**Effects of different predator stress on vulnerability to predation
and the underlying physiological and behavioral mechanisms of
this vulnerability in juvenile qingbo (*Spinibarbus sinensis*)**

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Abstract: Differences in the early life history would have a profound influence on the survival of the animal during the later life stages. We aimed to examine the effect of predator stress on vulnerability to predation in fish and the possible underlying behavioral and physiological mechanisms of this vulnerability. For this, we selected juvenile qingbo (*Spinibarbus sinensis*) as the prey and its natural predator, the southern catfish (*Silurus meridionalis*), as the predator. The juvenile qingbo were reared together with free predators (lethal group), caged predators (non-lethal group), and without predators (control group) for 4 weeks at 25 ± 0.5 °C. After predator acclimation, predation mortality was evaluated to understand the potential difference in vulnerability to predation among these treatments when prey fish from each treatment group was cultured together with the predator. To investigate the possible physiological and behavioral mechanisms of this vulnerability, we measured the following parameters under the same environmental conditions: metabolic response parameters such as routine metabolic rate (*RMR*), maximum metabolic rate (*MMR*), and metabolic scope ($MS = MMR - RMR$); routine activity level determined by measuring the total movement distance, percentage of time spent moving or hiding, and average velocity while moving; fast-start escape performance determined by measuring the response latency, maximum swimming speed (V_{max}), and maximum acceleration (A_{max}). Both the lethal and non-lethal group fish exhibited significantly lower predation mortality, greater boldness (as suggested by the percentage of time spent hiding), and shorter response latency of fast-start response compared to those of the control group. Furthermore, the lethal group exhibited higher activity level, as evidenced by the increased amount of time spent moving at higher average speed and higher *RMR*. However, neither the fast-start parameters (V_{max} and A_{max}) nor the maximum metabolic capacity (*MMR* and *MS*) of the two predator-stress groups differed from those of the control group. These results suggest that the *Spinibarbus sinensis* cultured under predation stress was less vulnerable to predators because of shorter response latency and more explorative routine activities. However, the increase in spontaneous activity and enhanced alertness resulted in further energy expenditure in qingbo. Thus, the plasticity in physiology and behavior in qingbo may be one of the main anti-predator strategies that can increase the survival rate of this fish species under predator stress conditions.

Keywords: predator stress; metabolism; fast-start response; spontaneous activity; vulnerability to predation; *Spinibarbus sinensis*

1. Introduction

Predation is one of the key factors governing patterns (e.g., energy and information) in natural systems, and experimental manipulations of predator have shown that they have important effects on prey populations [1]. Similarly, changes in predation pressures have dramatic influences on virtually all aspects of an animal's physiology, behaviour, morphology and even life-history traits [2]. To evade the predator, prey usually increase the detective activities to the predator and alter their anti-predator defences of behaviour, morphology and life history [3]. Upon encountering a predator, prey animals may show dramatic changes in their behaviour by fleeing or seeking refuge, which results in subtle changes in habitat choice and alterations in the timing of foraging [4]. Different experiences of the life history in the species have profound effects on the growth, reproductive patterns and longevity of the prey [5-7]. Surviving in the environments of different predator pressure, fish individuals possess their optimum fitness, and face the various challenges and generate new adaptive traits under the dramatic alterations of their habitat.

Generally, fish that have been exposed to predators often have stronger anti-predator responses than predator-naïve prey [8-12]. Furthermore, predator-encountered experience can improve the escape ability of prey by inducing alterations in behavior, morphology, physiology and even life histories [4, 13-16]. For example, compared to prey in an

environment without predators (or lower predator stress), the presence of predators (or higher predation stress) results in higher concentrations of adrenaline and cortisone [17], higher respiratory and metabolic responses [18-19], improved fast-start escape performance [20], increased spontaneous movements, increased boldness and higher vigilance [21-22]. These studies have focused on the physiological and behavioral responses to changes in predation stress [23-25]. However, whether such responses can reduce the vulnerability to predation and the corresponding underlying physiological and behavioral mechanisms of this vulnerability remains unclear.

Therefore, the aims of this study were to examine the effects of different predation intensities on escape ability of prey and to determine the underlying physiological and behavioral mechanisms. To achieve these aims, we selected the juvenile qingbo (*Spinibarbus sinensis*) and its natural predator, the southern catfish (*Silurus meridionalis*), as the prey and predator, respectively. The qingbo is a common commercial fish which widely distributed in the Yangtze River of China, while the southern catfish is an ambush and carnivorous predator in the same water body. These two fish species are important components of the food chain in the Yangtze River. The experimental incubated qingbo, which did not experience with any predator pressure, were reared without predators (the control group), with a caged southern catfish predation threat (the non-lethal group) or with predators (the lethal group) at 25 ± 0.5 °C for a period of 28 d. After the acclimation, the variables of the different treatment groups, including predator mortality, metabolic responses, fast-start performance and routine spontaneous activities, were evaluated to provide beneficial data for the fundamental research of the fish physiology and ecology.

2. Materials and methods

2.1. Experimental animals and acclimation

Experimental juvenile qingbo (4-8 g, $n = 240$) and southern catfish (20-26 g, $n = 8$) were obtained from local farmers (Hechuan district, Chongqing) and kept in dechlorinated, fully aerated 25 ± 0.5 °C water tanks (approximately 250 L) for three weeks before the experiments. The prey and predator were separately raised. All juvenile qingbo, which were predator naive before the experiment, were individually marked with passive integrated transponders (PIT) for observation of fish behavior using PIT antenna systems [26]. The fish were fed to satiation with a commercial diet once daily at 09:00 h. The chemical composition of the formulated diet was $41.2 \pm 0.9\%$ protein, $8.5 \pm 0.5\%$ lipid, $25.7 \pm 1.2\%$ digestible carbohydrate and $12.3 \pm 0.4\%$ ash. The southern catfish were fed to satiation with cutlets of freshly killed loach (*Misgurnus anguillicaudatus*) once daily at 09:00 h. Uneaten food and feces were removed with a siphon 1 h after feeding. All tanks were replaced approximately by 10% of total water volume every day. The water temperature was maintained at 25.0 ± 0.5 °C, and the oxygen content of the water was maintained above 7.0 mg/L. The tanks were kept under a natural light cycle.

2.2. Experimental design

After the acclimation, all tests were performed at a water temperature of 25 ± 0.5 °C. At the beginning of the experiment, body mass and body length were measured after the fish were slightly anesthetized with neutralized tricaine methanesulfonate (MS-222, 50 mg/L). These selected fish (5.88 ± 0.08 g, 6.52 ± 0.04 cm) were similar in the body mass and body length with a good healthy condition. The fish were randomly assigned to 3 groups: the non-

predator group (control group without predators, $n = 30$), non-lethal predator group (fish reared with the predation threat of 'non-lethal' caged southern catfish, $n = 30$) and lethal predator group (fish reared with direct predation by southern catfish, $n = 70$ at the beginning of the test). Because not all of the experimental fish could be held in one tank during the experimental period, similar physical conditions, including water temperature and dissolved oxygen levels, were maintained in the different tanks, and the fish density was kept the same by adjusting the rearing water volume, thus ensuring that the differences among different experimental groups were a reflection of treatment rather than tank variation.

Fish were reared for 4 weeks under the same conditions as in the acclimation period. All fish prey of three groups were fed to satiation with the same commercial diet once a day. The caged southern catfish in the non-lethal group were fed to satiation with freshly killed loach once three day, whereas those in the lethal group only fed on qingbo. After the 4 weeks' growth experiment, the body mass and body length of the qingbo were measured after slight anesthetization with MS-222 (50 mg/L) to evaluate the metabolism, behavior and predation mortality using randomly selected fish. The water temperature, dissolved oxygen level and light cycle of all tanks (length $\times$ width $\times$ height, 1.5 m $\times$ 0.6 m $\times$ 0.5 m) were kept the same as those during the acclimation period.

2.3. Experimental procedure

2.3.1. Metabolism

The metabolism parameters of present study included routine metabolic rate (RMR), maximum metabolic rate (MMR) and metabolic scope ($MS = MMR - RMR$). Ten experimental fish were selected from each of the treatment groups. For the measurement of RMR , each fish

was placed individually in the respirometer chamber and allowed to acclimate for 48 h. On the following two days, the oxygen consumption rate ($\dot{M}O_2$) of individual fish was measured at 09:00, 15:00 and 21:00 by using an oxygen meter (HQ₂₀, Hach Company, Loveland, CO, USA). Six measurement data were averaged for the *RMR* of each fish. The fish were used again for the measurement of *MMR* by the chasing method. Briefly, the experimental fish were chased to exhaustion with a hand net in a circular trough (Φ outside = 56 cm, Φ inside = 33 cm, water depth = 15 cm) and suffered a reflux with an approximate water velocity of 60 cm/s. The whole exercise process usually lasted within 5 min [27]. After the exhaustion, each fish was immediately placed into a respirometer chamber and $\dot{M}O_2$ of each fish was measured for an interval of one minute during a recovery period of 5 min. The flow rate of water through the respirometer chamber was measured by collecting the water outflow from each tube. The water flow rate of the respirometer chamber was approximately 0.6/L, and 99% of the water was replaced within 1 min in the 0.1 L chamber [28]. Therefore, the first measurement of $\dot{M}O_2$ was made 1 min after the fish was placed in the chamber. Maximal $\dot{M}O_2$ was usually observed immediately (1-2 min) after the fish was placed in the chamber and could be used as the *MMR* of experimental fish. The following formula was used to calculate the $\dot{M}O_2$ (mg/kg/h) of individual fish:

$$\dot{M}O_2 = \Delta O_2 \times v / m$$

where $\dot{M}O_2$ presents the metabolic rate of the experimental fish under different physiological status (resting or after exhaustion), ΔO_2 is the difference in the oxygen concentration (mg/L) between the experimental chamber and the control chamber (chamber without fish), v is the water flow rate in the experimental chamber (L/h) and m is the body

mass of the fish (kg).

2.3.2. Fast-start performance

The fast-start performance of experimental fish was examined by the self-made device [29]. According to a previous study [30], the center-of-mass (CM) were marked in the place which account for a 44.3% of the body length from lip. Ten experimental fish were randomly selected from each of the treatment groups. A small white plastic ball (diameter: 1 mm) was attached to the dorsal side of each fish at the CM position determined by a pilot experiment after the fish was slightly anesthetized with MS-222 (50 mg/L) [31-32]. The fish were transferred individually to the experimental glass tank and allowed to acclimate for 1 h. After 1 h of acclimation, the fast-start performance of each fish was conducted and then body mass and body length were also measured. The following parameters were calculated: response latency (Response time, ms), maximum linear velocity ($V_{\max}$, m/s) and maximum acceleration ($A_{\max}$, m/s^2). The environmental parameters (e.g., water temperature, dissolved oxygen level and photoperiod) of device were maintained the same as the acclimation period.

2.3.3. Spontaneous behaviour

Ten fish were randomly selected from each group, transferred to individual observation chambers (water depth = 12 cm) and acclimatized for 3 d. The chambers were cylindrical unplasticized polyvinyl chloride (UPVC) tanks with lids. Fish were observed through a window on the lid at 09:00, 15:00 and 21:00 h on the fourth day after they were transferred to the observation chamber. Individual fish were observed continuously for 15 min [27]. To compensate for the disturbance of fish by the observer's presence, the first 5 min and the final 2 min of each observation were omitted from further analyses. Videos were analyzed using

EthoVision XT 9.0 [33] to determine when fish were moving or frozen and when they moved among the different areas of the arena. These data were used to calculate the total movement distance, the average velocity during movement and the percent time spent moving.

After measuring spontaneous behaviour, the boldness of each fish was also examined. The percent time spent hiding (hiding time / the analyzed video time) was used as index for the boldness. The smaller the hiding time percent, the more bold the fish. We modified the experimental tank by adding an area of 'cover' consisting of a 15×10 cm opaque tile mounted on four 4-cm glass legs that was placed in a corner. After one hour of acclimation, individual fish were observed continuously for 25 min. To compensate for the disturbance of the fish by the observer's presence, the first 5 min and the final 5 min of each observation were omitted from further analyses. Videos (15 min) were analyzed using EthoVision XT 9.0. These data were used to calculate the proportion of time that juvenile qingbo individuals spent hiding under the tile in a 15-min observation period.

2.3.4. Predation mortality

Four fish from each group were randomly selected and transferred to one predation tank (length × width × height, 1.5 m × 0.8 m × 0.5 m) and reared together (12 prey) with two predators (southern catfish). Southern catfish were habituated to the predation tank for 3 d before the experiment. Once half the prey fish (i.e., six individuals) were killed by the predators, the experiment was terminated. The predation mortality was calculated as the percent of individuals killed at the end of the experiment. The above experiments were repeated three times.

2.4. Data handling and analysis

All experimental data were regularly calculated by the Excel (2003) and SPSS Statistics 17.0 was used for data analysis. All values are presented as means $\pm$ S.E., and $P < 0.05$ was used as the level of statistical significance. The effect of predator stress on metabolism (i.e., *RMR*, *MMR* and *MS*) was assessed by one-way analysis of covariance (ANCOVA; body mass was used as the covariate). The effect of predator stress on fast-start and behavior was also assessed by one-way ANCOVA, but body length was used as the covariate. The difference in predation mortality was performed by one-way analysis of variance (ANOVA). The ANOVA was followed by Duncan's multiple-comparison test when appropriate to identify differences among the experimental groups.

3. Results

3.1. Metabolism

The *RMR* was significantly higher in the lethal group than in the control and non-lethal groups ($F = 7.649$, $P = 0.002$, Fig. 1a). However, there was no significant difference in either *MMR* ($F = 0.612$, $P = 0.551$, Fig. 1b) or *MS* ($F = 1.849$, $P = 0.180$, Fig. 1c) among the three groups.

3.2. Fast-start performance

The response latency was significantly shorter in the lethal and non-lethal groups than in the control group ($F = 7.054$, $P = 0.004$, Fig. 2a). However, there was no significant difference in either $V_{\max}$ ($F = 0.329$, $P = 0.723$, Fig. 2b) or $A_{\max}$ ($F = 1.492$, $P = 0.243$, Fig. 2c) among the three groups.

3.3. Spontaneous behavior

The total movement distance (Total MD) ($F = 3.617$, $P = 0.042$, Fig. 3a), percent time spent moving (PTM) ($F = 4.944$, $P = 0.013$, Fig. 3b) and average speed ($F = 4.914$, $P = 0.014$, Fig. 3c) during movement were significantly higher in the lethal group than in the control group. However, there were no significant differences in the three variables between the non-lethal group and the other groups. Furthermore, the percent time spent hiding (PTH) was significantly lower in the non-lethal and lethal groups than in the control group ($F = 7.225$, $P = 0.003$, Fig. 3d).

3.4. Predation mortality

Predation mortality was significantly lower in the non-lethal and lethal groups compared to the control group ($F = 10.500$, $P = 0.011$, Fig. 4), whereas there was no significant difference in predation mortality between the two predator-treated groups.

4. Discussion

In the present study, we found that predator experience, whether non-lethal or lethal, decreased the susceptibility to predation in juvenile qingbo, suggesting predator pressure has a benefit to increase the survival of this fish species. The decreased predation mortality was related to up-regulated alertness, as suggested by increased explorative spontaneous movement and shorter fast-start escape response latency. However, such anti-predator strategies increased the routine energy expenditure and therefore had a negative effect on growth, suggesting an allocation trade-off between growth and anti-predator performance in juvenile qingbo. A possible acquisition trade-off between growth and predation risk might also occur but requires further investigation.

Lethal and non-lethal predator stress resulted in decreases in predator mortality of 44% and

56%, respectively, suggesting that predator experience was important in determining the outcome of subsequent predator-prey interactions in qingbo. This result is in agreement with a previous study of coral reef damselfish (*Pomacentrus amboinensis*) [34] and site-faithful damselfish (*Pomacentrus wardi*) [35], which revealed that experience gained by individuals enhanced their ability to avoid or escape predation and led directly to higher survival. Olfactory and visual cues can play equally important roles in affecting the survival of prey in the field [35-36]. Additionally, the release of chemicals from predators and damaged conspecifics leads to risk-averse behavior by prey [35, 37]. In the present study, the lethal treatment included predator stress by direct predation, the image and odor of the predator as well as the chemicals released from damaged conspecifics, whereas non-lethal treatment only included olfactory and visual predator stimuli. However, both two predator stress groups displayed similar improvements in susceptibility to predators. Thus, caged predator treatment with only olfactory and visual stimuli and direct predation attack had similar effects on the outcome of subsequent prey-predator interactions. Many studies have documented the effect of predation stress by either lethal [11-12, 25] or non-lethal [24, 38] methods on physiological and behavioral responses in fish species. However, this study represents the first direct comparison of the effects of lethal and non-lethal predation stress on a fish species. All fish in this study were predator-naïve before the experiment, and thus our results suggest that prey can experience predator stress even without witnessing predators attacking.

Fast-starts are brief, sudden accelerations used by fish during predator-prey encounters, and the kinematics and performance of fish during the fast-start response may determine the outcome of predator-prey interactions [39]. Misqueto fish (*Gambusia affinis*) in habitats with

predators exhibit a 20% higher bursting swimming speed compared to a predator-free population [40]. An improved fast-start performance profoundly increases the probability of successfully evading a predation strike [41]. However, in this study, neither $A_{\max}$ nor $V_{\max}$ changed among the different groups, which is reasonable because the linear performance of escaping fish is not always maximal [39], and did not correlated to the predator mortality of the juvenile qingbo. However, the response latency of both predator treatment groups decreased significantly compared to the predator-naïve group, and this decrease is at least partly responsible for the difference in predator-avoidance ability. The anti-predator strategies also involved behavioral response in terms of spontaneous movement, i.e., the fish spent more time moving, with faster average swimming speed. These results are in agreement with those of a previous study of poeciliids (*Brachyrhaphis episcopi*), which found that individuals from a highly predated population were more explorative and more active than those from a low-predation population [21]. In the present study, the time spent in hiding was much lower in both treatment groups, which indicated that predator-experienced individuals were bolder, in further agreement with the previous study in poeciliids [42-43] and a study in perch (*Perca fluviatilis*) [11, 21]. However, even though the spontaneous movement of the non-lethal group was not significantly different from that of the lethal group, the spontaneous movement of the non-lethal group was also not significantly different from that of the control group, suggesting that there was some difference in the effect of predator stress on the behavior of the non-lethal and lethal groups. A previous study found that 28 d of stress elicited a 40% increase in *RMR* in brown trout (*Salmon trutta*) but a 25% reduction in *MS* [44]. Similarly, in qingbo, the lethal group exhibited similar *MMR* but lower *MS* due to higher *RMR* compared to the control and

non-lethal groups. Thus, the higher routine energy expenditure is mainly due to increased spontaneous movement, which inevitably led to decreased growth performance in predator-stressed fish.

In conclusion, the present study clearly revealed adaptive physiological and behavioral anti-predator strategies under both indirect and direct predator stresses. The predator pressure (or experience) increased significantly the escape performance of the juvenile qingbo, hence resulting in an increase of the survival rate of the juvenile qingbo. The higher routine movement (predator exploring activities) and less time to forage caused an extra energy expenditure which might induce a depression on the growth of the juvenile qingbo. However, predator-experienced fish displayed higher anti-predator ability due to short escape latency and up-regulated alert effort. Thus, we clearly demonstrated a cost of anti-predator behavior.

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Captions

Fig. 1. Effects of predator stress on resting metabolic rate (*RMR*), maximum metabolic rate (*MMR*) and metabolic scope (*MS*) in qingbo ($n = 10$; means $\pm$ SE). Data with different letters were significantly different ($P < 0.05$).

Fig. 2. Effects of predator stress on response latency, maximum velocity ($V_{\max}$) and maximum acceleration ($A_{\max}$) during the escape response ($n = 10$; means $\pm$ SE). Data with different letters were significantly different ($P < 0.05$).

Fig. 3. Effects of predator stress on behavior in qingbo ($n = 10$; means $\pm$ SE). Data with different letters were significantly different ($P < 0.05$). Total MD = Total movement distance; PTM = Percent time spent moving; PTH = Percent time spent hiding.

Fig. 4. Effects of experimental treatment on predation mortality in qingbo ($n = 3$; means $\pm$ SE). Data with different letters were significantly different ($P < 0.05$).

Fig. 1

Fig. 2

Fig. 3

Fig. 4