in the ancestor of the genus and secondarily lost in a clade of sponge-dwellers (Taylor and Hellberg 2005; Côté and Soares 2011). The facultative cleaning behavior of *E. xanthiprora* was unknownst at the time but with it, both scenarios are equally parsimonious if we consider losses and gains of cleaning behaviors to be equally likely. Furthermore, our findings suggest that cleaning may be a more unidirectional evolutionary pathway where both dedicated

and facultative cleaner gobies rarely revert back to an entirely non-cleaning generalist state. The only deviation from cleaning that we found occurs with *E. atronasus*, where cleaning behavior transitioned toward planktivory. We interpret this transition as an additional step in the progression toward trophic specialization, rather than a true reversal or loss. Only two species of *Elacatinus* are planktivorous (*E. atronasus* and *E. jarocho*), which also live more epibenthic/pelagic lifestyles compared to their congeneric relatives (Taylor and Akins 2007).

Our data support the hypothesis that greater trophic specialization evokes more substantial and finely tuned phenotypic modifications (Ferry-Graham et al. 2002). With each origin of cleaning, species in the genera *Elacatinus* and *Tigrigobius* evolved similar morphological feeding adaptations that differed from their non-cleaning relatives. While our findings indicate that all cleaners have converged on similar changes in morphology, the dedicated cleaners did exhibit greater morphological specialization than the facultative cleaners, with their shorter jaws and greater mechanical advantage. The facultative cleaners deviate less from the non-cleaners in the morphospace and possess an intermediate or transitional form that reflects their more flexible feeding habits. Furthermore, a few species also vary intraspecifically (between sexes and within males) and exhibit both the archetypal cleaning

and generalist tooth morphologies. Interestingly, we recovered strong phenotypic convergence among the facultative cleaners despite their multiple evolutionary origins, suggesting that less specialized cleaning does not weaken the selective signals that shape morphology.

MORPHOLOGY OF CLEANER GOBIES

Our quantitative analysis of head shape in cleaner gobies revealed shape differences between trophic guilds that align well with the ecomorph assignments made by Colin (2010) and revised by Victor (2014). These head shapes correspond to inferior, subterminal, and terminal mouth positions—the definitions of which have been ambiguous in the literature. The inferior mouth in dedicated cleaners has long been suspected to represent a highly derived cleaning adaptation, which is typically associated with transitions toward feeding on more benthic, or sessile prey (Colin 1975). The behavior of cleaner gobies supports this trend because cleaners lie on the surface of their clients during cleaning interactions (Côté and Soares 2011). With this in mind, the degree of mouth modification also appears to vary with the degree of trophic specialization among facultative cleaners. For example, *E. phthirophagus* is a facultative cleaner with a subterminal mouth and partakes in longer and more frequent cleaning interactions compared to the closely related *E. figaro* or some of the facultative *Tigriobius* spp., which all have terminal mouths (Sazima et al. 2000; Francini-Filho and Sazima 2008; Quimbayo and Zapata 2018).

While skull morphology varied with the different levels of cleaning, a distinct tooth morphology was prominent across nearly all cleaner species, which we propose is specialized for scraping parasites off clients. Functionally, cleaning involves the dislodging of firmly attached objects from a substratum. This action is enacted by fishes that specialize in scraping algae or detritus off rocks and possess similar stockade-like dentitions (Parenti and Maciolek 1993; Streelman and Albertson 2006; Bellwood et al. 2014; Hundt and Simons 2018). Freshwater stream gobies of the *Stenogobius* group (formerly subfamily Sicydiinae; Thacker and Roje 2011; Agorreta et al. 2013) are exemplar analogs to dedicated cleaners due to their tightly packed rows of elongate teeth and inferior mouths (Parenti and Maciolek 1993). In both cleaners and stream gobies, each row of tightly packed teeth likely serves as a single scraping or cutting edge. However, instead of foraging on sticky biofilm and filamentous algae (Mochizuki and Fukui 1983), cleaners pursue prey items that are not so easily removed. Dedicated cleaners overcome this mechanical challenge by coupling their scraping dentition with increased jaw leverage to create a formidable tool for removing ectoparasites and the occasional fish scales (Whiteman and Côté 2002; personal observation). Meanwhile, the cleaner dental pattern is widely different from that of generalist gobies, which have large, widely spaced

canines that are well suited for grasping and or trapping more elusive prey.

CONSTRAINTS ON A CLEANER MORPHOTYPE

Previous attempts to unify cleaners across families as a single ecological guild based on body shape, coloration, and size have faced mixed results, and we suspect that grouping by feeding morphology is no different (Côté 2000; Arnal et al. 2006; Baliga and Mehta 2019). In fact, we posit that cleaning behavior is another instance of a putatively narrow niche that encompasses a broad range of taxa that exhibit diverse cleaning morphologies and behavioral strategies (Kolmann et al. 2018). Within Gobiidae, we found a strong relationship between functional morphology and cleaning, but these exact patterns are not representative of other cleaner fishes. For instance, cleaner wrasses (dedicated, facultative, and juvenile) are all converging on a “picking” morphology that trades jaw leverage for faster jaws and is different from the scraping morphology of cleaner gobies (Wainwright et al. 2004; Baliga et al. 2017). Given the subtle behavioral and ecological differences between cleaner fishes, it seems unreasonable to expect cleaners to converge on a single feeding morphotype (Côté 2000; Vaughan et al. 2017). While cleaner gobies lie on their clients, cleaner wrasses and other lineages of cleaner fishes remain suspended next to their hosts in the water column (Côté 2000). Cleaner fishes also differ in their preferred ectoparasites (Grutter 2002). While many cleaner species, including wrasses, consume a wide range of parasitic arthropods and leeches, gobies feed on smaller range of parasites (mostly gnathiid isopod larvae) that may pose different functional challenges and warrant different morphological adaptations (Arnal and Côté 2000; Grutter 2002).

In addition, evolutionary history and phylogenetic constraint may play equally important roles in determining the degree of phenotypic convergence between cleaning clades (Poulin and Grutter 1996; Futuyma 2010; Losos 2011). In terms of feeding morphology, gobies and labrids have very different ancestral starting points. Labrids process prey with their highly modified pharyngeal jaws, which liberates their oral jaws and allows them to specialize in prey capture (Westneat 1995; Wainwright et al. 2004). Meanwhile, most gobies have poorly developed pharyngeal jaws that might influence how their oral jaws are shaped in response to similar selective pressures. Furthermore, the two lineages differ in their ancestral feeding states because while cleaner gobies derived from trophic generalists, many cleaner labrids transitioned from one specialized feeding behavior (corallivory) to another (Cowman et al. 2009). Other cleaners like the leatherjacket, *Oligoplites saurus*, were pre-adapted for juvenile scale-feeding prior to cleaning and exhibit morphologies adapted for such (Lucas and Benkert 1983). It is highly likely that different pre-adaptive cranial traits and cleaning origins have prevented the morphological and

functional overlap between clades. However, in the case of facultative cleaner gobies, their shared ancestral morphologies and ecologies have allowed selection to shape their evolutionary trajectories in similar manners to produce nearly identical phenotypes. This underscores the importance of considering evolutionary history when evaluating the associations between ecological and morphological specialization.

AUTHOR CONTRIBUTIONS

J.M.H. and L.T. conceived the study. J.M.H. and L.T. provided financial support. J.M.H. collected the data. J.M.H. led the micro-computed tomography scanning and data collection. J.M.H. and C.T. analyzed the data with input from L.T. J.M.H. wrote the manuscript with crucial contributions from C.T. and L.T.

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DATA ARCHIVING

The raw data and R scripts used in this study can be found on Dryad (<https://doi.org/10.5061/dryad.qbzkh18d4>). The CT scans are available at MorphoSource.org.

CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of cleaner goby species and their respective color morphs, their degree of cleaning behavior, and cited text.

Table S2. List of examined species and respective museum lot numbers used in this study

Table S3. Loadings for the principal components axes from the pPCA performed on the cranial measurements. Bolded numbers indicate traits that are strongly associated with each PC axis.

Figure S1. A non-phylogenetic principal component analysis plot showing the variation in cranial morphology for all of the examined species.

Figure S2. A non-phylogenetic principal component analysis plot showing the variation in head shape for all of the examined species.

Figure S3. Barplots showing the frequency for the number of cleaning transitions observed across SIMMAP simulations performed on A) the single MCC BEAST tree and B) the 100 post-burn-in BEAST trees used to account for variation in branch lengths. In both analyses a scenario with four independent origins of facultative cleaning was the most common.