THE EVOLUTIONARY ECOLOGY OF VENOMOUS CATFISHES, WITH A FOCUS ON MEMBERS OF THE NORTH AMERICAN FAMILY ICTALURIDAE (TELEOSTEI: SILURIFORMES)

by

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ABSTRACT

The venom glands of fishes represent a widespread, putatively adaptive antipredatory trait, which have received little attention relative to those of other venomous organisms. I review the literature for ecologically and evolutionarily relevant information regarding venomous fishes and find poor support for an adaptive hypothesis, due to the lack of empirical evidence for an antipredatory function of venom glands in most groups of venomous fishes. Additional information, including phylogenies for venomous groups, the phylogenetic distribution of venom glands within these groups, intra- and interpopulational variation in the presence and/or structure of venom glands, and the functional design of venom constituents is also found to be lacking. Using histological and toxicological assays, I demonstrate that 1250-1625 catfish species should be presumed to be venomous, nearly doubling estimates of venomous fish species diversity. Interfamilial comparisons of venom toxicity and composition indicate that a broad range of venom activities and constituents exist in catfish venoms, which character optimization analyses suggest has resulted from a limited number of independent evolutionary origins of venom glands in the order Siluriformes. Behavioral experiments demonstrate that the venom glands of the tadpole madtom (*Noturus gyrinus*) provide a significant antipredatory advantage relative to individuals lacking venom glands, supporting an adaptive hypothesis for venom glands in catfishes. The broader examination of venom toxicity and composition in 22 ictalurid species reveals significant variation in levels of

venom toxicity, which appears to demonstrate correlated evolution with several aspects of ictalurid life history. Additional experiments demonstrate that the presence of venom glands can also influence the evolution of other aspects of a catfish species' biology. A model predatory species (*Micropterus salmoides*) quickly became conditioned to avoid attacking catfishes possessing the distinctive color pattern displayed by Lake Tanganyikan *Synodontis* species, and were unable to distinguish between different Tanganyikan *Synodontis* species, supporting a hypothesis of Müllerian mimicry in this group. Statistical examinations of *Synodontis* phylogenetic history and modern species' color patterns indicate that the color pattern conservatism seen in Lake Tanganyikan *Synodontis* is likely due to the protection provided by this color pattern, which is predicated on the defensive capabilities afforded by these species' venoms.

CHAPTER 1

THE VENOM GLANDS OF FISHES: A REVIEW AND DISCUSSION OF THEIR CLASSIFICATION AS ADAPTIVE TRAITS

ABSTRACT

The current state of knowledge regarding the venom glands of fishes and their products is examined in a review of morphological, pharmacological, and chemical studies of these structures. This information is investigated within the framework of several definitions of adaptive traits, in an effort to determine whether piscine venom glands should be considered to be adaptive in nature. Insufficient evidence is found to warrant the classification of the venom glands of fishes as adaptations in many cases, regardless of the criterion used, primarily due to the lack of any demonstrable evidence that these structures confer a selective advantage in their natural environment. The lack of additional information such as phylogenies for groups containing venomous taxa, knowledge of the phylogenetic distribution of venom glands within these groups, intra- and interpopulational variation in the presence and/or structure of venom glands, and the functional design of venom constituents also contribute to our current inability to confirm any type of adaptive hypothesis for these structures. Additional studies directly addressing these issues are necessary before investigations of the ecology and evolution of piscine venoms and delivery structures can meaningfully proceed.

INTRODUCTION

Venomous organisms produce toxic substances that are used in predatory, defensive, and competitive interactions with other organisms. These organisms differ from poisonous organisms in that they invariably possess specialized anatomical apparatus for the injection of the toxic compounds into the target organism, whereas poisons must be absorbed or ingested. Venomous representatives are known from several higher-level taxonomic groups, including squamates (Alagón et al., 1982; Jackson, 2003; Fry *et al.*, 2006), cnidarians (Fenner & Williamson, 1996), hymenopterans (Vetter & Visscher, 1998), lepidopteran larvae (Arocha-Piñago & Guerrero, 2001), arachnids (Coddington & Levi, 1991), mollusks (Gray et al., 1988; Bonnet, 1999), chondricthyans (Halstead, 1988), actinopterygians (Halstead, 1988; Smith & Wheeler, 2006; Wright, 2009), and even some mammals (Martin, 1981; de Plater et al., 1995). While most of these groups are the focus of many current ecological, toxicological, and evolutionary studies, the study of fish venoms in these contexts has been virtually ignored.

Species falling under the general classification of "fishes" (a paraphyletic assemblage including the classes Myxini (hagfishes), Petromyzontida (lampreys), Chondrichthyes (sharks, rays and chimaeras), Actinopterygii (ray-finned fishes), Sarcopterygii (coelacanths and lungfishes)) represent more than half of the world's known vertebrate species (Nelson, 2006). Due to the widespread use of the terms "fishes" and "piscine" in referring to this assemblage, I use them in the same manner here, despite their imprecision. A recent phylogenetic analysis of spiny-rayed fishes estimated that 585-650 of the species in this subdivision of fishes should be presumed to be venomous, a substantial increase from previous estimates of approximately 200 species (Halstead,

1988; Smith & Wheeler, 2006) (Fig. 1-1A). When other groups, such as catfishes and cartilaginous fishes are included, this estimate potentially jumps to well over 2500 species, just under 10% of all known fish species [(Wright, 2009), Fig. 1-1B]. Though this level of species diversity is much greater than that of many other venomous taxa, fish venoms have received comparatively little attention from researchers. As a particularly striking example, a recent (March 1, 2011) ISI Web of Science inquiry using the search parameters "snake venom" returned 6737 results while searching for "fish venom" yielded only 221, many of which concerned the effects of other species' venoms on fishes.

Most studies of fish venoms have focused on the presence and structure of venom glands in certain taxa and the pharmacology of particulary toxic species, with little direct examination of the ecological aspects and evolutionary history of the venoms produced or the species that produce them. This has not, however, prevented several authors from presenting hypotheses about the evolution of venom glands in fishes, and the ecological factors responsible for driving this process. Furthermore, the formulation of these hypotheses, often derived from a single line of investigation, may not account for potentially contradictory evidence produced by other areas of study.

One of the most widespread of these hypotheses, implicit in nearly all studies of fish venoms, and often explicitly stated, is that the spines and associated venom glands of fishes represent anti-predatory adaptations (Birkhead, 1967, 1972; Cameron & Endean, 1970, 1973; Magalhães et al., 2005; Bosher et al., 2006; Kiehl et al., 2006; Emmett & Cochran, 2010). Unfortunately, many of these authors provide no rigorous test for this hypothesis, nor do they acknowledge the definition of adaptation to which they subscribe.

This is not a trivial matter, as there has been much debate over the nature and prevalence of morphological adaptations (Dobzhansky, 1956; Williams, 1966; Gould & Lewontin, 1979; Bock, 1980; Gould & Vrba, 1982; Mayr, 1983; Krimbas, 1984; Sober, 1984; Baum & Larsen, 1991; Reeve & Sherman, 1993), leading to a proliferation of definitions as to what constitutes an adaptive trait (Table 1-1). A review of the existing information regarding piscine venom glands may reveal whether these structures fit one or more of the many definitions of adaptive traits that have been proposed.

The current work provides a brief review of the existing literature concerning the identification and anatomy of fish venom glands and venom delivery systems; the toxicology, pharmacology, and basic chemistry of the venoms of species that have thus far been investigated; and the few studies directly addressing the ecology and evolution of the venom systems of fishes. This information is then used to determine whether evidence exists to support the classification of piscine venom glands as adaptive in nature, as defined by the authors listed in Table 1-1. In cases where there is insufficient evidence to suggest that piscine venom glands fit a particular definition, future work that may serve to clarify the issue is suggested.

VENOM GLAND AND DELIVERY SYSTEM MORPHOLOGY

Gross Morphology

Venoms by definition require a method by which their bearer is able to introduce them into the body of a target organism. In all known venomous fishes (with the exception of *Meiacanthus* sp. and members of the deep-sea family Monogathidae), this is accomplished by spiny elements associated with the fins and/or opercular and cleithral bones (Fishelson, 1974; Halstead, 1988) (Fig. 1-2). These spiny elements contain grooves

to facilitate the flow of venom along the spine and in most cases the glandular tissue rests within the groove itself. The association of these venom glands with spiny elements led Perrière & Goudey-Perrière (2003) to name their toxic secretions acanthotoxins. In *Meiacanthus* (saber-toothed blennies) injection is achieved by the use of enlarged fangs in the bottom jaw with the buccal venom glands surrounding the proximal two-thirds of the fang (Fishelson, 1974). These fangs also possess grooves along the anterior margin, through which venom flows toward the tip and the site of envenomation. Monognathids, which lack upper jaws, apparently inject venom via a single, hollow rostral fang, which has paired glands at its base (Raju, 1974; Bertelsen & Nielsen, 1987). These species are unique among venomous fishes, in that they would appear to use their venoms to subdue their prey, shrimps that are very large relative to the size of these species (Bertelsen & Nielsen, 1987), and which would have the potential to cause significant damage to these relatively fragile fishes. This hypothesis, like that of the adaptive nature of venoms in other groups of fishes, lacks rigorous experimental confirmation, due to the great depths inhabited by monognathid species.

In some species, venom glands appear to be present only in juveniles, as is the case in some scatophagids in which the venom glands appear to atrophy with age (Cameron & Endean, 1970). Additional examples are found in the family Acanthuridae (surgeonfishes and tangs), in which the acronurus larvae of *Acanthurus sandvicensis* and juveniles of *Prionurus microlepidotus* have been shown to produce venom (Tange, 1955; Randall, 1961). Cameron & Endean (1973) stated that no venom glands were known to occur in adult acanthurids, but it is unknown whether adult specimens of these two species have been examined for this trait. Halstead (1988) provided photomicrographs of

dorsal spine cross sections of *Prionurus microlepidotus*, but did not give size data for the specimen from which the spine was taken. Smith & Wheeler's (2006) examination of four acanthurid species seems to support Cameron & Endean's findings, as none of the species examined (presumably adults) were found to possess venom glands. Potential ontogenetic loss of venom glands is an important consideration to make in future searches for venomous taxa, as it may lead to underestimation of the true number of venomous representatives from a particular group.

Cellular Morphology

The cellular morphology of venom glands in fishes is very similar across broad taxonomic categories, indicating possible widespread convergent evolution of these cells. Venom-producing cells are enclosed within an integumentary sheath composed of epithelial cells. The venom gland cells are large and polygonal, with prominent nucleoli and highly granulous cytoplasm (presumably due to high concentrations of venomous peptides) (Reed, 1907; Halstead et al., 1953; Halstead, 1988; Gopalakrishnakone & Gwee, 1993) (Fig. 1-3). In catfishes, the cells of the venom gland are binucleate (Reed, 1907; Halstead *et al.*, 1953; Halstead, 1988). As the cells mature, organelles and nuclear structures are lost and only the cytoplasmic granules are visible. Venomous secretions are held in the cells or the cells undergo holocrine secretion whereby the secreting cells are lysed, releasing the venomous secretions along with cellular fragments into the intercellular space, where they are held until being used. Venom is released upon tearing of the integumentary sheath and the venom producing cells within when the spine or fang enters another organism.

Cameron & Endean (1973) hypothesized that the venom gland cells of fishes and the acanthotoxins that they contain are derived from the clavate or club cells of the epidermis, which secrete proteins known as crinotoxins (Halstead, 1988). While crinotoxic secretions are released into the water when the cells are ruptured [ostensibly to repel predators (Randall, 1967; Randall et al., 1971) or fouling organisms (Cameron & Endean, 1973)], the injection of these compounds into other organisms has also been shown to have toxic effects (Al-Hassan et al., 1987a, 1987b; Shiomi et al., 1987, 1988). A preliminary study of the catfish *Plotosus lineatus* offers some support for Cameron & Endean's hypothesis, as the club cells of this species were found to produce a substance that is similar, and possibly identical to one of the toxic fractions found in the venom gland, based on immunological reactions (Shiomi et al., 1988).

Perrière & Goudey-Perrière (2003), however, point out that common production of a single toxic component is not sufficient evidence to prove the homology of these cell types. While certain crinotoxins and acanthotoxins produced by *P. lineatus* show similar histochemical and pharmacological activities, Whitear et al. (1991a), in their examination of the venom gland of *Heteropneustes fossilis* (Indian stinging catfish) found distinct differences in the ultrastructure and histochemistry of the venom gland cells and club cells of the epidermis that, in their estimation, precludes the homology of the two cell types. Specifically, club cells were found to contain helical filaments and a division of the cytoplasm into perinuclear and peripheral zones, both of which were lacking in the venom cells. Additionally, a previous study (Zaccone et al., 1990) had shown a positive immunohistochemical reaction for serotonin in the club cells of this species, but Whitear *et al.* found that this reaction was lacking in the venom cells.

Whitear et al. (1991a) do not, however, address why these differences should mean that the venom gland cells could not possibly have been derived from epidermal club cells. If venom glands are indeed adaptive structures, one might expect their cellular morphology and the secretions that they produce to be subject to selection pressures that differ from those experienced by secretory cells in other locations. The differences reported by Whitear *et al.* may simply reflect this history. Additional comparative studies of the morphology and secretions of venom gland cells and different types of secretory epidermal cells from different groups of venomous fishes will clarify these issues.

PISCINE AXILLARY GLANDS

In addition to the venom glands lining the spinous elements of the fins and/or operculae, several species (toadfishes of the subfamily Batrachoidinae and many catfishes) possess secretory glands situated in the axil of the pectoral fin (Wallace, 1893; Reed, 1907; Halstead & Smith, 1954; Greven et al., 2006). Various authors have considered these glands to be part of the venom apparatus (Reed, 1907; Citterio, 1926; Birkhead, 1967; Burgess, 1989). More recently however, several additional hypotheses have been proposed for the function of these structures.

Gross Morphology

The axillary glands of catfishes and toadfishes are small pouch-like structures that release their secretions via a pore located below the cleithrum, near the base of the pectoral fin spine (Fig. 1-4). Like the venom glands found in distantly related families of fishes, the axillary glands of catfishes and toadfishes show a high degree of convergence in their gross and cellular anatomy. The interior of the gland of batrachoidine toadfishes and ictalurid catfishes is divided into several lobes, with each lobe being separated from the

others by a layer of connective tissue (Wallace, 1893; Reed, 1907). Recent studies of callichthyid catfishes have revealed a simple, tubular morphology of the axillary gland in these species (Greven et al., 2006).

Cellular Morphology

The secretory cells of the axillary gland are located within further subdivisions of the axillary gland lobes (Fig. 1-5A). In all species thus far studied, these cells are large, polygonal, and contain large quantities of a granular, secretory product, which has been shown by multiple authors to be proteinaceous in nature (Vernick & Chapman, 1968; Cameron & Endean, 1971; Al-Hassan et al., 1987; Kiehl et al., 2006). In catfishes, the cellular ultrastructure resembles that of the venom gland, with the cells originating as binucleate cells with prominent nucleoli and large amounts of endoplasmic reticulum. The cells become completely filled with secretory product as they mature, to the point that most subcellular structures are no longer visible (Halstead et al., 1953; Cameron & Endean, 1971; Kiehl et al., 2006) (Fig. 1-5B). The cells of toadfish axillary glands have a similar structure, but differ noticeably in containing a single spherical nucleus (Vernick & Chapman, 1968; Maina et al., 1998). In both groups, release of the secretory product appears to be holocrine in nature, which is indicated by the presence of burst cells in secretions drawn directly from the axillary pore (Wallace, 1893; Reed, 1907; Cameron & Endean, 1971; Whitear et al., 1991b;) and lack of evidence for other methods of secretion (Vernick & Chapman, 1968).

Possible Function

The earliest mention of axillary glands in catfishes was made by Günther (1880), who assumed that secretions issuing from the axillary pore anoint the pectoral-fin spine,

allowing the secretions to be injected along with those from the pectoral venom glands. Many works that followed (Jordan & Gilbert, 1882; Jordan & Evermann, 1896; Jordan, 1904, 1905) accepted this statement without experimental confirmation. While later studies showed that axillary gland extracts are toxic when injected into other organisms (Cameron & Endean, 1971; Birkhead, 1972), the water soluble nature of axillary pore secretions is difficult to reconcile with the scenario envisioned by earlier authors. Current hypotheses regarding the function of the axillary gland secretions include antimicrobial (Maina et al., 1998; Kiehl et al., 2006), ichthyotoxic (Maina et al., 1998; Ng & Ng, 2001; Greven et al., 2006), pheromonal or alert substance (Maina et al., 1998), and ionoregulatory (Maina et al., 1998) roles, though only the first two are supported by empirical evidence.

While it appears that the axillary glands of fishes do not appear to function as part of the venom delivery apparatus, their function and the action of their products represents a potentially fruitful area for future research. Fairly simple procedures, such as comparative electrophoresis of venom and axillary gland extracts, could be used to more conclusively rule out the presence of axillary gland secretions on the pectoral spine. Further investigations of the antimicrobial and icthyotoxic hypotheses that have thus far received preliminary support are also warranted.

PHARMACOLOGY AND TOXICOLOGY OF PISCINE VENOMS

As naturally occurring substances which are able to elicit potent responses in vertebrate physiological systems, the venoms of fishes have come under increased scrutiny as possible sources of future biomedical compounds. As such, studies of their pharmacological qualities far outnumber those of any other aspect of their biology. As the

focus of this review is the venom glands of fishes in an evolutionary context, I will not undertake a full review of these pharmacological studies of fish venoms here [interested readers are referred to Sivan (2009) and Church & Hodgson (2002), which treat this subject in great detail]. Instead, I provide a brief overview of only those factors which are directly relevant to questions of fish venom evolution and ecology.

Studies of the toxic effects elicited by fish venoms in other organisms have revealed a high degree of similarity in these effects and the mechanism of their production, providing an additional example of apparent convergent evolution of fish venom glands and the substances they produce. The major symptom of envenomation by all known venomous fish species in humans is pain that is disproportionate to the size of the wound inflicted. The most common sites of human envenomation are the hands or feet, and in many cases the pain has been known to travel up the entire length of the affected appendage (Halstead et al., 1953; Calton & Burnett, 1975; Gwee et al., 1994; Lopes-Ferreira et al., 1998). That intense pain should be a common result of envenomation by fishes would not be surprising if the venoms had undergone selection in a defensive capacity, where the intent would be rapid deterrence of a predator.

The venoms of various species have been shown to have cardiovascular, neuromuscular, and general cytolytic effects in various assays (Church & Hodgson, 2002; Sivan, 2009; Gomes et al., 2010). The cytolitic action of these venoms is thought to produce the other negative effects, through forming pores in the plasma membranes of target cells, allowing the influx of Ca^{2+} which triggers the release of several biologically active compounds from the cell (Church & Hodgson, 2002). Such an action is also known from bee (Pawlak et al., 1991) and platypus venoms (Kourie, 1999), both of which are

primarily pain producing venoms, like those of fishes. That the activities of several fish species' venoms are neutralized by stonefish antivenom (Shiomi et al., 1989) speaks to a similar chemical structure and target of these substances, though the apparent lack of interaction between the venom of *Notesthes robusta* (bullrout) and stonefish antivenom (Hahn & O'Connor, 2000) suggests that the taxonomic range of this similarity may be limited.

While the results obtained from previous pharmacological studies are valuable, they may have limited ecological relevance and therefore, provide little evidence for the adaptive nature of fish venoms. This stems from the fact that most previous assays have utilized mammalian or amphibian test subjects that would not naturally be encountered and envenomated by the fishes tested. That investigations using different test species have often returned different activities from the same species' venom indicates that either (1) there is variation in the venom composition between individuals/populations of these species or (2) the structure of the venom causes it to have different effects on various species' physiology. This suggests that future studies of piscine venom pharmacology, in order to have greater applicability to studies of venom ecology, should test the particular venom's effect on a predator (or appropriate proxy species) that the species of interest would likely encounter naturally, while employing extensive intra- and interpopulational sampling of venomous individuals.

CHEMISTRY OF PISCINE VENOMS

Proteins

The toxic components of piscine venoms are likely to be proteinaceous, based on their lability to heat and pH (Schaeffer et al., 1971; Birkhead, 1972; Gwee et al., 1994; Lopes-

Ferreira et al., 1998). Some have also proven to be highly labile to other factors, as lyophilization, freezing and subsequent thawing, and storage for extended periods have all been shown to destroy the activity of *Scorpaena guttata* (California scorpionfish) and *Urobatis halleri* (round stingray) venoms (Halstead, 1988; Schaeffer et al., 1971). The sensitivity to these factors represents a significant problem for researchers hoping to work with these substances (in situations where long-term storage is necessary or desirable), and has yet to be adequately addressed.

The majority of existing information regarding the toxic proteins found in piscine venoms concerns the sizes of these compounds in various species. Of the 10 species detailed by Church & Hodgson (2002), the sizes of the toxic compounds ranged from 15–324 kDa. Catfishes generally fall within the lower end of this range (10–15 kDa) (Calton & Burnett, 1975; Auddy & Gomes, 1996), although Wright (2009) identified an additional putative toxin of approximately 110 kDa in the venoms of several species. Other venomous fishes show significantly larger toxin sizes (47–800 kDa). This type of information should be helpful in the identification of toxic peptide sequences determined from cDNA analyses of venom gland mRNAs. It has recently proven useful in the case of the toadfish *Thalassophryne nattereri*, in which the venomous secretions were characterized using cDNA libraries and expressed sequence tags (ESTs) (Magalhães et al., 2005, 2006). In these studies the amino acid sequence of natterin, the biologically active component of toadfish venom, was deduced from cDNA library data. The amino acid sequences corresponded closely in molecular weight to the toxic fraction recovered in an earlier study of toadfish venom (Lopes-Ferreira et al., 1998). Molecular weight data is especially important to studies utilizing fish venom cDNA libraries, because few

genetic sequences for fishes exist against which to search the cDNA sequences obtained from different species. Without this knowledge, there may be little hope for the identification of cDNA sequences that correspond to venom proteins.

Other Toxic Components

In addition to the abovementioned proteinaceous components, several other biologically active compounds have been discovered in the venoms of some species. The venoms of *Synanceia trachynis* (stonefish), *Pterois volitans* (lionfish), and *Potamotrygon motoro* (freshwater stingray) are thought, based on their pharmacological effects, to contain acetylcholine, or a substance that acts in a similar fashion (Rodrigues, 1972; Church & Hodgson, 2000). *Trachinus draco* (weeverfish) venom has been found to contain both histamines and catecholamines (Haavaldsen & Fonnum, 1963; Halstead, 1988). Catecholamines have also been discovered in the venoms of three stonefish species (*Synanceia horrida*, *S. trachynis*, and *S. verrucosa*) (Garnier et al., 1996), while the venom of *Urobatis halleri* (round stingray) has been shown to contain serotonin, 5'-nucleotidase, and phosphodiesterase (Russell & Van Harreveld, 1954; Halstead, 1988). Studies examining the contribution of these elements to the response elicited by these venoms and what interaction, if any, they have with the aforementioned toxic proteinaceous compounds are poorly represented in the literature.

Chemical Complexity of Piscine Venoms

In contrast to the venoms of terrestrial organisms and those of venomous marine organisms such as cone snails that can contain hundreds of toxic components per species (Gray et al., 1988; Bulaj et al., 2003), the venoms of fishes appear to contain only one or a few toxic components (Church & Hodgson, 2002). An interesting parallel is found in

the venoms of sea snakes, which have also been shown to contain few toxic components relative to other venomous snakes (Fry et al., 2003). Additional similarities are evident, as sea snake venoms show broad cross reactivity to antivenom developed from one or two species (Chetty et al., 2004), a similar result to that shown by the Shiomi *et al.* (1989) study of piscine venom cross reactivity to stonefish antivenom mentioned above. The similarities become all the more striking when one considers that venomous marine snakes represent two independent radiations (Slowinski & Lawson, 2002; Vidal & Hedges, 2002; Scanlon & Lee, 2004), while venoms have been independently derived in acanthomorph fishes no fewer than 11 times (Smith & Wheeler, 2006), and at least twice in catfishes (Wright, 2009).

The white catfish (*Ameiurus catus*) may represent an exception to the generalization that piscine venoms exhibit low toxin diversity. The venom of this species was found to contain two to eight fractions that showed lethal activity in mice (Calton & Burnett, 1975). The additional finding that *A. catus* venom lost little to no activity following treatment with trypsin and elevated temperature indicates that additional, non-proteinaceous compounds may be present in the venomous secretions of this species. These results are questionable however, as different methods of analysis yielded proteinaceous fractions of varying weights and biological activities. Until further analyses can be conducted on the venom of this species, the lower value from Calton & Burnett's estimate of the number of lethal fractions in the venom of *A. catus* is more consistent with what is known from other species. The low number of compounds found in fish venoms would appear to be an asset to studies of their evolution, as the problems

of homology inherent in evolutionary studies of species that produce many different toxic compounds should be easily addressed.

It is tempting to suggest that the parallel streamlining of these species' venoms is due to selection associated with a common target: piscine physiological systems. Little empirical evidence exists to support this hypothesis however, as few studies of the action of sea snake and piscine venoms on their (presumed) natural targets exist. The few studies of sea snake venoms that have been performed in this context have indicated that likely prey species possess high levels of resistance to sea snake venoms (Heatwole & Poran, 1995; Heatwole & Powell, 1998). This would appear to run counter to a selective streamlining hypothesis, as one might expect these species of sea snakes to possess more complex venoms to overcome prey resistances to particular toxic compounds. Preliminary results from studies on ictalurid catfishes (Wright, *in review*) indicate that the venoms of bullheads have little effect on potential predators with which they share a habitat type, suggesting that coevolution between predator and prey may be occurring in these systems and leading to these somewhat counterintuitive results. Further studies are clearly necessary to examine possible correlations between low number of toxic compounds in piscine venoms and the potential for coevolutionary interactions between venomous fishes and potential predators.

DO PISCINE VENOM GLANDS FIT EXISTING DEFINITIONS OF ADAPTIVE TRAITS?

Teleonomic Definitions

Teleonomic definitions identify adaptive traits based primarily on the detection of functional design of a trait that performs a task that solves some problem faced by an

organism, and is too complex to have arisen by chance (Williams, 1966; Thornhill, 1990; West-Eberhard, 1992). Implicit in these definitions is the historical, long-term role of natural selection in molding the trait for its current purpose. Incidental effects arising from physical and chemical laws are excluded from this definition of adaptation, as are traits that are not sufficiently complex, those that have arisen too recently to develop sufficient functional complexity, or those traits that have evolved less complexity since their origin, despite the fact that selection may have been acting in all of these situations.

The definition of Gould & Vrba (1982) is not strictly teleonomic, in that it makes no mention of trait complexity as a criterion for the recognition of adaptation. It does, however, state that an adaptive trait has been built by selection for its current purpose, which is an element present in all teleonomic arguments for the adaptive nature of a given trait. Their definition is therefore more similar to the teleonomic definitions outlined in Table 1-1 than the remaining definitions that incorporate the evolutionary history of a trait, and for lack of a better classification I include it there. Gould & Vrba introduce the term 'exaptation' for structures that originally arose for a purpose other than that for which they are currently used, recognizing as adaptations only those traits that were originally developed for the task that they perform now.

Gould & Vrba's definition has been criticized as non-operational, due to the difficulty of determining where an exaptation ends and an adaptation begins (Reeve & Sherman, 1993). This deficiency becomes apparent when one attempts to classify the venom glands of fishes according to Gould & Vrba's system. The cells of piscine venom glands are thought to have been derived from glandular cells of the epidermis (Cameron & Endean, 1973). The secretions of these epidermal cells have been shown to possess

antimicrobial (Robinette et al., 1998) and healing properties (Al-Hassan et al., 1983, 1985). Assuming that the compounds produced by these cells have been modified to produce venoms in the present, we must assume that (under Gould & Vrba's definition) the secretions of venom gland cells are exaptations. Should we also classify the cells that produce these secretions as exaptations since they are derived from cells that have a different function, or restrict the term to the lowest possible functional aspect of the trait in question? What about the aggregations of these cells that comprise the venom gland? The aggregation of secretory cells may have been advantageous because the integument of the spines is often injured in interactions with other organisms and even the environment, necessitating a greater concentration of the cells that produce antimicrobial and healing secretions. This scenario assumes that the aggregation of secretory cells precedes the production of venomous compounds, which may or may not be true. Answering this question of when a trait and/or its current function was derived is one way in which derived trait definitions attempt to make Gould & Vrba's definition more operational (see below).

The reliance of teleonomic definitions on trait complexity introduces a large degree of subjectivity into the identification of adaptive traits using functional design criteria. This has been evidenced in the study of piscine venom glands on at least one occasion. Though most authors have considered the glands to be adaptive in nature, Perrière & Goudey-Perrière (2003) considered the venom glands to be nonadaptive due to the passive nature of injection. While the venom glands of fishes would certainly appear to be less complex and well designed when compared to the venom glands found in other groups, this does not preclude the possibility that their morphology and the toxins that

they secrete have been influenced by natural selection. Nor must the venom glands of fishes necessarily be considered non-complex, as the aggregation of venom producing cells enclosed by an integumentary sheath is more complex than the scattered nature of the epidermal secretory cells from which they are thought to be derived.

The question of complexity and functional design is further confused by the level at which a trait is being examined. While the gross morphology of the glands themselves does not appear to support their classification as an adaptive trait in a teleonomic framework, the structure of the chemical compounds that they produce should also be examined for evidence of modification for their current purpose, relative to the compounds produced in the glandular epidermal cells found in the skin. For this reason (among others), information regarding piscine venom chemistry will be particularly important to future studies of the evolution of venom glands in fishes.

Derived Trait Definitions

Derived trait definitions apply cladistic methods to the detection of adaptive traits. These definitions universally state that adaptations are apomorphic traits that serve a current purpose, and like teleonomic definitions, invoke the historical action of natural selection in shaping the trait under examination for its current function (Fisher, 1985; Greene, 1986; Coddington, 1988; Baum & Larsen, 1991; Harvey & Pagel, 1991). As stated previously, these definitions allow a more operational concept of exaptation, though this concept is nearly the opposite of that proposed by Gould & Vrba (1982). Under a derived trait definition, a trait that was developed in an ancestral taxon for a particular function and remained unchanged through evolutionary time would be termed an exaptation. An

apomorphic trait with a new or improved function (resulting in improved fitness) relative to the plesiomorphic state is considered an adaptation.

Under the derived trait criterion, one can easily convert an exaptive trait into an adaptive one by expanding the scale of analysis to a more inclusive clade in which the trait becomes apomorphic relative to the newly included ancestral taxa. The continued ambiguity of what constitutes an exaptation or an adaptation led Reeve & Sherman (1993) to suggest that the two types of trait simply be referred to as adaptations. This seems a bit drastic, as studies of adaptation seek to understand the current utility of traits and frequencies of phenotypes in the context of natural selection theory. The original trait upon which natural selection has acted to produce the current phenotype is an important component of this process.

The application of a derived trait definition relies heavily on knowledge of the phylogeny of the taxon possessing the trait to be studied. In addition, the members of the taxon possessing that trait must be known. These criteria were fulfilled to some extent for spiny-rayed fishes by Smith & Wheeler's (2006) study of venomous representatives from this group. The phylogeny of several spiny-rayed fishes was determined using molecular data, and the presence of venom glands was mapped onto the phylogeny. The presence of venom glands was found to be apomorphic for the families in which they were found, but Smith and Wheeler's sampling method makes it difficult to determine whether venom glands are present in all members of the families where they have been found, or where in the family's phylogeny venom glands appear. It therefore becomes difficult to classify the venom glands of these species as adaptive under the derived trait criterion, because it

is unknown how the trait maps onto generic and species level phylogenies, necessitating additional sampling and phylogenetic studies.

Multiple phylogenies are available for catfishes based on both morphological (Mo, 1991; De Pinna, 1998; Diogo, 2004) and molecular (Hardman, 2005; Sullivan et al., 2006) data. Generic and species-level phylogenies are available for many (but by no means all) of the families included in the order Siluriformes (Diogo, 2003). A recent study (Wright, 2009) found that no fewer than 20 of the 36 currently recognized (Ferraris, 2007) families of catfishes contain venomous representatives. Venom glands have evolved independently within catfishes at least twice, once within the suborder Loricarioidei (in the family Callicthyidae), and at least once, probably relatively basally, within the Siluroidei, the suborder containing the remaining 19 venomous catfish families. Again, this study's sampling methodology was not detailed enough to provide information on the venomous nature of all of the species in all 20 of these families (which contain over 1500 different species), although more detailed examination of the family Ictaluridae was performed, and indicated several secondary losses of venom glands in this group.

At the family level, the venom glands of the Callichthyidae would appear to fulfill the apomorphic requirement of derived trait definitions, as these structures are found nowhere else within the Loricarioidei (a suborder containing over 1000 species), and show clear morphological differences from siluroid venom glands (Wright, 2009), supporting hypotheses of their independent evolution. Siluroid venom glands are apomorphic at an as yet undetermined point in the siluriform phylogeny (due to very poor resolution of basal siluroid relationships in most morphological and molecular studies).

At the family, generic, and species level, these venom glands would not be considered derived adaptive traits, unless they had undergone additional modification (assumed to be selectively advantageous) from their plesiomorphic (at the suborder level) state. Histological and preliminary biochemical analyses have indicated that venom gland morphologies and chemical profiles do differ between siluroid families, genera, and species, providing evidence for continued selection on, and modification of, these structures (Wright, 2009).

Selective Spread Definitions

The selective spread definition in its currently accepted form is attributed to Sober (1984). It requires both that a trait be prevalent in a population, and that the origin of the trait and its prevalence be the result of natural selection for the function performed by the trait. This definition would result in the counterintuitive recognition of currently disadvantageous traits as adaptations, so long as their initial spread was due to positive selection for a function performed by the trait. I suggest that this shortcoming may be circumvented by the modification of the definition given in Table I as follows: A trait that has become prevalent **and that has been maintained** in a population **due to continued** selection for said trait, where the selective advantage of the trait was**/is** due to the fact that the trait helped **and continues to help** perform a particular task. However, this definition remains subject to one of the criticisms leveled at the adaptationist program by Gould & Lewontin (1979) – namely that this definition would not include advantageous traits that became prevalent due to neutral processes such as population mixing or genetic drift.

Whether venom glands are widespread in populations of species that have been shown to possess them has not been addressed directly, though the consistent detection of venom glands in randomly selected individuals of venomous species indicates that they are widespread within and between populations. The limitation of venom glands to juvenile individuals in some scatophagid species (Cameron & Endean, 1970) may indicate that they do in fact perform a useful task at this point in the species' life history, thus leading to their prevalence in this subset of the population. If it could be demonstrated that the juvenile venom glands of scatophagids reduce predation on this life history stage, they could be considered adaptive under the selective spread criterion. However, the lack of evidence regarding the ecological function of venom glands in most groups of fishes (see below) confounds the detection of any selective advantage that the spread of venom glands may have conferred.

Nonhistorical Definitions

Nonhistorical definitions divorce the term adaptation from its association with natural selection and instead focus on determining phenotypic properties that characterize adaptations (though not in the same way as teleonomic definitions). This is done in order to avoid having a definition of a product (an adaptive trait) that relies on the process (natural selection) responsible for constructing the product, though nonhistorical definitions often cite phenotypic properties that are likely to cause a trait to be favored by selection as being indicative of adaptive traits. This usually takes the form of recognizing as adaptations those traits that currently increase the fitness or reproductive success of an individual (Dobzhansky, 1956; Endler, 1986; Reeve & Sherman, 1993; Vermeij, 1996; Table 1-1), though survival (Dobzhansky, 1956; Endler, 1986; Vermeij, 1996) or more

efficient performance of some ecologically related task (Bock, 1980; Williams & Neese, 1991; Table 1-1) may also be components of such definitions.

Increased survival would be the intuitive benefit to the possession of a venom gland by a fish species. The experimental demonstration of this increased survival would alone be sufficient to satisfy the basic requirement of most nonhistorical definitions of adaptation. The definition of Reeve & Sherman (1993; Table 1-1) would include the additional requirement of providing an appropriate set of phenotypes (individuals with venom glands vs. those without) to a predatory species to test the adaptive hypothesis of piscine venom glands. As the glands of most venomous species are found on external structures near the surface epidermal layer, their removal from some individuals prior to predator exposure should be a simple matter and would provide a phenotype lacking only the trait whose adaptive nature was being examined.

The environmental component present in many nonhistorical definitions (Dobzhansky, 1956; Endler, 1986; Endler & McLellan, 1988; Reeve & Sherman, 1993; Vermeij, 1996; Table 1-1) might be satisfied by exposing the venomous species in question to predatory species from varying habitats. The requirements of these definitions would be partially fulfilled if individuals of the venomous species having intact venom glands survived encounters with sympatric predators more often than with allopatric predator species. For results to fully comply with the requirements of these definitions, experiments would have to be performed in a completely natural setting, a nearly impossible proposition. In this respect, these definitions are not always as operational as their proponents may claim. It appears likely, however, that should current utility be

demonstrated for piscine venom glands, they might be found to closely approximate an adaptive trait in a nonhistorical sense.

Selective Advantage?

A significant problem presents itself when one attempts to classify piscine venom glands as adaptive structures, regardless of the category used: evidence that they provide any selective advantage in nature is ambiguous, at best. Given the pronounced effects demonstrated by fish venoms in pharmacological studies it might be assumed that the introduction of these noxious substances into a wound would offer an additional benefit to an individual possessing venom glands. As stated earlier, however, very few studies have examined the effect of piscine venoms on other fishes, particularly predatory species, or indeed, any aquatic predator.

Bosher et al. (2006) attempted to demonstrate the adaptive nature of the fin spines in *Ictalurus punctatus* (channel catfish), finding that intact individuals survived encounters with largemouth bass more frequently and were completely consumed much less frequently than individuals from which the fin spines had been removed. However, this study did not examine the separate contributions made by the presence of fin spines, themselves a potentially formidable defensive structure, vs. the presence of spines with associated venom glands. It would be fairly simple to incorporate a test of the utility of the venom glands in the channel catfish into Bosher et al.'s experimental framework. The tissue (including the venom gland) covering the fin spines of this species is thin and easily damaged, often being stripped completely off during routine histological preparations (Halstead, 1988; pers. obs.). Including individuals with stripped spines in an

experiment similar to Bosher et al. would provide preliminary evidence for the role of the venom gland in defense against predation.

A more recent study (Emmett & Cochran, 2010) examining a different venomous catfish species (*Noturus gyrinus* – tadpole madtom) from the same family also found that handling time was slightly higher for predators consuming catfishes vs. minnows, but also showed that largemouth bass (an ecologically relevant potential predator of this species) were able to completely consume all catfish with which they were presented. This casts serious doubt on the adaptive benefit of the venom glands of this species, as even with intact venom glands, an individual's fitness drops to zero when encountering a predator, the same (presumably) as a species lacking these defensive structures. Bass did show signs that consuming madtoms was more difficult than consuming minnows, but nevertheless remained willing to consume madtoms when they were presented, thus eliminating potential group selectionist arguments based on predators learning to select against suboptimal prey. It is possible that size plays a role in the selective advantage that may be conferred by venom glands in fishes. Smaller bass than those used by Emmett & Cochran may have experienced a greater physiological response to the venom delivered by the madtoms used, leading to their rejection, as observed with *Ictalurus punctatus* by Bosher et al. (2006). It is also possible that the bass used had previous experience feeding on this species, potentially developing some level of resistance to its venom, as they were wild individuals caught on hook and line. Nevertheless, the results as presented provide evidence that the venom glands of this species (and by possible extension, those of other species) are potentially ineffective in an anti-predatory capacity.

The lack of information regarding the actual use of venom glands in nature and the possibility that they do not protect against predation are sufficient to prevent the current classification of piscine venom glands as adaptive traits, no matter what the type of definition used. While evidence that venom glands confer some advantage to fishes possessing them should be demonstrated before studies of the ecology and evolution of the venom glands of fishes proceed, it will still be useful to examine other categories of adaptive trait definitions, in order to determine whether, assuming an advantageous function is discovered for piscine venom glands, these structures may eventually be considered an adaptive trait under one or more criteria.

SUMMARY

The venom glands of fishes are composed of aggregations of venom-producing cells that are wrapped in an integumentary sheath, and are nearly always associated with spinous elements of the fins or opercular area. The venom apparatus of catfishes and toadfishes in all likelihood does not include the axillary gland. Venom-producing cells are likely derived from epidermal secretory cells, based on preliminary immunohistochemical studies, though the exact type of epidermal cell from which they are derived remains uncertain. The toxic components produced by these cells are proteinaceous in nature, and have significant cytolytic effects that can lead to secondary cardiovascular and neurotoxic effects, and severe pain in envenomated organisms. The number of toxic compounds produced by piscine venom glands is very low relative to other venomous organisms. Venom glands have been independently developed multiple times in fishes and are very similar in structure and the effect of their products, though finer scale chemical analyses have revealed large variation in the molecular weights of different species' venoms.

Studies regarding the actual utility of venom glands, whether in laboratory or natural settings, are lacking.

The lack of evidence that venom glands confer a selective advantage to those species that possess them precludes their current classification as adaptive traits, despite previous authors' claims to the contrary. In addition to results confirming that piscine venom glands contribute to the deterrence of potential predators, information regarding the phylogeny of groups containing venomous representatives, the phylogenetic distribution of venom glands within these groups, intra- and interpopulational studies to determine the prevalence of venom glands, and chemical characterization of the toxic components of piscine venoms must be gathered in order to confirm or refute the applicability of various types of adaptive trait definitions to the venom glands of fishes. Future studies of piscine venom glands will undoubtedly encounter difficulties due to our current lack of knowledge regarding their ecological use and evolutionary history. It is due to this very lack of knowledge, however, that they also represent an exciting and potentially fruitful area of research.

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Table 1-1. Adaptive trait definitions, arranged according to author and type of evidence primarily used in identification and delineation of adaptive traits.

Definition Type	*Author(s)*	*Definition*
Teleonomic		
	Williams (1966); Thornhill (1990)	A feature that performs a function or purpose with sufficient precision, economy, efficiency, etc. to rule out pure chance as an adequate explanation for the presence of said feature
	Gould & Vrba (1982)	Any feature that promotes fitness and was built by selection for its current role
	West-Eberhard (1992)	A trait that has evolved in specific ways to make it more effective in the performance of a task, the change having occurred due to the fitness increase that results
Derived Trait		
	Fisher (1985)	A derived trait that enhances the current reproductive potential of most individuals bearing it
	Greene (1986)	A shared, derived trait that appears at the same point within a particular phylogeny as the advantage it confers
	Coddington (1988)	A trait with an apomorphic function promoted by natural selection
	Baum & Larson (1991)	A derived trait with enhanced utility relative to its antecedent state, with the evolutionary transition having been found to have occurred within the selective regime of the focal taxon

Table 1-1. continued

Definition Type	*Author(s)*	*Definition*
Derived Trait		
	Harvey & Pagel (1991)	A derived character that evolved in response to a specific selective agent
Selective Spread		
	Sober (1984); Futuyma (1998)	A trait that has become prevalent in a population because there was selection for said trait, where the selective advantage of the trait was due to the fact that the trait helped to perform a particular task
Nonhistorical		
	Dobzhansky (1956); Endler (1986)	An aspect of the developmental pattern which facilitates the survival and/or reproduction of its carrier in a certain succession of environments
	Bock (1980); Williams & Neese (1991)	A feature having properties of form and function which permit the organism to maintain successfully the synergy between a biological role of that feature and a stated selectional force
	Endler & McLellan (1988)	A trait that improves an organism's fit with its environment, or improves the efficiency or responsiveness of its internal machinery, whether originally developed for its current function or not

Figure 1-1. Phylogenetic reconstructions of groups of teleost fishes containing venomous lineages (indicated in red). (A) Phylogeny of spiny-rayed fishes, redrawn from Smith & Wheeler (2006), showing multiple independent derivations of venom glands in this group. (B) Phylogeny of Siluriformes (catfishes), redrawn from Sullivan et al. (2006), showing the widespread presence of venomous lineages within the order. Note the lack of resolution of basal relationships within the Siluroidei, a common problem in reconstructions of siluriform phylogeny.

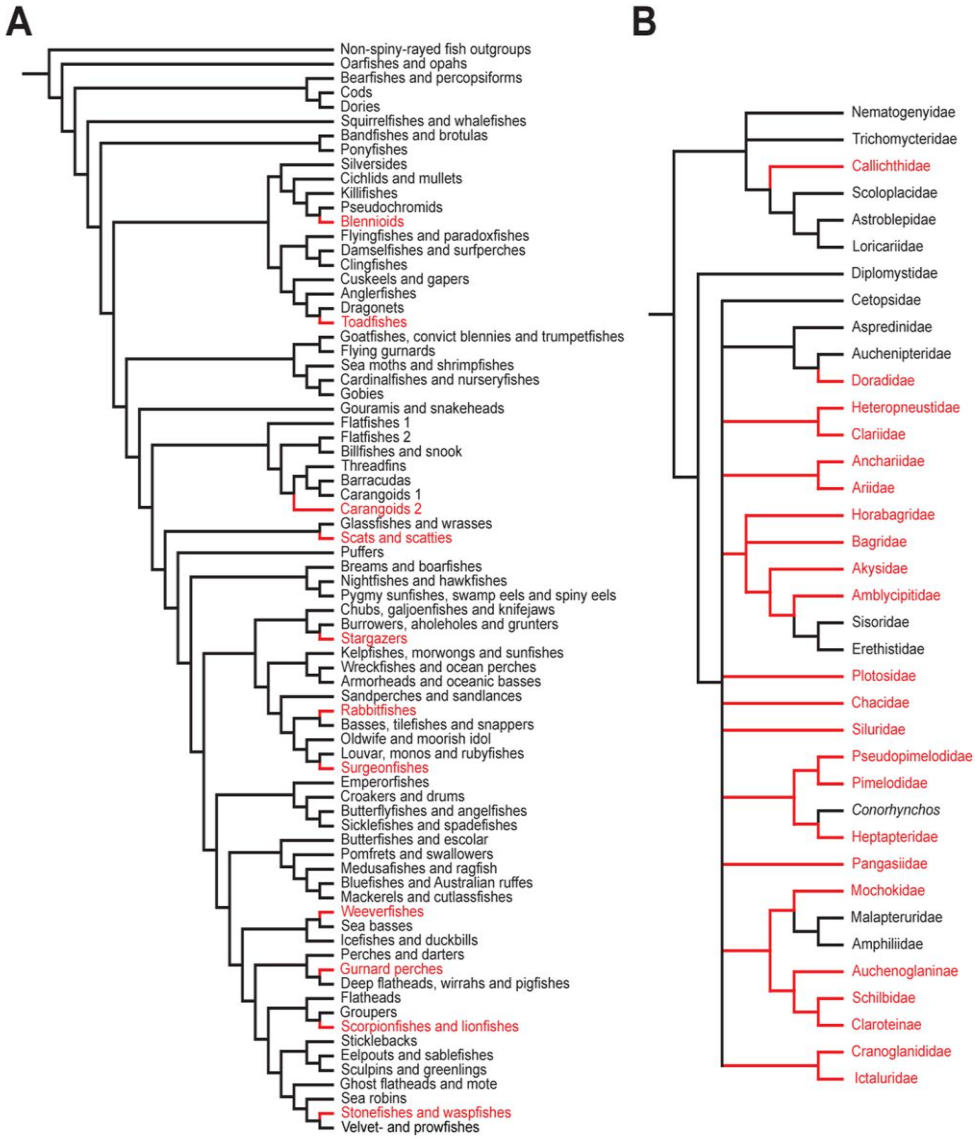


Figure 1-2. Gross morphology of the venom apparatus of selected spiny-rayed fishes. (A) Venomous dorsal spines of the toadfish *Thalassophryne amazonica*. (B) Venomous dorsal spine of the velvetfish *Ptarmus jubatus*. (C) Dorsal spine of scorpionfish *Neomerinthe hemingwayi*, showing a possible venom gland on the caudal margin of the spine. (D) Venomous dorsal spine of the rabbitfish *Siganus stellatus*. (E) Venomous dorsal spine with enlarged venom glands in the stonefish *Synanceia verrucosa*. (F) Venomous opercular spine of the toadfish *Thalassophryne amazonica*. (G) Venomous opercular spine of a weeverfish *Trachinus araneus*. (H) Venomous fang from the lower jaw of a saber-toothed blenny *Meiacanthus grammistes*. Abbreviations: ag, anterodorsal groove; os, opercular spine; and vg, venom gland. Figures reproduced from Smith and Wheeler (2006), with permission of Oxford University Press.

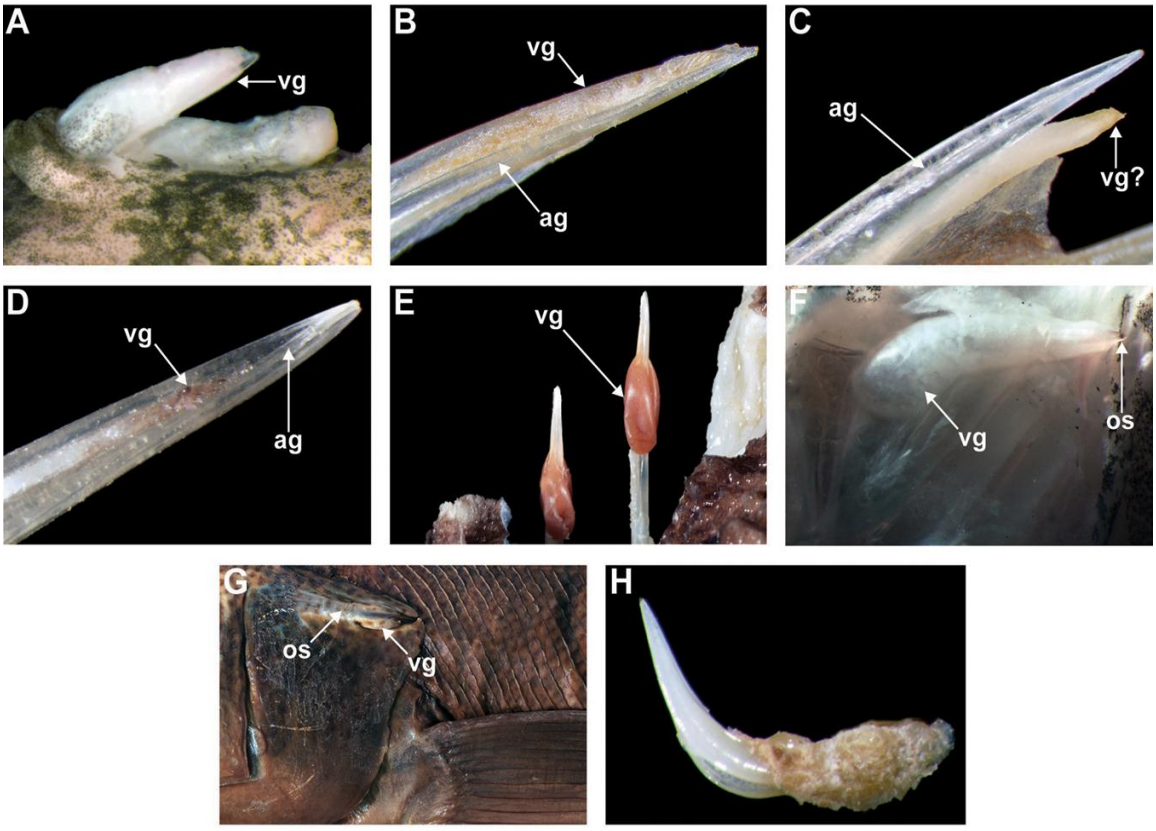


Figure 1-3. Representative photomicrographs of venom glands from different groups of fishes. Histological sections of the tail spine of (A) *Urobatis jamaicensis* (yellow stingray), (B) the pectoral spine of *Noturus gyrinus* (tadpole madtom), and (C) the third dorsal spine of *Scorpaena plumieri* (spotted scorpionfish). Abbreviations: g, glandular tissue; s, spine.

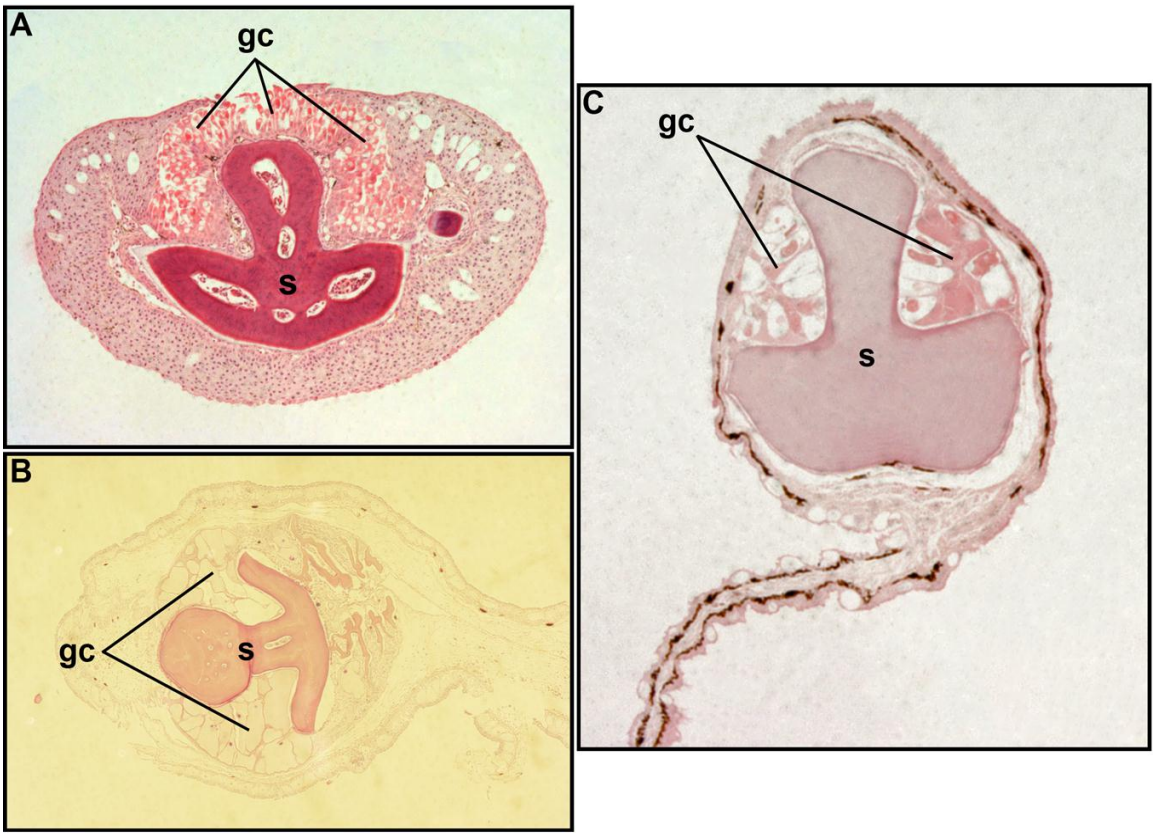


Figure 1-4. Gross morphology of the axillary glands and associated structures in catfishes. (A) Anterior half of *Ariopsis felis*, with cleithral region and axillary pore indicated by white box. (B) Close up of cleithral region from the same specimen, with the axillary pore indicated by the white arrow. (C) Cleithral region of *Bagre marinus* with skin removed, showing the position of the axillary gland relative to the cleithrum. Black arrow indicates glandular tissue, which extends further upward behind the cleithrum. (D) The axillary gland of the same specimen, removed from behind the cleithrum.

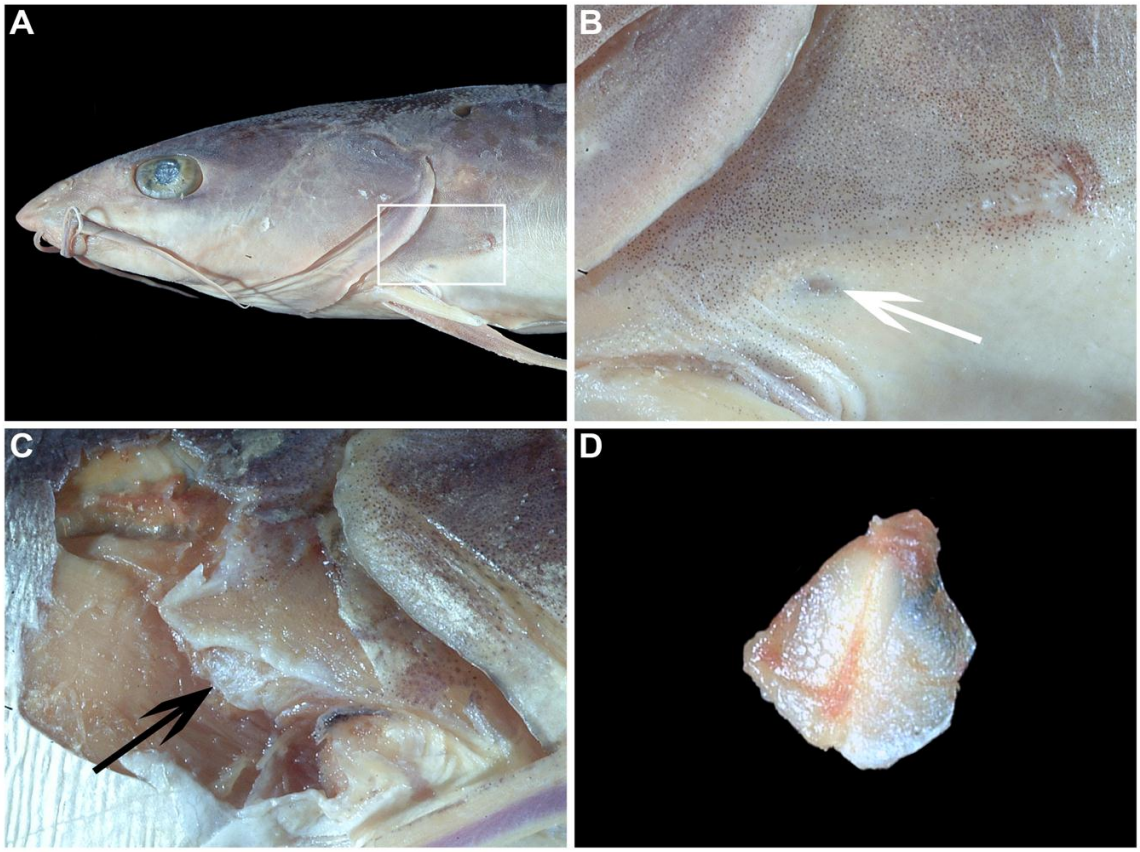
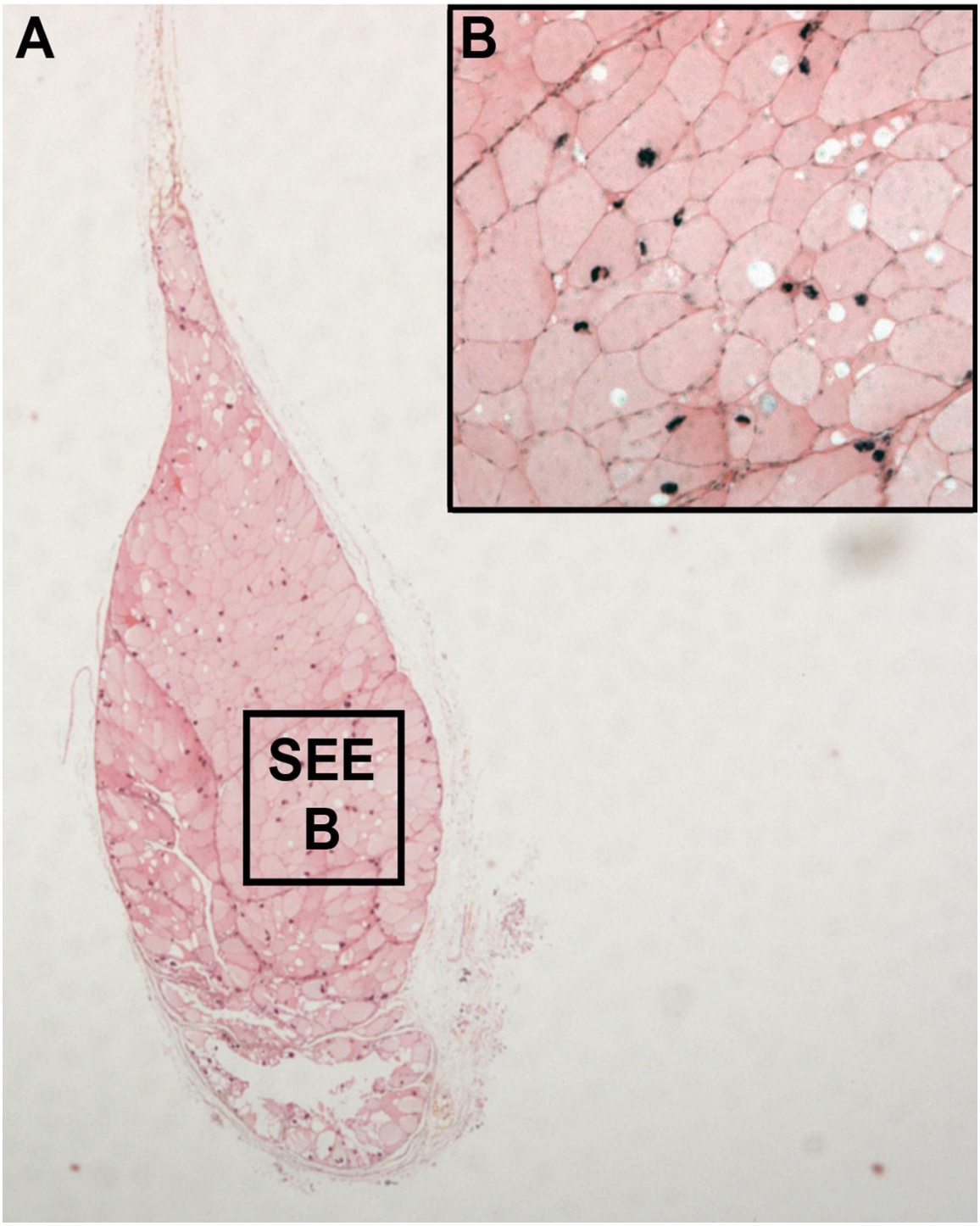


Figure 1-5. Cellular morphology of the axillary gland of *Bagre marinus*. Photomicrographs of (A) a histological section of the axillary gland pictured in Fig. 4D and (B) a close-up view of the glandular cells.



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CHAPTER 2

DIVERSITY, PHYLOGENETIC DISTRIBUTION, AND ORIGINS OF VENOMOUS CATFISHES[1]

ABSTRACT

The study of venomous fishes is in a state of relative infancy when compared to that of other groups of venomous organisms. Catfishes (Order Siluriformes) are a diverse group of bony fishes that have long been known to include venomous taxa, but the extent and phylogenetic distribution of this venomous species diversity has never been documented, while the nature of the venoms themselves also remains poorly understood. In this study, I used histological preparations of over 100 catfish genera, basic biochemical and toxicological analyses of fin spine extracts from several species, and previous systematic studies of catfishes to examine the distribution of venom glands in this group. These results also offer preliminary insights into the evolutionary history of venom glands in the Siluriformes. Histological examinations of 158 catfish species indicate that approximately 1250-1625+ catfish species should be presumed to be venomous, when viewed in conjunction with several hypotheses of siluriform phylogeny. Unambiguous parsimony character optimization analyses indicate two to three independent derivations of venom glands within the Siluriformes. A number of putative toxic peptides were identified in the venoms of catfish species from many of the families determined to contain venomous representatives. These peptides elicit a wide array of physiological

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effects in other fishes, though any one species examined produced no more than three distinct putative toxins in its venom. The molecular weights and effects produced by these putative toxic peptides show strong similarities to previously characterized toxins found in catfish epidermal secretions. Venom glands have evolved multiple times in catfishes (Order Siluriformes), and venomous catfishes may outnumber the combined diversity of all other venomous vertebrates. The toxic peptides found in catfish venoms may be derived from epidermal secretions that have been demonstrated to accelerate the healing of wounds, rather than defensive crinotoxins. The reduced diversity of toxic peptides found in catfish venoms, relative to venomous terrestrial organisms, likely reflects differences in venom function and other selective factors influencing the evolution and diversification of these compounds.

INTRODUCTION

The venoms produced by cnidarians, mollusks, snakes, arachnids, insects, and some mammals have been the subject of multiple studies of chemical structure (Gray et al., 1988; Escoubas et al., 2000; Kita et al., 2004), pharmacology (Grotendorst & Hessinger, 1999; Escoubas et al., 2000; Arocha-Piñago & Guerrero, 2001; Kita et al., 2004), and toxicology (Fletcher et al., 1996; Arocha-Piñago & Guerrero, 2001; Saminathan et al., 2006), in addition to several evolutionary studies (Duda & Palumbi, 1999; Kordiš & Gubenšek, 2000; Fry & Wüster, 2004; Lynch, 2007; Whittington et al., 2008), but information regarding these aspects of fish venoms is relatively sparse (Birkhead, 1972; Gwee et al., 1994; Hahn & O'Connor, 2000; Church & Hodgson, 2002; Magalhães et al., 2005; Smith & Wheeler, 2006). Until recently, even reliable estimates of the number of venomous fish species have been unavailable. Morphological examinations, combined

with phylogenetic analyses have suggested that 585-650 species of spiny-rayed fishes are venomous, a number which rivals the known diversity of venomous snakes and is significantly higher than previous estimates of about 200 venomous spiny-rayed fish species (Smith & Wheeler, 2006). We still lack estimates, however, for catfishes (Order Siluriformes), a diverse, monophyletic group with 34 recognized extant families and over 400 genera containing more than 3,000 known species (Ferraris, 2007). The historical lack of such basic information may be largely responsible for the paucity of research on venomous fishes in general, and venomous catfishes in particular.

The venom glands of catfishes are found in association with sharp, bony spines along the leading edge of the dorsal and pectoral fins, which can be locked into place when the catfish is threatened (Fig. 2-1). When a spine enters a potential predator, the integument surrounding the venom gland cells is torn, releasing venom into the wound. Catfish venoms have been shown to display neurotoxic and hemolytic properties and can produce a variety of additional effects such as severe pain, ischemia, muscle spasm, and respiratory distress; though any single species' venom may not display all of these properties (Halstead, 1978). These effects are produced in a wide range of taxonomic classes of vertebrates, including mammals, reptiles, birds, and amphibians (Toyoshima, 1918). In humans, the primary symptoms are severe pain and swelling at the site of envenomation, though fatalities have been reported in cases involving *Plotosus lineatus* and *Heteropneustes fossilis* (Halstead, 1978). Complications arising from secondary infection of the wound are also frequently encountered (Murphey et al., 1992; Carty et al., 2010; Roth et al., 2010).

The chemical nature of piscine venoms is poorly known, though the loss of toxicity seen when these venoms are subjected to common denaturing agents suggests that proteins constitute the major toxic component of these secretions (Church & Hodgson, 2002). Thus far, detailed examinations of these proteins in catfishes have been limited to the venoms of *Plotosus canius*, a particularly toxic marine species found in Southeast Asia, and *Ameiurus catus*, a freshwater species found in the eastern United States. The neurotoxic and hemolytic properties of *P. canius* venom have been attributed solely to a 15 kDa protein, termed toxin-PC (Auddy & Gomes, 1996). The venom of *A. catus* was thought to contain anywhere from two to eight toxic proteins with approximate molecular weights of 10 kDa (Calton & Burnett, 1975). Both the mechanism by which these toxins act and their physiological targets are very poorly understood. It is thought that cytolytic activity due to pore formation in cell membranes is a likely explanation, as this activity is present in other 'pain-producing' venoms, such as those produced by bees (Pawlak et al., 1991) and platypus (Kourie, 1999), and reactions consistent with this mechanism have been observed in response to piscine venoms (Church & Hodgson, 2002).

As a globally distributed and thus, biogeographically interesting group, catfishes have recently been a topic of interest in several phylogenetic studies (Diogo, 2004; Hardma, 2005; Sullivan et al., 2006; Lundberg et al., 2007). When combined with these data, information regarding the distribution of venom glands within the Siluriformes can be examined in an evolutionary context, and we can begin to build a foundation to advance the studies of venom evolution in this group to the level seen in other venomous organisms. In this work, I use histological and toxicological techniques to elucidate the

diversity and taxonomic distribution of venomous catfishes and examine these findings within the phylogenetic framework established by previous authors to provide a broad-scale hypothesis for the evolutionary origin of venom glands in catfishes. These examinations are further integrated with preliminary biochemical characterizations of venoms from several catfish species to highlight several intriguing parallels between the evolution of venoms in catfishes and other venomous organisms.

METHODS AND MATERIALS

Venom Gland Survey and Histological Techniques

The right pectoral-fin spine was removed from 158 catfish specimens (see Table 2-1), housed in the fish collection of the University of Michigan Museum of Zoology. Spines were decalcified in CalEx® according to the manufacturer's instructions, after which segments from the distal third of the spine of an appropriate size for histological preparation were removed. These segments were subjected to automated dehydration and paraffin infiltration and embedding at the Tissue Core Facility of the University of Michigan Comprehensive Cancer Center. Serial sections of 0.7 microns were then obtained from each spine sample. Sections were stained with hematoxylin and eosin and mounted on glass slides.

Spines were examined for the presence of venom glands using a Nikon YS2-T compound microscope. Morphological confirmation of the presence of venom gland cells was achieved by comparisons with previously published photomicrographs of venom glands in catfishes and spiny rayed fishes (Halstead et al., 1953; Cameron & Endean, 1973; Halstead, 1978; Whitear et al., 1991), descriptions of piscine venom gland cellular anatomy (Halstead, 1978), and sections obtained from the spines of catfish species that

have been shown to secrete venomous substances by previous studies (Birkhead, 1972; Halstead, 1978). When a representative of a particular genus was found to possess venom glands, all members of that genus were presumed to be venomous, except in the case of the ictalurid genus *Ameiurus*, where the examination of multiple species within the genus indicated otherwise. These generic counts of venomous species formed the basis for the minimum estimate of venomous catfish species (Table 2-2). The number of species contained in unexamined genera from families containing venomous representatives was added to the minimum estimate to give a maximum estimate of venomous catfish species (Table 2-2).

Venom Gland Extract Preparation and Assay

Representatives of the catfish families Ariidae, Bagridae, Callichthyidae, Ictaluridae, Mochokidae, Pangasiidae, Pimelodidae, Pseudopimelodidae, and Plotosidae were obtained either from field collections (Ictaluridae) or the aquarium trade (other families). Specimens were euthanized using MS-222 at a concentration of 300 mg/L in fresh water. All further preparations were carried out either on ice or under refrigeration at 4°C. Spines and caudal fin tissue were removed from each specimen, rinsed in physiological saline and gently scraped with a microspatula in order to remove any excess epidermal secretions, and weighed to the nearest 0.001 g using a GeneMate digital balance. Spines were minced and then further homogenized in a 2 mL Dounce homogenizer along with either marine (Plotosidae) or freshwater (other families) euteleost physiological saline (Hoar & Hickman, 1975) at a volume of 2 mL/g of tissue. The homogenate was then centrifuged at 6,000 rpm at 4°C for 20 minutes and the supernatant collected. The

supernatant served as the crude venom extract. Control extracts prepared from caudal fin tissue were prepared in the same manner.

Largemouth Bass were collected from Boyden Creek, Washtenaw Co., MI in October of 2008. Bass were anesthetized in MS-222 at a concentration of 75 mg/L of fresh water and weighed to the nearest 0.1 g. They were then placed in 10 G experimental aquaria in a room with natural light and allowed to acclimate for a period of 72 hours. After the 72 hour acclimation period, bass were injected in the caudal peduncle at a depth of 2 mm with 2 μL/g body weight of either crude venom extract or control extract. Individuals were then observed at one minute, one hour, and 24 hours after injection for symptoms consistent with envenomation (Table 2-3). For each species of catfish tested, two bass were injected with venom extract and two were injected with caudal fin control extract.

Character Optimization Analyses

Several previously published phylogenetic hypotheses for the order Siluriformes (Diogo, 2004; Sullivan et al., 2006; Mo, 1991) were examined using MacClade 4.0 PPC (Maddison & Maddison, 2000). Presence and absence of venom glands was traced onto the trees using the criterion of maximum parsimony. Specific taxa that were present in the phylogenetic reconstruction but which were not examined in the current study were coded as ambiguous (?) within the data matrix.

SDS-PAGE Analyses

Crude extracts were prepared for SDS-PAGE analysis by reduction with NuPAGE® reducing agent and loading buffer, according to manufacturer's instructions. Reduced samples were subjected to electophoresis in NuPAGE® precast 4-12 % Bis-Tris

polyacrylamide gels in 1X MES running buffer for 35 minutes, at 200V in an x-Cell SureLock™ Mini Cell. Reduced peptides were visualized using SimplyBlue™ SafeStain according to manufacturer's instructions. Molecular weights of venom and caudal fin extracts were estimated by comparison with Novex® Sharp Protein Standard. Proteins unique to venom extracts (relative to caudal-fin extracts) were treated as putative toxins, pending further characterization.

RESULTS

To establish a preliminary estimate of the number and phylogenetic distribution of venomous catfish species, 159 species from over 100 genera, representing 32 of the 34 siluriform families were examined for the presence of venom glands (Table 2-1). Material for representatives of the families Austroglanididae and Lacantuniidae was unavailable for study, but their omission from this study has little effect on estimates of the number of venomous catfish species, due to the low species diversity of these families (three species and one species, respectively). Structures identified as venom glands were observed in 20 families. Venom gland size, orientation, and cellular morphology were found to vary considerably between, and sometimes within, families (Table 2-1; Figs. 2-2, 2-3). Based upon the generic identity of the venomous species identified, the number of species contained within those genera, and the number of remaining unexamined species in those families shown to contain venomous representatives, an estimate of 1234–1625 venomous catfish species was developed (Table 2-2).

The production of toxic compounds by representatives from several siluriform families was confirmed through analysis of effects of crude fin-spine extracts on a predatory fish species. The injection of fin-spine extracts caused symptoms of

envenomation in all cases; in all cases but one (*Plotosus lineatus*), injection with control extracts prepared from fin tissue yielded no appreciable effect. Symptoms produced by the venoms tested included chromatophore expansion at the injection site, loss of coloration elsewhere on the body, hemorrhage, loss of equilibrium, muscle spasm, and in one instance (*Plotosus lineatus*), rapid mortality (Table 2-3). Symptoms of envenomation occurred immediately and were resolved within an hour in most trials. Though representatives from several families were not examined, species in those families possess cells associated with their fin spines that have similar, if not identical, morphologies to the venomous species tested, suggesting that these cells produce toxic substances in the untested families as well.

The evolution of venom glands within the order Siluriformes was examined by performing unambiguous parsimony character optimization analyses on several previously published siluriform phylogenies that were reconstructed from both morphological (Diogo, 2004; Mo, 1991) and molecular (Sullivan et al., 2006) data. Multiple phylogenies were analyzed due to the fact that the relationships of some siluriform families are either poorly resolved or vary between reported phylogenies. Given the widespread presence of venom glands in catfishes, it was expected that these previous systematic studies, in conjunction with the results presented above, would offer some insight into broader phylogenetic patterns of siluriform venom gland evolution in spite of the poor resolution of familial relationships found in these phylogenies.

Character optimization anlyses of these phylogenies indicate that this trait has arisen at least twice (Figs. 2-4, 2-5) and potentially three or more times (Fig. 2-6). Venom glands evolved once within the Loricarioidei, a diverse and exclusively Neotropical

suborder of armored catfishes, in the family Callichthyidae. They also appear independently at least once basally within the Siluroidei, a clade containing all other non-loricarioid catfishes with the exception of the Diplomystidae. A recent molecular phylogeny based on nuclear gene sequences (RAG1 and RAG2) implies an additional evolution of venom glands within the Doradidae, owing to their placement within a clade of South American catfishes including the apparently nonvenomous Aspredinidae and Auchenipteridae (Sullivan et al., 2006; Fig. 2-6).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to identify venom proteins with similar molecular weights that are shared between species (and families), potentially reflecting homology of these proteins. Comparisons with extracts prepared from caudal-fin tissue were used to identify putative toxin peptides. The composition of different species' venoms was found to vary considerably, but some strong similarities were also evident. A putative toxin peptide of approximately 110 kDa was found in very high concentrations in the venom extracts of eight of the nine species examined (Fig. 2-7). Although a protein with a similar molecular weight was also found in the caudal-fin extracts of several species, it was generally found in much lower concentrations, and previous authors have stated that at least some toxin producing cells may be present in the fin tissue of catfishes (Birkhead, 1972). In addition to the siluroid species tested, a 110 kDa peptide also appears to be present in the venom extracts of several species of *Corydoras*. *Corydoras* is distantly related to the remaining species analyzed, and the possession of venom glands by members of the family Callichthyidae appears to represent an independent evolution of these structures. A protein having this molecular weight was not found in the fin-spine extracts of *Pimelodus pictus*, a species

shown by the current study to be venomous, reflecting a secondary loss of this putative toxic peptide. Additionally, nearly every siluroid species examined displayed at least one (and often more) putative toxic peptide(s) of approximately 10-20 kDa in weight. These peptides appear to vary significantly within this range however, and no molecular weight was represented with the same frequency as the 110 kDa peptide described above.

DISCUSSION

Venomous Catfish Diversity

Examinations of histological sections of pectoral-fin spines, in conjunction with character optimization analyses of previously published siluriform phylogenies and toxicological assays, imply that approximately 1250-1625 species of catfishes from at least 20 families are venomous. These numbers are much higher than previous estimates, based largely on anecdotal evidence, which suggested a maximum of 1000+ venomous catfish species (Smith & Wheeler, 2006). Of these families, 14 (Akysidae, Anchariidae, Callichthyidae, Chacidae, Claroteidae, Cranoglanididae, Doradidae, Heptapteridae, Mochokidae, Pangasiidae, Pimelodidae, Pseudopimelodidae, Schilbidae, Siluridae) are shown to contain venomous taxa for the first time; six (Amblycipitidae, Ariidae, Bagridae, Clariidae, Ictaluridae, Plotosidae) have previously been demonstrated to contain venomous representatives (Halstead, 1978). The approximation of 1250 species of venomous catfishes is undoubtedly an underestimate, as many genera in siluriform families containing venomous taxa remain to be examined. New species of catfishes are also continuously being discovered and described [958 species described in the last 10 years according to the Catalog of Fishes (Eschmeyer & Fricke, 2009)], with some

venomous genera such as *Chiloglanis* (Mochokidae) containing an estimated 25 or more undescribed species (J.P. Friel, pers. comm.).

The apparently low incidence of independent venom gland evolution in catfishes stands in stark contrast to the results obtained for venomous spiny-rayed fishes, in which venom glands appear to have evolved independently no fewer than nine times (Smith & Wheeler, 2006). The exact number of times that venom glands arose within the Siluroidei remains ambiguous, though the majority of possible resolved topologies would require only a single derivation. However, the hypothesis of an additional derivation of venom glands in the family Doradidae that would be necessitated by the results of recent molecular phylogenetic analyses (Sullivan et al., 2006; Lundberg et al., 2007) does warrant further investigation. The venom glands found in doradid species differ morphologically from those seen in other siluroid families, by virtue of their structure (discrete clusters of glandular tissue internally subdivided into pockets of glandular cells by integumentary septa vs. continuous single sheaths of glandular cells) (Figs. 2-2 and 2-3), orientation (limited to spaces between posterior serrae of dorsal and pectoral-fin spines vs. being found along the entire length of the spines), and visibility without magnification (Fig. 2-8). Future studies of doradid venom composition should help to clarify this issue.

The loss of venom glands appears to be a common phenomenon within catfishes, which is not surprising given that bony fin spines have been lost in some families (Malapteruridae, most amphiliids). Genera in several families that contain venomous representatives (Heptapteridae, Pimelodidae, Siluridae) have also lost bony dorsal and/or pectoral-fin spines. Without an effective delivery system, there would seem to be no

selection pressure for the maintenance of venom producing structures, leading to their reduction and eventual loss. The apparent loss of venom glands in groups that have maintained bony fin spines (Aspredinidae, Auchenipteridae, Sisoridae, some ictalurids; see Table 2-1) is more unexpected, and explanations for these losses are not immediately apparent.

Inter- and intrageneric loss of venom glands was also found within the family Ictaluridae (Table 2-1). Both *Ameiurus melas* and *Pylodictus olivaris* lack any structures that could be identified as venom glands based on histological examination. Additionally, SDS-PAGE analysis detected no putative venom peptides in either species (Fig. 2-7). This finding was particularly surprising for *A. melas*, which had previously been considered venomous and quite virulent, based upon toxicological and histological work (Birkhead, 1972; Halstead, 1978). This discrepancy may be attributable to geographic variation in venom production; *A. melas* is a widely distributed species and the specimens examined in the current study were collected in Michigan, while those used in the previous toxicological study came from Texas. A potentially important factor in the case of *Pylodictus* is that this species can reach adult sizes that would presumably prohibit predation by even the largest North American predatory fishes (all of which are gape-limited predators), possibly weakening or eliminating selection for the maintenance of venom glands through adulthood.

The number of venomous catfishes estimated by this study (when combined with estimates of venomous spiny-rayed and cartilaginous fishes) supports previous claims that venomous fishes far outnumber all other venomous vertebrates (Smith & Wheeler, 2006), and also demonstrates that venomous catfish diversity likely equals or exceeds

that of all other venomous vertebrates (including other fishes) combined (Table 2-4). Recently, some lizards and snakes traditionally considered to be non-venomous have been shown to produce several of the same toxic compounds as their venomous relatives (Fry et al., 2006). Many of these species appear to lack a specialized mechanism for transmitting these compounds, possibly preventing them from being classified as venomous in the traditional sense (Zug et al., 2001), due to a potential inability to effectively utilize these compounds in feeding. However, recent work has shown that venom is likely to play a previously unsuspected, but major role in the feeding ecology of *Varanus komodoensis* (Komodo Dragon) (Fry et al., 2009). This finding strongly indicates that such a role will be found for venom in other groups of lizards as well, potentially vastly increasing the estimate of venomous reptile diversity.

Evolution of Catfish Venoms

Cameron & Endean (1973) hypothesized that the venom glands of fishes are derived from glandular epidermal cells that secrete toxic proteinaceous compounds (termed "ichthyocrinotoxins") when fishes are threatened or injured. While it is true that these compounds are secreted in these situations, the hypothesis that they serve in an antipredatory capacity in catfishes appears flawed. With the exception of ichthyocrinotoxins associated with the epidermis of the dorsal and pectoral fin, there is no effective delivery device for these compounds, which are produced all over the body. This is of particular importance, as all assays demonstrating toxicity of epidermal secretions of catfishes have relied on intravenous injection of these compounds as a toxicological assay (Shiomi et al., 1986, 1987; Alnaqeeb et al., 1989; Thomson et al., 1998). Furthermore, the presence of epidermal secretions does not appear to be a significant deterrent to potential predators, as they will attack and feed on distressed

catfishes, as well as other baits coated with catfish epidermal secretions (Al-Hassan, 1985; pers. obs.).

That venom glands in catfishes produce similar compounds to epidermal glandular cells has been indicated by immunocytochemical assays (Shiomi et al., 1988). The results of SDS-PAGE analyses presented here offer additional support for the similarity of these secretions. The major toxic factor of the skin secretion of *Arius bilineatus* has been isolated and shown to have a molecular weight of approximately 39 kDa (Thomson et al., 1998). The venom of *Arius jordani* clearly shows a strong band at approximately 39 kDa which is found in low concentration in the control lane (Fig. 2-7). The presence of this protein in the control sample is likely due to the presence of epidermal secretory cells in the tissue sample used, while the low concentration is due to the removal of most of the epidermal secretions before sample preparation. While these cells were also probably present in spine samples, the large difference in concentration indicates that venom gland cells are likely responsible for production of most of this protein band. A similar case is seen in the electrophoretic profile of *Plotosus lineatus,* which shows major toxin bands at 15-16 kDa and 13-14 kDa (Fig. 2-7). While the larger band is similar in weight to toxin-PC, as characterized by Auddy & Gomes (1996), the lower band is very similar in weight to a toxic fraction isolated from the skin secretions of this species (Shiomi et al., 1986, 1987), with the slight discrepancy in estimated size possibly being due to differences in sample preparation and analysis.

While the venom gland cells in catfishes (and other fishes as well) are likely to be derived from epidermal secretory cells, an alternative scenario to Cameron and Endean's antipredatory hypothesis is also able to explain their origin. Studies of the epidermal

secretions of several *Arius* species have indicated that these compounds are able to accelerate healing of wounds and may also have some antimicrobial properties (Al-Hassan et al., 1983, 1985, 1987). The spines of catfishes act to effectively increase their cross-sectional circumference when locked into place, and would likely be the first structures to contact a gape-limited predator's tissues during an attack. As such, the spines would often be damaged, and individuals with larger numbers of epidermal secretory cells surrounding the spine could gain a selective advantage due to decreased healing time and a corresponding decreased chance of infection of exposed tissues. This selection may have led to increased aggregations of these cells around the fin spines, with the toxic effects of their secretions being an epiphenomenon to their primary healing benefits. Once the toxic secretions had become associated with an effective delivery device, selection for increased toxicity, as seen in some plotosid and clariid species, could begin to operate. Explicit tests of this scenario will require more detailed structural and genetic characterizations of these compounds.

The symptoms of envenomation produced by a diverse array of catfish species' venoms are very similar and a large number of putative toxins appear to fall within a well-defined molecular weight range. The conserved molecular weight patterns and toxic effects of catfish venom peptides suggest two possible scenarios for the evolution of venoms in catfishes: widespread convergent evolution of catfish venom toxins with similar targets and thus similar molecular characteristics and effects, or common origins of toxic peptides with subsequent species-specific alterations. The widespread presence of venom glands shown by the character optimization evidence discussed above strongly

suggests that the latter case is the more parsimonious and likely scenario, even in cases where phylogenetic resolution of basal siluriform divergences is lacking.

Conclusions

This study utilizes several lines of investigation to increase our knowledge of several poorly understood areas of the biology of venomous catfishes. These investigations have demonstrated that at least 1250, and possibly over 1600 species of catfishes may be venomous, a number far greater than any previous estimate of venomous catfish diversity. In conjunction with previous systematic studies, these findings also offer insight into the evolutionary history of venom glands in the order Siluriformes, indicating at least two independent evolutionary origins of these structures. Finally, the symptoms of catfish envenomation, along with preliminary biochemical characterizations of toxic catfish venom peptides, may suggest a novel selective explanation for the evolution of catfish venom glands and their secretions.

Finer-scale studies of venom gland evolution in fishes will require continued systematic studies of venomous fish families to elucidate the relationships of the species contained therein. Additionally, examinations of the chemical composition of fish venoms and the identities and structures of their constituents will provide valuable insight into the mechanisms and potential selective factors driving venom evolution in fishes, as well as their potential for biomedical research and pharmaceutical bioprospecting.

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Table 2-1. List of species examined over the course of this study, with brief description of the presence/absence of potential venom delivery system (Bony Spine), venom gland condition, and voucher specimen catalog number. Names and familial memberships follow Ferraris (2007).

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Akysidae			
Acrochordonichthys rugosus	Y	Paired, in anterior grooves	UMMZ 245670
Akysis hendricksoni	Y	Paired, in anterior grooves	UMMZ 238793
Breitensteinia cessator	Y	Spine complete, but lacking glandular cells and anterior grooves	UMMZ 243238
Parakysis anomalopteryx	Y	Glands stripped, well-developed anterior grooves present	UMMZ 209923
Pseudobagarius inermis	Y	Paired, in anterior grooves	UMMZ 234709
Pseudobagarius similis	Y	Paired, in anterior grooves	UMMZ 241324
Amblycipitidae			
Amblyceps mangois	Y	Paired, in anterior grooves	UMMZ 244760
Liobagrus mediadiposalis	Y	Paired, in anterior grooves	UMMZ 238983
Liobagrus reini	Y	Paired, in anterior grooves	UMMZ 183862
Amphiliidae			
Amphilius uranoscopus	N	No discernible glandular cells	UMMZ 199996
Leptoglanis rotundiceps	Y	No discernible glandular cells	UMMZ 200020
Anchariidae			
Gogo ornatus	Y	Multiple bundles of glandular cells along posterior half of spine	UMMZ 244995

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Ariidae			
Arius aguadulce	Y	Paired anterior glands	UMMZ 143460
Bagre marinus	Y	Paired anterior glands	UMMZ 244720
Batrachocephalus mino	Y	Spine damaged, anterior glandular cell remnants visible	UMMZ 155787
Cochlefelis danielsi	Y	Spine damaged, posterior glandular cells visible	UMMZ 214019
Hemipimelodus borneensis	Y	Small, paired, posterior glands	UMMZ 214617
Osteogeniosus militaris	Y	Spine damaged, posterior glandular cells visible	UMMZ 245436
Potamarius nelsoni	Y	Spine damaged, anterior glandular cells visible	UMMZ 143498
Aspredinidae			
Bunocephalus rugosus	Y	No discernible glandular cells	UMMZ 206289
Dysichthys bifidus	Y	No discernible glandular cells	UMMZ 204374
Astroblepidae			
Astroblepus chotae	N	No discernible glandular cells	UMMZ 179260
Auchenipteridae			
Ageneiosus sp.	Y	No discernible glandular cells	UMMZ 240342
Centromochlus sp.	Y	No discernible glandular cells	UMMZ 214828
Entomocorus benjamini	Y	No discernible glandular cells	UMMZ 204709
Parauchenipterus striatulus	Y	No discernible glandular cells	UMMZ 216166
Trachelyopterus coriaceus	Y	No discernible glandular cells	UMMZ 216161

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Bagridae			
Bagrichthys majusculus	Y	Paired anterior and posterior glands	UMMZ 241720
Bagrus docmac	Y	Anterior glandular cells present	UMMZ 187332
Batasio affinis	Y	Small, paired, posterior glands	UMMZ 245967
Hemibagrus spilopterus	Y	Paired anterior and posterior glands	UMMZ 238651
Hyalobagrus flavus	Y	Spine damaged, posterior glandular cells visible	UMMZ 248500
Mystus mysticetus	Y	Spine damaged, anterior and posterior glandular cells visible	UMMZ 232730
Nanobagrus nebulosus	Y	Single circumferential gland	UMMZ 238794
Olyra longicaudata	Y	Glandular cells near anterior groove	UMMZ 243657
Pseudomystus siamensis	Y	Paired hemispherical glands	UMMZ 224812
Rama chandramara	Y	Single circumferential gland	UMMZ 247463
Rita rita	Y	Spine damaged, large, paired, posterior glands visible	UMMZ 244943
Sperata aor	Y	Spine stripped, no glandular cells visible	UMMZ 208359
Tachysurus crassilabris	Y	Paired anterior and posterior glands	UMMZ 232107
Tachysurus intermedius	Y	Paired anterior and posterior glands	UMMZ 245073
Callichthyidae			
Aspidoras taurus	Y	Circumferential glandular cells	UMMZ 236693
Callichthys callichthys	Y	Circumferential glandular cells	UMMZ 235769
Corydoras aeneus	Y	Circumferential glandular cells	UMMZ 205959

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Callichthyidae (cont.)			
Corydoras splendens	Y	Spine stripped, no glandular cells visible	UMMZ 235764
Dianema longibarbis	Y	Paired posterior glands, a few anterior glandular cells	UMMZ 235768
Hoplosternum littorale	Y	Unorganized anterior glandular cells	UMMZ 207376
Cetopsidae			
Cetopsis plumbea	N	No discernible glandular cells	UMMZ 203882
Helogenes marmoratus	N	No discernible glandular cells	UMMZ 232086
Chacidae			
Chaca chaca	Y	Paired anterior glands	UMMZ 244665
Clariidae			
Clarias theodorae	Y	Paired hemispherical glands	UMMZ 200160
Dinotopterus cunningtoni	Y	Circumferential glandular cells	UMMZ 199927
Encheloclarias velatus	Y	Spine stripped, no glandular cells visible	UMMZ 243883
Gymnallabes typus	Y	Circumferential glandular cells	UMMZ 243235
Heterobranchus longifilis	Y	Spine broken, glandular cells present	UMMZ 189155
Heteropneustes fossilis	Y	Small, paired, posterior glands	UMMZ 209199
Tanganikallabes sp.	Y	Single large, circumferential gland	UMMZ 196021
Xenoclarias holobranchus	Y	Spine damaged, ventral hemispherical gland visible	UMMZ 187331

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Claroteidae			
Auchenoglanis occidentalis	Y	Paired posterior glands, small anterior gland also visible	UMMZ 200182
Bathybagrus tetranema	Y	Spine damaged, posterior glands visible	UMMZ 196092
Chrysichthys mabusi	Y	Spine damaged, posterior glands visible	UMMZ 200184
Clarotes laticeps	Y	Paired, anterior glands visible, posterior portion of spine stripped	UMMZ 195024
Lophiobagrus cyclurus	Y	Spine damaged, large, hemispherical glands evident	UMMZ 199932
Cranoglanididae			
Cranoglanis henrici	Y	Spine damaged, lateral gland remnants visible	UMMZ 238763
Diplomystidae			
Diplomystes nahuelbutaensis	Y	No discernible glandular cells	UMMZ 227170
Doradidae			
Amblydoras hancockii	Y	Glandular tissue between posterior serrae	UMMZ 66314
Doras micropoeus	Y	Glandular tissue between posterior serrae	UMMZ 216217
Leptodoras nelsoni	Y	Glandular tissue between posterior serrae	UMMZ 245737
Lithodoras dorsalis	Y	Glandular tissue between posterior serrae	UMMZ 230776
Opsodoras humeralis	Y	Glandular tissue between posterior serrae	UMMZ 216894
Physopyxis lyra	Y	Spine stripped, no glandular cells visible	UMMZ 204520
Trachydoras paraguayensis	Y	Glandular tissue between posterior serrae	UMMZ 207842

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Heptapteridae			
Imparfinis lineata	N	Posterior glandular cells visible	UMMZ 194201
Myoglanis sp.	Y	Single large, circumferential gland	UMMZ 231759
Pimelodella mucosa	Y	Spine damaged, paired, large, circumferential glands visible	UMMZ 207033
Rhamdia guatemalensis	Y	Paired anterior and posterior glands	UMMZ 193940
Ictaluridae			
Ameiurus brunneus	Y	No discernible glandular cells	UMMZ 156102
Ameiurus melas	Y	No discernible glandular cells	UMMZ 243064
Ameiurus natalis	Y	Paired, in anterior grooves	UMMZ 233519
Ameiurus nebulosus	Y	Paired, in anterior grooves	UMMZ 230890
Ameiurus platycephalus	Y	Paired, in anterior grooves	UMMZ 225986
Ameiurus serracanthus	Y	4 distinct pairs, surrounding spine	UMMZ 186261
Ictalurus furcatus	Y	Paired, in anterior grooves	UMMZ 201496
Ictalurus pricei	Y	Spine damaged anteriorly, anterior groove visible, lacking glandular cells, glandular tissue visible posteriorly	UMMZ 161510
Ictalurus punctatus	Y	Paired, in anterior grooves	UMMZ 114940
Noturus albater	Y	Paired, in anterior grooves	UMMZ 167170
Noturus elegans	Y	Paired, in anterior grooves	UMMZ 165395

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Ictaluridae (cont.)			
Noturus eleutherus	Y	Spine damaged anteriorly, well-developed anterior groove visible, lacking glandular cells, glandular tissue visible posteriorly	UMMZ 66624
Noturus flavus	Y	Paired, in poorly-developed anterior groove	UMMZ 199203
Noturus furiosus	Y	Large hemispherical glands	UMMZ 107097
Noturus insignis	Y	Paired, in anterior grooves	UMMZ 36151
Noturus leptacanthus	Y	Paired, in anterior grooves	UMMZ 242770
Noturus munitus	Y	Paired, in anterior grooves	UMMZ 181771
Noturus nocturnus	Y	Paired, in anterior grooves	UMMZ 242696
Noturus placidus	Y	Paired, in anterior grooves	UMMZ 167656
Noturus stigmosus	Y	Paired, in multiple grooves	UMMZ 248603
Pylodictis olivaris	Y	Spine damaged, no glands evident	UMMZ 226324
Loricariidae			
Ancistrus cirrhosus	Y	No discernible glandular cells	UMMZ 204398
Aphanotorulus unicolor	Y	No discernible glandular cells	UMMZ 205129
Farlowella kneri	Y	No discernible glandular cells	UMMZ 206541
Hemipsilichthys sp.	Y	No discernible glandular cells	UMMZ 215265
Hypostomus boulengeri	Y	No discernible glandular cells	UMMZ 207649
Loricaria cataphracta	Y	Possible glandular cells in posterior groove	UMMZ 207475

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Loricariidae (cont.)			
Otocinclus vittatus	Y	No discernible glandular cells	UMMZ 216577
Malapteruridae			
Malapterurus tanganyikaensis	N	No discernible glandular cells	UMMZ 199858
Mochokidae			
Chiloglanis productus	Y	Paired anterior and posterior glands	UMMZ 199817
Euchilichthys astatodon	Y	No discernible glandular cells	UMMZ 195064
Microsynodontis batesii	Y	Spine stripped, no glands visible	UMMZ 248519
Mochokiella paynei	Y	Spine stripped, no glands visible	UMMZ 248513
Synodontis irsacae	Y	Paired anterior and posterior glands	UMMZ 199829
Synodontis zambesensis	Y	Paired anterior glands	UMMZ 200003
Nematogenyidae			
Nematogenys inermis	N	A few possibly glandular cells	UMMZ 212697
Pangasiidae			
Helicophagus leptorhynchus	Y	Spine damaged, paired, hemispherical glands evident	UMMZ 214467
Pangasianodon hypophthalmus	Y	Paired, hemispherical glands	UMMZ 232681
Pangasius bocourti	Y	Paired, hemispherical glands	UMMZ 234583
Pseudolais pleurotaenia	Y	Paired, hemispherical glands	UMMZ 214260

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Pimelodidae			
Cheirocerus eques	Y	Spine stripped, no glands visible	UMMZ 187223
Hypopthalmus marginatus	Y	Spine stripped, no glands visible	UMMZ 231726
Megalonema platinum	N	No discernible glandular cells	UMMZ 216638
Parapimelodus valencienesi	Y	Spine stripped, no glands visible	UMMZ 218468
Pimelodus clarias	Y	Paired anterior and posterior glands	UMMZ 211343
Sorubim lima	Y	Paired anterior and posterior glands	UMMZ 242595
Plotosidae			
Paraplotosus albilabris	Y	Large, paired, dorsal and ventral glands	UMMZ 100219
Plotosus canius	Y	Large, paired, hemispherical glands	UMMZ 245508
Pseudopimelodidae			
Pseudopimelodus zungaro	Y	Paired, small, anterior glands	UMMZ 206076
Schilbidae			
Ailia coilia	Y	Spine stripped, no glands visible	UMMZ 244694
Clupisoma garua	Y	Spine stripped, no glands visible	UMMZ 208292
Eutropiichthys vacha	Y	Spine stripped, no glands visible	UMMZ 208330
Laides longibarbis	Y	Spine damaged, small, paired, posterior glands visible	UMMZ 235391
Neotropius atherinoides	Y	Small, paired, posterior glands	UMMZ 208591
Pseudeutropius brachypopterus	Y	Spine damaged, paired posterior glands visible	UMMZ 243440

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Schilbidae (cont.)			
Schilbe mystus	Y	Spine damaged, large hemispherical gland visible	UMMZ 200312
Silonia silondia	Y	Spine damaged, glandular cells visible	UMMZ 208460
Siluranodon auritus	Y	Spine stripped, no glands visible	UMMZ 195044
Scoloplacidae			
Scoloplax empousa	Y	No discernible glandular cells	UMMZ 214696
Siluridae			
Belodontichthys truncatus	N	No discernible glandular cells	UMMZ 217151
Hito taytayensis	Y	Large, posterior gland	UMMZ 100557
Kryptopterus bicirrhis	Y	Spine damaged, posterior glandular cells visible	UMMZ 241757
Ompok bimaculatus	Y	Spine damaged, posterior glands visible	UMMZ 240771
Ompok krattensis	Y	Spine damaged, posterior glands visible	UMMZ 238655
Pterocryptis berdmorei	Y	Paired, large, posterior glands	UMMZ 246494
Pterocryptis cochinchinensis	Y	Paired, large, posterior glands	UMMZ 248529
Silurichthys schneideri	Y	Paired, large, posterior glands	UMMZ 243747
Silurus asotus	Y	Spine damaged, paired anterior glands visible, posterior glandular cells also visible	UMMZ 180202
Silurus mento	Y	Spine stripped, no glands visible	UMMZ 214491
Wallago micropogon	Y	No discernible glandular cells	UMMZ 186807

Table 2-1. continued

Taxon	*Bony Spine*	*Venom Gland Condition*	*Museum Voucher*
Sisoridae			
Ayarnangra estuarius	Y	No discernible glandular cells	UMMZ 248520
Bagarius yarelli	Y	No discernible glandular cells	UMMZ 241095
Caelatoglanis zonatus	Y	No discernible glandular cells	UMMZ 247116
Conta conta	Y	No discernible glandular cells	UMMZ 247195
Erethistes pusillus	Y	No discernible glandular cells	UMMZ 247198
Gagata sexualis	Y	No discernible glandular cells	UMMZ 244895
Glyptothorax panda	Y	No discernible glandular cells	UMMZ 246004
Sisoridae (cont.)			
Glyptothorax platypogonides	Y	No discernible glandular cells	UMMZ 235704
Gogangra viridescens	Y	No discernible glandular cells	UMMZ 243717
Hara hara	Y	No discernible glandular cells	UMMZ 247446
Sisor rabdophorus	Y	No discernible glandular cells	UMMZ 240013
Trichomycteridae			
Trichomycterus areolatus	N	No discernible glandular cells	UMMZ 215412
Incertae sedis			
Horabagrus brachysoma	Y	Paired anterior and posterior glands	UMMZ 247478

Table 2-2. Taxonomic distributions and estimates of venomous catfish diversity. Basic estimates of family diversity used to generate these figures are taken from Ferraris (2007) and were supplemented through consultation of species descriptions that have been published since the completion of that study.

Taxon	*# Presumed Venomous*
Siluriformes – Catfishes	≈1250-1625 species
Akysidae – Asian stream catfishes	48
Amblycipitidae – Torrent catfishes	26-28
Anchariidae – Madagascan catfishes	4-6
Ariidae – Sea catfishes	67-134
Bagridae – Bagrid catfishes	176-198
Callichthyidae – Armored catfishes	182-194
Chacidae – Angler catfishes	3
Clariidae – Labyrinth catfishes	79-114
Claroteidae – Claroteid catfishes	56-84
Cranoglanididae – Armorhead catfishes	3
Doradidae – Thorny catfishes	48-81
Heptapteridae – Shrimp catfishes	91-160
Ictaluridae – North American catfishes	57-64
Mochokidae – Squeakers	166-189
Pangasiidae – Shark catfishes	27-30
Pimelodidae – Antennae catfishes	41-79
Plotosidae – Eeltail catfishes	17-37
Pseudopimelodidae – Bumblebee catfishes	21-31
Schilbidae – Glass catfishes	48-62
Siluridae – Sheat catfishes	74-83

Table 2-3. The effects of several catfish species' venoms on Largemouth Bass. X denotes that the effect was observed in bass injected with 2 µL/g body weight of crude venom extract. In no case except that of *Plotosus lineatus* did injection of caudal fin extract produce any of the symptoms below. In this species, injection of fin extract caused color loss, tetanus, loss of equilibrium, and eventual mortality.

	Venom Effect					
Species	Color loss	Myoclonus	Tetanus	Hemorrhage	Loss of Equilibrium	Mortality
Arius jordani (Ariidae)	X		X		X	
Corydoras paleatus (Callichthyidae)	X					
Horabagrus brachysoma (*incertae sedis*)	X		X	X	X	
Microglanis iheringi (Pseudopimelodidae)	X			X		
Noturus gyrinus (Ictaluridae)	X	X		X	X	
Pangasius hypophthalmus (Pangasiidae)		X			X	
Pimelodus pictus (Pimelodidae)	X			X		
Plotosus lineatus (Plotosidae)			X			X
Synodontis multipunctata (Mochokidae)	X	X		X		

Table 2-4. Taxonomic distributions and estimates of venomous vertebrate diversity**.** Estimates for acanthomorphs, chondrichthyans, and mammals are from Smith & Wheeler (2006). Estimates for venomous snakes and lizards are from Fry et al. (2006) and Fry et al. (2009).

Taxon	*# Presumed Venomous*
Actinopterygii – Ray-finned fishes	≈1835 – 2275 species
Siluriformes – Catfishes	≈1250-1625 species
Acanthomorpha – Spiny-rayed fishes	≈585-650 species
Chondrichthyes – Cartilaginous fishes	≈200 species
Sarcopterygii – Lobe-finned fishes and tetrapods	≈685+ species

Figure 2-1. The venom delivery system of catfishes. (A) Northern madtom (*Noturus stigmosus*) with dorsal and pectoral fin spines indicated by red arrows. (B) Pectoral girdle of *Noturus stigmosus* with articulated pectoral fin spines. Abbreviations: ps = pectoral fin spine, cle = cleithrum, cor = coracoid, cor-pp = posterior process of coracoid. (C) Cross section of the pectoral-fin spine of *Noturus stigmosus* showing the association of venom gland cells with the fin spine. Abbreviations: ps = pectoral spine, vgc = venom gland cells.

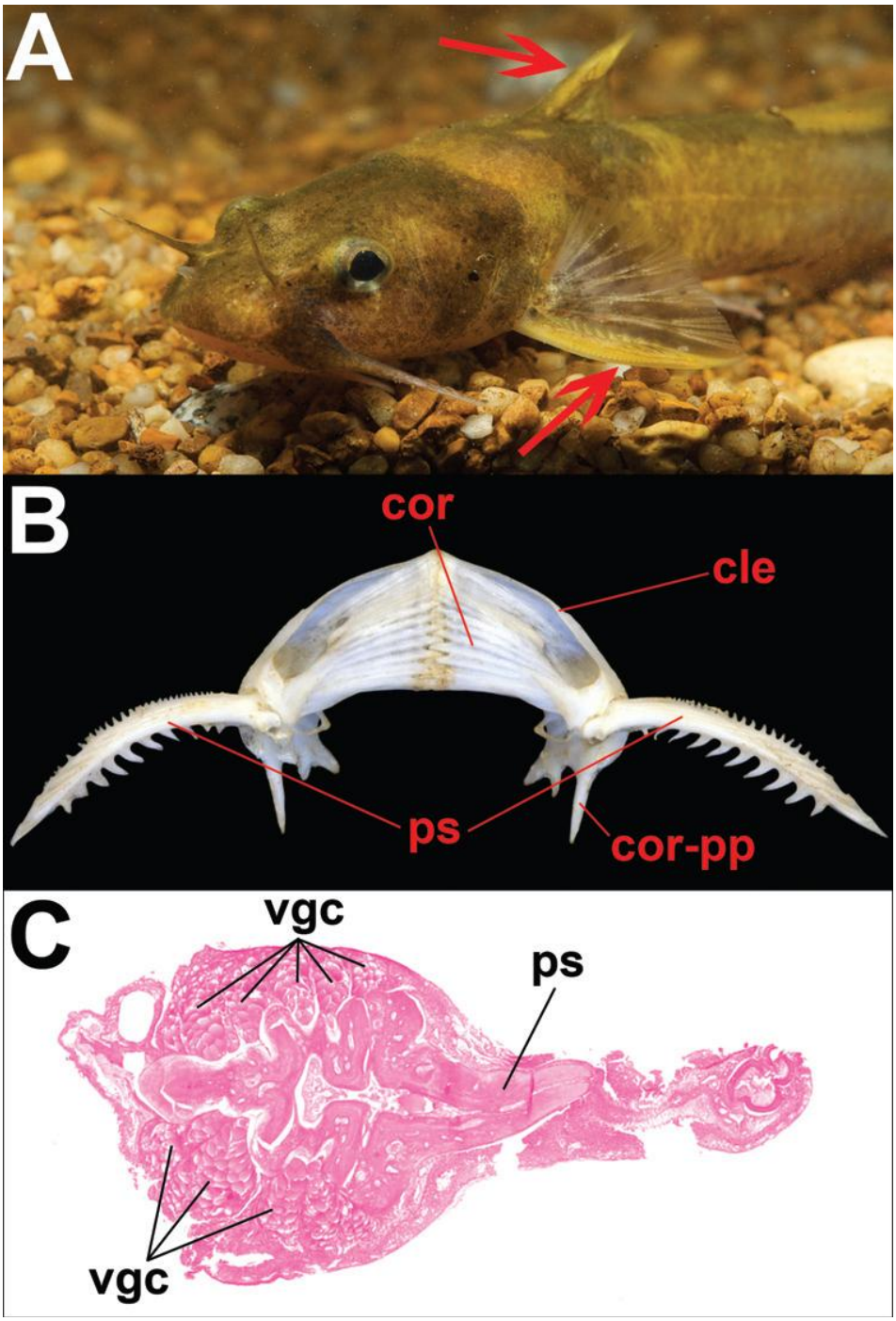


Figure 2-2. Histological preparations of fin spines from several venomous catfish species. (A) *Acrochordonichthys rugosus* (Akysidae), (B) *Liobagrus reini* (Amblycipitidae), (C) *Dianema longibarbis* (Callichthyidae), (D) *Chaca chaca* (Chacidae), (E) *Lophiobagrus cyclurus* (Claroteidae), (F) *Lithodoras dorsalis* (Doradidae). Abbreviations: ps = pectoral fin spine, vgc = venom gland cells. Scale bars, 0.5 mm.

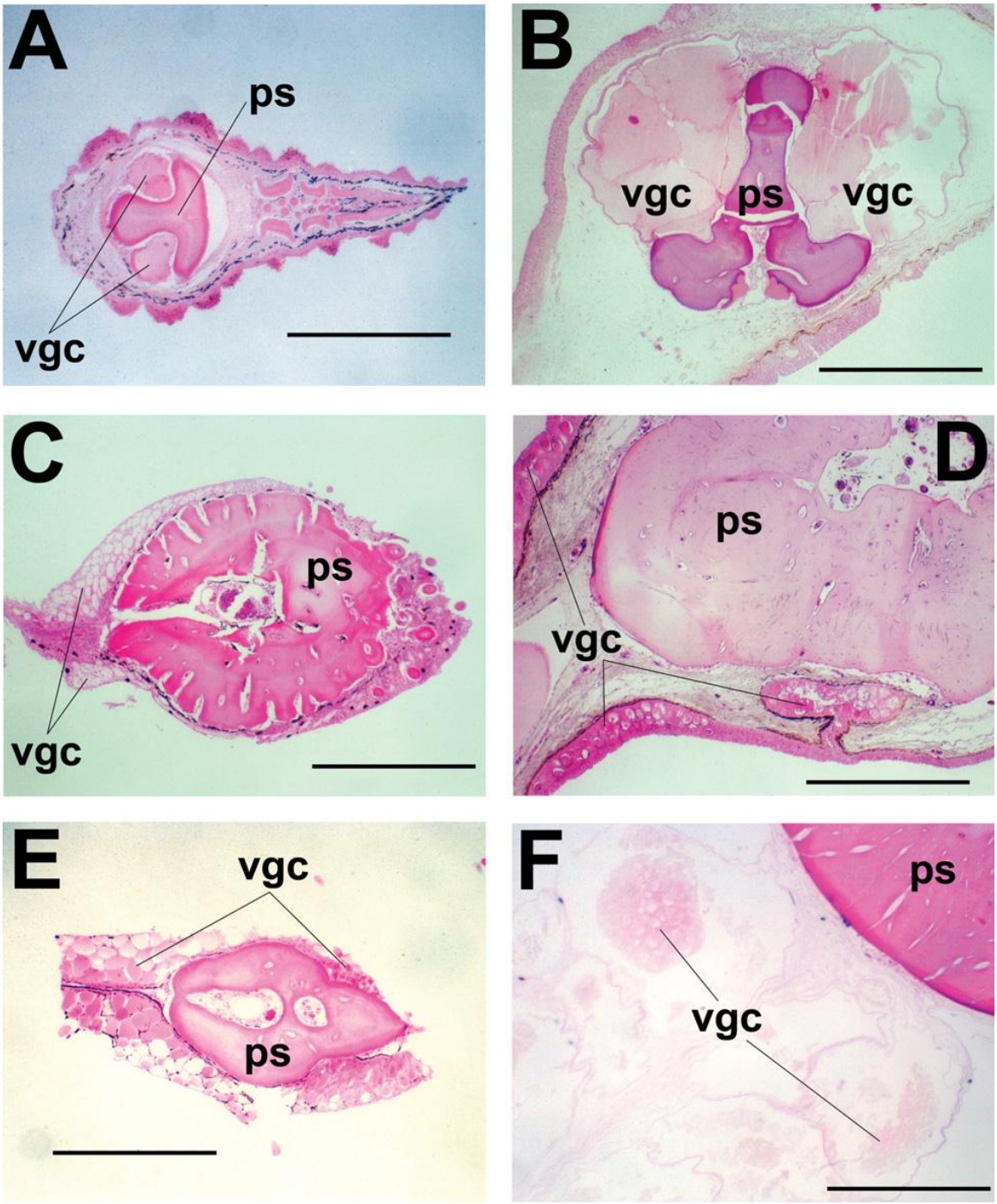


Figure 2-3. Additional histological preparations of fin spines from venomous catfish species. (A) *Pimelodella mucosa* (Heptapteridae), (B) *Chiloglanis productus* (Mochokidae), (C) *Pseudolais pleurotaenia* (Pangasiidae), (D) *Plotosus canius* (Plotosidae), (E) *Schilbe mystus* (Schilbidae), (F) *Horabagrus brachysoma* (*incertae sedis*). Abbreviations: ps = pectoral fin spine, vgc = venom gland cells. Scale bars, 0.5 mm.

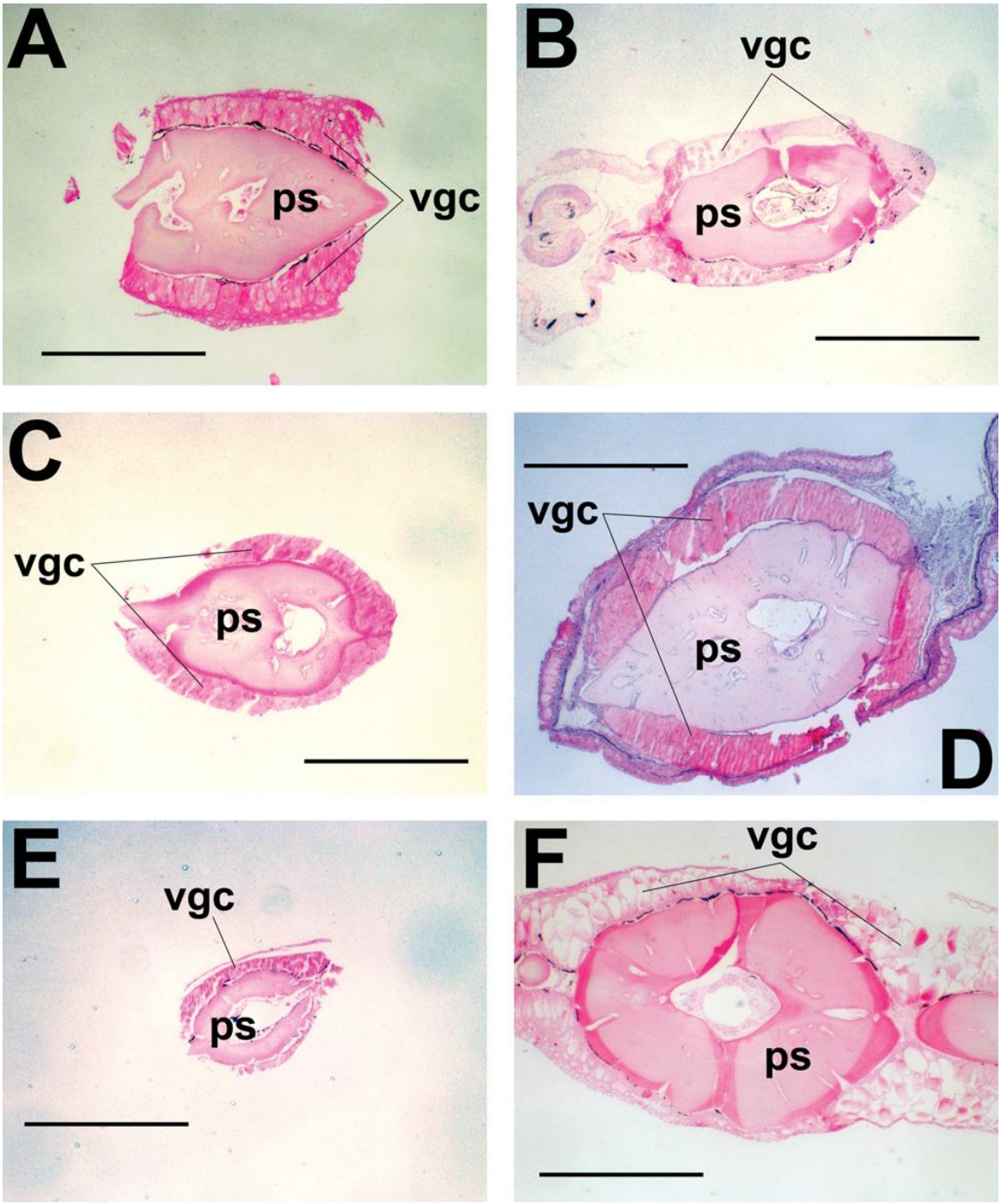


Figure 2-4. Venom glands have evolved multiple times in catfishes. The results of a character optimization analysis of a siluriform phylogeny generated from 440 morphological characters indicate the independent evolution of venom glands within the Loricarioidei as well as within the Siluroidei, leading to the majority of venomous catfish diversity. Phylogeny redrawn from Diogo (2004). Red branches indicate venomous lineages, black branches indicate non venomous lineages, yellow branches indicate lineages not examined in this study.

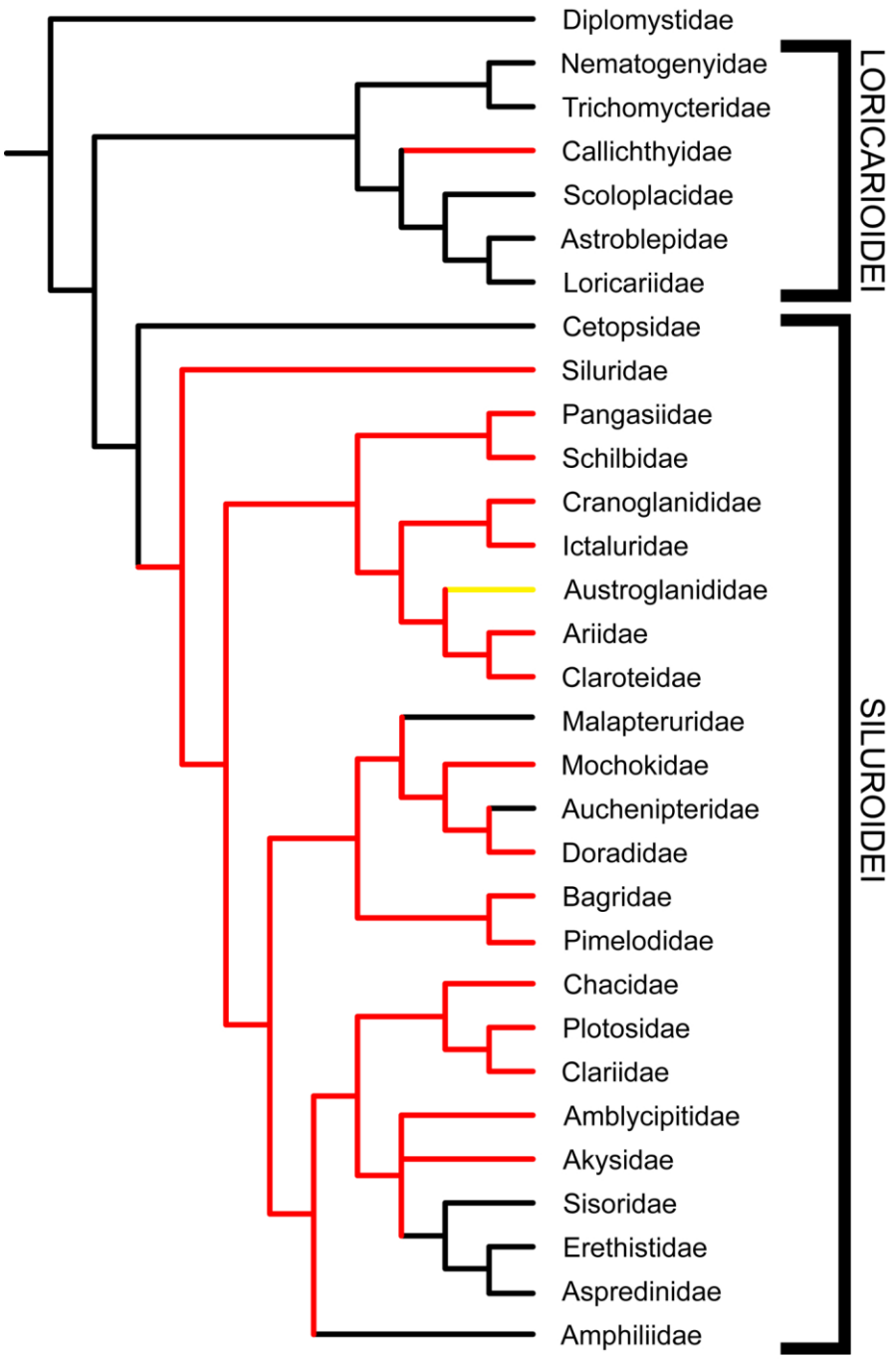


Figure 2-5. Results of character optimization analysis using an alternative morphology-based phylogeny. Phylogeny redrawn from Mo (1991), based on 126 morphological characters. Red branches indicate venomous lineages, black branches indicate non venomous lineages, and yellow branches indicate groups not examined in this study. As in Figs. 4 and 6, the independent evolution of venom glands is indicated in the Loricarioidei [*sensu* Diogo (2004) and Sullivan et al. (2006)], in the family Callichthyidae. Patterns of venom gland evolution in the Siluroidei are obscured, due to the poor resolution of basal relationships. Given the broad range of siluroid families in which venom glands are found and similarities in venom composition between these families, a single, relatively basal development of venom glands seems the most parsimonious and likely scenario.

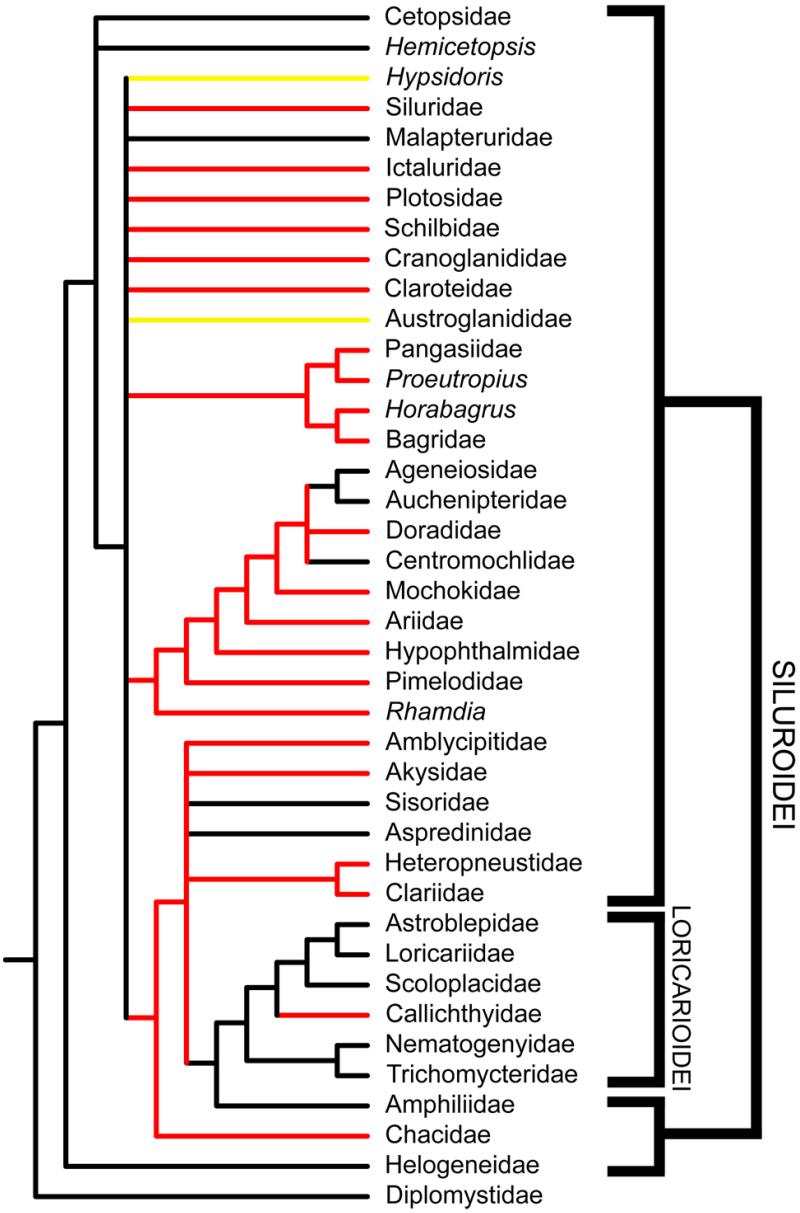


Figure 2-6. Results of character optimization analysis using a recent molecular siluriform phylogeny. Phylogeny redrawn from Sullivan et al. (2006), based on RAG 1 and RAG 2 nuclear data. Red branches indicate venomous lineages, black branches indicate non venomous lineages. Again, the independent evolution of venom glands is found in the Loricarioidei, in the family Callichthyidae. Independent evolution of venom glands must also be ascribed to the family Doradidae, due to its nesting within a clade containing the non-venomous Aspredinidae and Auchenipteridae. Similarly to Figure 2-5, the evolution of venom glands at the base of the Siluroidei is obscured, due to poor resolution of basal relationships.

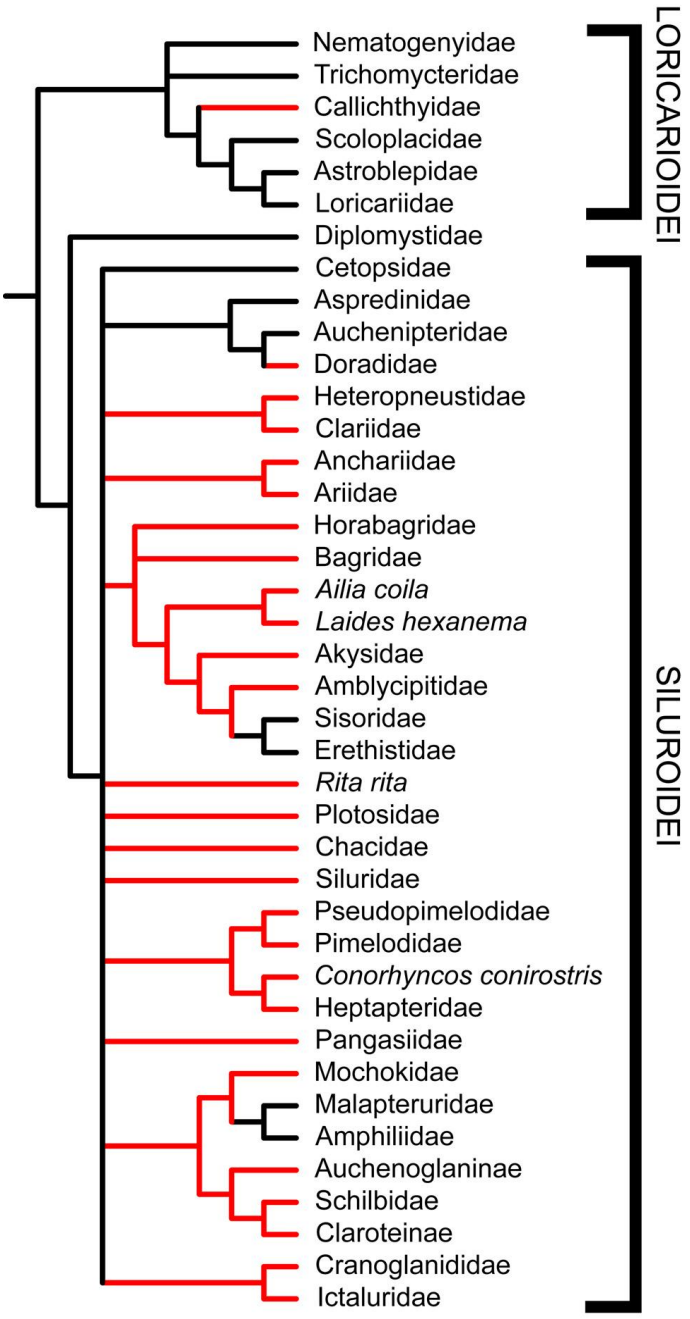


Figure 2-7. SDS-PAGE analyses of venom extracts from several catfish species. Left lanes represent venom extracts, right lanes represent extracts prepared from fin tissue. Arrows indicate positions of unique venom protein bands or proteins found in greater concentrations in venom extracts than in fin tissue extracts. (**?**) represents ambiguity between smearing and an additional, unique venom peptide band. Large quantities of a 110 kDa peptide are found in the venom extracts of nearly all species shown, with the exception of *Pimelodus*. The presence and variation of venom peptides in the size range of 10-20 kDa is also clearly visible.

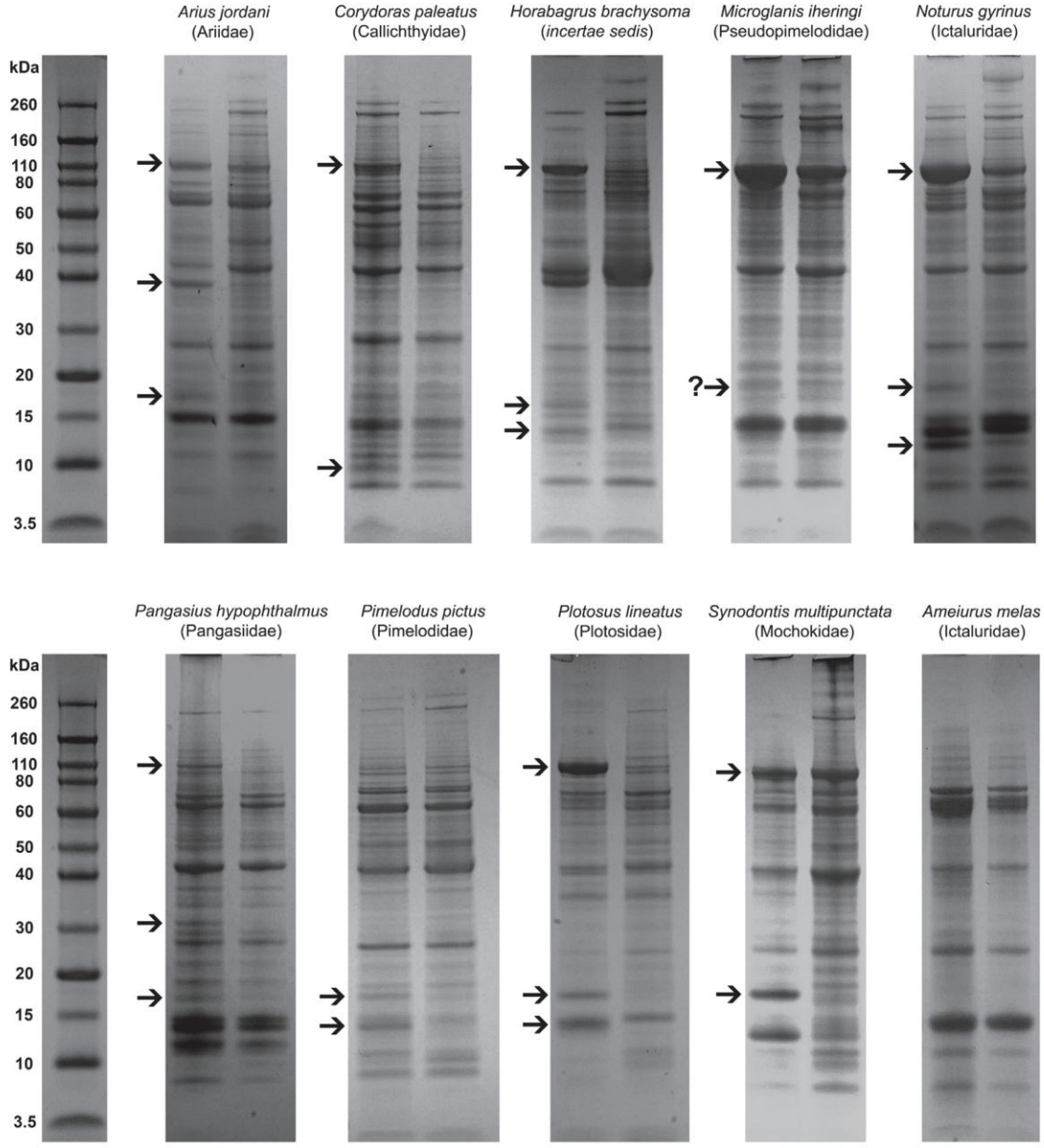
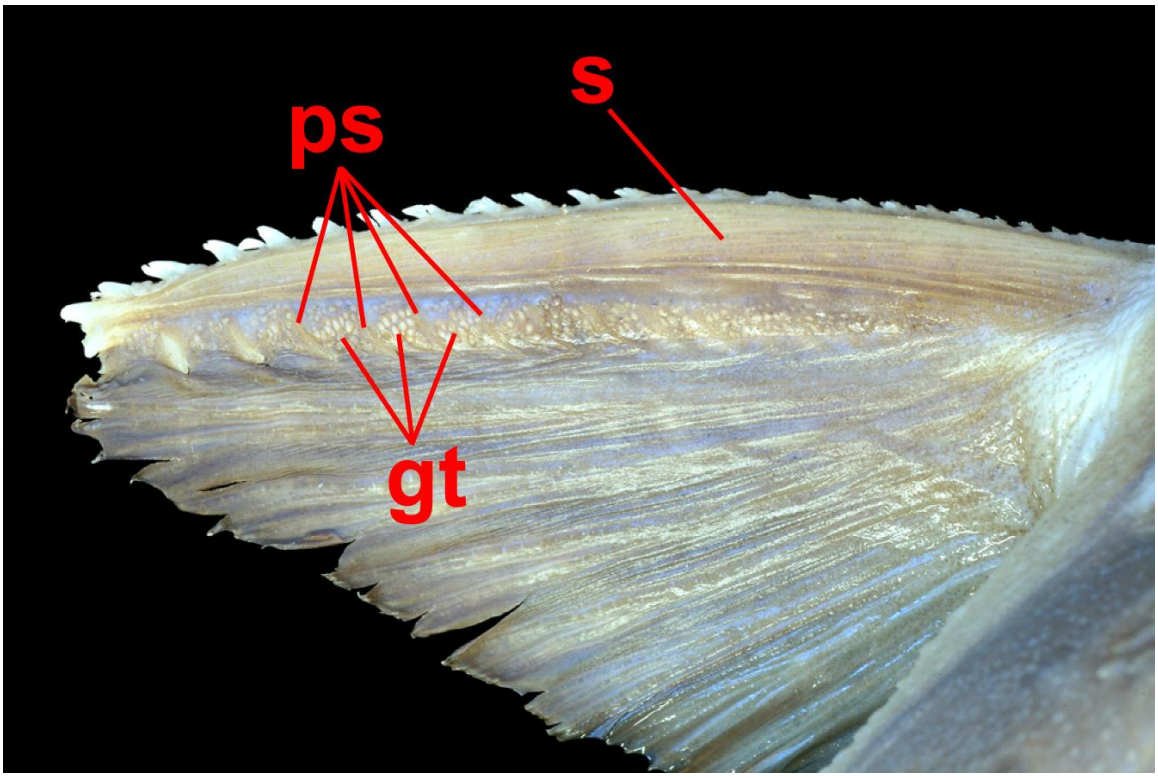


Figure 2-8. The distinctive venom delivery apparatus of a doradid catfish. Rather than forming longitudinal bundles along the spine, as in other siluroid catfishes, the glandular tissue in doradids is found in macroscopically visible aggregations between the posterior serrae of the fin spine. Abbreviations: s = pectoral spine, ps = posterior serrae, gt = glandular tissue.



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CHAPTER 3

ADAPTIVE SIGNIFICANCE OF VENOM GLANDS IN THE TADPOLE MADTOM *NOTURUS GYRINUS* (SILURIFORMES: ICTALURIDAE)[2]

ABSTRACT

Piscine venom glands have implicitly been assumed to be anti-predatory adaptations, but direct examinations of the potential fitness benefits provided by these structures are relatively sparse. Previous experiments examining this question have not presented alternative phenotypes to ecologically relevant predators, and their results are thus potentially confounded by the presence of sharp, bony fin spines in these species, which may also represent significant deterrents to predation. Here, I present the results of experiments exposing *Micropterus salmoides* (largemouth bass) to tadpole madtoms (*Noturus gyrinus*) with one of several fin spine phenotypes (intact, stripped, absent), which indicate that the venom glands of this species do provide a significant fitness benefit, relative to individuals having fin spines without venom glands, or no spines at all. Intact madtoms were repeatedly rejected by the bass and were almost never consumed, while alternative phenotypes were always consumed. Madtoms with stripped fin spines showed increases in predator rejections relative to spineless madtoms and control minnows, but non-significant increases in handling time, contrasting with previous results and predictions regarding the adaptive benefit of these structures. Comparisons with a less venomous catfish species (*Ameiurus natalis*) indicate that a

[2]A version of this chapter was previously published as: Wright, J. J. 2012. Adaptive significance of venom glands in the tadpole madtom *Noturus gyrinus* (Siluriformes: Ictaluridae). *Journal of Experimental Biology* **215**, 1816-1823.

single protein present in the venom of *N. gyrinus* may be responsible for providing the significant selective advantage observed in this species. These results, considered in conjunction with other studies of ictalurid biology, suggest that venom evolution in these species is subject to a complex interplay between predator behavior, phylogenetic history, life history strategy, and adaptive responses to different predatory regimes**.**

INTRODUCTION

The study of adaptation addresses some of the most fundamental mechanisms driving evolution and, by extension, every other aspect of an organism's biology. Research focusing on this process generally takes the form of examinations of individual traits (whether morphological, behavioral, physiological or genetic, real or simulated) and their use and variation in nature. Multiple lines of evidence have been used to infer the past action of adaptation on different traits, with the type of evidence varying according to the adaptive trait definition to which the researcher subscribes (Williams, 1966; Gould & Vrba, 1982; Sober, 1984; Fisher, 1985; Endler, 1986; Thornhill, 1990; Harvey & Pagel, 1991; Reeve & Sherman, 1993). Though adaptive trait definitions differ in their specific requirements, they all share a common criterion for classifying a trait as adaptive: the presence of the trait in question must provide a selective advantage relative to an alternative phenotype. For many putatively adaptive traits, however, the fitness benefits that are required for selection to act have not been explicitly demonstrated through realistic experimental comparisons with alternative phenotypes.

Recent work (Smith & Wheeler, 2006; Wright, 2009) has indicated that over 2,500 fish species (or ≈ 10% of all known species) possess venom glands associated with specialized teeth (*Meiacanthus* sp., *Monognathus* sp.) or fin (acanthurids, apistids,

aploactinids, batrachoidids, caracanthids, gnathanacanthids, neosebastids, scatophagids, scorpaenids, sebastids, setarchids, siganids, siluriforms, synanceids, tetrarogids), opercular (batrachoidids, trachinids), or cleithral (uranoscopids) spines. The secretions produced by these glands elicit a wide array of physiological effects in vertebrate organisms, including severe pain (Halstead et al., 1953; Calton & Burnett, 1975; Gwee et al., 1994; Lopes-Ferreira et al., 1998), cardiovascular, neuromuscular, and general cytolytic effects (Church & Hodgson, 2002). However, these effects have largely been demonstrated in mammalian or amphibian test subjects that would not naturally be encountered and envenomated by the fishes tested. Furthermore, these assays utilized prepared (occasionally purified) venom extracts that were introduced to test subjects via an unnatural delivery device, possibly resulting in the injection of larger volumes or concentrations of toxins than might be encountered during a predation attempt on a single individual. The venom glands of fishes thus represent a widespread, putatively adaptive trait for which selective benefits have yet to be demonstrated via ecologically relevant comparative experiments.

To date, the only studies examining predator response to venomous fish species have been performed on catfishes (Bosher et al., 2006; Emmett & Cochran, 2010; Wright, 2011). The venom glands of catfishes are associated with sharp, bony spines that are found along the anterior margin of the dorsal and pectoral fins. The bases of these spines, along with their associated musculature, are modified in such a way that the spines can be erected and locked into place when the fish is threatened, and in many species pectoral spine stridulation is also responsible for producing sounds that appear to be important for intraspecific communication (Fine & Ladich, 2003; Fine et al., 1997;

Kaatz et al., 2010). The presence of fin-spines effectively increases the cross-sectional circumference of catfishes, offering intuitive protection from gape-limited predators by making the catfish too large to consume, or at least significantly increasing handling times, making catfishes a less energetically favorable prey choice. This latter scenario has been formally described as the "Dangerous Prey Hypothesis" (Forbes, 1989), and has gained empirical support from studies of zooplankton (Barnhisel, 1991; Kolar & Wahl, 1998), as well as being the focus of the studies of catfishes (for which the hypothesis was originally formulated) mentioned above (Bosher et al., 2006; Emmett & Cochran, 2010). While the possible benefits to dangerous individuals and prey species are clear, the way in which natural predator sensitization and choice is affected by differing levels of prey "dangerousness", has yet to be examined. Such a consideration is particularly relevant for catfishes, which can show high degrees of overlap in habitat use between species (for instance, both species of catfish used in the present study were collected from the same stretch of beach and vegetation), but high degrees of variability in venom composition and toxicity (Wright, 2009).

While the catfish studies mentioned above did offer limited support for the Dangerous Prey Hypothesis, neither separated the possible deterrent effects (and adaptive benefit) of the venoms produced by these species from those of the delivery apparatus itself. Bosher et al. (2006) confirmed that the presence of spines increased the difficulty of ingestion of *Ictalurus punctatus* (channel catfish) by a gape-limited predator *Micropterus salmoides* (largemouth bass), also noting decreased aggression in experienced predators and increased survivability in catfishes with their spines intact, all of which were taken to indicate apparent fitness benefits conferred by the presence of

these structures. This study, while clearly demonstrating these effects, utilized a catfish species (*Ictalurus punctatus*) that possesses venom glands associated with its fin spines (Birkhead, 1969, 1972; Halstead, 1988; Wright, 2009), and therefore may have confused deterrence due to the presence of venom as an adaptive benefit of the spines themselves [although it should be noted that the catfish in Bosher et al.'s experiments were taken from an aquaculture population that had been released from predation pressure for many generations, and Birkhead (1972) also indicated that the venom of *I. punctatus* may be relatively innocuous]. Emmett & Cochran (2010) documented a similar effect on handling time in the tadpole madtom (*Noturus gyrinus*), although their comparisons were made with minnows rather than catfishes with modified phenotypes, leaving open the question of the relative contribution of the spines and venom glands to higher handling times. Furthermore, the increased survivorship and predator sensitization observed by Bosher et al. was not found in Emmett & Cochran's study. In their experiments, the predator species (again *Micropterus salmoides*) completely consumed all of the madtoms with which they were presented (with each bass receiving multiple exposures), thus casting doubt on the presence of any appreciable individual fitness benefit conferred by their venomous fin spines. Such a result was unexpected, as a previous study (Wright, 2009) has shown *N. gyrinus* venom to elicit a number of harmful effects when injected intramuscularly into largemouth bass.

Here, I present the results of experiments in which an ecologically relevant predatory species (*Micropterus salmoides*; Fig. 3-1A) was presented with fully intact, venomous catfishes (*Noturus gyrinus* – Tadpole Madtom; Fig. 3-1B), as well as with individuals that were lacking their venomous fin spines, following the example of Bosher

et al. (2006). Additionally, bass were presented with *N. gyrinus* that still possessed fin spines, but lacked venom glands, due to their removal via microdissection of the fin spines. This allowed for the separation of selective benefits associated with the presence of fin spines versus those associated with the presence of venom glands. I also explored the adaptive nature of variation in venom protein composition and toxicity, by comparing the results of experiments involving intact *N. gyrinus* to those using a separate, naturally co-occuring catfish species (*Ameiurus natalis* – Yellow Bullhead; Fig. 3-1C), which was first examined for differences from *N. gyrinus* in measures of venom potency and protein composition. These comparisons provide an opportunity for the examination of an understudied adaptive trait and alternative phenotypes at additional levels of biological organization, potentially providing valuable insight into the ecology and evolution of defensive venoms.

MATERIALS AND METHODS

Animal Acquisition and Care

Largemouth bass (*Micropterus salmoides*, (Lacépède 1802)) were collected from Boyden Creek, Washtenaw Co., MI and ranged from, 12-18 mm in standard length when captured. Bass were assumed to be naïve to other fish as a potential source of food based on their small size and the lack of appropriately sized potential forage fish in collections. Twelve bass were euthanized using MS-222 at a concentration of 300 mg/L in fresh water, and their stomach contents examined to confirm that an ontogenetic dietary shift to piscivory had not yet occurred. This was desirable, as bass would not yet have attempted to prey on any local catfishes, which would potentially have influenced the results obtained from predation experiments. Bass were maintained in aquaria under natural light

conditions and were fed a diet of frozen mosquito larvae and krill, only being allowed to shift to piscivory when they had reached an appropriate size for experiments to begin.

Individuals of *Noturus gyrinus*, (Mitchill 1817) and *Ameiurus natalis*, (Leseuer 1819) were collected from Clark Lake, Jackson Co., MI., using a 12' minnow seine. All specimens of *Pimephales vigilax* (Baird and Girard 1853) were obtained from a local pet store and consisted of individuals displaying the wild type coloration for *P. vigilax*, as well as the "Rosy Red" variety widely available in the pet trade. Catfishes and minnows were also maintained in aquaria under natural light conditions until they were required for experiments, and were fed a diet of frozen mosquito larvae.

Predation Experiments

Prior to being used in experiments, all catfishes (n=24 *N. gyrinus*, 8 *A. natalis*) and minnows (n=8) were anesthetized in MS-222 at a concentration of 75 mg/L of water. Dial calipers were used to measure the width (at pectoral spine origin) and depth (at dorsal spine origin) of catfish bodies, as well as the lengths of their dorsal and pectoral-fin spines, to ensure that bass would be physically capable of consuming the catfish with which they were presented (values for measurements given in Table 3-1). The dorsal and pectoral-fin spines were completely removed from eight madtoms while under anesthesia. The venom glands were removed from the spines of eight additional madtoms under a Wild M5A stereo microscope, using scissors and fine forceps to remove the integumentary sheaths covering the spines, and the tip of a microsyringe needle to remove the glandular tissue from the anterior grooves in the spines. Histological preparations were made from several individuals with intact or stripped fin spines, to demonstrate the efficacy of this procedure in removing venom gland tissue from the fin

spines before experiments commenced (Fig. 3-2). Sham surgery was performed on intact catfishes and minnows by touching the forceps and scissors used for surgical procedures to the pectoral fin and spines (in catfishes). Removal of the venom glands took longer than other procedures, thus all catfishes and minnows were kept under anesthesia for the average amount of time required for venom gland removal. After measurement and any required surgical procedures, catfish and minnows were allowed to recover from anesthesia for a period of 24 hours in clean, well-aerated water.

Individual bass were removed from communal holding tanks and lightly anesthetized in MS-222 at a concentration of 75 mg/L of fresh water, and their standard length and horizontal and vertical gape were measured to the nearest 0.1 mm with dial calipers. Each bass (n=40, standard length = 107.9–130.0 mm, horizontal gape = 20.0–26.4 mm, vertical gape = 21.4–28.7 mm) was then placed in its own, 40L experimental aquarium and allowed to acclimate to its new environment for a period of five days. Bass were fed a single minnow [*Pimephales vigilax* ("rosy red" or wild type color pattern)] each day for the first three days of this period. Bass were not fed during the final two days of this acclimation period to ensure that they would be hungry when first exposed to a catfish or control minnow (*P. vigilax*). When the acclimation period had ended, a cardboard blinder (with 70 mm x 15 mm viewing slit) was placed around each tank to eliminate the potential influence of the observer on experiments. The bass were then given two hours to recover from any stress associated with placing the blinder around the experimental aquarium.

After the two hour recovery period had ended, bass were presented with one of the following: a fully intact madtom (n=8), a "stripped" (lacking an integumentary sheath

and venom glands, but with otherwise intact fin-spines) madtom (n=8), a madtom with the spines completely removed (n=8), a bullhead (n=8), or a minnow (n=8). Bass were observed for one hour, and all predation attempts performed on the catfish or minnows in this time period were recorded. A predation attempt was defined as any attack during which the bass engulfed a portion of the prey item's head, body, or caudal region– instances of bass performing aggressive motions with a partially opened mouth, or nipping at fins were not counted. The amount of time required for the bass to consume the prey item was also recorded. Completion of consumption could easily be observed by the appearance of a noticeable distension in the ventral region of the bass due to the presence of the prey item in the stomach, which also corresponded to the cessation of movements of the mouth and operculae associated with conveyance of the prey item into the stomach.

Toxicity and Venom Composition Comparisons

Specimens of *Noturus gyrinus* and *Ameiurus natalis* were euthanized using MS-222 at a concentration of 300 mg/L in fresh water. All further preparations were carried out either on ice or under refrigeration at 4°C. Spines and caudal fin tissue were removed from each specimen, rinsed in physiological saline and gently scraped with a microspatula in order to remove any external epidermal secretions, and weighed to the nearest 0.001 g using a GeneMate digital balance. Spines were minced and then further homogenized in a 2 mL Dounce homogenizer along with euteleost physiological saline at a volume of 2 mL/g of tissue. The homogenate was then centrifuged at 6,000 rpm at 4°C for 20 minutes and the supernatant collected. The supernatant served as the crude venom extract. Control extracts were prepared from caudal fin tissue in the same manner.

Largemouth bass (n = 32) were anesthetized in MS-222 at a concentration of 75 mg/L of fresh water and weighed to the nearest 0.1 g. They were then placed in 40L experimental aquaria in a room with natural light and allowed to acclimate for a period of 72 hours. After the 72 hour acclimation period, bass were injected (using a 10 µL syringe with 26S gauge needle) in the caudal peduncle at a depth of 2 mm with 2 µL/g body weight of crude venom extract (Tadpole madtom n=8, Yellow bullhead n=8), or 2.0 µL/g control extract (Tadpole madtom n=8, Yellow bullhead n=8). Individuals were then observed at one minute, one hour, and 24 hours after injection for symptoms associated with extract injections. Venom toxicity was scored using a six point toxicity index [modified from Birkhead (1972)] which has been developed during a concurrent study of the comparative toxicity of ictalurid catfish venoms (Table 3-2).

Venom and control extracts were prepared for SDS-PAGE analysis by reduction with NuPAGE® reducing agent and loading buffer, according to manufacturer's instructions. Reduced samples were subjected to electrophoresis in NuPAGE® precast 4-12 % bis-tris polyacrylamide gels in, 1X MES running buffer for 35 minutes, at 200V in an x-Cell SureLock™ Mini Cell. Reduced peptides were visualized using SimplyBlue™ SafeStain according to manufacturer's instructions. Molecular weights of venom and caudal fin extract proteins were estimated by comparison with Novex® Sharp Protein Standard. Proteins unique to venom extracts (relative to caudal-fin extracts) were identified as putative toxins.

Data Analysis

All statistical analyses of experimental data were performed using PASW Statistics, 18, Release Version, 18.0.0 (=D3 SPSS, Inc., 2009, Chicago, IL, www.spss.com). Numbers

of attacks and handling times for alternative phenotypes were first compared using a Friedman two-way ANOVA to identify the presence of significant variation in the results from different test groups. Post-hoc, nonparametric Tukey's HSD tests were then performed to identify significant pairwise differences in number of attacks and handling time between phenotypes. Data from toxicity assays were evaluated using two-tailed Mann-Whitney *U*-tests to compare both pairwise differences between toxicities of catfish species' venoms, as well as differences between the toxicity of venom extract versus control injections.

RESULTS

Outcomes of Bass Encounters with Alternate Prey Phenotypes

Bass showed significant differences in both the number of attacks on different prey phenotypes (Fig. 3-3A; Friedman's ANOVA, χ^2 (4, n=8) = 28.81, $P < 0.001$) and the handling times for these prey types (Fig. 3-3B; Friedman's ANOVA, χ^2 (4, n=8) = 27.50, $P < 0.001$). Fully intact *N. gyrinus* were subject to significantly higher numbers of attacks than any other prey phenotype (Non-parametric Tukey's HSD, $P < 0.05$), due to repeated attacks and post-capture rejections of the madtoms. These rejections were accompanied by a number of reactions that indicated discomfort on the part of the bass, including repeated head shakes, flaring of the operculae, and "coughing" behavior (rapid, repeated expansion of the buccal cavity). Stripped madtoms and bullheads, while eliciting fewer numbers of attacks than intact madtoms, still received a higher number of predation attempts than spineless madtoms or minnows (Non-parametric Tukey's HSD, $P < 0.05$). Repeated attacks on stripped madtoms or bullheads appeared to be associated with attempts to reposition prey items with erected fin spines rather than responses to injuries

inflicted by those spines, as head shakes, gill flaring, and coughing behavior were never observed in these cases. Additionally, bass would often retain and continue to attempt to consume these prey items even as the fin spines had visibly pierced completely through the mouth. Finally, bass required only one or two attempts to consume spineless madtoms and minnows, occasionally ejecting the somewhat bulkier madtoms when they had been engulfed in a lateral position, rather than head or tail first.

A similar qualitative pattern was observed in the handling times of different prey phenotypes, although the statistical significance ascribed to pairwise comparisons of prey types differed from the data set discussed above. The handling time for fully intact *N. gyrinus* was again significantly higher than all other prey phenotypes (Non-parametric Tukey's HSD, $P < 0.05$), with only one individual being consumed within the one hour experimental window. This individual was, in fact, the only intact madtom consumed by bass at all, as intact madtoms left in aquaria with their bass predator overnight were still present the following morning, with no signs of additional predation attempts. In contrast, all stripped (n = 8) and spineless (n = 8) madtoms, yellow bullheads (n = 8), and minnows (n = 8) were consumed by the bass within the experimental period. The handling times of stripped madtoms and bullheads did not differ significantly from that of spineless madtoms (Non-parametric Tukey's HSD, $P = 0.27$ for stripped madtoms, $P = 0.21$ for yellow bullheads). Handling times for minnows were significantly lower than for all three of these prey phenotypes (Non-parametric Tukey's HSD, $P < 0.05$). In the case of spineless madtoms, this difference again appears to be attributable to differences in size and strength of madtoms as a prey item, relative to the minnows used.

Differences in Venom Toxicity and Composition

Injections of venom extracts from both catfish species examined showed significantly higher toxicity indices in largemouth bass than injections of control extracts prepared from fin tissue (Fig. 3-4; Mann-Whitney $U = 64$, n_1, $n_2 = 8$, $P < 0.001$ two-tailed for *N. gyrinus*, $U = 63$, n_1, $n_2 = 8$, $P < 0.01$ two-tailed for *A. natalis*). Symptoms associated with madtom venom injection were similar to those reported by Wright (2009), including color loss (except for a black spot formed at the injection site), muscle spasms, loss of equilibrium, and hemorrhage at the base of the fins. These effects resulted in significantly greater levels of toxicity being ascribed to this species' venom (Fig. 3-4; Mann-Whitney $U = 64$, n_1, $n_2 = 8$, $P < 0.001$ two-tailed) relative to that of the yellow bullhead, in which color loss and chromatophore expansion at the injection site were the only consistently observed symptoms of envenomation.

The venom protein compositions of both catfish species were highly similar, with venom-specific proteins of approximately, 100 kDa and, 18 kDa being identified in both cases (Fig. 3-5A). The higher molecular weight peptide was initially difficult to distinguish from bands that were also observed in control extracts prepared from fin tissue due to multiple proteins in this size range being found in venom extracts. Tris-HCl gels were employed to gain better separation of these proteins, and confirmed the presence of a unique peptide in venom extracts (Fig. 3-5B). Additionally, the venom of *N. gyrinus* was found to possess a vonom specific band at approximately 12 kDa, which was not found in the venom extract of *A. natalis*. These results are consistent with those reported for *N. gyrinus* by Wright (2009), as well as the observation by that study that the

number and weight of venom peptides in the range of, 10-20 kDa can vary widely between catfish species, including those within the same family.

DISCUSSION

The significantly higher number of rejections and handling times of intact tadpole madtoms provide the first experimental evidence that piscine venom glands are able to effectively function as a deterrent to natural predators, supporting previous assumptions regarding their adaptive nature. Although the actual degree of differences in individual fitness and selection coefficients were not explicitly quantified, it is clear that a significant advantage exists for those individuals having venom glands, as all other individuals were completely consumed, reducing their fitness to zero. In contrast to the conclusions of previous studies (Bosher et al. 2006; Emmet & Cochran 2010), the spines of the species examined here were not themselves found to significantly increase handling times, and did not deter a gape-limited predator from eventually consuming catfishes. This does not necessarily refute the Dangerous Prey Hypothesis, as the presence of spines still resulted in greater numbers of rejections by bass relative to spineless catfishes and minnows, and in fact, when the anti-predatory contributions of spine-associated venom glands are taken into account, support for the hypothesis is greatly increased. The results do imply, however, that in the case of catfishes (for which this hypothesis was originally conceived), predator sensitization and avoidance are disproportionately influenced by the presence of venom glands, rather than the spine itself. It should be acknowledged that in both of the species examined here (particularly in *N. gyrinus*), the spines are relatively simple in terms of serrations on the anterior and posterior margins of the spines, which are known to vary significantly between madtom

species (Fig. 3-6), often in conjunction with venom gland morphology (Egge and Simons 2011). Increased mechanical damage due to greater size and numbers of spine serrations could increase predator deterrence, possibly with a concomitant trade-off of lower venom toxicity resulting from the greater effect of the spines in these species.

The data presented here also have implications for understanding the development of predator foraging strategy and sensitization to suboptimal prey. Naïve bass were able to learn almost immediately (within a one hour session) to avoid a naturally occurring noxious prey source, a finding that has been paralleled in a previous study (Wright, 2011) utilizing *Micropterus salmoides* as a model predator species (but on a non-native, aposematically-colored prey species). In previous studies of predation on catfishes, much larger, experienced bass were used and showed limited ability to discriminate between potentially harmful and less well-defended prey, in one case requiring approximately 20 sessions with each prey item to establish an individual preference between intact and spineless prey catfish (Bosher et al., 2006) and in another (Emmett & Cochran, 2010), never showing a preference at all. These findings suggest that initial negative experience with a relatively large and dangerous prey species is a powerful reinforcement that may become somewhat diminished in larger experienced prey, adding a previously unconsidered, but potentially powerful ontogenetic component to the Dangerous Prey Hypothesis. Further experiments are necessary, however, to determine the temporal extent of avoidance behavior instigated by these single exposures [although no extinction of avoidance behavior has been seen over a period of several weeks following limited exposures to the catfish species examined by Wright (2011)].

Unexpectedly, *Ameiurus natalis*, a species that has been confirmed through histological and toxicological examinations to be venomous, did not yield significantly different results from madtoms that had had their venom glands removed. This may indicate a coevolutionary relationship that has reduced bass susceptibility to bullhead venom or, perhaps more likely, a life history trade-off in ictalurid catfishes in which predation effects on larger-bodied, longer-lived, less toxic bullhead species are mediated through increased fecundity and/or growth rates rather than the increased venom toxicity that is generally seen in madtom species. While phylogenetic inertia is no doubt at least partially responsible for the consistent differences in relative fecundity between these genera, it is nevertheless tempting to suggest that the smaller body size, shorter life span and much lower fecundity of madtoms [among the lowest of all North American groups of freshwater fishes (Mayden & Walsh, 1984)] may be historically linked with the evolution of greater venom toxicities, which would make these life history strategies more feasible. Testing such a hypothesis will require a much broader sample of comparative toxicity measurements of different ictalurid species' venoms.

The differences observed in venom toxicity and protein composition, when considered in conjunction with the results of behavioral experiments, suggest that single defensive venom toxins can provide significant adaptive benefits. The possible selective advantages of diversification and modification of venom components have been well established in organisms that use these substances for prey capture, as diverse or novel dietary regimes may require similarly complex venom compositions to effectively subdue a variety of prey types (e.g. Daltry et al., 2006; Lynch, 2007; Duda & Lee, 2009; Gibbs & Mackessy, 2009; Barlow et al., 2009), or co-evolutionary relationships necessitate

modification of venoms to overcome increasing prey resistance (e.g. Poran et al., 1987; Heatwole & Poran, 1995; Biardi et al., 2006). Due to the complex nature of these venoms, however, the contribution of any one toxin to the overall selective benefit that an organism receives via their use may be relatively small.

Venoms as defensive traits, in contrast to their efficacy in subduing prey, have been comparatively poorly studied. In fishes, which, with a single probable exception [*Monognathus* sp., a family (Monognathidae) of deep-sea saccopharyngifrom fishes (Bertelsen & Nielsen, 1987)], employ venoms exclusively in the deterrence of predators, venom toxin complexity is apparently rather low, consisting of only one or a few (mainly) proteinaceous, components (Church & Hodgson, 2002; Wright, 2009). This may suggest an evolutionary scenario in which a few, broad-scale toxins act on conserved vertebrate (the major potential predators of venomous fishes) physiological targets. Venoms have historically been viewed as metabolically "expensive" (McCue, 2006; Nisani et al., 2007) and the production of fewer toxic components in cases where general deterrence is all that is required may be a more energetically favorable strategy than that seen in organisms which rely on venom to capture prey. In the former case, the generation of novel venom peptides or the neofunctionalization of an existing toxin is likely to have a proportionally greater effect on the overall fitness benefit conferred by these substances, as seen in the results presented above.

The results of this study, when considered in their entirety, indicate that the variation observed in the toxicity and composition of venoms across a wide distribution of catfish species (Wright 2009) likely represents an ecologically and evolutionarily complex situation, incorporating predator behavior, phylogenetic history, life history

variation, and adaptive responses to different predatory regimes. Within each of these areas, additional details are likely to contribute further complications to any generalizations that might be made, as is becoming more widely recognized in other areas of venom research (e.g. Barlow et al., 2009; Gibbs & Mackessy, 2009). Future examinations of catfish venoms within their natural context thus appear to represent a potentially fruitful, but as yet untapped, area of ecological and evolutionary study.

ACKNOWLEDGMENTS

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Table 3-1. Measurements for the different classes of prey items offered to largemouth bass over the course of experiments (all measurements in mm). Abbreviations: WPFO = width at pectoral-fin origin; WPS = width with pectoral spines; HDFO = height at dorsal-fin origin; HDS = height with dorsal spine; NA = not applicable.

Prey type	*Standard length*	*WPFO*	*WPS*	*HDFO*	*HDS*
Intact madtom	40.1–51.9	11.0–13.5	19.9–25.3	9.5–11.9	12.4–16.1
"Stripped" madtom	39.6–50.2	10.8–12.9	19.8–24.6	9.3–12.5	11.9–16.6
Spineless madtom	41.6–52.3	10.9–14.9	NA	9.6–13.3	NA
Yellow bullhead	41.3–54.7	11.9–14.7	21.4–26.2	9.9–13.8	14.1–17.6
Minnow	30.4–38.0	NA	NA	NA	NA

Table 3-2. Toxicity index used to score effects of *N. gyrinus* and *A. natalis* venom and control extract injections. Note the additive nature of envenomation symptoms, likely due to the presence of both shared and novel putative toxins in different ictalurid species' venoms.

Toxicity Index	*Symptom(s)*
0	No effect
1	Chromatophore expansion
2	As with 1 + color loss
3	As with 2 + loss of equilibrium
4	As with 3 + muscle spasm
5	As with 4 + hemorrhage
6	Death

Figure 3-1. The model predator and two potentially "dangerous" prey species examined in this study. (A) Largemouth bass (*Micropterus salmoides*). (B) Tadpole madtom (*Noturus gyrinus*). (C) Yellow bullhead (*Ameiurus natalis*).

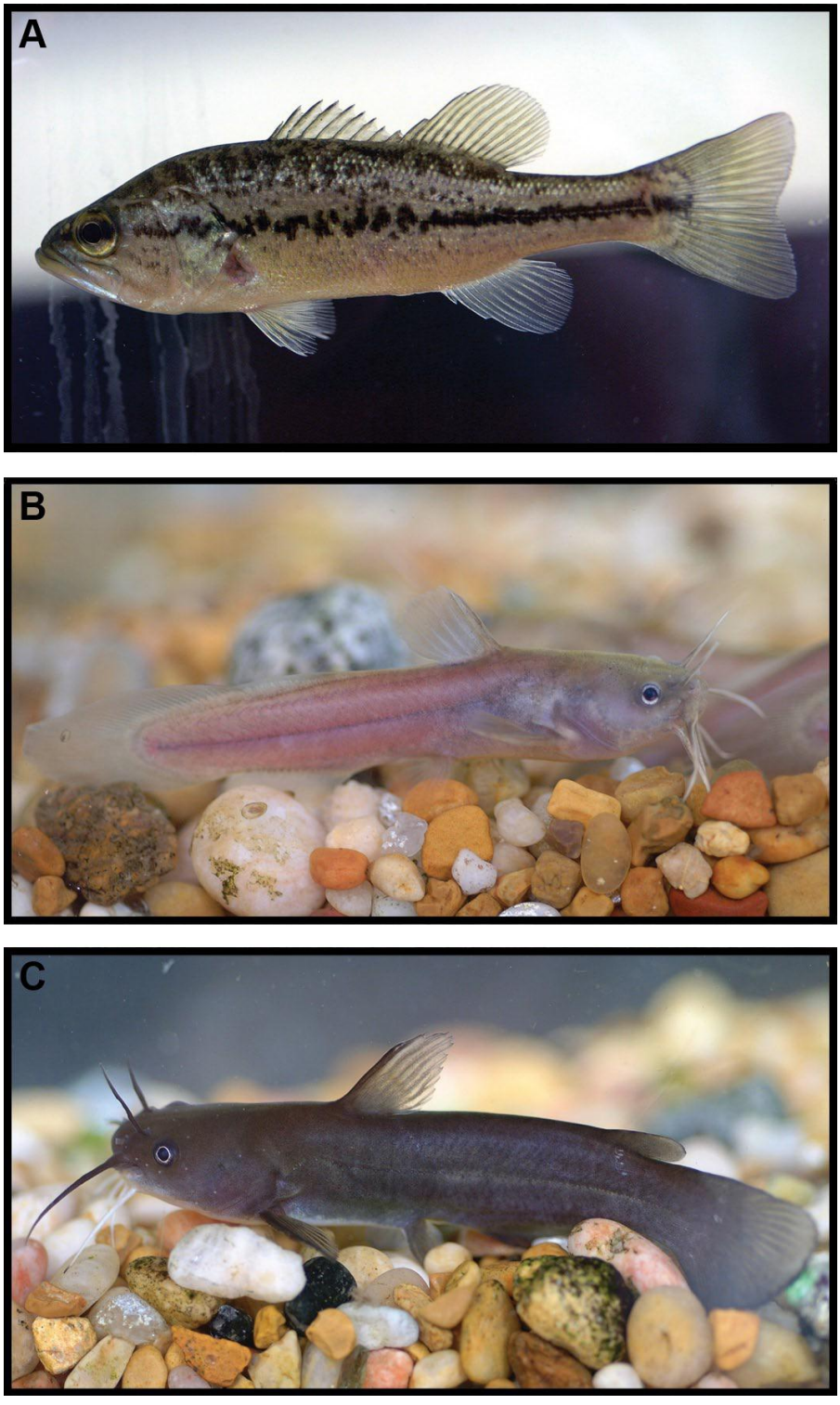


Figure 3-2. Histological preparations of *Noturus gyrinus* fin spines confirmed the efficacy of procedures for removing venom gland material from fin spines. (A) Cross section of *N. gyrinus* pectoral-fin spine prior to venom gland removal. (B) Cross section of *N. gyrinus* pectoral-fin spine after dissection. Note the almost complete removal of venom gland tissue. Abbreviations: ps = pectoral spine; gc = glandular cells.

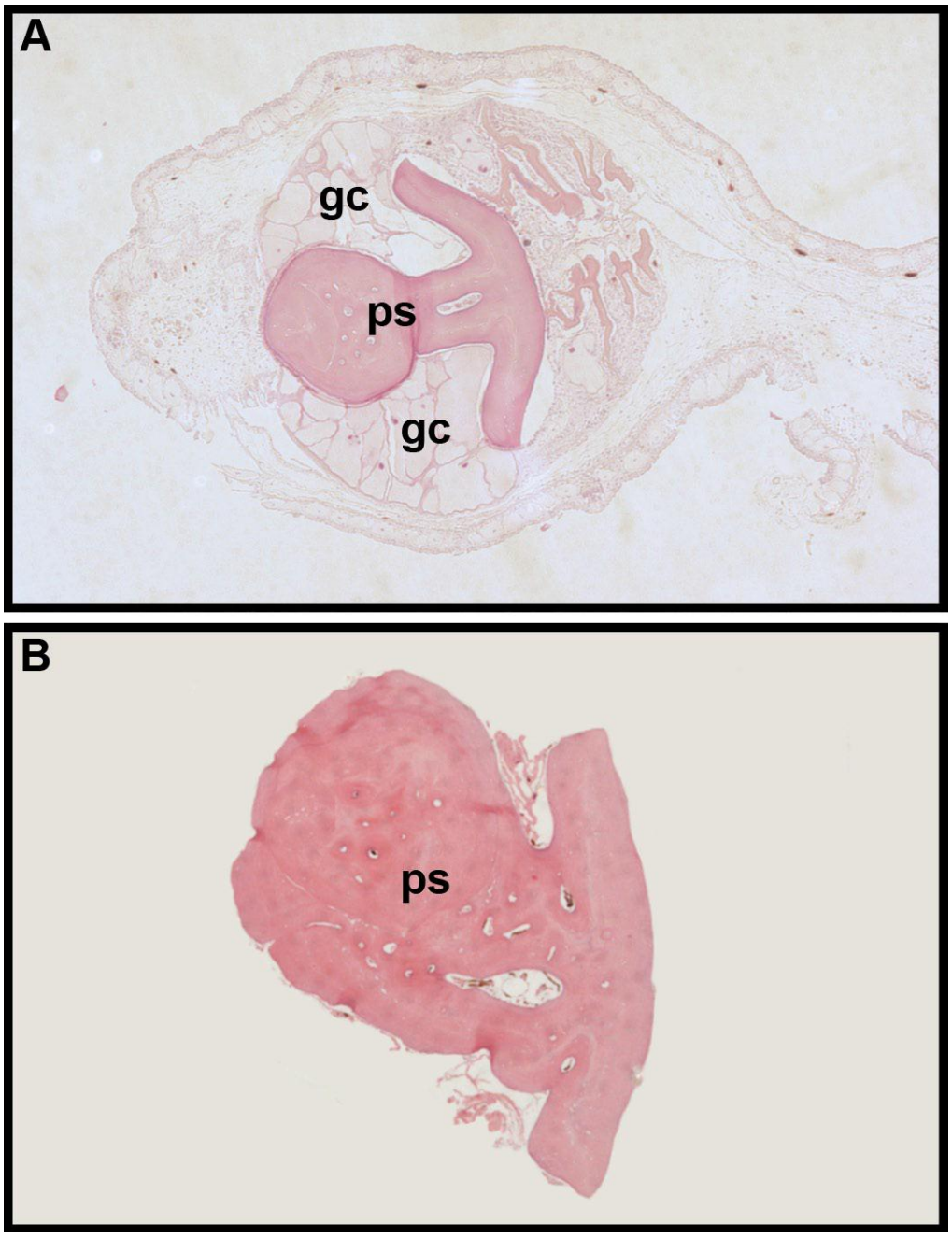


Figure 3-3. Significant differences were found in both (A) the number of attacks performed by bass on different prey phenotypes (Friedman's ANOVA, χ^2 (4, n=8) = 28.81, $P < 0.001$) and (B) bass handling times for those phenotypes (Friedman's ANOVA, χ^2 (4, n=8) = 27.50, $P < 0.001$). Fully intact madtoms elicited significantly higher numbers of attacks than other prey types due to repeated rejections by bass, which is also reflected in significantly higher handling times for this prey phenotype. Stripped madtoms and yellow bullheads produced significantly higher numbers of rejections than spineless madtoms or minnows, but did not differ significantly from these prey types in total handling time. Different letters between prey types indicate significant differences ($P < 0.05$) as determined by post-hoc, non-parametric Tukey's HSD tests. Error bars represent 95% confidence intervals.

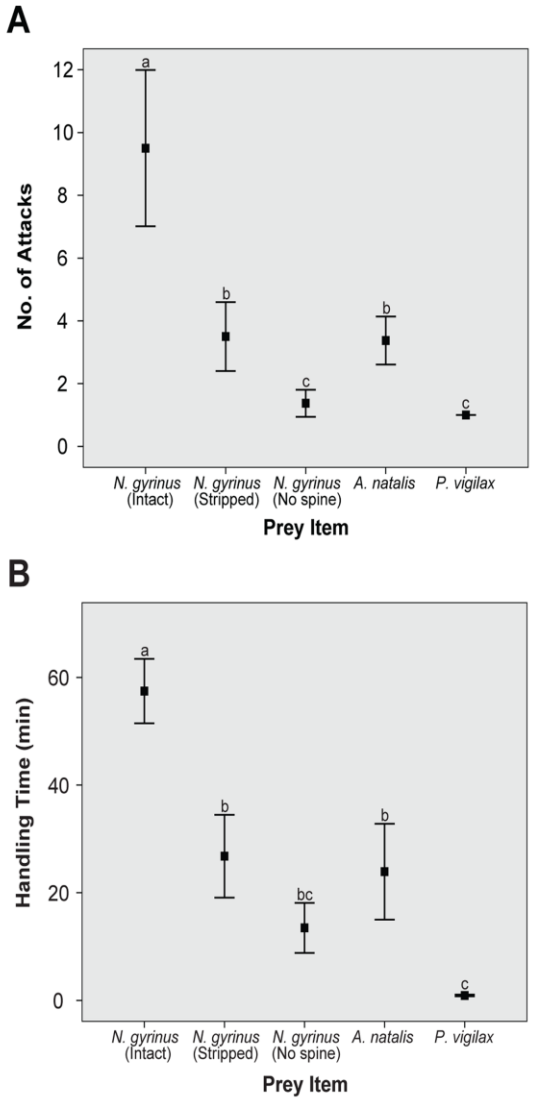


Figure 3-4. Comparisons of venom toxicity indicated that the venom of *N. gyrinus* is significantly more noxious than that of *A. natalis* (Mann-Whitney $U = 64$, n_1, $n_2 = 8$, $P < 0.001$ two-tailed). Solid lines indicate the results of venom extract injections, dashed lines indicate control extract injections.

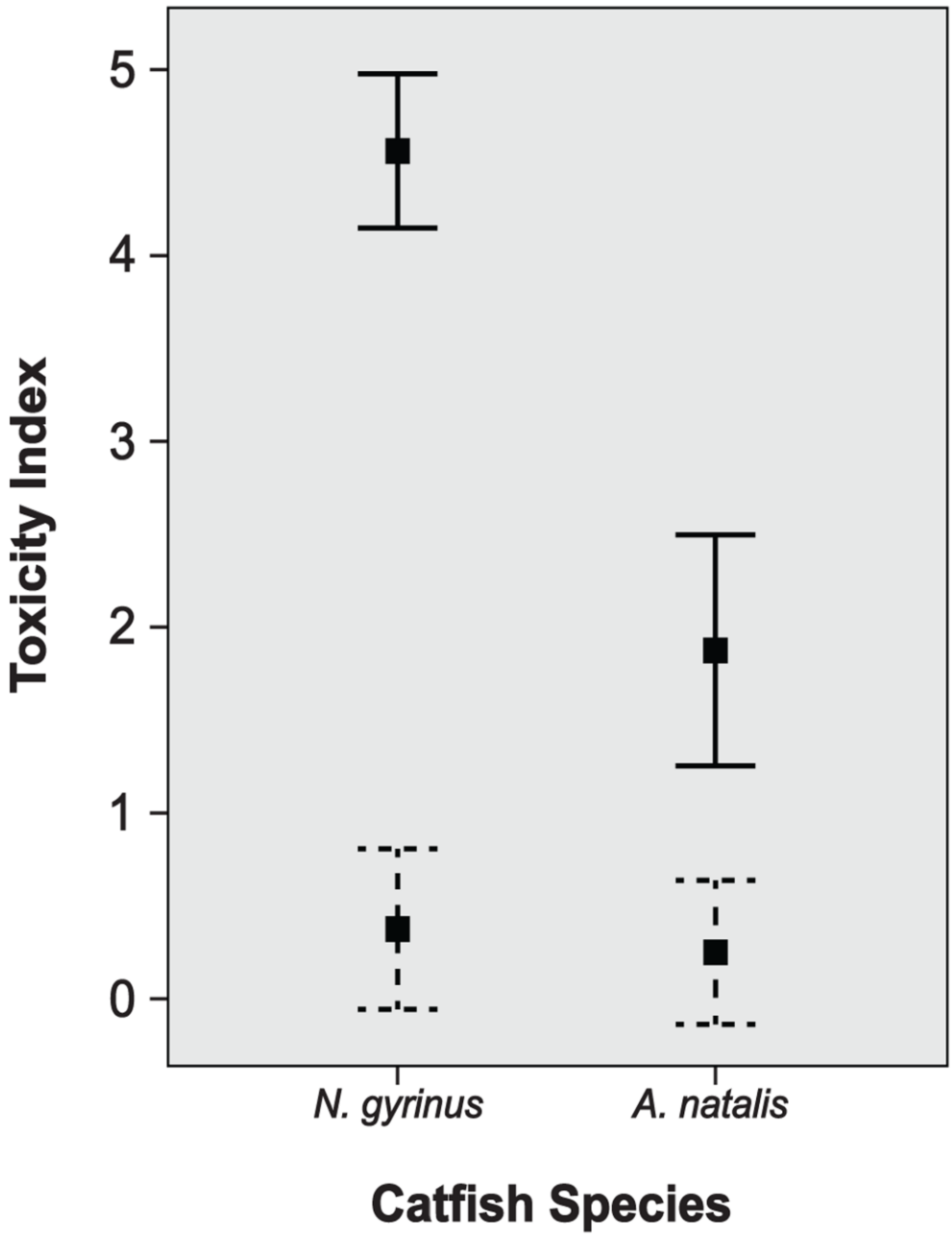


Figure 3-5. SDS-PAGE gels of venom extracts (left lanes) and fin tissue extracts (right lanes) of *N. gyrinus* and *A. natalis*, with putative venom toxins indicated by arrows. (A) Venom extract protein composition of *N. gyrinus* and *A. natalis* was found to be quite similar, with putative toxic peptides being identified at approximately 100 and 18 kDa. An additional putative toxin at approximately 12 kDa distinguishes the venom of *N. gyrinus* from that of *A. natalis*. (B) The 100 kDa protein band as viewed on a Tris-HCl gel, more clearly showing the presence of a putative toxin in the venom extract lane (left) vs. the fin tissue extract lane (right).

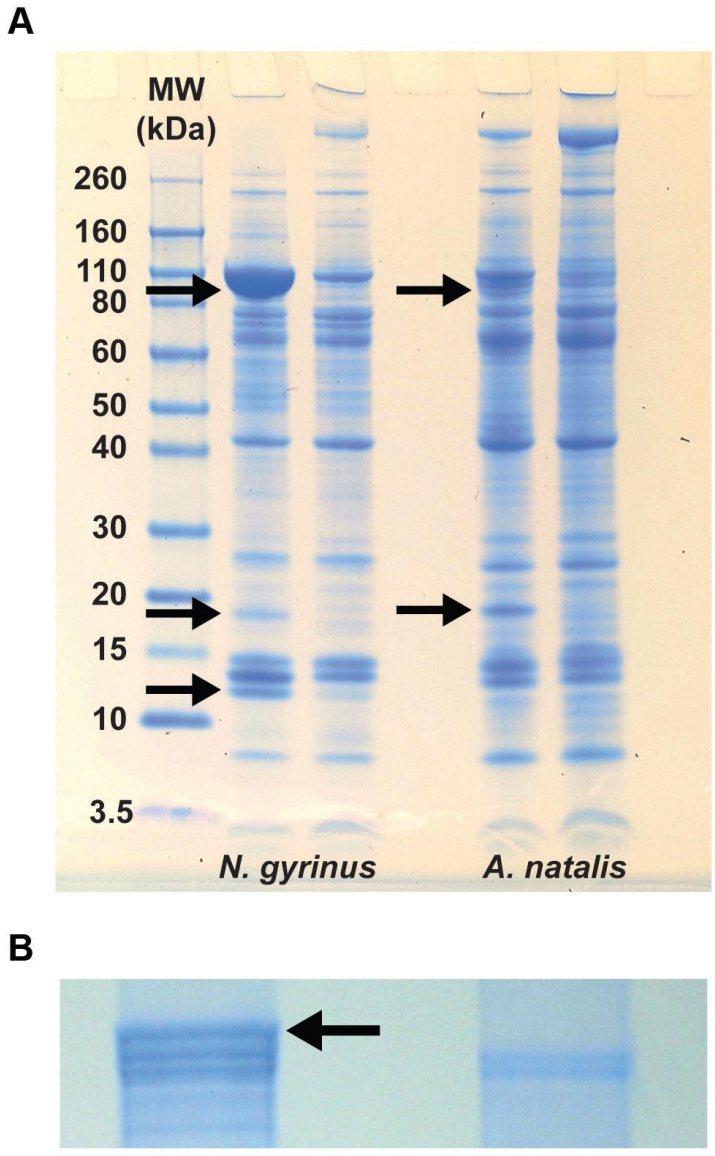
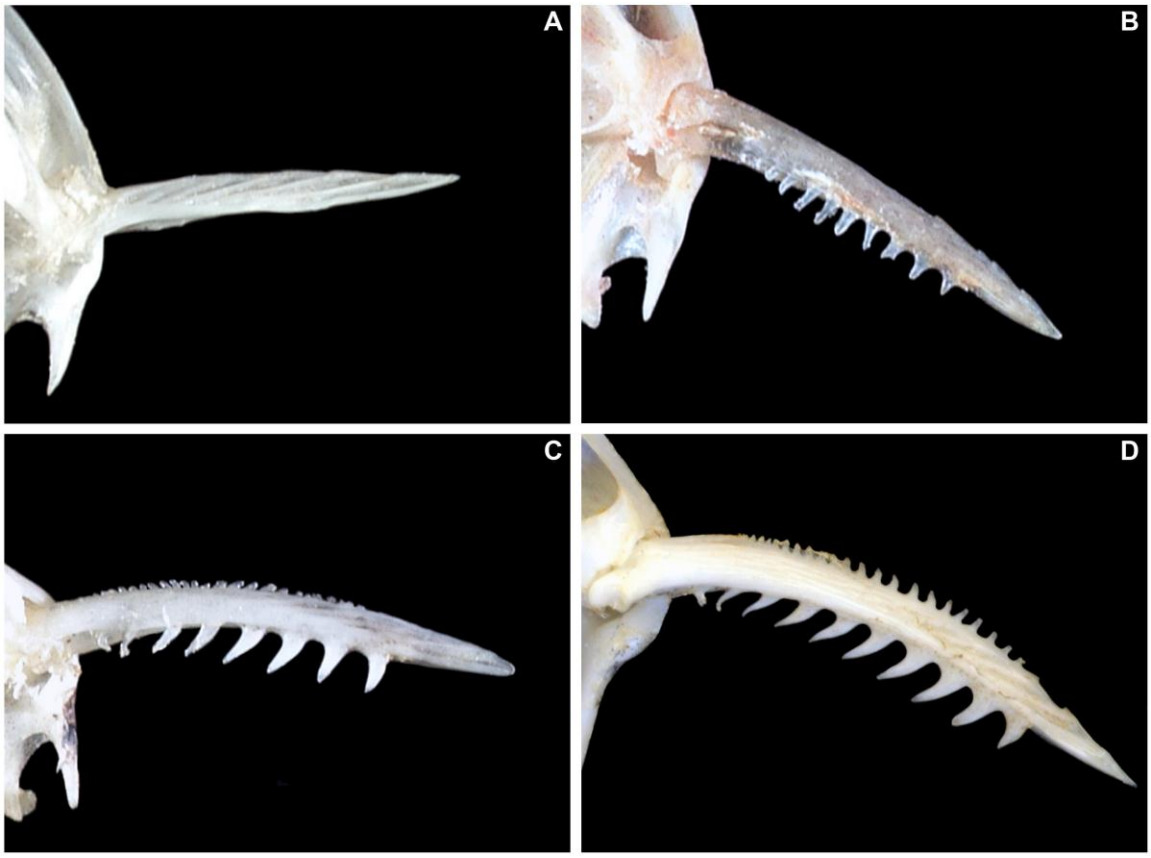


Figure 3-6. The pectoral-fin spines of several *Noturus* species, demonstrating the variation in fin spine morphology and potential for mechanical damage to predators found in this genus. (A) *N. gyrinus*, which displays the simplest of *Noturus* spine morphologies. (B) *N. exilis*, which possesses numerous, moderately sized serrae along the posterior margin of the spine. (C) *N. miurus*, in which small serrae are also present along the anterior margin of the spine, along with larger posterior serrae than in *N. exilis*. (D) *N. stigmosus*, which possesses both larger anterior serrae than *N. miurus* and larger posterior serrae than *N. exilis*.



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CHAPTER 4

COMPARATIVE TOXICITY AND LIFE HISTORY CORRELATES OF ICTALURID CATFISH VENOMS (SILURIFORMES: ICTALURIDAE)

ABSTRACT

Fish venoms represent a widespread antipredatory adaptation, but relatively little is known about their toxicity, composition, and correlated evolution with other aspects of species biology. Here I report the results of toxicity and toxin diversity assays in nearly two dozen species of ictalurid catfishes, the only exclusively North American representatives of a group comprising nearly two-thirds of global venomous fish species diversity. The results of these assays are then examined using ahistorical correlations and phylogenetically independent contrasts with sting morphology and life history data derived from the primary literature. These examinations reveal a high degree of venom toxicity and protein diversity, which appear to have evolved in concert with increases in sting morphology complexity. Ahistorical correlations with nearly all examined life history characteristics are significant, though significant phylogenetically independent correlations are observed only with early growth characteristics and, in the case of the genus *Noturus*, differences in the degree of maternal provisioning. The results presented here suggest an evolutionarily important relationship between increased venom toxicity and aspects of life history in ictalurids. Studies of additional venomous fish groups for similar relationships, as well as other potentially important factors such as regional

variation in venom toxicity, differences in predator regime and resistance, and habitat preference, will provide much needed insights into the factors influencing the evolution of an important antipredatory trait in a globally ubiquitous group of aquatic organisms, as well as the development of venoms in a unique ecological context.

INTRODUCTION

In perhaps no case are the impacts of a single trait on all aspects of an organism's biology (and, by extension, a species' evolutionary history) more evident than in that of venomous organisms, which utilize toxic secretions introduced by the means of a specialized morphological apparatus to aid in prey capture (e.g. Bub & Bowerman, 1979; Theusen et al., 1988; Terlau et al., 1996; Wigger et al., 2002; Fry et al., 2006), defense (e.g. Fry et al., 2006; Haight & Tschinkel, 2003; Kutsukake et al., 2004; Wright, 2009, 2012), and intraspecific competition (Temple-Smith, 1973; Whittington et al., 2008). Studies of the adaptive benefits of intra and interspecific variation in venom toxicity and composition, particularly as it relates to the capture of different prey species, are well-represented in the recent literature (e.g. Daltry et al., 1996; Jakubowski et al., 2005; Sanz et al., 2006; Abdel-Rahman et al., 2009; Barlow et al., 2009; Duda et al., 2009). In contrast, very little attention has been given to the influence of variability in venom potency and constituents on the defensive capabilities of these organisms. Such considerations are potentially of great importance to studies of selective factors driving venom evolution, as evidenced by the growing recognition that observed variation in venom efficacy and organization is likely the result of a complex interplay between a number of ecological and evolutionary factors (Barlow et al., 2009; Gibbs & Mackessy, 2009; Wright, 2012).

Venomous actinopterygians represent one of the most diverse groups of venomous vertebrates [> 2,500 species (Smith & Wheeler, 2006; Wright, 2009)]. With few exceptions [e.g. deep-sea species of the family Monognathidae (Raju, 1974; Bertelsen & Nielsem, 1987)], the toxic secretions produced by these species are used exclusively in the deterrence of predators. Catfishes (order Siluriformes) represent the majority of venomous fish species [1250-1650 venomous species (Wright, 2009)] and have been the focus of recent research to determine the efficacy of venoms in an antipredatory capacity (Wright, 2012). To date, however, very few studies have examined the effect of interspecific variation of venom toxicity and composition within venomous fish families (including nearly all venomous catfish families) on predator deterrence, or the potential ecological and evolutionary influences leading to these differences.

The exception to this generalization is the North American catfish family Ictaluridae (Fig. 4-1), which was the focus of a series of toxicological experiments in the late 1960s and early 1970s (Birkhead, 1967, 1972). In these experiments, fin-spine and caudal-fin extracts were prepared from 12 ictalurid species and their toxic effects evaluated in mosquitofish (*Gambusia affinis*). The injection of fin-spine extracts was found to elicit a number of symptoms, including edema, hemorrhage, and chromatophore expansion around the wound site, as well as tissue necrosis and, in some cases, mortality (with the species *Ameiurus melas* and *Noturus exilis* being found to possess by far the most virulent venoms). While the results of these studies provided valuable preliminary information about variation in venom toxicity between related catfish species, they are of limited ecological relevance, as they were obtained from a non-predatory assay organism. Furthermore, though these early examinations offered speculative scenarios to explain

variation in venom toxicity, no explicit tests for correlations between venom potency and other variables (venom complexity, delivery apparatus morphology, phylogenetic history, life history variables, etc.) have yet been performed.

Venom composition, spine morphology, and life history traits such as body size, growth rate, life span, and reproductive characteristics (fecundity, egg size, parental care, etc.) vary widely between ictalurid genera (Burr & Stoeckel, 1999; Wright, 2012; Table 4-1). Madtoms (genus *Noturus*) in particular are notable for their generally diminuitive size, short life spans and low fecundities [among the lowest of all North American groups of freshwater fishes (Mayden and Walsh, 1984)], and are well known for their generally higher levels of venom toxicity relative to other ictalurid species (Birkhead, 1967, 1972; Burr & Stoeckel, 1999). It has been suggested that increases in ictalurid venom toxicity may be evolutionarily linked with changes in these life history characteristics in ictalurid species, affording these smaller-bodied, less fecund, shorter-lived species greater protection from predation, which might otherwise be gained from the larger sizes and predation dilution effects potentially attained by confamilial species (Wright, 2012). This suggestion, however, was based on a two-species comparison and has yet to be examined using information on an appropriate range of ictalurid species' venoms and natural histories.

Here, I present the results of venom toxicity assays of 22 ictalurid species in a geographically widespread, ecologically relevant predatory species (Largemouth bass – *Micropterus salmoides*). Predation by this species has been shown to be prevented by the venom of at least one ictalurid species, with levels of aversion being affected by the toxicity of the venom employed (Wright, 2012). In addition to experimental measures of

toxicity, I provide preliminary surveys of candidate toxin proteins within the venoms of different ictalurid species, in an effort to relate putative toxin diversity to the observed toxicity assay results. I also examine relationships between venom toxicity and delivery apparatus morphology [using a recently developed classification of ictalurid sting morphology (Egge & Simons, 2011)], life history traits (adult body size, growth rate, fecundity) and phylogeny. Taken together, the data and comparisons presented here provide valuable insights into factors influencing venom evolution in catfishes and potentially the defensive capabilities of venoms in other organisms as well. Due to the global ubiquity of catfishes in freshwater habitats, these results also have possible ramifications for trophic interactions and community structure in aquatic ecosystems.

MATERIAL AND METHODS

Animal Acquisition and Care

Micropterus salmoides (largemouth bass) were collected from Boyden Creek, Washtenaw County, Michigan (U.S.A.) and ranged from 12-18 mm in total length when captured. Bass were assumed to be naïve to other fish as a potential source of food based on their small size and the lack of appropriately sized potential forage fish in the collections made. Twelve bass were euthanized using MS-222 at a concentration of 300 mg/L in fresh water, and their stomach contents examined to confirm that an ontogenetic dietary shift to piscivory had not yet occurred. This was desirable, as bass would not yet have attempted to prey on any local catfishes, which may have resulted in exposure and possible acquired resistance to the toxic components of ictalurid venoms. Bass were maintained in aquaria under natural light conditions and were fed a diet of frozen mosquito larvae and krill.

Catfishes were collected from various locations throughout the United States using various methods, including 12' minnow seines, 30' bag seines, backpack and boat electrofishing, and snorkeling. Captured individuals were transported to the University of Michigan Museum of Zoology in plastic-lined Styrofoam™ coolers with aeration supplied by battery-powered aquarium pumps. Catfishes were maintained in species-specific common aquaria until they were used in toxicity assays.

Venom Toxicity and Protein Composition

Preparation of crude venom and caudal-fin extracts was performed immediately prior to their use in toxicity assays, as previously described (Wright, 2009, 2011, 2012). Prior to being used in assays, all largemouth bass (n=16 per ictalurid species analyzed) were anesthetized in MS-222 at a concentration of 75 mg/L of water. Live weight was determined to the nearest 0.1 g using a GeneMate digital balance. Bass were then placed in 10 G experimental aquaria and allowed to acclimate for a period of 72 hours. After the acclimation period, eight bass per ictalurid species examined were injected in the caudal peduncle at a depth of 2 mm with 2.0 μL/g body weight of crude venom extract. For each ictalurid species, eight bass were also injected with 2.0 μL/g body weight of caudal fin extract to serve as negative controls, and eight additional bass were injected with 2.0 μL/g body weight of freshwater teleost physiological saline (Hoar & Hickman, 1975) to assess effects of the injection procedure.

Bass were observed one minute, one hour, and 24 hours post-injection to determine any adverse effects associated with injection of the prepared venom and control extracts. The toxicity index of Birkhead (1972) was to be used to score the effects of venom extract injection in order to quantitatively compare virulence of ictalurid species' venoms. However, it immediately became obvious that this system is unsuitable

for use, as many of the symptoms elicited by injection of ictalurid venoms into largemouth bass were not directly comparable to those observed in earlier experiments. Number and severity of venom extract effects varied between ictalurid species, but, in cases where more than one symptom was observed, these effects were always additive and always followed an ordered progression. This facilitated the creation of a new toxicity index, which was used to assign a numerical value to the venom toxicity of each ictalurid species examined (see Chapter 3, Table 3-2).

Significance of venom toxicity was evaluated by comparison of the results from crude venom, caudal-fin extract, and physiological saline injections, using nonparametric Mann-Whitney *U*-tests. Variation in the toxicity of different ictalurid species' venoms was first examined using a Friedman two-way ANOVA to identify significant variation in results from different species, followed by Post-hoc, nonparametric Tukey's HSD tests to identify significant pairwise differences between the toxicity of different species' venoms. These and all subsequently described statistical analyses (with the exception of phylogenetically independent contrasts) were implemented in PASW Statistics, 18, Release Version, 18.0.0 (=D3 SPSS, Inc., 2009, Chicago, IL, www.spss.com).

The relationship between protein composition and venom extract toxicity was assessed through visualization of extract protein profiles using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Venom and fin extracts were prepared for SDS-PAGE analysis by reduction with NuPAGE® reducing agent and loading buffer, according to manufacturer's instructions. Reduced samples were subjected to electophoresis in NuPAGE® precast 4-12 % Bis-Tris polyacrylamide gels in 1X MES running buffer for 35 minutes, at 200V in an x-Cell SureLock™ Mini Cell. Reduced

peptides were visualized using SimplyBlue™ SafeStain according to manufacturer's instructions. Molecular weights of venom and caudal fin extract proteins were estimated by comparison with Novex® Sharp Protein Standard. Proteins unique to venom extracts (relative to caudal-fin extracts) were treated as putative toxins.

Toxicological Relationships with Protein Diversity, Sting Morphology, and Life History Traits

The sting (fin spine and associated venom gland) morphology of the species examined was scored as previously described (Egge & Simons, 2011) – in the case of *Noturus* species, *Ameiurus natalis*, *Ictalurus punctatus*, and *Pylodictis olivaris*, scores were taken directly from this publication. Scores for *Ameiurus* species not included in Egge & Simons (2011) were determined based on previous examinations of spine and venom gland morphology (Wright, 2009).

Several life history characteristics, including fecundity, fertilized egg chorion diameter, maximum body size, and growth (both in length and weight) were obtained via a survey of literature concerning ictalurid life history (see Table 4-1 for values and references). Ahistorical correlations between all pairs of traits (including venom toxicity, protein diversity, and spine morphology) were first examined, following ln (x+1) transformation of all variables to meet assumptions of normality. To address the non-independence of the data analyzed (owing to the shared phylogenetic history of the species from which the data were derived), phylogenetically independent contrasts (PICs) were also performed to address the influence of species' relationships on the correlations observed (Felsenstein, 1985; Garland et al., 1992). As madtom species constituted over half of the toxicological data set (14 of 22 species), ahistorical and PIC analyses were

also performed for a subset of the data including only *Noturus* species, to examine possibly important correlations that would potentially be obscured due to large differences in trait values between genera.

To provide a framework for PIC analysis, sequence data for one nuclear (RAG2) and two mitochondrial (cytochrome b and ND5) genes that have previously been used in studies of ictalurid phylogeny (Hardman & Page, 2003; Hardman, 2004; Egge & Simons, 2011) were downloaded from GenBank [see Egge & Simons (2011) for accession numbers] and aligned using Se-Al v.2.0a11 Carbon (Rambaut, 1996). In addition to the taxa for which toxicity data were available, *Prietella phreatophila* and *Noturus gilberti* were included in this phylogenetic data set, as both species have been found to lack venom glands (Egge & Simons, 2011), thus making it possible to assign approximate values for toxicity (using a mean value calculated from species found to be non-venomous in the present study) and putative venom toxin diversity. The genetic alignment was subjected to a partitioned Bayesian phylogenetic analysis using MrBayes 3.1.12 (Ronquist & Huelsenbeck, 2003), following likelihood model selection using the Akaike Information Criterion (AIC) in jModelTest (Posada, 2008). Four independent Markov chain Monte-Carlo (MCMC) analyses were run for 5×10^7 generations, with three heated (0.2 temperature) and one cold chain, and tree sampling frequency of 1,000 generations. Trees sampled before convergence (average standard deviation of split frequencies < 0.01) were discarded as burn-in.

PIC analysis was conducted using a consensus tree constructed from the remaining sampled trees using the PDAP:PTREE module (v. 1.16, Midford et al., 2011) in MESQUITE v. 2.75 (Maddison & Maddison, 2011), with branch lengths estimated

from MrBayes. Because PIC analysis requires a completely resolved phylogeny, polytomies were randomly resolved using near-zero branch lengths, and the data examined using the PDAP:PTREE module for violations of the assumptions of Felsenstein's independent contrasts model (FIC; Felsenstein, 1985). Where violations were found, various branch length transformations were applied to examine their effect on the fit to the FIC model, with subsequent calculations of independent contrasts using optimally transformed branch lengths.

RESULTS

Toxicity Assays and Protein Diversity

As expected, significant variation was observed in the toxicity of ictalurid species' venoms (Fig. 4-2, Table 4-2). In nearly all cases, Mann-Whitney *U*-tests indicated significantly greater toxicity indices resulting from injections of extracts prepared from fin spines and associated tissues than from injections of fin-tissue controls or physiological saline (Fig. 4-2), confirming the venomous nature of the majority of ictalurid species. Non-significant differences were found in only three species (*Ameiurus melas, A. brunneus, Pylodictis olivaris*), which were considered to be non-venomous [in agreement with previous studies indicating that these species lack venom glands associated with their fin spines (Wright, 2009)]. Effects of injection were most evident at one minute post-injection, and had, in nearly all cases, been completely resolved within 24 hours.

Four species (*Noturus stigmosus*, *N. gyrinus*, *N. furiosus* and *N. miurus*) were found to possess markedly higher levels of venom toxicity than the other ictalurid species tested, though the level of significance of these differences as indicated by post-hoc,

pairwise comparisons varied (see Fig. 4-2 for details of all pairwise comparisons). The other *Noturus* species examined showed overlapping levels of venom toxicity, with species at the upper end of this range (i.e. *N. insignis*, *N. hildebrandi*) showing significantly higher levels of toxicity than those at the lower end (*N. albater*, *N. leptacanthus*), and the remaining species showing comparable levels of venom toxicity.

The larger bodied *Ictalurus punctatus*, *Ameiurus* species, and *Pylodictis olivaris* all showed generally lower levels of venom toxicity than *Noturus* species, although the magnitude and statistical significance of this difference was dependent on the species being compared. *Ameiurus natalis*, *A. nebulosus*, and *A. catus* showed higher levels of venom toxicity than the remaining large-bodied species, though values were not significantly higher than those found for *A. platycephalus* and *I. punctatus*. The three remaining species (*A. brunneus, A. melas, P. olivaris*) did not show significantly higher levels of toxicity associated with injection of their fin-spine extracts versus caudal-fin extracts or physiological saline as determined by Mann-Whitney *U*-tests (Fig. 4-2) and were thus considered to be non-venomous.

Tracing of log-transformed venom toxicity values onto the phylogeny used for PIC analyses (Fig. 4-3) did not reveal any apparent phylogenetic pattern in ictalurid venom toxicity, besides the aforementioned tendency for members of the *Ameiurus* clade to possess generally lower levels of venom toxicity than *Noturus* species. While two highly toxic species (*N. stigmosus* and *N. furiosus*) were resolved as sister species (due to the exclusion of additional *Noturus* species for which venom data were lacking) and included within a clade containing an additional, relatively noxious species (*N. miurus*), another highly toxic species (*N. gyrinus*) was not found to be closely related to these

species, with subtending nodes giving rise to species with varying levels (low to moderate) of venom toxicity. Patterns within the *Ameiurus* clade were similarly variable, generally lower levels of venom toxicity notwithstanding.

SDS-PAGE profiles of venom extracts and caudal-fin extracts were highly similar, with venomous species showing one to four venom-specific peptides (Fig. 4-4, Table 4-2), and non-venomous species showing identical venom and caudal-fin profiles. A unique venom protein was found in all venomous species at an approximate molecular weight of 100 kDa, which has nearly always been found in other studies of catfish venoms (Wright, 2009, 2011, 2012). When additional candidate toxins were present, they were consistently found in the range of 10-20 kDa, with only one species (*Noturus hildebrandi*) showing a unique venom protein outside of this range (at approximately 35 kDa; Fig. 4-4).

Ahistorical Correlations

Correlation coefficients and significance levels for pairwise ahistorical comparisons of the complete ictalurid data set can be found in Table 4-3. For the complete toxicological data set, venom toxicity showed statistically significant correlations with sting morphology, putative toxin diversity, and nearly all included life history traits. The exception was fertilized egg chorion diameter, which showed no significant relationship with venom toxicity. Toxicity was positively correlated with toxin diversity and sting morphology, and negatively correlated with maximum body size and fecundity. Standard length and weight at one year of age were found to be negatively related to species' venom toxicity, while the percentage of a species' total standard length and weight represented by these values were negatively correlated with venom toxicity, owing to the

tendency of relatively innocuous species to gain relatively large adult body sizes (a much smaller fraction of which is attainable in one year of growth). Restriction of analyses to *Noturus* species had almost no effect on significance levels associated with ahistorical correlations of venom toxicity and other characteristics (or pairwise life history comparisons; data not shown), with the exception of fertilized egg chorion diameter, which, for *Noturus*, was found to exhibit a significant negative relationship with venom toxicity (N = 11, r = -0.707, p = 0.015).

Significant relationships were found between nearly all of the life history traits examined (the exception being that no life history characteristic demonstrated a significant correlation with fertilized egg chorion diameter; Table 4-3). Beyond their significant correlation with venom toxicity, sting morphology showed a significant positive correlation with putative toxin diversity, while toxin diversity was negatively correlated with standard length at one year of age. All remaining ahistorical correlations for these two traits were non-significant.

Phylogeny Reconstruction and PIC Analysis

Model selection for cyt *b*, *RAG2*, and *ND5* (GTR + I + Γ, K80 +Γ , GTR + I + Γ, respectively) was identical to that indicated for each gene in earlier work using these genes (Egge & Simons, 2011). Bayesian phylogenetic analysis for included taxa returned a fairly well-resolved phylogeny that was largely congruent with that presented by this earlier study, though some notable differences were present. In addition to the inclusion of additional *Ameiurus* species, a polytomy was observed involving *Noturus insignis*, *N. gilbert*, and the species pair of *N. gyrinus* and *N. flavus* (Fig. 4-3). This was not unexpected, as the relationships reported by Egge & Simons (2011) for these species

were characterized by poor nodal posterior probability support. These species' relationships were alternately arbitrarily resolved using near zero (0.0001) branch lengths, and forced to match those reported in Egge & Simons (2011), again using near-zero branch lengths for re-arranged relationships, with the exception that the root trichotomy was left unresolved and was ignored in PIC calculations. No discernible difference in PIC correlations calculated using these topologies was found, and all values given here were calculated using the randomly resolved phylogeny.

Branch lengths provided by MrBayes did not provide an adequate standardization of contrasts as indicated by plots of the absolute value of standardized contrasts against the standard deviation of contrasts generated by MESQUITE. Evaluation of all available methods of branch length transformation indicated that the branch lengths method of Nee (Purvis, 1995) provided the best fit of all data to the assumptions of independent contrasts (Garland et al., 1992). This transformation was applied and used in all subsequent calculations.

Correlation coefficients and significance levels for PIC analyses of the complete ictalurid data set can be found in Table 4-3. Values for PIC correlations of venom toxicity and sting morphology and venom protein diversity did not differ markedly from ahistorical comparisons (Fig. 4-5A, B). In contrast, relationships between toxicity and nearly all of the life history traits examined differed greatly in magnitude and statistical significance from ahistorical correlations. The ahistorically significant correlation between putative toxin diversity and standard length at the end of the first year was found to be non-significant in PIC analyses, although the relationship between sting morphology and toxin diversity was still found to be significant (Fig. 4-5C). Standard

length attained by the end of the first year was the only life history characteristic that maintained a significant relationship with venom toxicity when phylogenetic non-independence was considered (though correlations between venom toxicity and putative toxin diversity and sting morphology were still significant; Fig. 4-5D). A similar situation was observed in PIC analysis of the *Noturus*-only data set, though in addition to standard length at year 1 (N = 10, r = -0.615, p = 0.044), venom toxicity maintained a significant correlation with fertilized egg chorion diameter (Fig. 4-5E; N = 10, r = -0.660, p = 0.027), while a relationship with sting morphology was no longer found to be significant (N = 14, r = 0.307, p = 0.265).

Nearly all of the examined life history characteristics maintained significant correlations in PIC analyses. Again, fertilized egg chorion diameter was not significantly correlated with any of the examined life history traits. Additionally, correlations between 1) standard length and weight at the end of the first year, 2) percentage of total length attained in the first year and weight attained in the first year, 3) percentage of the total maximum standard length attained in the first year and weight attained in the first year, and 4) weight at year one and percentage of total weight attained in year one, were the only relationships between life history traits that were found to be non-significant in PIC analyses.

DISCUSSION

The wide range in the toxicity of ictalurid venoms reported here is consistent with that observed in previous examinations (Birkhead, 1967, 1972), as are the general levels of venom toxicity observed in ictalurid genera (with *Noturus* species generally possessing higher venom toxicity than other ictalurid genera). This variation in venom toxicity likely

represents real differences in the antipredatory capabilities of the species examined, as a previous two-species study (Wright, 2012) showed a relationship between venom toxicity (as measured here) and predator deterrence. Differences between the present and previous studies were observed, however, in the identities of species exhibiting high levels of venom toxicity, as well as the symptoms associated with envenomation by certain species. In particular, levels of venom toxicity observed in *Ameiurus melas* and *Noturus exilis*, which have previously been indicated to be the most toxic representatives of their respective genera (Birkhead, 1967, 1972), were found to be quite different from those indicated by previous experiments. *Ameiurus melas* was found here to be nonvenomous, confirming the results of recent assays (Wright, 2009). *Noturus exilis*, while confirmed to be venomous, possessed a mean venom toxicity that was significantly lower than that found in four other *Noturus* species. It must be noted, however, that several of these species were not included in previous studies, owing to their lack of formal description at the time of those experiments.

Qualitative differences between the present and previous studies were also observed in the toxic effects elicited by all of the venomous ictalurid species examined. The most notable of these was the body-wide color-loss (never mentioned in previous studies of ictalurid venoms) that was observed in bass that had been injected with several ictalurid species' venoms (Fig. 4-6), but which had not previously been reported in ictalurid venom studies (Birkhead, 1967, 1972). Such a disparity (as well as those seen in overall toxicity) may be explained by the differences in assay organism used (largemouth bass versus mosquitofish), as is the loss of predator equilibrium that was only observed in the present study. The lack of venom necrotic activity, which was frequently encountered

in previous work (Birkhead, 1967, 1972), may also be a result of differences in the test organisms used. It seems just as likely, however, that this effect was the result of bacterial infection resulting from these earlier assays, as secondary infections mimicking necrotizing fasciitis have been observed not only in injuries to humans by catfishes (Murphey et al., 1992; Carty et al., 2010; Roth & Geller, 2010), but also by carp (Calif et al., 2002), which have no venom glands associated with their fin spines.

The strong correlation observed between ictalurid venom toxicity and putative venom toxin diversity is perhaps not surprising, but does serve to demonstrate the likely toxic nature of the unique proteins found in the venom extracts examined. Such information is valuable, as these proteins are poorly studied and have proven difficult to examine for protein sequence information and comparison with known venom toxins from other organisms, with N-terminal sequencing and mass spectrometric analyses of putative toxic proteins thus far proving unsuccessful (Wright, unpublished). Comparative transcriptomic analyses of venom gland and fin tissues represent a promising approach to this problem, and are expected to confirm the identity and toxic nature of many, if not all of these proteins. Additionally, significant correlations (ahistorical and PIC) with sting morphology for both venom toxicity and toxin diversity indicate that in ictalurids, these three characteristics have largely developed in concert. In many cases this has resulted in species possessing venom apparatuses which, owing to the noxious nature of their venoms and extensively serrated spines, represent highly effective antipredatory structures.

A potential ontogenetic shift in venom production was considered as a possible explanation for the three species that were found to be completely non-venomous

(*Ameiurus brunneus*, *A. melas*, *Pylodictis olivaris*), as they are all relatively large-bodied species, as were the specimens from which venom extracts were prepared (125.0-126.0 mm SL, 167.1-175.3 mm SL, and approximately 800 mm SL, respectively), perhaps indicating that these individuals had been released from most predation pressure, and had therefore ceased venom production. A similar argument could conceivably be made for the low venom toxicities seen in other large-bodied ictalurid species, which may have been in the process of reducing venom production, though it had not ceased entirely. Such a scenario seems unlikely, however, in light of the fact that a previous study (Wright, 2009) examined histological sections from much smaller specimens of all of these species (well under 100 mm SL), and found either no evidence of venom glands in the three species specifically mentioned above, or similar venom gland morphologies to the venom-producing, large-bodied species examined here. Furthermore, an examination of histological preparations prepared from a size series (50-400 mm SL) of *A. natalis*, a species found to possess moderately toxic venom, shows no deterioration in venom glands over this size range, although it must be noted that the venom glands themselves do not show a concomitant increase in size with the rest of the spine-related structures in larger bodied specimens (Wright, unpublished data).

The interpretation of interactions between other life history characteristics and venom evolution are less straightforward, as phylogenetic non-independence was found to have a large influence on the correlations between venom toxicity and many of the life history characteristics examined. The negative correlation between venom toxicity and standard length after the first year, which was the only significant post-PIC correlation for the whole data set, indicates a trade-off between size and venom toxicity that has been

suggested in previous work (Wright, 2012). This correlation was also present when considering *Noturus* species only, along with a negative correlation between venom toxicity and fertilized egg chorion diameter. Together, these relationships suggest that *Noturus* species with lesser maternal provisioning and smaller amounts of growth in the first year (though no differences in final adult body size are apparent), may have compensated by developing higher levels venom toxicity, which would compensate for the potential advantages to predator avoidance of greater maternal investment and more rapid attainment of larger body size. The significant PIC correlations in venom toxicity and these life history traits in *Noturus* species echoes earlier suggestions (Burr & Stoeckel, 1999) that predation may exert a greater influence on aspects of life history evolution in madtoms than in larger-bodied ictalurid species.

Despite the clear differences between ictalurid genera in examined life history characteristics, the results of PIC analyses nonetheless do not support the hypothesis of phylogenetic constraint in the evolution of ictalurid body size, growth, and fecundity. Nearly all of these life history variables included showed strong correlations (both ahistorical and PIC) with each other, likely due to physical constraints (particularly in terms of total fecundity) associated with small body size in *Noturus* species. The relationships of various growth-related variables are also intimately tied to body size differences in ictalurid species and, while showing clear phylogenetic trends, were nonetheless significant when phylogenetic non-independence of data was considered. Such correlations suggest that selection has strongly influenced the development of smaller body size and associated growth characteristics in ictalurids. The markedly higher levels of species diversity in *Noturus* relative to other ictalurid genera suggest that these

changes in body size, coupled with the increased levels of venom toxicity discussed above, may have contributed to the greater levels of diversification in this genus. This would be due both to the possibility of exploiting habitats that are unavailable to larger bodied confamilial species, as well as the greater protection from predation that these habitats [due to both structure and cryptic coloration in many madtom species (Armbruster & Page, 1996; Burr & Stoeckel, 1999)], and intrinsic antipredatory defenses would supply.

The observed correlations and trade-offs between venom toxicity and life history traits imply that predator-mediated coexistence may play a role in the high degree of syntopy observed between many ictalurid species [which can often represent the most locally abundant representatives of regional ichthyofaunas (Burr & Stoeckel, 1999)], many of which share significant overlap in habitat and trophic preferences. While this mechanism is likely an important influence on ictalurid composition in a given habitat, its use as a sole explanation is overly simplistic, due to the fact that many additional factors, such as historical species distributions, environmental parameters and stresses, structural heterogeneity, and indirect effects from other species [both predator and prey, piscine and otherwise) also play roles in predation and community structure (Sih et al., 1985)]. Additionally, the present experiments utilized a single generalist predator species, with individuals drawn from a single regional population (Southeast Michigan); regionally specific predatory species, with different degrees of trophic generalization doubtlessly influence the presence of multiple ictalurid species in a particular location. Notably, three Michigan *Noturus* species were among the most toxic species assayed, while two *Ameiurus* species collected in Michigan were the most toxic species examined from that

genus. Geographical variation in the susceptibility or resistance of largemouth bass (and other predators) to particular ictalurid venoms is thus a possibility that merits future investigation.

Though this study represents an important step in our understanding of the factors influencing venom evolution and ecology in this group (and likely others as well), several gaps remain in the present state of our knowledge. High quality, standardized life history and venom toxicity and composition data is absent for many ictalurid species and, owing to the threatened or endangered status of many members (nearly 40%) of this family at the state or federal level, will likely be difficult to acquire. Additionally, much of the life history information examined here was obtained from single populations within widely distributed species, and thus likely does not represent the variation that exists within these species in its entirety. Nonetheless, the results presented demonstrate the presence of widespread, ecologically relevant variation in the toxicity and composition of North American catfish venoms and offer multiple insights into the relationships between venom toxicity and composition, sting morphology, life history evolution, and community composition in an important component of freshwater ecosystems. Hundreds of additional venomous fish groups, representing over 10% of all fish species (Smith & Wheeler, 2006; Wright, 2009), await examinations of venom production, its effect on predator interactions, and possible correlations with other aspects of biological evolution at multiple levels of taxonomic classification. Such studies will not only provide insights into the factors influencing the evolution of venoms in a rather unique ecological context (defense only), but also the development and evolution of a widespread adaptation in the world's largest and most broadly distributed group of aquatic vertebrates.

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Table 4-1. Size, fecundity, and growth metrics from past life history studies of ictalurid species used in ahistorical and PIC correlations. * all maximum TL estimates are taken from Page & Burr (1991).

Species	*Maximum TL (mm)*[*]	*Fecundity*	*Fertilized Yolk Diameter (mm)*	*% Max SL at Yr 1*	*Actual SL at Yr 1 (mm)*	*% Max Wt at Yr 1*	*Actual Wt at Yr 1 (g)*	*Reference(s)*
Noturus								
N. hildebrandi	69	17-38 (mean = 29.9)	3.4	56.5%	39.0	44.1%	0.75	Mayden & Walsh, 1984
N. elegans	89	19-42 (mean = 30.5)	4.2	N/A	N/A	N/A	N/A	Burr & Dimmick, 1981
N. miurus	130	42-90 (mean = 66.2)	3.5	43.2%	56.1	25.2%	3.1	Burr & Mayden, 1982
N. exilis	150	26-150 (mean = 83.6)	4.5	33.2%	49.8	27.6%	2.2	Mayden & Burr, 1981 Vives, 1987
N. phaeus	150	108-128 (mean = 118)	N/A	42.8%	56.5	N/A	3.1	Simon & Wallus, 2003 Chan & Parsons, 2000
N. insignis	150	53-223 (mean = 138)	3.8	32.9%	≈52.1	10.2%	2.7	Clugston & Cooper, 1960 Simon & Wallus, 2003
N. stigmosus	130	89-141 (mean = 115)	3.2	38.5%	50.0	N/A	N/A	Simon & Wallus, 2003
N. funebris	150	85-167 (mean = 108.8)	N/A	38.2%	57.3	N/A	N/A	Bennett & Kuhadja, 2008
N. albater	120	109-116 (mean = 111.7)	3.7	44.2%	53.0	N/A	N/A	Mayden et al., 1980

Table 4-1. continued

Species	*Maximum TL (mm)*[*]	*Fecundity*	*Fertilized Yolk Diameter (mm)*	*% Max SL at Yr 1*	*Actual SL at Yr 1 (mm)*	*% Max Wt at Yr 1*	*Actual Wt at Yr 1 (g)*	*Reference(s)*
N. furiosus	120	79-298 (mean = 126.3)	3.9	48.3%	58.0	N/A	N/A	Burr et al., 1989
N. gyrinus	130	48-323 (mean = 151.3)	3.0	49.9%	21.9	24.6%	2.8	Whiteside & Burr, 1986
N. flavus	180	189-570 (mean = 377.8)	3.4	27%	50.5	2.2%	1.9	Walsh & Burr, 1985
N. leptacanthus	94	14-45 (mean = 24.0)	5.5	N/A	N/A	N/A	N/A	Burr & Stoeckel, 1999
Ameiurus								
A. catus	620	1000-4000 (mean = 2500)	≈3.7	16.4%	83.2	0.2%	10.2	Carlander, 1969 Simon & Wallus, 2003
A. platycephalus	290	207-1742 (mean = 825.5)	≈3.0	33.2%	79.4	4.1%	9.1	Olmstead & Cloutman, 1979
A. nebulosus	500	1797-9870 (mean = 4154)	≈2.05	21.4%	78.5	0.9%	11.7	Carlander, 1969 Harvey & Fortin, 1982
A. melas	620	1917-5730 (mean ≈ 3500)	N/A	23.4%	75.6	1.9%	17.5	Carlander, 1969 Dennison & Bulkley, 1972
A. natalis	470	1650-7000 (mean = 4325)	N/A	24.9%	109.9	2.1%	23.0	Carlander, 1969 Simon & Wallus, 2003

Table 4-1. continued

Species	*Maximum TL (mm)*[*]	*Fecundity*	*Fertilized Yolk Diameter (mm)*	*% Max SL at Yr 1*	*Actual SL at Yr 1 (mm)*	*% Max Wt at Yr 1*	*Actual Wt at Yr 1 (g)*	*Reference(s)*
Ictalurus								
I. punctatus	1270	2395-12859 (mean = 6117)	≈3.75	13.0%	80.4	0.09%	18.0	Jearld & Brown, 1971 Wahlquist, 1974
Pylodictis								
P. olivaris	1550	7961-26923 (mean = 13250)	≈4.0	10.7%	119.5	0.08%	14.94	Carlander, 1969 Simon & Wallus, 2003

Table 4-2. Venom toxicity, candidate venom toxin diversity, and collection information for the ictalurid species examined in this study. Standard length range values are given in millimeters. Abbreviations: TI = toxicity index; SD = standard deviation of toxicity index; SL = standard length.

Species	*Mean TI*	*SD*	*Putative Toxins*	*Collection Locality*	*SL Range*
Ictalurus punctatus	1.13	±0.35	3	Sandy Creek, Bibb Co., AL	82.0 – 95.7
Pylodictis olivaris	0.25	±0.46	0	Tar River, Edgecombe Co., NC	≈ 800
Ameiurus brunneus	0.38	±0.52	0	Deep River, Chatham-Lee Co., NC	125.0 – 126.0
A. catus	1.63	±0.44	2	Lake Gaston, Halifax Co., NC	≈ 230
A. melas	0.25	±0.46	0	Huron River, Washtenaw Co., MI	167.1 – 175.3
A. natalis	1.88	±0.74	2	Huron River, Washtenaw Co., MI	94.0 – 141.2
A. nebulosus	1.63	±0.74	2	Huron River, Washtenaw Co., MI	141.8
A. platycephalus	1.13	±0.35	2	Lake Gaston, Halifax Co., NC	114.4 – 115.0
Noturus albater	1.31	±0.65	1	Middle Fork White River, Washington Co., AR	63.2 – 68.1
N. elegans	1.69	±0.37	1	Trace Fork (Green River), Casey Co., KY	46.5 – 64.5
N. exilis	1.75	±0.60	2	Middle Fork White River, Washington Co., AR	98.7 – 118.8
N. fasciatus	1.56	±0.68	1	Brushy Fork Creek, Hickman Co., TN	52.4 – 58.5
N. flavus	2.06	±0.62	2	Huron River, Washtenaw Co., MI	71.7 – 81.2
N. funebris	2.31	±0.53	2	White Oak Creek, Clay Co., AL	76.4 – 92.7
N. furiosus	4.13	±0.69	3	Contentnea Creek, Wilson Co., NC	64.2 – 65.7
N. gyrinus	4.56	±0.50	3	Clark Lake, Jackson Co., MI	50.0 – 67.9
N. hildebrandi	2.44	±0.62	4	Terrapin Creek, Henry Co., TN	41.1 – 53.4

Table 4-2. continued

Species	*Mean TI*	*SD*	*Putative Toxins*	*Collection Locality*	*SL Range*
N. insignis	2.50	±0.46	3	Deep River, Chatham-Lee Co., NC	54.7 – 74.2
N. leptacanthus	1.13	±0.23	2	Sachs Systems Aquaculture, FL	48.2 – 68.1
N. phaeus	1.44	±0.50	1	Terrapin Creek, Henry Co., TN	84.8 – 105.9
N. miurus	3.75	±0.46	3	Huron River, Washtenaw Co., MI	82.8 – 88.6
N. stigmosus	5.06	±0.68	3	Huron River, Washtenaw Co., MI	97.3 – 103.6

Table 4-3. Ahistorical and PIC correlation coefficients and *P*-values for pairwise comparisons of all venom and life history variables examined. Statistically significant relationships are indicated by bold font. * indicates correlations that were significant in the *Noturus*-only data set.

			Ahistorical correlation		PIC correlation	
Variable 1	*Variable 2*	*N*	*(r)*	*P value*	*(r)*	*P value*
Venom toxicity	Sting morphology	24	**0.561**	**0.002**	**0.481**	**0.017**
Venom toxicity	Toxin diversity	24	**0.875**	**<0.001**	**0.867**	**<0.001**
Venom toxicity	Fecundity	20	**-0.535**	**0.015**	-0.211	0.371
Venom toxicity	Maximum SL	24	**-0.561**	**0.007**	-0.098	0.650
Venom toxicity	SL at Yr 1	18	**-0.658**	**0.002**	**-0.496**	**0.036**
Venom toxicity	%SL at Yr 1	18	**0.665**	**0.001**	0.361	0.141
Venom toxicity	Wt at Yr 1	14	**-0.636**	**0.007**	-0.352	0.217
Venom toxicity	%Wt at Yr 1	13	**0.678**	**0.005**	0.448	0.125
Venom toxicity	Yolk diameter	16	-0.258	0.334	-0.366	0.163
Sting morphology	Toxin diversity	24	**0.523**	**0.009**	**0.419**	**0.042**
Sting morphology	Fecundity	20	-0.150	0.528	0.116	0.626
Sting morphology	Maximum SL	24	0.046	0.830	0.380	0.067
Sting morphology	SL at Yr 1	18	0.107	0.673	0.189	0.453
Sting morphology	%SL at Yr 1	18	0.115	0.649	-0.256	0.305
Sting morphology	Wt at Yr 1	14	-0.035	0.907	0.001	0.997
Sting morphology	%Wt at Yr 1	13	0.089	0.773	-0.196	0.520
Sting morphology	Yolk diameter	16	0.029	0.916	0.067	0.804
Toxin diversity	Fecundity	20	-0.313	0.179	-0.121	0.612
Toxin diversity	Maximum SL	24	-0.166	0.439	0.008	0.969
Toxin diversity	SL at Yr 1	18	**-0.494**	**0.037**	-0.287	0.248
Toxin diversity	%SL at Yr 1	18	0.463	0.053	0.297	0.231
Toxin diversity	Wt at Yr 1	14	-0.494	0.073	-0.410	0.145
Toxin diversity	%Wt at Yr 1	13	0.515	0.072	0.303	0.313
Toxin diversity	Yolk diameter	16	-0.258	0.335	-0.332	0.208
Fecundity	Maximum SL	20	**0.976**	**<0.001**	**0.940**	**<0.001**

Table 4-3. continued

			Ahistorical correlation		PIC correlation	
Variable 1	*Variable 2*	*N*	*(r)*	*P value*	*(r)*	*P value*
Fecundity	SL at Yr 1	18	**0.769**	**<0.001**	**0.642**	**0.004**
Fecundity	%SL at Yr 1	18	**-0.917**	**<0.001**	**-0.851**	**<0.001**
Fecundity	Wt at Yr 1	14	**0.934**	**<0.001**	**0.831**	**<0.001**
Fecundity	%Wt at Yr 1	13	**-0.943**	**<0.001**	**-0.866**	**0.001**
Fecundity	Yolk diameter	16	-0.369	0.160	-0.193	0.473
Maximum SL	SL at Yr 1	18	**0.765**	**<0.001**	**0.695**	**0.001**
Maximum SL	%SL at Yr 1	18	**-0.969**	**<0.001**	**-0.941**	**<0.001**
Maximum SL	Wt at Yr 1	14	**0.869**	**<0.001**	**0.709**	**0.004**
Maximum SL	%Wt at Yr 1	13	**-0.952**	**<0.001**	**-0.922**	**<0.001**
Maximum SL	Yolk diameter	16	-0.215	0.423	0.032	0.907
SL at Yr 1	%SL at Yr 1	18	**-0.755**	**<0.001**	**-0.671**	**0.002**
SL at Yr 1	Wt at Yr 1	14	**0.804**	**0.001**	0.473	0.087
SL at Yr 1	%Wt at Yr 1	13	**-0.776**	**0.002**	**-0.756**	**0.003**
SL at Yr 1	Yolk diameter	14	0.071	0.810	0.286	0.322
%SL at Yr 1	Wt at Yr 1	14	**-0.767**	**0.001**	-0.527	0.053
%SL at Yr 1	%Wt at Yr 1	13	**0.927**	**<0.001**	**0.949**	**<0.001**
%SL at Yr 1	Yolk diameter	14	-0.071	0.810	-0.288	0.319
Wt at Yr 1	%Wt at Yr 1	13	**-0.813**	**0.001**	-0.482	0.096
Wt at Yr 1	Yolk diameter	11	-0.173	0.612	-0.090	0.791
%Wt at Yr 1	Yolk diameter	11	0.121	0.723	-0.216	0.524

Figure 4-1. Representatives of the three ictalurid genera from which venomous representatives are currently known. (A) *Ictalurus punctatus*. (B) *Ameiurus melas*. (C) *Noturus hildebrandi*.

Figure 4-2. Assays revealed a wide range of variation in the toxicity of ictalurid species' venoms (Friedman's ANOVA, χ^2 (20, n=8) = 130.68, $P < 0.001$). Error bars represent 95% confidence intervals and different letters between ictalurid species indicate significant differences ($P < 0.05$) in venom toxicity as determined by post-hoc, non-parametric Tukey's HSD tests. * by species name indicates significant differences ($P < 0.05$) between mean toxicity index of venom extract injections and caudal-fin extract injections.

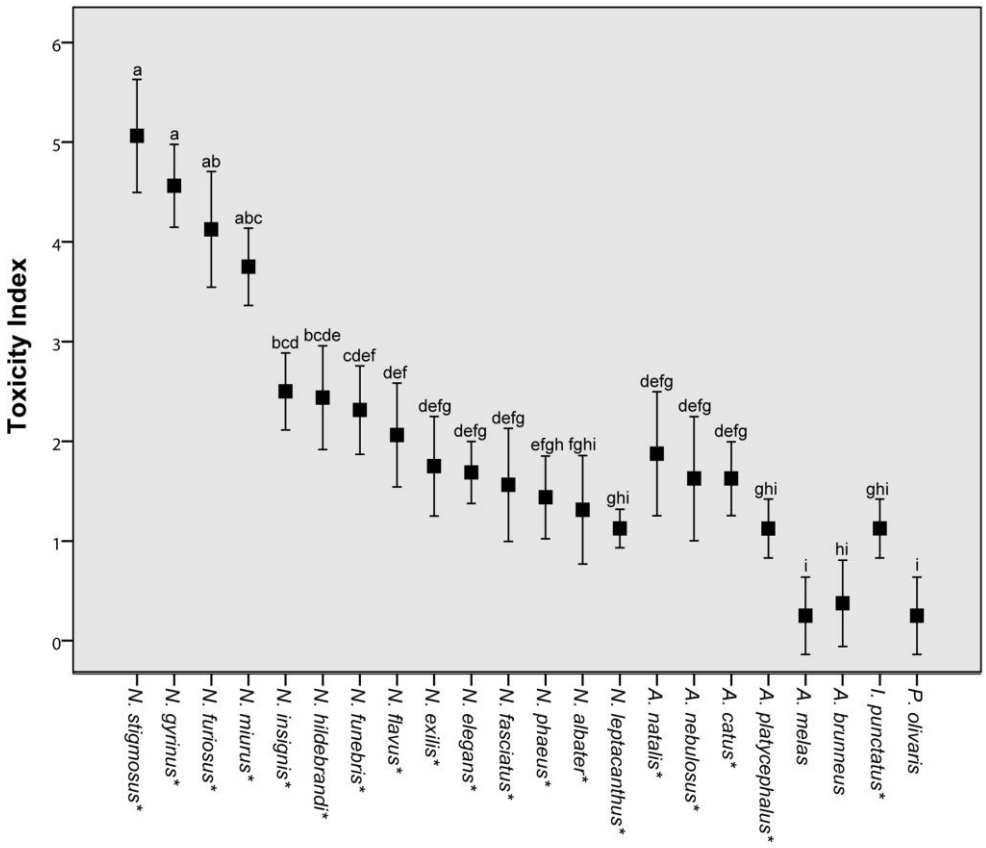


Figure 4-3. The transformed mean venom toxicity index of ictalurid species included in this study, as mapped onto the phylogeny used in PIC analyses. Though two highly venomous species (*N. stigmosus* and *N. furiosus*) were found to be closely related, distribution of venom toxicity showed little apparent pattern with respect to particular clades.

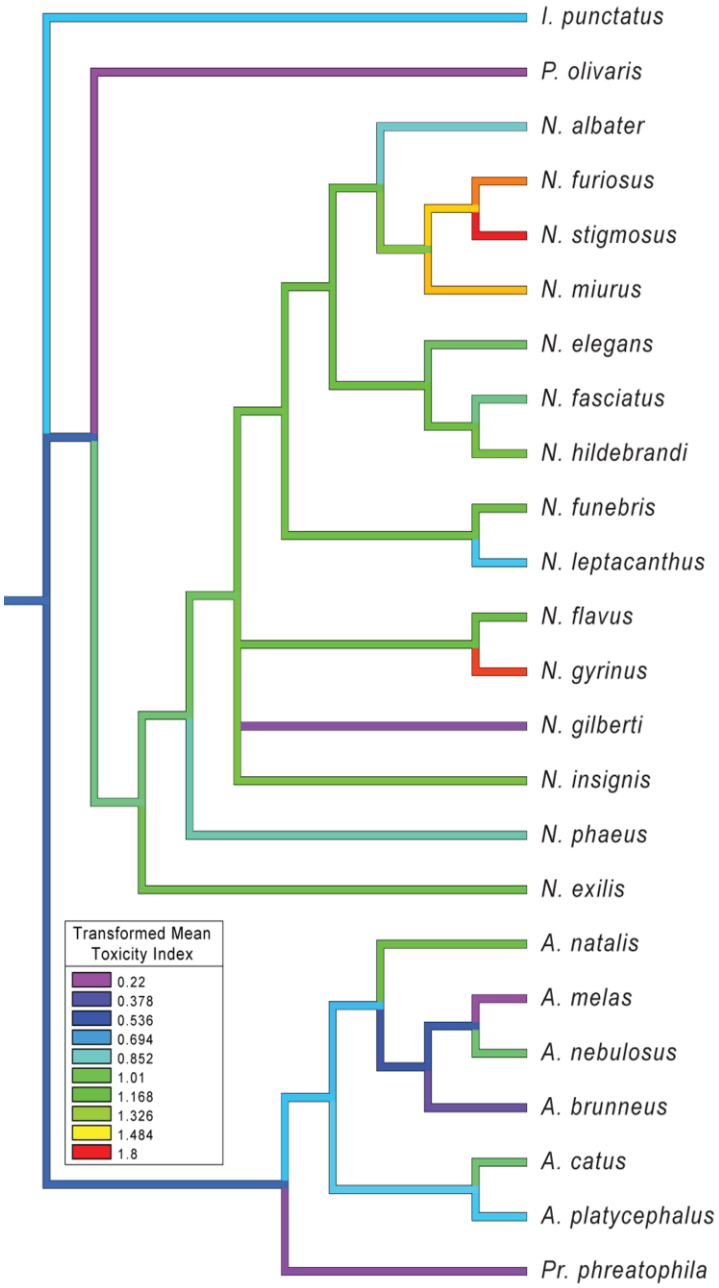


Figure 4-4. Representative venom and caudal-fin extract profiles from several *Noturus* species. The candidate venom toxin at 110 kDa was found in all venomous ictalurids examined, while the number and molecular weight of additional putative toxin peptides varied widely from species to species. V, venom extract lane; C, caudal-fin extract lane.

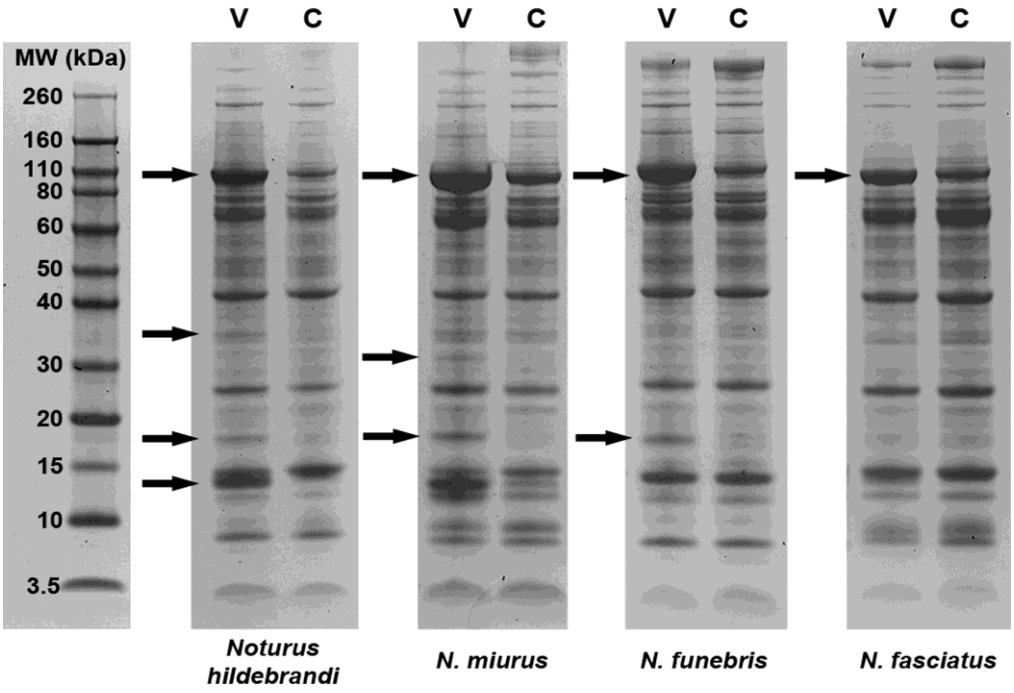


Figure 4-5. Plots of transformed (A) venom toxicity index vs. venom toxin diversity, (B) venom toxicity index vs. sting morphology, (C) sting morphology vs. venom toxin diversity, (D) venom toxicity index vs. standard length attained in the first year of life, and (E) venom toxicity index vs. fertilized egg chorion diameter (*Noturus* only). These venom-related ahistorical correlations were the only ones to maintain statistical significance in PIC analyses.

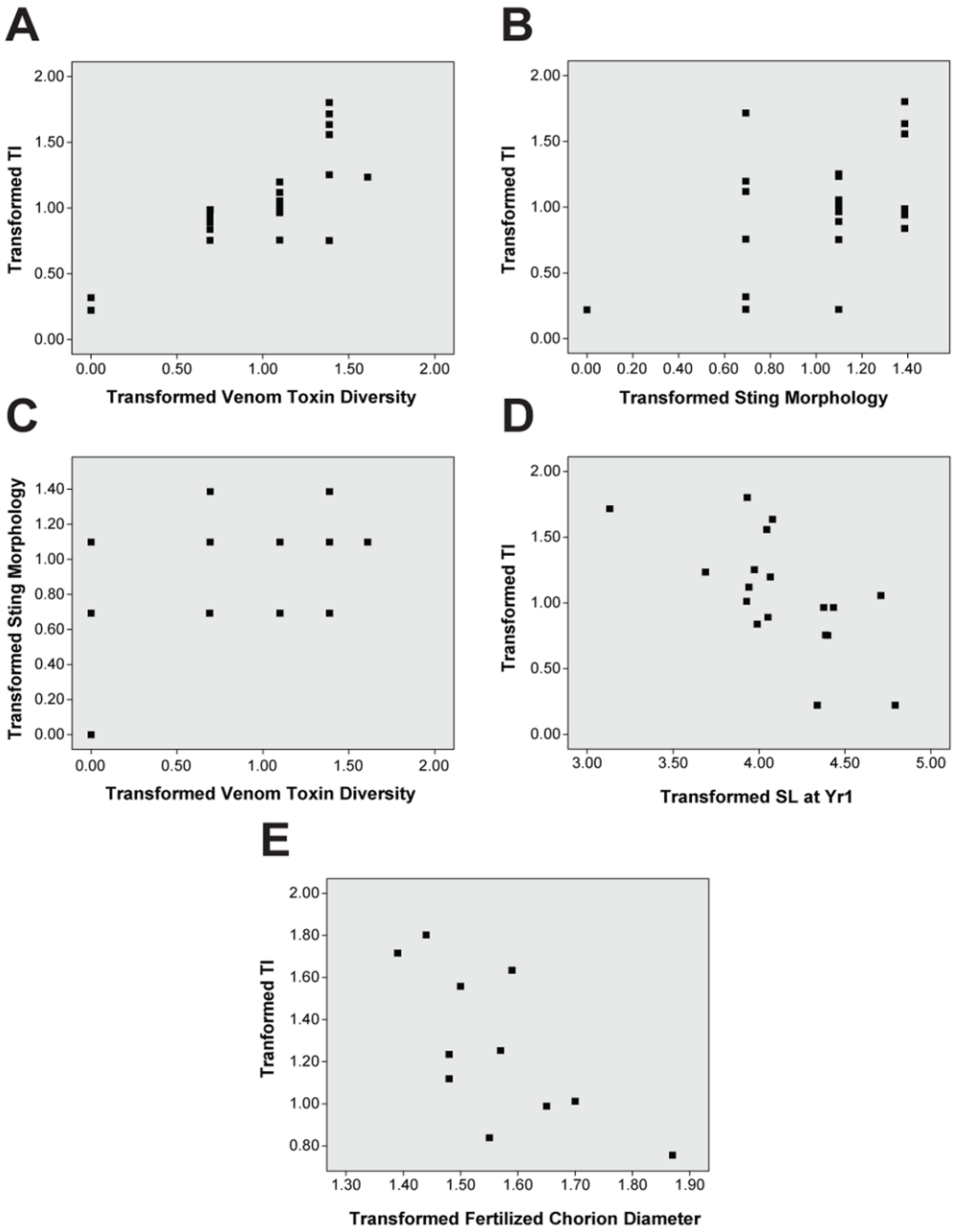
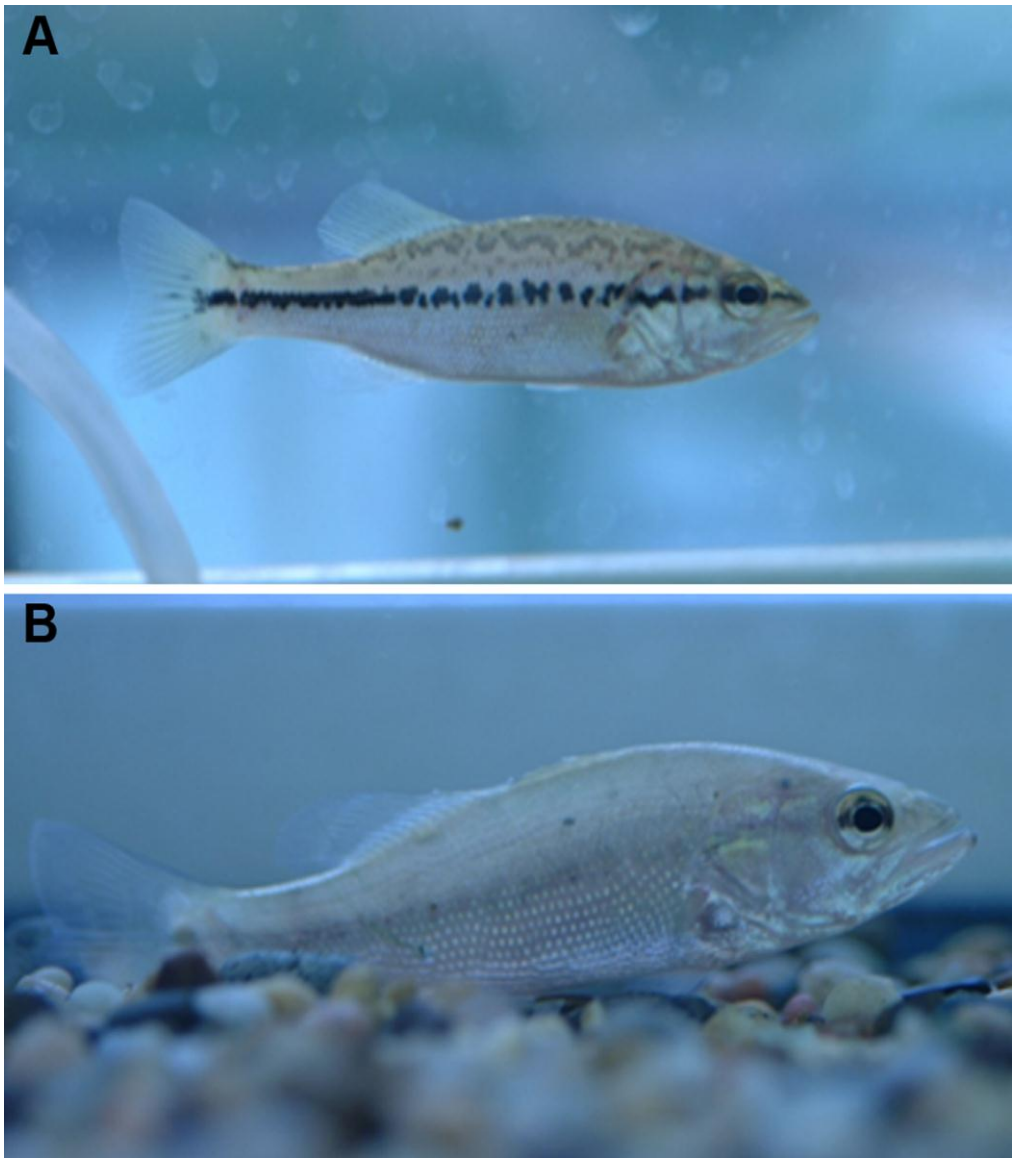


Figure 4-6. Symptoms of ictalurid catfish envenomation varied widely from those reported in earlier studies. (A) Largemouth bass prior to injection. (B) The same individual one minute after injection with *Noturus flavus* crude venom extract. This nearly complete loss of body coloration was commonly observed, but has not been reported in prior studies of ictalurid venoms.



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CHAPTER 5

CONSERVATIVE COEVOLUTION OF MÜLLERIAN MIMICRY IN A GROUP OF RIFT LAKE CATFISHES[3]

ABSTRACT

Biological mimicry has long been viewed as a powerful example of natural selection's ability to drive phenotypic evolution, though continuing debates surround the mechanisms leading to its development and the nature of these mimetic relationships. Müllerian mimicry, in which unpalatable species derive a mutual selective benefit through evolved phenotypic similarity, has alternatively been proposed to evolve through either a two-step process initiated by a large mutational change, or through continuous gradual evolution toward a common aposematic phenotype. I exposed a model predatory fish species to two species of endemic Lake Tanganyikan *Synodontis* to provide evidence for aposematism and the presence of Müllerian mimicry in these species. Predators quickly became conditioned to avoid the venomous catfishes and did not discriminate between the two species when they were switched, supporting a hypothesis of functional Müllerian mimicry in this group of similarly colored fishes. Ancestral state reconstructions and statistical comparisons of color pattern divergence in Tanganyikan *Synodontis* indicate that Müllerian mimicry in these catfishes has developed through diversification of an aposematic common ancestor with subsequent conservative mutualistic coevolution among its daughter lineages, rather than advergent evolution of a

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mimic toward a non-related model, as assumed by widely accepted models of Müllerian mimicry evolution.

INTRODUCTION

Biological mimicry has been a subject of fascination for biologists since its first formal description nearly 150 years ago (Bates 1862). The processes responsible for the evolution and maintenance of mimetic relationships are consequently a topic of great interest and discussion, and their elucidation has ramifications for many other areas of biological study (Mallet 2001). Müllerian mimicry, in which two defended species share a mutual selective benefit from a shared color pattern, has been a particularly widely debated concept among evolutionary biologists. Contentions have historically centered on both the nature of the relationships between differently defended co-mimics (Malcolm, 1990; Speed, 1993; Joron & Mallet, 1998; Mallet & Joron, 1999; Speed & Turner, 1999; Mallet, 2001; Ruxton & Speed, 2005; Sherratt, 2007, 2008) and the evolutionary mechanisms responsible for initial establishment of mimicry between phenotypically dissimilar, unpalatable species.

The evolutionary origin of traditional Müllerian mimicry has widely been held to be the result of a two-step process. This process is initiated by a mutational change in appearance of an unpalatable organism that results in a resemblance to a formerly dissimilar, aposematic species, with subsequent fine-tuning of the resulting phenotypic similarity through either convergent or continued one-sided, advergent evolution (Nicholson, 1927; Turner, 1984; Sheppard et al., 1985; Mallet, 2001). Purifying selection against individuals of unpalatable species that vary from their own species' average appearance is the major theoretical agent that has been used to support this model, as

opposed to a model of gradual evolution towards an alternative aposematic phenotype. This latter model, first proposed by Fisher (1927), has gained support from recent simulations that verify its feasibility when the species involved are initially similar in appearance (Franks & Noble, 2002; Balogh & Leimar, 2005), or exposed to predators showing certain forms of prey generalization (Ruxton et al., 2008).

These two models of Müllerian mimicry evolution share common ground, however, in that they both assume that an incipient mimic undergoes advergent evolution resulting in increased similarity to a preexisting, aposematic model organism. However, a third potential mechanism for the evolution of Müllerian mimicry exists that circumvents theoretical concerns arising from this assumption: diversification of an unpalatable, aposematic species with subsequent conservative mutualistic co-evolution of its daughter lineages. Such a mechanism was advocated by Brower et al. (1964), and has been proposed to underlie the aposematic similarity of *Cauliognathus* beetles (Machado et al., 2004), although it appears to have received little additional consideration.

The phenomenon of biological mimicry can be classified in two separate, although not necessarily exclusive, contexts. Functional mimicry requires the use of predatory species to confirm that the phenotypic similarity of mimetic species is sufficient to protect them from predation by an individual with experience of the aposematic phenotype under examination (Wickler, 1968). Adaptive mimicry presupposes the presence of functional mimicry, but also requires evidence of either convergence or one-sided advergence of an incipient mimic on a pre-existing model. Early descriptions of mimicry rings relied on regional co-occurrence of aposematic color patterns in species that occupied large geographical ranges to infer the presence of local

selection leading to convergent or advergent evolution of phenotypic similarity. More recently, evidence for these processes has come largely from studies of phylogeny, demonstrating the polyphyletic nature of putative mimicry complexes (Brower, 1996; Miller, 1996; Choi, 2001; Dumbacher & Fleischer, 2001; Symula et al., 2001; Marek & Bond, 2009). However, empirical tests (either in the lab or field) of the functional mimicry that is assumed to exist in these cases have been relatively sparse (Platt et al., 1971; Field, 1974; Ritland, 1991; Kapan, 2001; Pinheiro, 2003).

Here I provide, to my knowledge, the first experimental evidence for the presence of functional Müllerian mimicry in a group of fishes. Furthermore, I provide evidence from phylogenetic and statistical examinations of species' color patterns to demonstrate that this mimicry ring has likely arisen via the abovementioned evolutionary model of Brower et al. (1964). My model system in this case is the Lake Tanganyikan *Synodontis* catfish species flock, a group that has not previously been investigated for the presence of mimicry. At some point in their ontogeny, the endemic *Synodontis* species of Lake Tanganyika all display a distinct and striking body coloration pattern, consisting of black spots of varying size over a solid background (usually yellow to greenish bronze), with the rayed fins displaying well-defined black bases and highly contrasting white borders (Fig. 5-1A-C). Recent molecular phylogenies (Day & Wilkinson, 2006; Koblmüller et al., 2006; Day et al., 2009) indicate that *Synodontis victoriae* (Fig. 5-1D), a non-Tanganyikan species that does not share this color pattern, is closely allied with the endemic Tanganyikan *Synodontis* species, rendering them paraphyletic (Fig. 5-1E). This phylogenetic pattern indicates that selective factors associated with residence in Lake Tanganyika may be responsible for the continued presence of this color pattern in its

endemic *Synodontis* species. Additionally, there is an ontogenetic loss of this color pattern in two large (adult size > 50 cm) Tanganyikan species [*S. dhonti* and *S. tanganaicae* (Wright & Page, 2006)], and all of the endemic Tanganyikan species thus far examined (as well as *S. victoriae*) possess well-developed venom glands (Fig. 5-2). These circumstances, when viewed in light of behavioral observations of these fishes in their natural environment [diurnal activity of Tanganyikan *Synodontis* vs. mainly nocturnal in other catfishes, in addition to movement over large stretches of open substrate vs. restriction to local rock outcroppings in many cichlid species (Brichard, 1978; Brichard, 1989)], suggest that the highly contrasting color pattern of these species is aposematic.

In this study, I first used behavioral experiments to demonstrate the aposematic nature of the color pattern of the *Synodontis* catfish species of Lake Tanganyika and that the resemblance between these species is sufficient to prevent attacks by a conditioned predator. The model predator organism chosen for this study (*Micropterus salmoides* – Largemouth bass) is a large, visually oriented generalist predator of other fishes, and is thus an appropriate proxy for the predators that Tanganyikan *Synodontis* species might naturally encounter (*Lates* sp., large, piscivorous cichlids). In order to establish the aposematic nature of the Tanganyikan *Synodontis* color pattern and functional Müllerian mimicry due to phenotypic similarity between species, naïve bass were exposed, under a variety of conditions, to Tanganyikan *Synodontis* specimens, as well as dissimilar catfishes and artificial *Synodontis* models. I then performed biochemical and toxicological assays to confirm the venomous nature of the *Synodontis* species used in behavioral experiments. Finally, I enlisted ancestral state reconstruction and contingency

table analyses using previously published molecular phylogenetic evidence to establish the likelihood of the evolutionary mechanism introduced above.

MATERIALS AND METHODS

Animal Acquisition and Care

Tanganyikan *Synodontis* specimens used for histological study were collected in the field by Peter McIntyre (School of Natural Resources and the Environment, University of Michigan) in July and August of 2009. Specimens were euthanized, fixed in 10% formalin, and transferred to 70 % ethanol prior to histological preparation.

Micropterus salmoides (largemouth bass) were collected from Boyden Creek, Washtenaw Co., MI in May of 2008. Bass ranged from 12-18 mm in total length when collected, and were assumed to be naïve to other fish as a potential source of food based on their small size. Twelve bass were euthanized using MS-222 at a concentration of 300 mg/L in fresh water, and their stomach contents examined to confirm that an ontogenetic dietary shift to piscivory had not yet occurred. This was desirable, as bass would not yet have attempted to prey on any local catfishes or developed preferences for a particular type of prey species. Bass were maintained in aquaria under natural light conditions from May 2008 to May 2009 on a diet of frozen mosquito larvae and krill, only being allowed to shift to piscivory when experiments began. All individuals of *Synodontis multipunctata* and *S. petricola* used in experiments were captive bred individuals obtained through the aquarium trade. All specimens of *Pimephales vigilax* were obtained from a local pet store and consisted of individuals displaying the wild type coloration for *P. vigilax*, as well as the "Rosy Red" variety widely available in the pet trade.

Experimental Setup and Equipment

Individual bass were removed from communal holding tanks and lightly anesthetized in MS-222 at a concentration of 75 mg/L of fresh water, and their horizontal and vertical gapes, as well as standard lengths were measured to the nearest 0.1 mm with dial calipers. Each bass (n=24) was then placed in its own 10 gallon experimental aquarium and allowed to acclimate to its new environment for a period of five days. Bass were fed a single minnow [*Pimephales vigilax* ("rosy red" or wild type color pattern)] each day for the first three days of this period. The same coloration type of prey fish was never offered for two consecutive days, in an attempt to avoid familiarization of bass with any one prey phenotype. Bass were not fed during the final two days of this acclimation period to ensure that they would be hungry when first exposed to a *Synodontis* individual or control minnow (*P. vigilax*).

When the acclimation period ended, a clear barrier was placed in each experimental aquarium to divide the area in half. The barrier consisted of a frame made from ½" plastic grid, which had been cut to fit each individual aquarium. Plastic wrap was stretched across this frame and held in place with cotton thread. A cardboard blinder (with 70 x 15 mm viewing slit) was placed around each tank to eliminate the potential influence of the observer on experiments. The bass were then given two hours to recover from any stress associated with placing the barrier in, and the blinder around, the experimental aquarium.

Aposematic Conditioning Experiments

After the recovery period, an individual of either *Synodontis multipunctata* or *S. petricola* was placed in the experimental aquaria, separated from the bass by the clear plastic

barrier. Catfishes had previously been anesthetized and measured using dial calipers to ensure that the bass to which they would be exposed would be able to consume them when their pectoral and dorsal-fin spines were erected, based on previous measurements of bass gape sizes. The number of times that bass struck the plastic barrier was used to gauge the relative interest of bass in consuming the potential prey organism. All strikes on the plastic barrier occurring during a period of five minutes were counted.

After the five minute observation period, the plastic barrier was removed, and the bass were allowed to attempt to feed on the potential prey individual. All attempts to consume the prey individual, and the resulting behavior of the bass were observed for evidence of noxious stimuli. Bass were provided access to the potential prey for a period of five minutes, after which the prey, if not ingested, was removed. For each bass, this procedure (exposure with barrier, barrier removal) was repeated every 24 hours, with each bass being exposed to its assigned prey type a total of five times.

To show that the color pattern of *Synodontis multipunctata* and *S. petricola* is sufficiently similar to prevent distinction by conditioned bass, the experiments described above were repeated, with *S. multipunctata*-conditioned bass now being presented with *S. petricola*, and vice-versa. Control bass were exposed to a randomly selected *Synodontis* specimen. All strikes on the barrier in a five minute period and all attacks on the potential prey fish after removal of the barrier were observed (in the case of control bass, the barrier was not removed in order to avoid negative experience with Tanganyikan *Synodontis* individuals, allowing control bass to be used in subsequent experiments). The experiment was repeated the following day to eliminate introduction of an unfamiliar prey species or lack of hunger as possible confounding factors.

Control Experiments

Control experiments were performed to eliminate experimental manipulations and non-visual sensory information as possible influences on bass behavior. Only one control experiment was performed within a given 24 hour period. The first of these experiments followed the procedure of the conditioning experiments detailed above, except that *Synodontis*-conditioned bass were exposed to *Pimephales vigilax* as a prey item. These experiments were performed to demonstrate that repeated experiences with the plastic barrier did not lead to the decrease in attacks that was seen in bass conditioned using *Synodontis* specimens.

To eliminate olfactory cues as a possible means of discrimination, conditioned and control bass were sequentially presented with a *Synodontis petricola* and a *Pimephales vigilax* in a one quart, waterproof, plastic zip-top bag, filled with fresh water. For each exposure, the number of strikes on the bag in a five minute period was recorded.

Body shape and movement cues were eliminated as discriminatory factors by presenting *Synodontis*-conditioned bass with an appropriately sized *Ameiurus natalis*, a North American catfish that lacks the distinctive coloration pattern of Tanganyikan *Synodontis* species, but which has a similar body shape and swimming pattern. Bass were exposed to a specimen of *A. natalis* in the same manner as in the conditioning experiments described above, with both the number of attacks on the plastic barrier, and the outcome of direct attacks on the *A. natalis* specimen being recorded.

Models were prepared from foam board material and enamel paint to resemble a Tanganyikan *Synodontis* and the "Rosy Red" color form of *Pimephales vigilax*. Models were soaked in several changes of distilled water to remove chemical odors associated

with the paints. The models were then impaled on the end of 12' bamboo skewers, and alternately presented to conditioned bass for a five minute period. The number of attacks on the plastic barrier in a five minute period was recorded, but bass were not (intentionally) allowed to directly attack the models, due to potential harm associated with model ingestion.

Toxicity Confirmation

Aquarium specimens of *Synodontis multipunctata* and *S. petricola* were euthanized using MS-222 at a concentration of 300 mg/L in fresh water. All further preparations were carried out either on ice or under refrigeration at 4°C. Spines and caudal fin tissue were removed from each specimen, rinsed in physiological saline and gently scraped with a microspatula in order to remove any external epidermal secretions. All tissues were weighed to the nearest 0.001 g using a GeneMate digital balance. Spines were minced and then further homogenized in a 2 mL Dounce homogenizer along with euteleost physiological saline at a volume of 2 mL/g of tissue. The homogenate was then centrifuged at 6,000 rpm at 4°C for 20 minutes and the supernatant collected. The supernatant served as the crude venom extract. Control extracts prepared from caudal fin tissue were prepared in the same manner.

Largemouth bass (n = 32) were anesthetized in MS-222 at a concentration of 75 mg/L of fresh water and weighed to the nearest 0.1 g. They were then placed in 10 G experimental aquaria in a room with natural light and allowed to acclimate for a period of 72 hours. After the 72 hour acclimation period, eight bass each were injected in the caudal peduncle at a depth of 2 mm with 2 μL/g body weight of crude venom extract, or

2.0 μL/g control extract. Individuals were then observed at one minute, one hour, and 24 hours after injection for symptoms consistent with envenomation.

Venom and control extracts were prepared for SDS-PAGE analysis by reduction with NuPAGE® reducing agent and loading buffer, according to manufacturer's instructions. Reduced samples were subjected to electophoresis in NuPAGE® precast 4-12 % Bis-Tris polyacrylamide gels in 1X MES running buffer for 35 minutes, at 200V in an x-Cell SureLock™ Mini Cell. Reduced peptides were visualized using SimplyBlue™ SafeStain according to manufacturer's instructions. Molecular weights of venom and caudal fin extracts were estimated by comparison with Novex® Sharp Protein Standard. Proteins unique to venom extracts (relative to caudal-fin extracts) were treated as putative toxins.

Color Pattern Analyses

Ancestral states for coloration characters were reconstructed using Mesquite v. 2.71 (Maddison & Maddison, 2009), using a previously published molecular phylogeny (Day et al., 2009). For reconstruction purposes, color pattern was split into two components: body pattern and rayed fin pattern. Body pattern was coded as follows: 0 = unmarked, 1 = spotted, 2 = barred. Rayed fin pattern was coded as follows: 0 = unmarked, 1 = spotted, margins indistinct, 2 = solid, dark patches at base, distinct margins. Both maximum parsimony and likelihood optimizations were used to examine ancestral character states. Because little is known about rates of color or other character evolution in *Synodontis* species, the likelihood model used was the default Markov k-state one-parameter model (Mk1) provided in Mesquite, which assumes equal rates of change between character states.

Levels of color pattern divergence in Tanganyikan *Synodontis* species in comparison to non-Tanganyikan species were examined using the phylogenetic topology provided by Day et al. (2009). Each species included in that analysis was first classified as either Tanganyikan (including *S. victoriae*) or non-Tanganyikan. Nodes with branches leading to these taxa were counted, and in cases where differences in components of body or fin pattern and/or coloration were found to exist between taxa related at these nodes, they were coded as divergent. In cases where the color patterns were largely the same (variation in general size of spots included in color patterns was allowed, other variations were not), the nodes were coded as non-divergent. Differences in color pattern were determined using descriptions of species provided by Poll (1971), supplemented with my own experience examining nearly all of the included species. Nodal counts were then used to assess the statistical significance of color pattern conservatism in Tanganyikan *Synodontis* species.

Statistical Analyses

Results from conditioning, mimicry, and control experiments were examined for statistical significance using Mann-Whitney *U*-tests, implemented in SPSS Statistics 17.0. Results of conditioning and mimicry ecperiments, as well as barrier and body shape control experiments were examined using pairwise tests between *Synodontis*-conditioned and control groups. Statistical significance of olfactory and model controls was assessed via pairwise comparisons of the two different treatments for each conditioning group.

Nodal counts from the molecular phylogeny of Day et al. (2009) were used to construct a 2 x 2 contingency table for the performance of two-tailed Fisher's Exact Tests in SPSS Statistics 17.0, which provided an assessment of the statistical significance of the

lack of color pattern divergence in Tanganyikan *Synodontis* species. An additional analysis was performed that excluded nodes predating the Tanganyikan *Synodontis* radiation, to examine the possible effect of greater ages of some *Synodontis* lineages on these results.

RESULTS

Conditioning and Mimicry Experiments

Bass quickly became conditioned to avoid attacking the two Tanganyikan *Synodontis* species (*Synodontis multipunctata* and *S. petricola*) with which they were presented. During initial exposures, bass vigorously attacked the barrier separating them from the Tanganyikan *Synodontis* specimen, showing no significant difference in number of attacks from control group bass presented with a minnow (Mann-Whitney $U = 34.5$, $n_1 = n_2 = 8$, $P = 0.834$ two-tailed for *S. petricola*, $U = 28$, $n_1 = n_2 = 8$, $P = 0.713$ two-tailed for *S. multipunctata*; Fig. 5-3A). When offered unobstructed access to the catfish, all bass performed at least one attack, and often attacked multiple times. Responses of bass following these attacks included ejection of the *Synodontis* from the oral cavity and accompanying signs of discomfort such as head shakes, rapid gaping, and flaring of the gills. Attacks directed at the separating barrier decreased significantly after the first exposure (Mann-Whitney $U = 64$, $n_1 = n_2 = 8$, $P < 0.001$ two-tailed for *S. petricola*, $U = 58$, $n_1 = n_2 = 8$, $P < 0.01$ two-tailed for *S. multipunctata* on second exposure; Fig. 5-3A), and no bass showed aggression towards a Tanganyikan *Synodontis* specimen after the third exposure (Fig. 5-3A). When conditioned bass were presented with an unfamiliar Tanganyikan *Synodontis* species, no attacks by any individual were recorded (Fig. 5-3B). This suggests that the similarity in color pattern of the two *Synodontis* species was

sufficient to significantly prevent predation attempts (Mann-Whitney $U = 64$, $n_1 = n_2 = 8$, $P < 0.001$ two-tailed for *S. petricola*, $U = 64$, $n_1 = n_2 = 8$, $P < 0.001$ two-tailed for *S. multipunctata*) and supports a hypothesis of Müllerian mimicry in these species.

Subsequent control experiments sought to demonstrate that avoidance behavior displayed by *Synodontis*-conditioned bass was based solely on the color pattern of the catfishes to which they had been exposed. *Synodontis*-conditioned bass vigorously attacked the plastic barrier when a minnow was placed on the other side, showing no significant difference in number of attacks from minnow-conditioned control group bass (Mann-Whitney $U = 29$, $n_1 = n_2 = 8$, $P = 0.793$ two-tailed for *S. petricola*, $U = 22$, $n_1 = n_2 = 8$, $P = 0.32$ two-tailed for *S. multipunctata*; Fig. 5-3C), demonstrating that the repeated presence of the plastic barrier was not acting as a cue that bass would be unable to access the prey on the other side, leading to reduction in number of attacks.

Potential olfactory cues leading to discrimination by conditioned bass were examined by alternately exposing bass to a Tanganyikan *Synodontis* and a minnow, both of which were enclosed in a clear, waterproof plastic bag. *Synodontis*-conditioned bass performed a significantly lower number of attacks (zero for all but one individual in each group) on the bag containing the Tanganyikan *Synodontis* (Mann-Whitney $U = 64$, $n_1 = n_2 = 8$, $P < 0.001$ two-tailed for *S. petricola*, $U = 58$, $n_1 = n_2 = 8$, $P < 0.001$ two-tailed for *S. multipunctata*; Fig. 5-3C), while minnow-conditioned control bass showed no significant difference in attacks on the two prey types (Mann-Whitney $U = 33$, $n_1 = n_2 = 8$, $P = 0.958$ two-tailed).

Body shape was considered as another difference potentially leading to avoidance of Tanganyikan *Synodontis* by conditioned bass, and was tested as a discriminatory cue

by presenting bass with a similarly shaped catfish (juvenile *Ameiurus natalis*) that lacked the characteristic Tanganyikan color pattern. *Synodontis*-conditioned bass again tenaciously attacked the barrier, showing no significant difference in mean number of attacks from control group bass (Mann-Whitney $U = 32$, $n_1 = n_2 = 8$, $P = 1.0$ two-tailed for *S. petricola*, $U = 26$, $n_1 = n_2 = 8$, $P = 0.563$ two-tailed for *S. multipunctata*; Fig. 5-3C). Furthermore, when the barrier was removed, *Synodontis*-conditioned bass repeatedly attacked the unpatterned catfish, leading to greater than 50% total mortality of the catfish specimens used, often due to complete consumption by the bass.

Finally, experiments using models (Fig. 5-4) eliminated any other uncontrolled cues that may have been present. Again, *Synodontis*-conditioned bass performed significantly fewer attacks on the plastic barrier when presented with a Tanganyikan *Synodontis* model, as opposed to a model resembling a minnow (Mann-Whitney $U = 64$, $n_1 = n_2 = 8$, $P < 0.001$ two-tailed for *S. petricola*, $U = 64$, $n_1 = n_2 = 8$, $P < 0.001$ two-tailed for *S. multipunctata*; Fig. 5-3C), while control bass performed a similar number of attacks when presented with either model type (Mann-Whitney $U = 28.5$, $n_1 = n_2 = 8$, $P = 0.752$ two-tailed). When taken together, the results of these control experiments indicate that conditioned bass were responding solely to the color pattern common to Tanganyikan *Synodontis* species.

Toxicity Confirmation

Conditioning experiments revealed no significant difference in the number of exposures necessary for bass to show avoidance of either Tanganyikan *Synodontis* species (Fig. 5-2A), indicating a similar degree of unpalatability in these species. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of venoms from *S.*

multipunctata and *S. petricola*, when compared to control extracts prepared from fin tissue, indicated that putative toxic peptides from both species exhibit similar molecular weights, including putative toxins at approximately 100, 18, and 12-13 kDa (Fig. 5-5). The injection of these venom extracts into largemouth bass elicited several symptoms, including the rapid appearance of a bilateral dark spot that extended from the injection site to the end of the caudal peduncle, loss of coloration over the remainder of the body, and pronounced lethargy. In contrast, injection with sterile saline and solutions prepared from fin tissue elicited no appreciable effect. Assays did not reveal any noticeable differences in the toxicity of the two species' venoms. These results confirm the presence of toxic compounds associated with the spines of these species, and also indicate that the venoms *S. multipunctata* and *S. petricola* possess no appreciable difference in potency.

Color Pattern Analyses

Reconstruction of *Synodontis* color pattern characters using the phylogeny of Day et al. (2009) indicated that the common ancestor of each respective subclade of endemic Tanganyikan *Synodontis* possessed the two main characteristics of the common Tanganyikan *Synodontis* color pattern [spotted body and characteristic fin pattern (Fig. 5-6)]. However, both parsimony and likelihood-based reconstructions returned ambiguous results for the common ancestor of all Tanganyikan *Synodontis* species plus *S. victoriae*. While both methods suggested that this ancestor had a spotted body, parsimony and likelihood reconstructions returned ambiguous results between an ancestor with spotted fins, and one with the characteristic pattern found in Lake Tanganyika. Thus, the question of whether an aposematic Tanganyikan ancestor occurred independently at the base of

each subclade, or the base of the entire Tanganyikan *Synodontis* clade (inclusive of *S. victoriae*) requires further investigation.

Examination of the phylogeny of Day et al. (2009) resulted in 7 nodes within the Tanganyikan *Synodontis* radiation being labeled "non-divergent" and one (the node leading to *S. victoriae*) being labeled "divergent" (Fig. 5-7). The remainder of the phylogeny contained 5 nodes that were classified as "non-divergent" and 15 nodes that were classified as "divergent" (Fig. 5-7). Tanganyikan *Synodontis* species were found to show a significant lack of divergence in color pattern relative to other members of the genus ($P < 0.01$, Fisher's Exact Test). Accounting for the greater age of some lineages by removing those nodes with age estimates exceeding that of the most basal node in the Tanganyikan *Synodontis* radiation did not lead to the elimination of statistical significance ($P = 0.011$).

DISCUSSION

The behavioral data reported by this study strongly support a hypothesis of aposematism, as well as functional and potentially adaptive Müllerian mimicry in Tanganyikan *Synodontis*. This would represent a rare case of Müllerian mimicry for vertebrates in general [the only other putative cases occurring in snakes (Greene & McDiarmid, 1981; Sanders et al., 2006), dendrobatid frogs (Symula et al., 2001), and certain populations of New Guinean birds (Dumbacher & Fleischer, 2001)]. The reaction of largemouth bass to both direct attacks on Tanganyikan *Synodontis* and the injection of venom extracts prepared from their fin spines indicates that these fishes deliver a thoroughly unpleasant, though not significantly harmful stimulus to potential predators. Thus, the strong similarity in appearance between Tanganyikan *Synodontis* species

represents a case of concrete homotypy [where a model is an identifiable species or group of species, as opposed to abstract homotypy, where the model is a general category of organism (e.g., a snake)], as predicted by previous models inversely relating degree of noxiousness to precision of mimicry (Pasteur, 1982; Pough, 1988).

More difficult to reconcile with traditional assumptions of Müllerian mimicry is the phylogenetic relatedness of Tanganyikan *Synodontis* species. My experiments have shown that the similarity of these catfishes would be functionally mimetic in an ecological context (Greene, 1977; Pough, 1988). However, if mimicry also requires an evolutionary sequence in which selection establishes and increases similarity of a mimic to a model organism, this system would, at first glance, not qualify as an example, due to the pre-existing close similarity of an aposematic daughter lineage to its ancestor. Additionally, the relatedness of putatively mimetic daughter lineages is problematic, as past studies have used the polyphyletic nature of the mimetic assemblages being studied as phylogenetic evidence for adaptive mimicry. However, if selectively driven coevolution among initially similar daughter lineages could be inferred to have *maintained* the similarity of these species to an ancestral model, then this could be viewed as an evolutionary analogue to directional selection of a mimic towards a model, as more variable individuals in the daughter lineages would face purifying selection towards the ancestral phenotype due to increased predation. Evidence for this selective maintenance of color pattern similarity in Tanganyikan *Synodontis* species is provided by the above analyses of color pattern within the genus, which show that those species confined to the selective regime of Lake Tanganyika display a significantly lower degree of color pattern divergence than do *Synodontis* species occurring outside of the lake. The

examination of this lack of color pattern divergence within the context of the geological history of Lake Tanganyika lends further credence to a hypothesis of selective maintenance of color similarity in Tanganyikan *Synodontis* species.

The geological history of Lake Tanganyika has been implicated as a factor related to the evolution of many of its endemic species flocks (Salzburger et al., 2005; Marijnissen et al., 2006; Day et al., 2008) and its *Synodontis* species are no exception. Recent work dates the origin of this group at about 5-6 million years ago, during a period of deepening in Lake Tanganyika (Day et al., 2009). The divergence and diversification of the two endemic subclades contained within this radiation corresponds to a period of aridification and low lake levels, which lead to the cleavage of Lake Tanganyika into two or more sub-basins (Cane & Molnar, 2001; Day et al., 2009). The parallel maintenance (or independent origin) of the Tanganyikan color pattern in isolated species groups during their diversification due to similar selective influences (i.e. predation), represents an additional argument for the selective maintenance of Müllerian mimicry in Tanganyikan *Synodontis*. Upon the rejoining of the two sub-basins, additional mutualistic co-evolution would be possible, evidence of which is shown here by the ability of a member of one of these subclades (*S. multipunctata*) to reduce or eliminate attacks on a member of the other (*S. petricola*), and vice-versa. It is this coevolutionary maintenance of a similar color pattern which would allow this system to be considered a case of Müllerian mimicry in not only a functional, ecological sense, but in an evolutionary context as well.

Further phylogenetic evidence that coevolution has maintained this color pattern is offered by *Synodontis victoriae*. This species has emigrated from Lake Tanganyika to the adjacent Malagarasi River and Lake Victoria, and has either secondarily lost the

characteristic Tanganyikan color pattern, or diverged prior to the establishment of the characteristic Tanganyikan color pattern in its nearest relatives. Either option supports the labeling of the Tanganyikan *Synodontis* system as a case of Müllerian mimicry in the sense of an evolutionary process leading to increased similarity of mimic and model. The first option implies that release from the selective regime of Lake Tanganyika has allowed *S. victoriae* to diverge in color pattern from its nearest relatives, while the related endemic Tanganyikan *Synodontis* species have maintained a similar appearance due to shared selective influences. The second option necessitates two independent origins of the Tanganyikan color pattern in isolated sub-basins during a period of aridification. The independent origin of a highly similar, multi-component color pattern between two geographically isolated groups that nonetheless share very similar environments also suggests a selective explanation for the development of the species' phenotypic similarity.

The functional mimicry of the *Synodontis* species of Lake Tanganyika illustrates a simple, yet overlooked mechanism for the development of Müllerian mimicry systems. Such a mechanism should also facilitate the occurrence of phylogenetic codivergence, due to the presence of a similar, selectively advantageous color pattern in populations undergoing local species radiations. The demonstration of Müllerian mimicry in this group of catfishes, with its concomitant implications for the ecology and evolutionary history of these species may also have ramifications for studies of speciation and adaptive radiation in rift lakes, which have traditionally focused on cichlids (Kornfield & Smith, 2000; Kocher, 2004; Seehausen, 2006), and for which the endemic Tanganyikan

Synodontis species are becoming the focus of comparative studies (Day & Wilkinson, 2006; Koblmüller et al., 2006; Day et al., 2009;).

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Figure 5-1. Color pattern and phylogeny of Lake Tanganyikan *Synodontis* species. (A) *Synodontis multipunctata*, (B) *S. petricola*, and (C) *S. irsacae*. (D) *S. victoriae*, which lacks the characteristic color pattern seen in (A-C). (E) Phylogeny of Tanganyikan *Synodontis* species redrawn from Day et al. (2009), based on nuclear (rpS7) and mitochondrial (cyt *b*, tRNA) data.

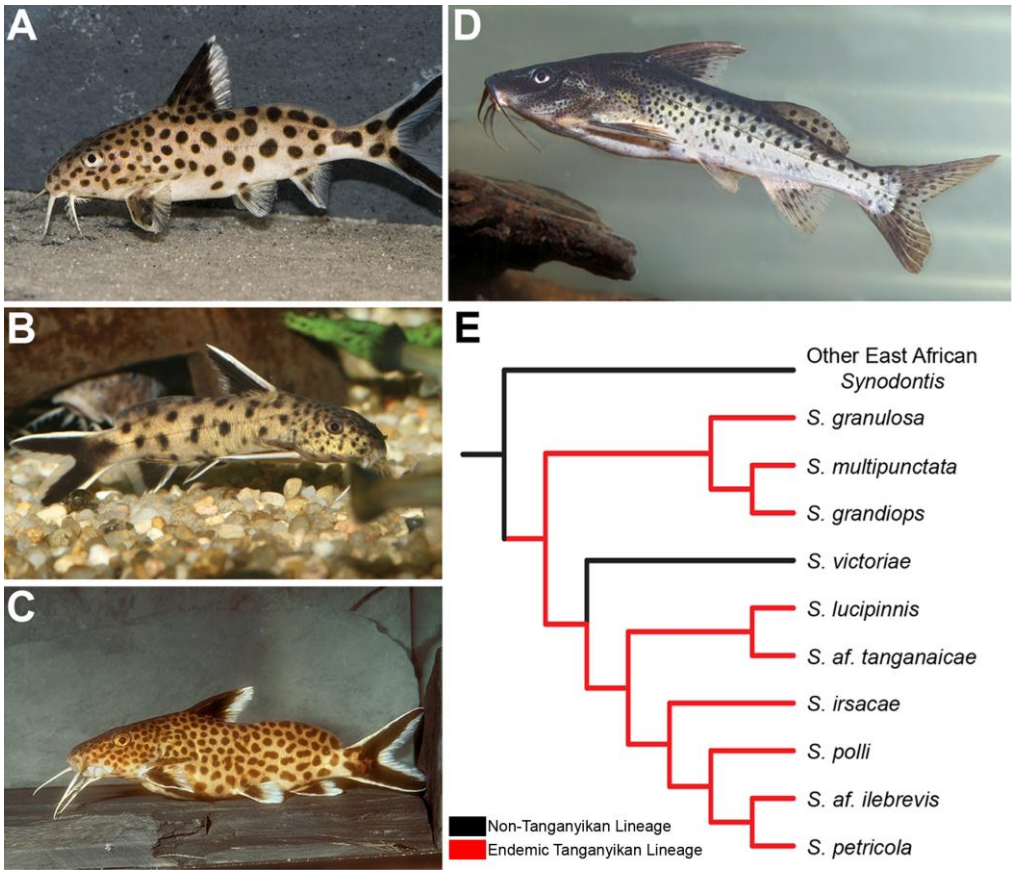


Figure 5-2. Histological preparations of Tanganyikan *Synodontis* fin spines indicate ubiquity of venom glands in this group. (A) *S. multipunctata*, (B) *S. petricola*, (C) *S. irsacae*, (D) *S. lucipinnis* (E) *S. grandiops*, (F) *S. polli*. Although all species examined had identifiable venom glands, variation was observed in venom gland arrangement and size. gc = glandular cells.

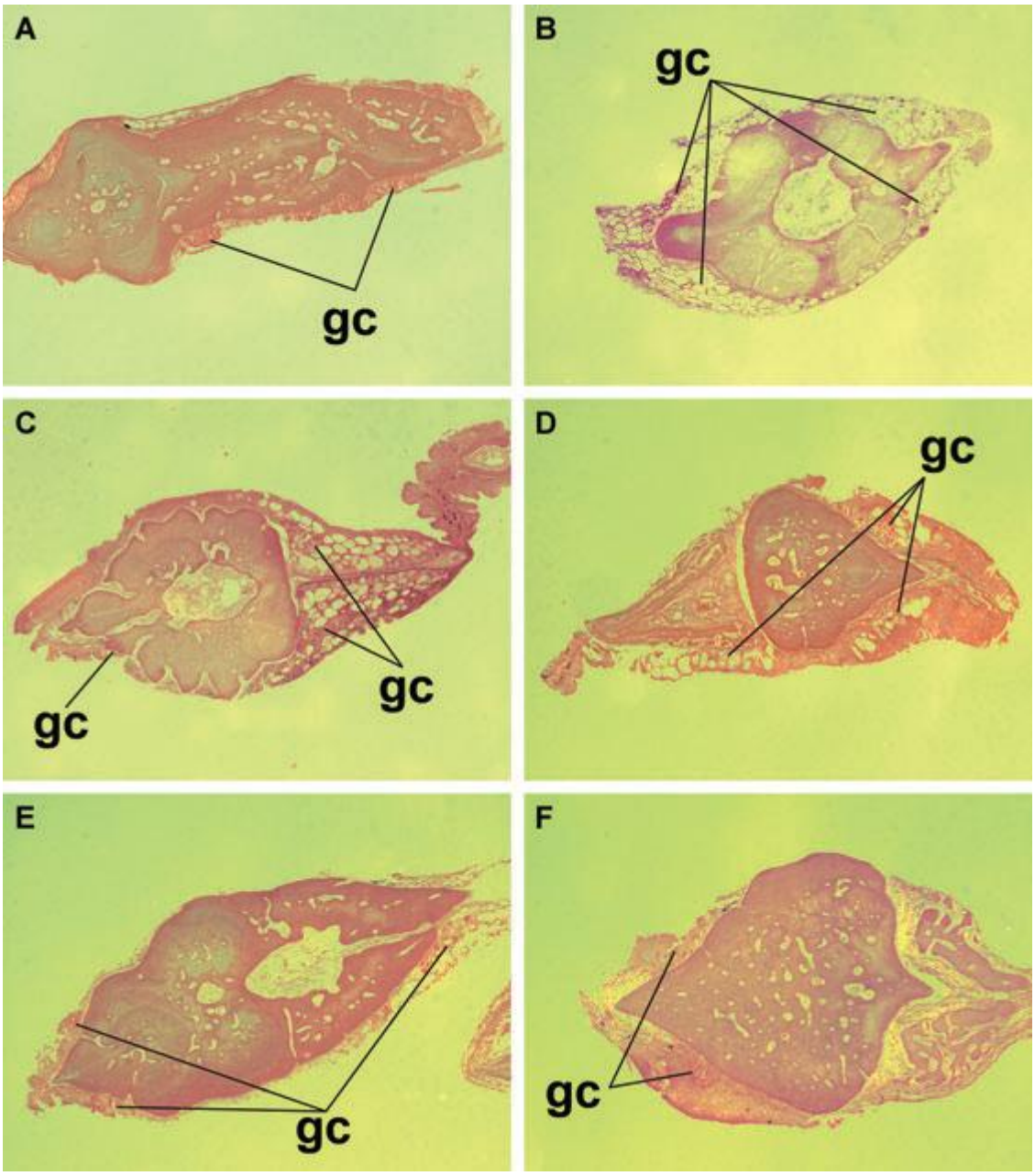


Figure 5-3. Largemouth bass quickly became conditioned to avoid two Tanganyikan *Synodontis* species based on their appearance. (A) Results of exposing bass to *S. petricola* (*N* = 8), *S. multipunctata* (*N* = 8), and *Pimephales vigilax* (Control, *N* = 8) over a five-day period. (B) Results of exposing *S. petricola*-conditioned bass to *S. multipunctata* and vice versa. Controls consisted of Pimephales-conditioned bass that were exposed to one of the two Tanganyikan *Synodontis* species available (randomly selected). (C) Results of control experimentsto determine potential effects of other noncolor-related discriminatory cues. Error bars in all graphs represent 95% confidence intervals. Symbols and Abbreviations: NS = nonsignificant (*P*-value > 0.05), $* = P < 0.05$, $** = P < 0.001$, $*** = P < 0.0001$. M = bass presented with *P. vigilax* individual or model, S = bass presented with Tanganyikan *Synodontis* individual or model.

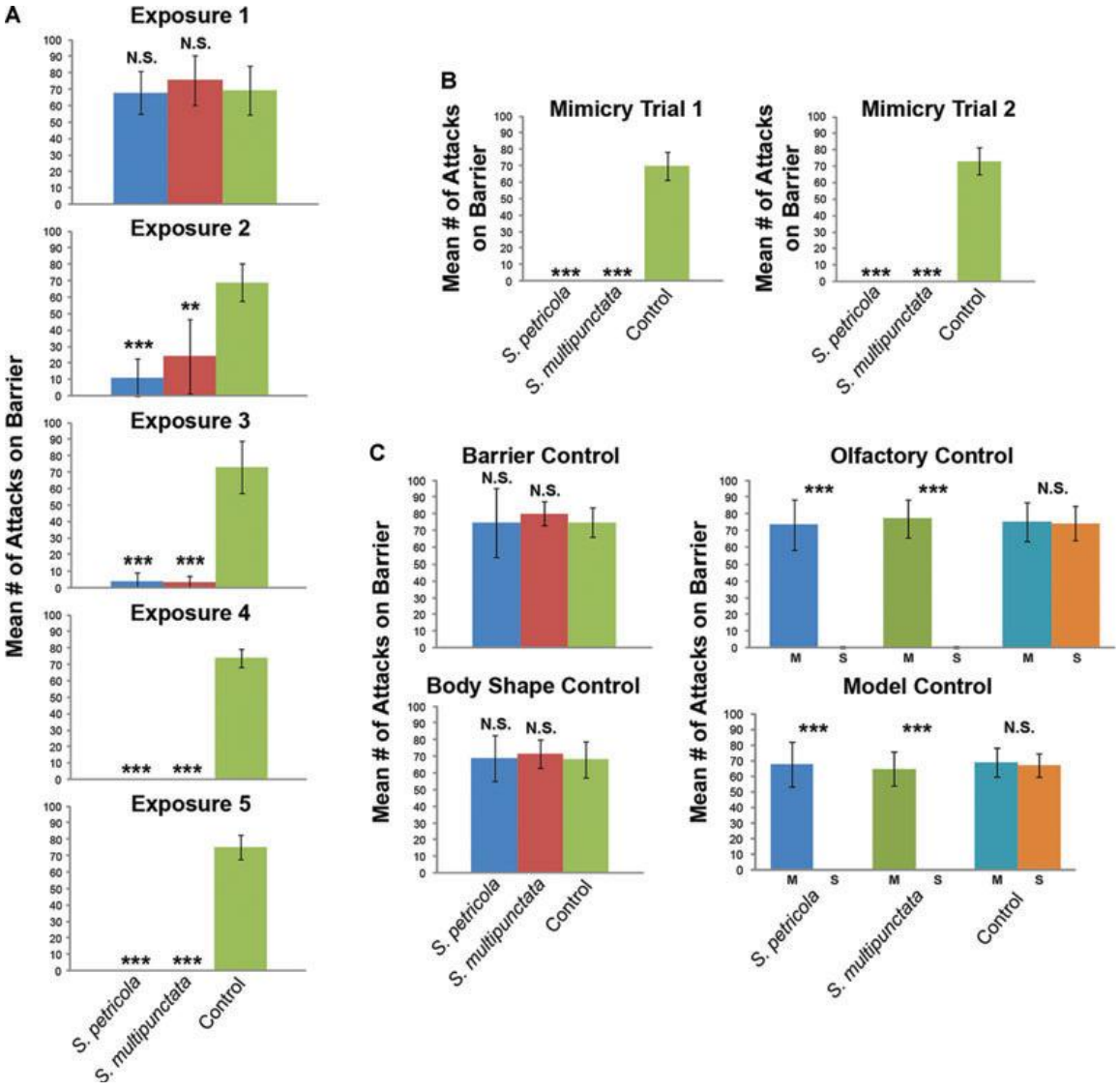


Figure 5-4. Models used in Model Control experiments. (A) Tanganyikan *Synodontis* model, (B) *Pimephales vigilax* model. (C) Tanganyikan *Synodontis* model used in a control group model experiment. In the case of (C), the bass was able to break through the plastic barrier, attacked, and attempted to swallow the model, attesting to the ability of these models to effectively imitate living, potentially viable prey items.

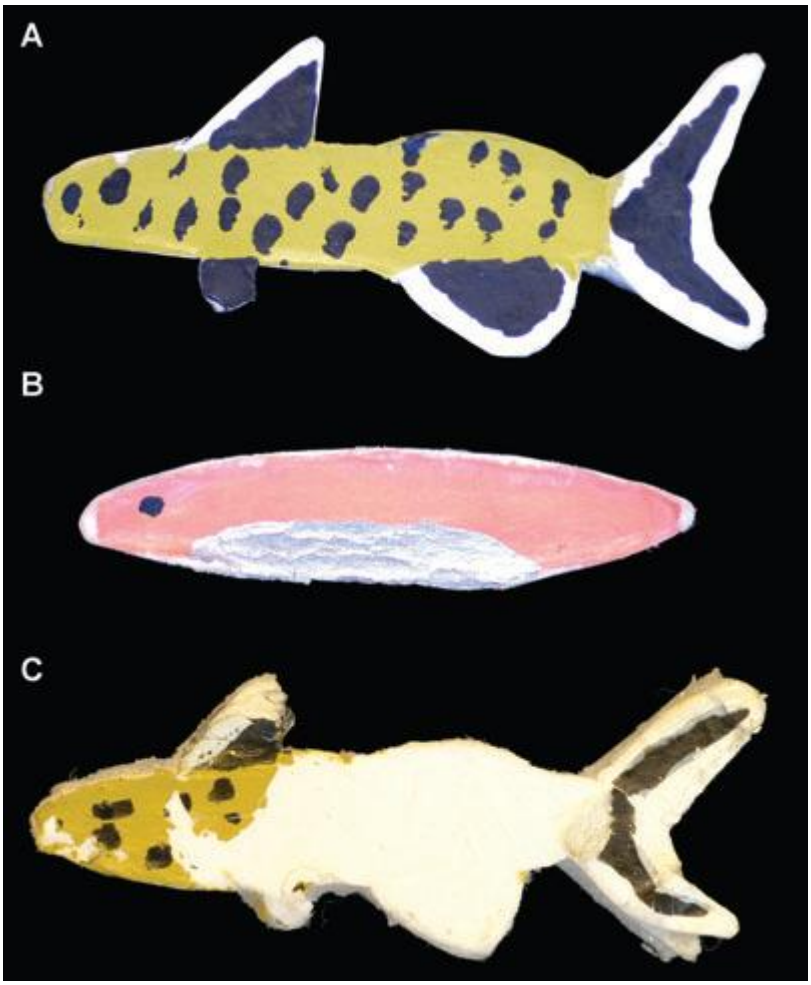


Figure 5-5. SDS-PAGE analysis indicates a high degree of similarity in the venom compositions of Tanganyikan *Synodontis*. (A) Venom and control extract profiles for *S. multipunctata*. Left panel contains venom extract, whereas the right contains control extract. (B) Venom and control extract profiles for *S. petricola*. Putative toxic venom peptides are indicated with arrows.

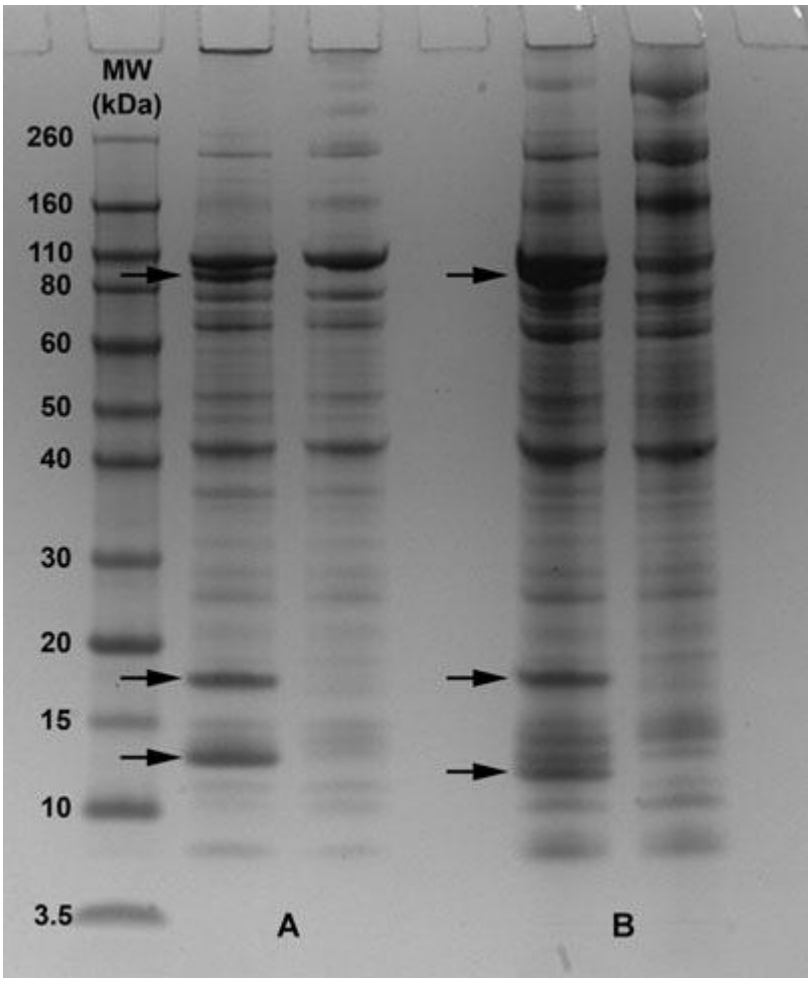


Figure 5-6. Likelihood-based ancestral state reconstructions of Tanganyikan *Synodontis* fin color patterns. Basal nodes for Tanganyikan *Synodontis* subclades are indicated with arrows. Proportional probabilities for nodes of interest are given in the following order: unpatterned fins (when applicable)/spotted fins/Tanganyikan fins.

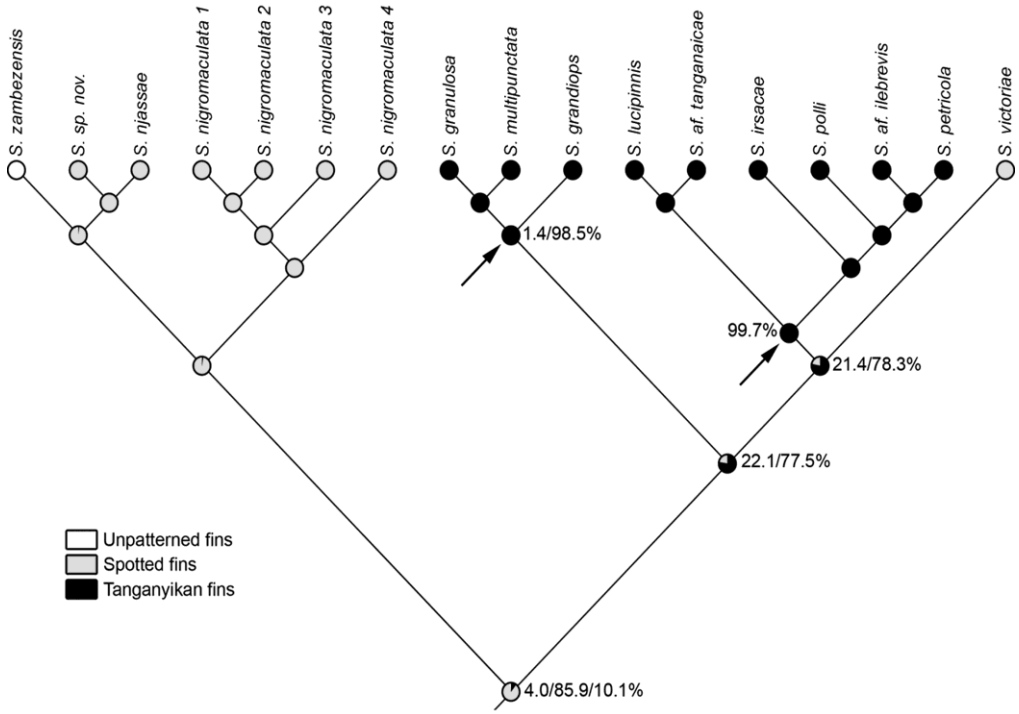
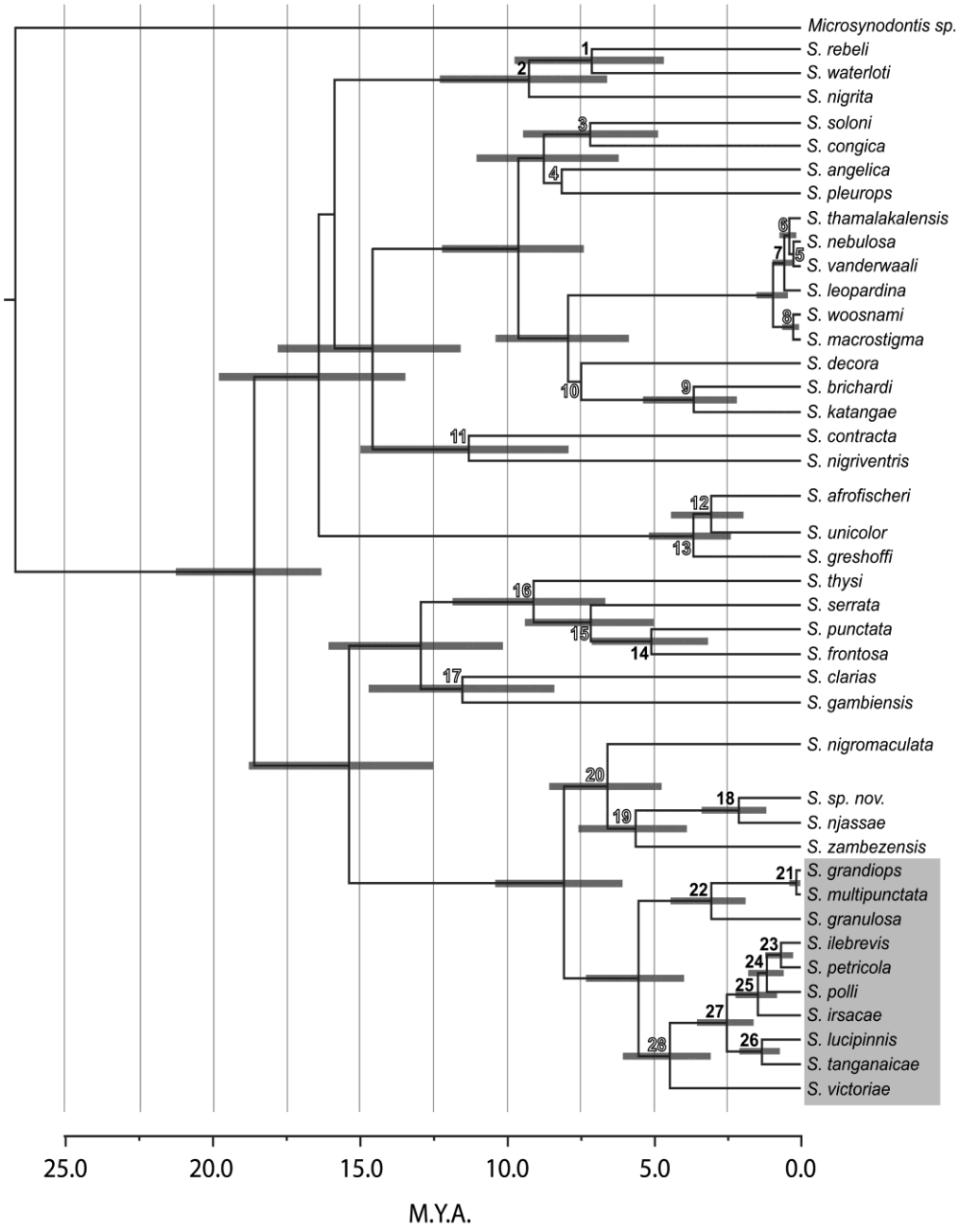


Figure 5-7. Chronogram of Synodontis species examined by Day et al. (2009), used to examine nodes in comparisons of color patterndivergence. Gray bars indicate nodal age estimates. Gray box indicates Tanganyikan *Synodontis* radiation. Nodes with solid numbers = nondivergent, outlined = divergent.



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