RESEARCH ARTICLE

Adaptive significance of venom glands in the tadpole madtom *Noturus gyrinus* (Siluriformes: Ictaluridae)

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SUMMARY

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. In previous experiments examining this question, alternative phenotypes have not been presented to ecologically relevant predators, and the 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 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.

Key words: catfish, venom, adaptation, predation, defense.

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 examination 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 and Vrba, 1982; Sober, 1984; Fisher, 1985; Endler, 1986; Thornhill, 1990; Harvey and Pagel, 1991; Reeve and 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 and Wheeler, 2006; Wright, 2009) has indicated that over 2500 fish species (or ~10% of all known species) possess venom glands associated with specialized teeth (*Meiacanthus* spp., *Monognathus* spp.), fin spines (acanthurids, apistids, aploactinids, batrachoidids, caracanthids, gnathanacanthids, neosebastids, scatophagids, scorpaenids, sebastids, setarchids, siganids, siluriforms, synanceids, tetrarogids), opercular spines (batrachoidids, trachinids) or cleithral spines (uranoscopids). The secretions produced by these glands elicit a wide array of physiological effects in vertebrate organisms, including severe pain (Halstead et al., 1953; Calton and Burnett, 1975; Gwee et al., 1994; Lopes-Ferreira et al., 1998), and cardiovascular, neuromuscular and general cytolytic effects (Church and 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 and 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 and Ladich, 2003; Fine et al., 1997; Kaatz et al., 2010). The presence of fin spines effectively increases the cross-sectional circumference of the catfish, 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 scenario has been formally described as the 'dangerous prey hypothesis' (Forbes, 1989), and has gained empirical support from studies of zooplankton (Barnhisel, 1991; Kolar and 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 and 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, the two 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 (Bosher et al., 2006; Emmett and Cochran, 2010) 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 and colleagues 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 (Bosher et al., 2006). Their study, while clearly demonstrating deterrent effects, utilized a catfish species (*I. punctatus*) that possesses venom glands associated with its fin spines (Birkhead, 1967; Birkhead, 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 (Bosher et al., 2006) were taken from an aquaculture population that had been released from predation pressure for many generations, and Birkhead (Birkhead, 1972) also indicated that the venom of *I. punctatus* may be relatively innocuous]. Emmett and Cochran (Emmett and 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 time. Furthermore, the increased survivorship and predator sensitization observed by Bosher and colleagues was not found in Emmett and Cochran's study. In their experiments, the predator species (again *M. 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) had 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 (*M. salmoides*; Fig. 1A) was presented with fully intact, venomous catfishes (*N. gyrinus* – tadpole madtom;
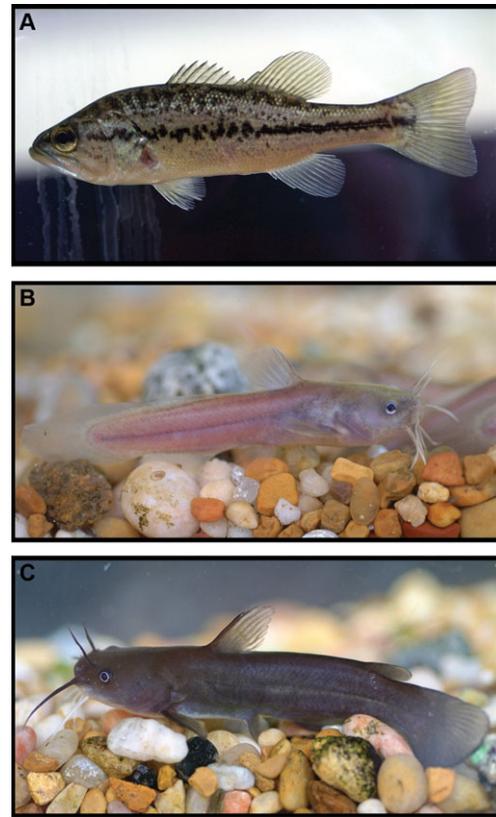

Fig. 1. The model predator and two potentially 'dangerous' prey species examined in this study. (A) Largemouth bass (*Micropterus salmoides*). (B) Tadpole madtom (*Noturus gyrinus*). (C) Yellow bullhead (*Ameiurus natalis*).

Fig. 1B), as well as with individuals that were lacking their venomous fin spines, following the example of Bosher et al. (Bosher et al., 2006). Additionally, bass were presented with *N. gyrinus* that still possessed fin spines but lacked venom glands as a result of their removal *via* microdissection of the fin spines. This allowed 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* with those using a separate, naturally co-occurring catfish species (*Ameiurus natalis* – yellow bullhead; Fig. 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

Micropterus salmoides (Lacépède 1802) were collected from Boyden Creek, Washtenaw Co., MI, USA, and ranged from 12 to 18 mm in standard length when captured. Bass were assumed to be naive 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 killed using MS-222 at a concentration of 300 mg l^{-1} in fresh water, and their stomach contents examined

Table 1. Measurements for the different classes of prey items offered to largemouth bass over the course of experiments

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

All measurements are 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.

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 *N. gyrinus* (Mitchill 1817) and *A. natalis* (Leseuer 1819) were collected from Clark Lake, Jackson Co., MI, USA, using a 12 in (~30.5 cm) 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.

Procedures utilizing live fishes were approved by the University of Michigan Committee on Use and Care of Animals (protocol nos 09713 and 10504).

Predation experiments

Prior to being used in experiments, all catfishes (*N*=24 *N. gyrinus*, *N*=8 *A. natalis*) and minnows (*N*=8) were anesthetized in MS-222 at a concentration of 75 mg l^{-1} 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 are given in Table 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 stereomicroscope, 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. 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 h 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^{-1} 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, 40 l experimental aquarium and allowed to acclimate to its new environment for a period of 5 days. Bass were fed a single minnow (*P. vigilax* – 'rosy red' or wild-type color pattern) each day for the first 3 days of this period. Bass were not fed during the final 2 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 screen (with a 70×15 mm viewing slit) was placed around each tank to eliminate the potential influence of the observer on experiments. The bass were then given 2 h to recover from any stress associated with placing the screen around the experimental aquarium.

After the 2 h 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 1 h, and all predation attempts performed on the catfish or minnows in this time period were recorded. A
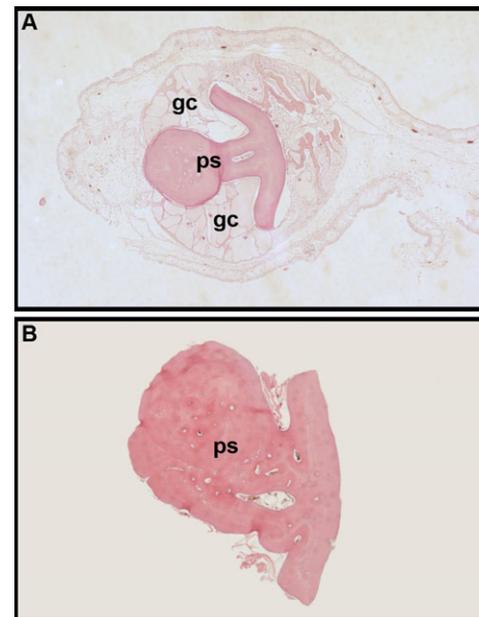

Fig. 2. Histological preparations of *N. gyrinus* fin spines confirmed the efficacy of procedures for removing venom gland material from fin spines. (A) Cross-section of *N. gyrinus* pectoral fin spine prior to venom gland removal. (B) Cross-section of a stripped *N. gyrinus* pectoral fin spine after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

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 *N. gyrinus* and *A. natalis* were killed using MS-222 at a concentration of $300\,mg\,l^{-1}$ 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, then weighed to the nearest 0.001 g using a GeneMate digital balance (BioExpress, Kaysville, UT, USA). Spines were minced and then further homogenized in a 2 ml Dounce homogenizer along with euteleost physiological saline at a volume of $2\,ml\,g^{-1}$ of tissue. The homogenate was then centrifuged at $2000\,g$. at 4°C for 20 min 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 $75\,mg\,l^{-1}$ of fresh water and weighed to the nearest 0.1 g. They were then placed in 40 l experimental aquaria in a room with natural light and allowed to acclimate for a period of 72 h. After the 72 h 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 $2\,\mu l\,g^{-1}$ body mass of crude venom extract (tadpole madtom *N*=8, yellow bullhead *N*=8), or $2.0\,\mu l\,g^{-1}$ control extract (tadpole madtom *N*=8, yellow bullhead *N*=8). Individuals were then observed at 1 min, 1 h and 24 h after injection for symptoms associated with extract injections. Venom toxicity was scored using a six-point toxicity index (modified from Birkhead, 1972), which was developed during a concurrent study of the comparative toxicity of ictalurid catfish venoms (Table 2).

Venom and control extracts were prepared for SDS-PAGE analysis by reduction with NuPAGE® reducing agent and loading buffer, according to the manufacturer's instructions (Invitrogen, Grand Island, NY, USA). Reduced samples were subjected to electrophoresis in NuPAGE® precast 4–12% bis-tris polyacrylamide gels in 1× MES running buffer for 35 min, at 200 V in an x-Cell

Fig. 3. Significant differences were found in both (A) the number of attacks performed by bass on different prey phenotypes [Friedman's ANOVA, χ^2 (d.f.=4, *N*=8)=28.81, *P*<0.001] and (B) bass handling times for those phenotypes [Friedman's ANOVA, χ^2 (d.f.=4, *N*=8)=27.50, *P*<0.001]. Fully intact madtoms elicited significantly higher numbers of attacks than other prey types because of repeated rejections by bass, which is also reflected in significantly higher handling times for this prey phenotype. Stripped madtoms (*N. gyrinus*) and yellow bullheads (*A. natalis*) produced significantly higher numbers of rejections than spineless madtoms or minnows (*P. vigilax*), 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.

Table 2. Toxicity index used to score effects of *Noturus gyrinus* and *Ameiurus natalis* venom and control extract injections

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

Note the additive nature of envenomation symptoms, likely due to the presence of both shared and novel putative toxins in different ictalurid species' venoms.
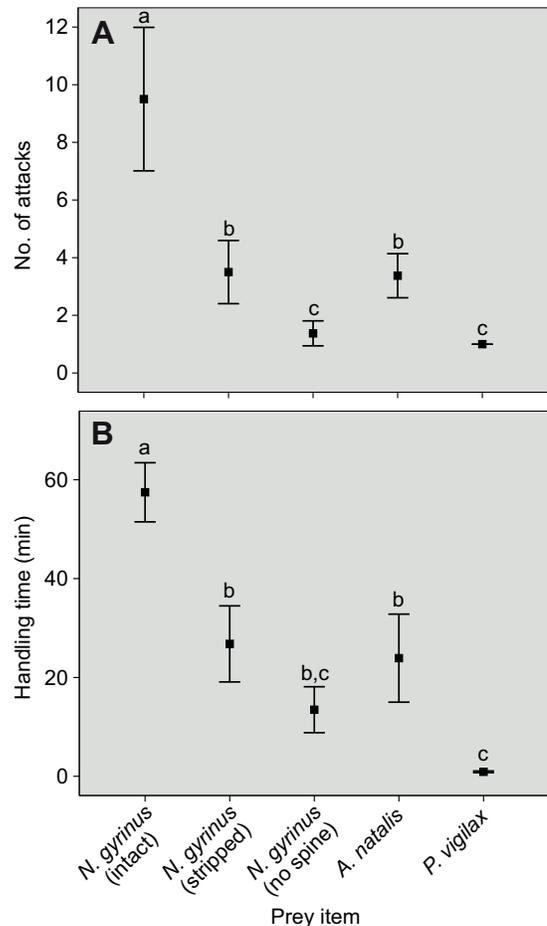
SureLock™ Mini Cell. Reduced peptides were visualized using SimplyBlue™ SafeStain according to the manufacturer's instructions (Invitrogen). Molecular masses of venom and caudal fin extract proteins were estimated by comparison with Novex® Sharp Protein Standard. Proteins unique to venom extracts (relative 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, USA, 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*, non-parametric Tukey's HSD tests were then performed to identify
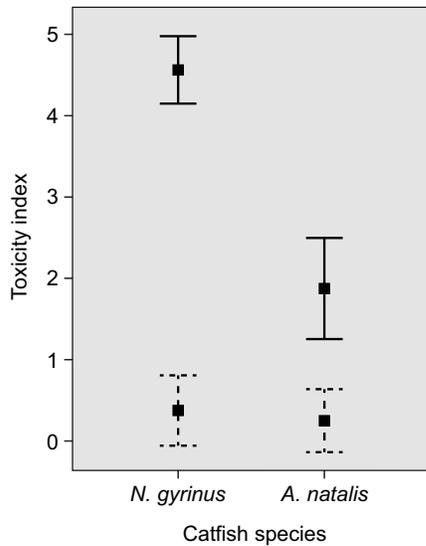
Fig. 4. Comparisons of venom toxicity indicate that the venom of *N. gyrinus* is significantly more noxious than that of *A. natalis* (Mann–Whitney U=64, N_1,N_2=8, P<0.001 two-tailed). Solid lines indicate the results of venom extract injections; dashed lines indicate control extract injections.
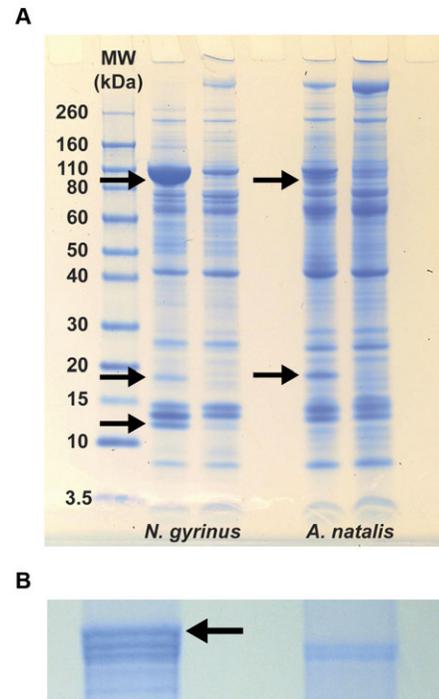

Fig. 5. SDS-PAGE gels of venom extracts (left lanes) and fin tissue extracts (right lanes) of *N. gyrinus* and *A. natalis*, with putative venom toxins indicated by arrows. (A) The venom extract protein composition of *N. gyrinus* and *A. natalis* was found to be quite similar, with putative toxic peptides 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) *versus* the fin tissue extract lane (right).

significant pairwise differences in the 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' venom and differences between the toxicity of venom extract *versus* control injections.

RESULTS
Outcomes of bass encounters with alternative prey phenotypes

Bass showed significant differences in both the number of attacks on different prey phenotypes [Fig. 3A; Friedman's ANOVA, χ^2 (d.f.=4, N=8)=28.81, P<0.001] and the handling times for these prey types [Fig. 3B; Friedman's ANOVA, χ^2 (d.f.=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) because of repeated attacks and post-capture rejections. 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 that of all other prey phenotypes (non-parametric Tukey's HSD, P<0.05), with only one individual being consumed within the 1 h 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 sign 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 time 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 time for minnows was 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. 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
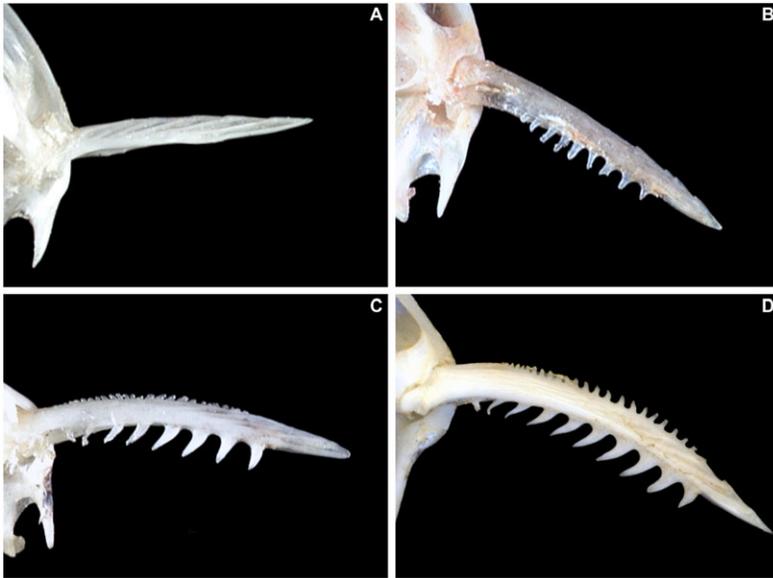
Fig. 6. The pectoral fin spines of several *Noturus* species, demonstrating the variation in fin spine morphology and potential for mechanical damage to predators found in this genus. (A) *Noturus gyrinus*, which displays the simplest of *Noturus* spine morphologies. (B) *Noturus exilis*, which possesses numerous, moderately sized serrae along the posterior margin of the spine. (C) *Noturus 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) *Noturus stigmosus*, which possesses both larger anterior serrae than *N. miurus* and larger posterior serrae than *N. exilis*.

reported previously (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. 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 composition of the two catfish species was highly similar, with venom-specific proteins of approximately 100 and 18 kDa being identified in both cases (Fig. 5A). The higher molecular mass peptide was initially difficult to distinguish from bands that were also observed in control extracts prepared from fin tissue because of 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. 5B). Additionally, the venom of *N. gyrinus* was found to possess a venom-specific band at approximately 12 kDa, which was not found in the venom extract of *A. natalis*. These results are consistent with those reported previously for *N. gyrinus* (Wright, 2009) as well as the observation in that study that the number and mass of venom peptides in the range 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 and Cochran, 2010), the spines of the species examined here were not themselves found to significantly increase handling time, 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 a greater number 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, which are known to vary significantly between madtom species (Fig. 6), often in conjunction with venom gland morphology (Egge and Simons, 2011). Increased mechanical damage due to a greater size and number 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. Naive bass were able to learn almost immediately (within a 1 h session) to avoid a naturally occurring noxious prey source, a finding that has been paralleled in a previous study (Wright, 2011) utilizing *M. 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 and Cochran, 2010) never showing a preference at all. These findings suggest that an 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 was seen over a period of several weeks following limited exposures to other catfish species examined previously (Wright, 2011)].

Unexpectedly, *A. 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 co-evolutionary 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 and Walsh, 1984)] may be historically linked with the evolution of greater venom toxicity, 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., 1996; Lynch, 2007; Duda and Lee, 2009; Gibbs and Mackessy, 2009; Barlow et al., 2009), or co-evolutionary relationships necessitate modification of venom to overcome increasing prey resistance (e.g. Poran et al., 1987; Heatwole and Poran, 1995; Biardi et al., 2006). Because of 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* spp., a family (Monognathidae) of deep-sea saccopharyngiform fishes (Bertelsen and 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 and 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 presented here, 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 and 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.

REFERENCES

Barlow, A., Pook, C. E., Harrison, R. A. and Wüster, W. (2009). Coevolution of diet and prey-specific venom activity supports the role of selection in snake venom evolution. *Proc. R. Soc. B* **276**, 2443-2449.

Barnhisel, D. R. (1991). Zooplankton spine induces aversion in small fish predators. *Oecologia* **88**, 444-450.

Bertelsen, E. and Nielsen, J. G. (1987). The deep sea eel family Monognathidae (Pisces, Anguilliformes). *Steenstrupia* **13**, 141-198.

Biardi, J. E., Chien, D. C. and Coss, R. G. (2006). California ground squirrel (*Spermophilus beecheyi*) defenses against rattlesnake venom digestive and hemostatic toxins. *J. Chem. Ecol.* **32**, 137-154.

Birkhead, W. S. (1967). The comparative toxicity of stings of the ictalurid catfish genera *Ictalurus* and *Schilbeodes*. *Comp. Biochem. Physiol.* **22**, 101-111.

Birkhead, W. S. (1972). Toxicity of stings of ariid and ictalurid catfishes. *Copeia* **1972**, 790-807.

Bosher, B. T., Newton, S. H. and Fine, M. L. (2006). The spines of the channel catfish, *Ictalurus punctatus*, as an anti-predator adaptation: an experimental study. *Ethology* **112**, 188-195.

Calton, G. J. and Burnett, J. W. (1975). Catfish (*Ictalurus catus*) fin venom. *Toxicon.* **13**, 399-403.

Church, J. E. and Hodgson, W. C. (2002). The pharmacological activity of fish venoms. *Toxicon* **40**, 1083-1093.

Daltry, J. C., Wüster, W. and Thorpe, R. S. (1996). Diet and snake venom evolution. *Nature* **379**, 537-540.

Duda, T. F. and Lee, T. (2009). Ecological release and venom evolution of a predatory marine snail at Easter Island. *PLoS ONE* **4**, e5558.

Egge, J. J. D. and Simons, A. M. (2011). Evolution of venom delivery structures in madtom catfishes (Siluriformes: Ictaluridae). *Biol. J. Linn. Soc.* **102**, 115-129.

Emmett, B. and Cochran, P. A. (2010). The response of a piscivore (*Micropterus salmoides*) to a venomous prey species (*Noturus gyrinus*). *J. Freshwat. Ecol.* **25**, 475-479.

Endler, J. A. (1986). *Natural Selection in the Wild*. Princeton: Princeton University Press.

Fine, M. L. and Ladich, F. (2003). Sound production, spine locking and related adaptations. In *Catfishes* (ed. B. G. Kapoor, G. Arratia, M. Chardon and R. Diogo), pp. 248-290. Enfield: Science Publishers, Inc.

Fine, M. L., Friel, J. P., McElroy, D., King, C. B., Loesser, K. E. and Newton, S. (1997). Pectoral spine locking and sound production in the channel catfish (*Ictalurus punctatus*). *Copeia* **1997**, 777-790.

Fisher, D. C. (1985). Evolutionary morphology: beyond the analogous, the anecdotal, and the ad hoc. *Paleobiology* **11**, 120-138.

Forbes, L. S. (1989). Prey defences and predator handling behavior: the dangerous prey hypothesis. *Oikos* **55**, 155-158.

Gibbs, H. L. and Mackessy, S. P. (2009). Functional basis of a molecular adaptation: prey-specific toxic effects of venom from *Sistrurus* rattlesnakes. *Toxicon* **53**, 672-679.

Gould, S. J. and Vrba, E. S. (1982). Exaptation – a missing term in the science of form. *Paleobiology* **8**, 4-15.

Gwee, M. C. E., Gopalakrishnakone, P., Yuen, R., Khoo, H. E. and Low, K. S. Y. (1994). A review of stonefish venoms and toxins. *Pharmacol. Ther.* **64**, 509-528.

Halstead, B. W. (1988). *Poisonous and Venomous Marine Animals of the World*, second revised edition. Princeton: The Darwin Press, Inc.

Halstead, B. W., Kuninobu, L. S. and Hebard, H. G. (1953). Catfish stings and the venom apparatus of the Mexican catfish, *Galeichthys felis* (Linnaeus). *Trans. Am. Microsc. Soc.* **72**, 297-314.

Harvey, P. H. and Pagel, M. D. (1991). *The Comparative Method in Evolutionary Biology*. New York: Oxford University Press.

Heatwole, H. and Poran, H. S. (1995). Resistances of sympatric and allopatric eels to sea-snake venoms. *Copeia* **1995**, 136-147.

Kaatz, I. M., Stewart, D. J., Rice, A. N. and Lobel, P. S. (2010). Differences in pectoral fin spine morphology between vocal and silent clades of catfishes (Order Siluriformes): ecomorphological implications. *Curr. Zool.* **56**, 73-89.

Kolar, C. S. and Wahl, D. H. (1998). Daphnid morphology deters fish predators. *Oecologia* **116**, 556-564.

Lopes-Ferreira, M., Barbaro, K. C., Cardoso, D. F., Moura-da-Silva, A. M. and Mota, I. (1998). *Thalassophryne nattereri* fish venom: biological and biochemical characterization and serum neutralization of its toxic activities. *Toxicon* **36**, 405-410.

Lynch, V. J. (2007). Inventing an arsenal: adaptive evolution and neofunctionalization of snake venom phospholipase A$_2$ genes. *BMC Evol. Biol.* **7**, 2.

Mayden, R. L. and Walsh, S. J. (1984). Life history of the least madtom *Noturus hildebrandi* (Siluriformes: Ictaluridae) with comparisons of related species. *Am. Midl. Nat.* **112**, 349-368.

McCue, M. D. (2006). Cost of producing venom in three North American pitviper species. *Copeia* **2006**, 818-825.

Nisani, Z., Dunbar, S. G. and Hayes, W. K. (2007). Cost of venom regeneration in *Parabuthus transvaalicus* (Arachnida: Buthidae). *Comp. Biochem. Physiol.* **147A**, 509-513.

Poran, N. S., Coss, R. G. and Benjamini, E. (1987). Resistance of California ground squirrels (*Spermophilus beecheyi*) to the venom of the northern Pacific rattlesnake (*Crotalus viridis oreganus*): a study of adaptive variation. *Toxicon* **25**, 767-777.

Reeve, H. K. and Sherman, P. W. (1993). Adaptation and the goals of evolutionary research. *Q. Rev. Biol.* **68**, 1-32.

Smith, W. L. and Wheeler, W. C. (2006). Venom evolution widespread in fishes: a phylogenetic road map for the bioprospecting of piscine venoms. *J. Hered.* **97**, 206-217.

Sober, E. (1984). *The Nature of Selection: Evolutionary Theory in Philosophical Focus*. Cambridge: The MIT Press.

Thornhill, R. (1990). The study of adaptation. In *Interpretation and Explanation in the Study of Animal Behavior*, Vol. 2 (ed. M. Bekoff and D. Jamieson), pp. 31-62. Boulder: Westview Press, Inc.

Williams, G. C. (1966). *Adaptation and Natural Selection*. Princeton: Princeton University Press.

Wright, J. J. (2009). Diversity, phylogenetic distribution, and origins of venomous catfishes. *BMC Evol. Biol.* **9**, 282.

Wright, J. J. (2011). Conservative coevolution of Müllerian mimicry in a group of rift lake catfish. *Evolution* **65**, 395-407.