Temperature-dependent effects of PFOS on risk recognition and fast-start performance in juvenile *Spinibarbus sinensis*

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ABSTRACT: Perfluorooctane sulfonate (PFOS) is a synthetic chemical substance that has become a ubiquitous environmental contaminant. It has been used in both industrial and consumer applications for over 50 yr, resulting in high levels of contamination worldwide. The potential ecotoxicity of PFOS has recently become a focus of interest and concern. The aim of this study was to investigate the impact of PFOS on risk recognition and escape performance of juvenile qingbo *Spinibarbus sinensis*. Fish were exposed to a range of PFOS concentrations (0, 0.32, 0.8, 2 and 5 mg l^{-1}) at different temperatures (18 and 28°C) for 4 wk, at which point their antipredator behavior and fast-start swimming performance were assessed. We found that PFOS exposure caused qingbo to increase the time they spent in the 'risky area' (the area of the experimental aquarium closest to the predator tank) and reduce their average distance from the predator, as well as resulting in a noticeable increase in latency time and a significant decline in maximum linear velocity, maximum linear acceleration and escape distance. Many of these effects were more pronounced at the higher temperature. Our results indicate that exposure to PFOS could have deleterious effects on survival-related behavior in fish.

KEY WORDS: Risk recognition · Fast-start · Ecotoxicology · Perfluorooctane sulfonate · PFOS · Temperature

INTRODUCTION

Perfluorinated compounds (PFCs) are a class of synthetic chemical compounds that have been used in a vast array of industrial and commercial applications for the past 50 yr, including surface protection (e.g. water-, oil-, and soil-resistant coatings for textiles, carpets, and upholstery), food-package treatments, fire retardants, lubricants and insecticides (Giesy & Kannan 2001, Fuentes et al. 2007, Pereiro et al. 2014). The high-energy carbon–fluorine bonds are among the strongest in organic chemistry, and this stability make these compounds resistant to photolysis, hydrolysis and defluorination, and therefore practically nonbiodegradable (Key et al.

1997, Beach et al. 2006). As one of the most commonly detected perfluorinated alkylated substances in aquatic ecosystems, perfluorooctane sulfonate (PFOS) has become the target of public concern and has been declared a persistent organic pollutant (Stockholm Convention 2009) due to its potential toxicity to animals, persistence in the environment and global occurrence (Ferrey et al. 2012, Hagenaars et al. 2014).

The ecological effects of PFOS on the fitness of aquatic organisms have been a subject of great interest. Most toxicological tests concerning PFOS in fish have focused on metabolic, reproductive and developmental aspects (Hagenaars et al. 2008, Han & Fang 2010, Dorts et al. 2011). However, there is limited but

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growing evidence linking PFOS exposure to the toxic contamination of the central nervous system (Austin et al. 2003, Johansson et al. 2009, Chen et al. 2014). As reported by Johansson et al. (2009), PFOS exposure can affect processes linked to neurodegeneration, thereby affecting cognitive function. In this context, it is plausible to suppose that certain behaviors could be adversely affected by PFOS. However, to date few studies have assessed the effects of PFOS on fish behavior (e.g. antipredator behavior). In a previous publication, we reported that short-term PFOS exposure could cause behavioral defects in fish (manifested as reduced spontaneous activity), although no significant changes in swimming capabilities were observed (Xia et al. 2013). Are the fitness-related behavioral traits of fish susceptible to chronic PFOS exposure? One goal of this study was to determine whether PFOS exposure has an effect on risk recognition and escape performance. Importantly, many fish in natural water bodies are subjected to large seasonal changes in temperature. An altered thermal environment can also change the effects of a toxicant (Langberg 2014); thus, another goal of this study was to determine whether the effects of PFOS on risk recognition and fast-start performance were temperature-dependent.

In general, prey species can demonstrate an antipredator response upon encountering a predator. The ability of a prey fish to recognize and respond appropriately to predation risk decreases its probability of being captured (Kiesecker et al. 2002). Conversely, the impairment of antipredator traits could directly reduce prey survival when encountering predators (Janssens & Stoks 2012). Given the high impact and frequency at which predation events occur in ecosystems, studies focusing on how a given pollutant affects key antipredator traits in prey species are important to increase our understanding of the ecotoxicological effects of pollutants in nature (Trekels et al. 2012). Following exposure to a perceived risk, fish usually resort to escape behavior using a 'fast-start' (Domenici et al. 2007). Fast-start swimming, which lasts for less than approximately 1 s, is crucial for most fish in predator–prey interactions (Domenici & Blake 1997, Tudorache et al. 2008). Ecologically, decreased antipredator response or impairment of the fast-start performance in a polluted environment (such as that caused by PFOS contamination) may render animals more vulnerable to predation. Therefore, the extent to which fish can maintain their risk recognition and escape performance would be closely related to their fitness.

MATERIALS AND METHODS

Animals and test chemicals

The qingbo *Spinibarbus sinensis* is a fish endemic to China that is highly sensitive to changes in environmental conditions in its habitat and is mainly distributed in the upper reaches of the Yangtze River (Pang et al. 2011). Juvenile fish of uniform size (2.69 ± 0.04 g, 5.39 ± 0.03 cm, n = 200) were obtained from local farmers in Chongqing Municipality, China. Prior to the experiment, the fish were reared in a 120 l recirculating water tank system for 2 wk. The rearing water was dechlorinated and filtered through activated carbon. Water temperature was maintained at 22 ± 1°C, water oxygen content was kept above 7 mg l^{-1}, pH ranged from 6.8 to 7.5, ammonia-N ranged from 0.005 to 0.02 mg l^{-1}, and the rearing system was maintained under a 15 h light:9 h dark cycle. The fish were fed to satiation daily with commercial *Tubifex* spp. After the acclimation period, healthy fish of similar sizes were selected for the study.

We used juvenile southern catfish *Silurus meridionalis* (95.0 to 97.4 g, 19.6 to 20.2 cm) to provide the predator stressor in this study. This species was chosen mainly because (1) it is a predator that qingbo frequently encounter in nature and (2) it is a typical ambush (i.e. sit-and-wait) predator that usually remains stationary for long times (Fu et al. 2009). The southern catfish were obtained from local fisheries and acclimated in a rearing system for 2 wk. The conditions of the rearing system were similar to those of qingbo described above, except that the water temperature was 24 ± 1°C and the fish were fed with juvenile qingbo.

Heptadecafluorooctanesulfonic acid potassium salt (PFOS; purity > 99%) was purchased from Tokyo Kasei Kogyo; other chemicals were obtained from Sigma-Aldrich. PFOS was initially dissolved in dimethyl sulfoxide (DMSO), and the stock solution (0.5 g ml^{-1}) was stored at 4°C until preparation of the final exposure solutions in water.

Experimental design and protocol

We used a semi-static exposure experiment apparatus for waterborne PFOS exposure. The apparatus consisted of 10 glass aquaria (length × width × height: 42 × 22 × 42 cm), each with a capacity of approximately 22 l.

Prior to exposure, the juvenile qingbo were randomly selected and divided into 10 groups (n = 16 for

each group) and were gently transferred to the aquariums. Fish were maintained at conditions similar to those described above for 1 wk to eliminate stress effects. For each group, the same amount of food (approximately 10% of body weight) was provided daily during this period and during subsequent processing. After that, the water temperature was increased or decreased by 1°C d^{-1} until it reached a prescribed temperature (18 or 28°C). The selected temperatures were chosen based on the habitat/season temperature. For qingbo, the habitat temperature is approximately 18°C in spring and autumn and approximately 28°C in summer. We hypothesized that an increase in the seasonal temperature would enhance the ecotoxicity of PFOS to the fish.

Once the water temperature reached the prescribed values, the toxicants were administered. Fish were exposed to a range of PFOS concentrations (0, 0.32, 0.8, 2 and 5 mg l^{-1}) under the 2 different temperatures (18 and 28°C) for 4 wks. The concentration of DMSO in the water did not exceed 0.001% (v/v). During the experimental period, 50% of the exposure solution was renewed daily (Shi et al. 2009). Water disposal from the aquaria was filtered through activated carbon before being delivered into the municipal sewage system. After termination of PFOS exposure, feeding was withheld for 48 h and the antipredator behavior and fast-start performance of the fish were examined successively.

The experimental protocols used in this study were in strict accordance with current guidelines for the care of laboratory animals and ethical standards permissible for investigations in conscious animals (Zimmermann 1983). At the end of the experiment, fish were anesthetized in tricaine methanesulfonate (MS-222, 50 mgl^{-1}).

Antipredator behavior

Tests to the assess antipredator behavior of the fish were carried out in an interference-free behavioral observation chamber. The system consisted of 3 rectangular glass aquariums, a video camera and a computer (Fig. 1). The testing tank (length × width × height: 72 × 12 × 42 cm) was placed in the middle position. A second tank (20 × 12 × 42 cm) containing a predator (southern catfish) was closely placed on one side of the testing tank, and an identical empty fish tank (containing only water, used as the control tank) was placed on the other side. The depth of water in all aquaria was 16 cm, and the dissolved oxygen level was maintained above 7 mg l^{-1}. The

water temperature was maintained at 18 or 28°C in the testing tank, which was consistent with the temperature during the exposure period. For the predator, the water temperature was maintained at 24°C, which is the value used for acclimation. A video camera (Sony) was installed 1.5 m in front of the testing tank and was operated by remote control.

Prior to the start of the test, the experimental fish were fasted for 48 h and the predators were fed to satiation. The head of the predator was positioned close to the testing tank. The behavioral assays were conducted after the light had been on for at least 1 h. Individual fish were carefully moved into the testing arena and the operator left the room immediately. The testing tank was initially obscured by opaque Plexiglas barriers (12 ×16 cm) at the ends of the chamber. Fish were allowed to habituate to the novel environment for 15 min. After that, the barriers were carefully removed at the same time. Thus, the antipredator behavior of the test fish would be provoked by the appearance of the predator. The behaviors of the fish were then recorded on video for 5 min. The predators were relatively stationary (the centroid speed was not greater than 1.0 cm s^{-1}) during the experimental trials and made no attempts to attack the prey or to escape their containers. If the predator did not remain calm during the measurement, data from that trial were discarded. Fish were tested in a randomized order across all treatment groups. The water in the testing tank was replaced with each subsequent test fish.
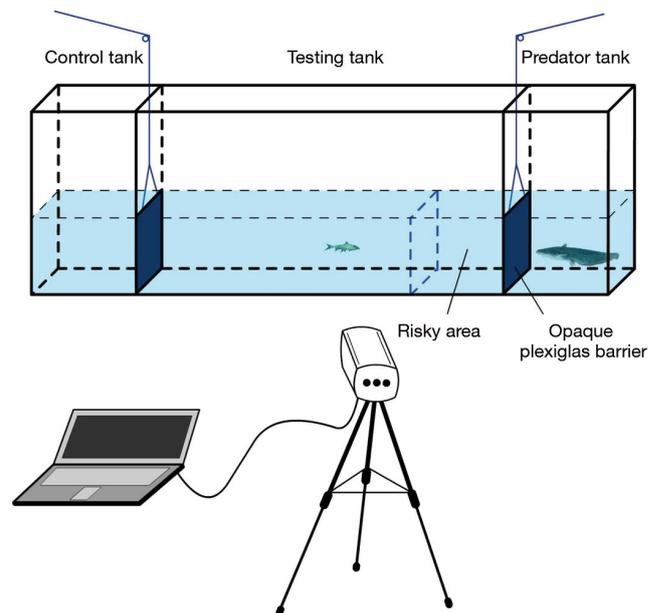

Fig. 1. Experimental chamber used to assess antipredator behavior of juvenile qingbo *Spinibarbus sinensis*

EthoVision XT 9.0 video tracking software (Noldus) was used to assay and quantify the antipredator behavior of the fish. The program compared each incoming image sample with the original background. Image samples were taken at a rate of 30 frames s^{-1}. For each fish, the percentage of time spent in the 'risky area', the use of the risky area (times), and the average distance to the predator were calculated. The risky area was defined as the quarter of the length of the aquarium closest to the predator tank (Fig. 1) based on results of pre-experiments (data not shown). Quantification of all of the distance measures was achieved after calibration of the EthoVision software by inputting the actual dimensions of the testing tank.

Fast-start performance

The fast-start swimming performance of the fish was assessed with a device developed by our laboratory (Xia et al. 2013), which consisted of a quadrate testing tank (40 × 40 × 15 cm), a direct current, an electrical pulse generator, a high-speed camera (BASLER A504K) and a computer. A reference grid of 1 cm squares and an LED matrix light source were positioned at the bottom of the testing tank.

The fish were dorsally marked at the center of the mass (CM) position with titanium oxide before the experiment. Individual fish were gently placed in the testing tank and were allowed to habituate to the novel environment for 30 min. The depth of the water in the tank was 8 cm, the dissolved oxygen level was maintained above 7 mg l^{-1}, and the temperature was maintained at the values used for the exposure treatment of the fish (18 or 28°C). The water in the tank was replaced with each fish to rid the tank of the potential impact of the previously tested fish.

Fast-start responses were elicited by an electrical pulse (0.75 V cm^{-1}; 50 ms), which was administered when the fish was stationary and maintaining a position at the center of the filming zone (Xia et al. 2013, Fu 2015). A high-speed camera (500 frames s^{-1}) was used to record the escape trajectory of the fish. The resulting images were initially processed using nEOiMAGING and ACDSee 12 software and subsequently digitized using TpsUnil and TpsDig software (http://life.bio.sunysb.edu/morph/) to examine the displacement of the CM of the fish during the escape response. The following parameters were calculated based on the locomotion track of CM: latency time ($T_{latency}$), maximum linear velocity (V_{max}), maximum linear acceleration (A_{max}), and distance ($D_{120\,ms}$) cov-

ered by each fish within 120 ms after the stimulus onset, considering both response latency and cumulative distance. Here, the parameter $D_{120\,ms}$ was determined according to the results of pre-experiments, in which we determined that the main escape process of the fish ended at approximately 120 ms.

Statistical analyses

Statistical analysis was performed using the software program SPSS v.16.0. The data were first examined for normality and homogeneity of variance. The effects of PFOS and temperature on the measured variables were detected using a 2-way ANOVA. The ANOVA was followed by a Tukey's test (PFOS effect) or t-test (temperature effect), if necessary, to determine statistical significance among different PFOS treatment groups. Additionally, if the data did not show homogeneity of variances, a Kruskal-Wallis test followed by a Dunnett's T3 test was conducted. All values are presented as means ± SE. Results were considered significant at p < 0.05.

RESULTS

Antipredator behavior

Both PFOS treatment ($F_{4,110}$ = 6.33, p < 0.001) and temperature ($F_{1,110}$ = 12.9, p < 0.001) had significant effects on the time individuals spent in the 'risky area,' which increased with increased PFOS concentration (Fig. 2A). The percentage of time that the fish spent in the risky area was significantly affected by PFOS exposure ($F_{4,110}$ = 4.78, p = 0.001) and temperature ($F_{1,110}$ = 7.72, p = 0.006) (Fig. 2B). The average distance to predator was also significantly affected by PFOS treatment ($F_{4,110}$ = 7.62, p < 0.001) and temperature ($F_{1,110}$ = 4.09, p = 0.046) (Fig. 2C). In addition, significant differences in the above parameters were observed between 28 and 18°C after exposure to 2 mg l^{-1} PFOS. Both the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) of PFOS for the above parameters were lower at 28°C than that at 18°C (Table 1).

Fast-start performance

Both PFOS ($F_{4,110}$ = 5.34, p = 0.001) and temperature ($F_{1,110}$ = 115.9, p < 0.001) had significant effects on $T_{latency}$ (Fig. 3A). V_{max} was also significantly af-
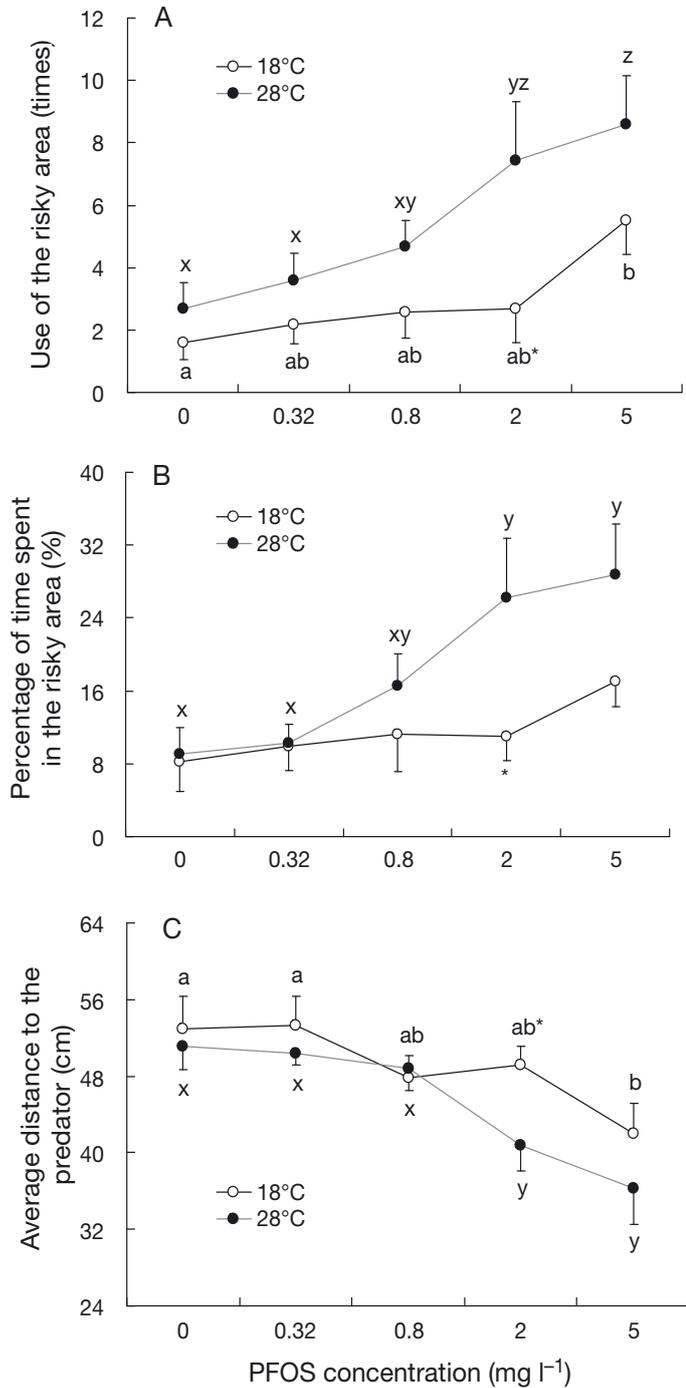
Fig. 2. Effects of perfluorooctane sulfonate (PFOS) on risk recognition by juvenile qingbo *Spinibarbus sinensis* at different temperatures, showing (A) use of the risky area (cumulative number of times), (B) percentage of time spent in the risky area and (C) average distance to the predator. Data are mean ± SE. Values in the 18°C groups (n = 12) without a common lowercase letter (a,b) are significantly different (p < 0.05); values in the 28°C groups (n = 12) without a common lowercase letter (x,y,z) are significantly different (p < 0.05); *significant difference between the 18 and 28°C groups (p < 0.05)

Table 1. No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values of perfluorooctane sulfonate (PFOS) for the parameters of antipredator behavior and fast-start performance in juvenile qingbo *Spinibarbus sinensis* at different temperatures. $T_{latency}$: latency time; V_{max}: maximum linear velocity; A_{max}: maximum linear acceleration; $D_{120\,ms}$: distance covered by each fish within 120 ms of stimulus onset

	NOEC (mg l^{-1})		LOEC (mg l^{-1})	
	18°C	28°C	18°C	28°C
Antipredator behavior				
Increased use of risky area	2	0.8	5	2
Increased % time spent in risky area	–	0.8	–	2
Decreased avg. distance to predator	2	0.8	5	2
Fast-start performance				
Increased $T_{latency}$	2	0.8	5	2
Decreased V_{max}	2	2	5	5
Decreased A_{max}	0.32	0.32	0.8	0.8
Decreased $D_{120\,ms}$	2	2	5	5

fected by PFOS exposure ($F_{4,110}$ = 9.67, p < 0.001) and temperature ($F_{1,110}$ = 10.6, p = 0.001) (Fig. 3B), as was A_{max} (PFOS treatment: $F_{4,110}$ = 12.3, p < 0.001; temperature: $F_{1,110}$ = 21.7, p < 0.001; Fig. 3C) and $D_{120\,ms}$ (PFOS treatment: $F_{4,110}$ = 8.27, p < 0.001; temperature: $F_{1,110}$ = 31.4, p < 0.001; Fig. 3D). The V_{max}, A_{max}, and $D_{120\,ms}$ of the fish all decreased with increasing PFOS concentration, whereas the $T_{latency}$ increased. Both LOEC and NOEC of PFOS for $T_{latency}$ were lower at 28°C than that at 18°C (Table 1).

DISCUSSION

Fish exposed to PFOS showed a decrease in antipredator behavior and fast-start performance, suggesting that PFOS, a common PFC contaminant, may have significant negative effects on the risk recognition and escape behavior in prey species. Furthermore, the high temperature treatment caused enhanced toxicity of PFOS, suggesting that ecological risk assessments in the field should be based, in part, on the results of toxicity tests conducted under given seasonal conditions.

Studies have shown that PFOS exposure can cause neurotoxicity and cognitive behavior defects, such as the recently-described 'hyperactive/impulsive motor phenotype'. As reported by Spulber et al. (2014), zebrafish larvae exposed to 1 mg l^{-1} PFOS displayed a disorganized pattern of spontaneous activity and persistent hyperactivity. In the current study, we clearly demonstrated that 2 mg l^{-1} PFOS exposure at
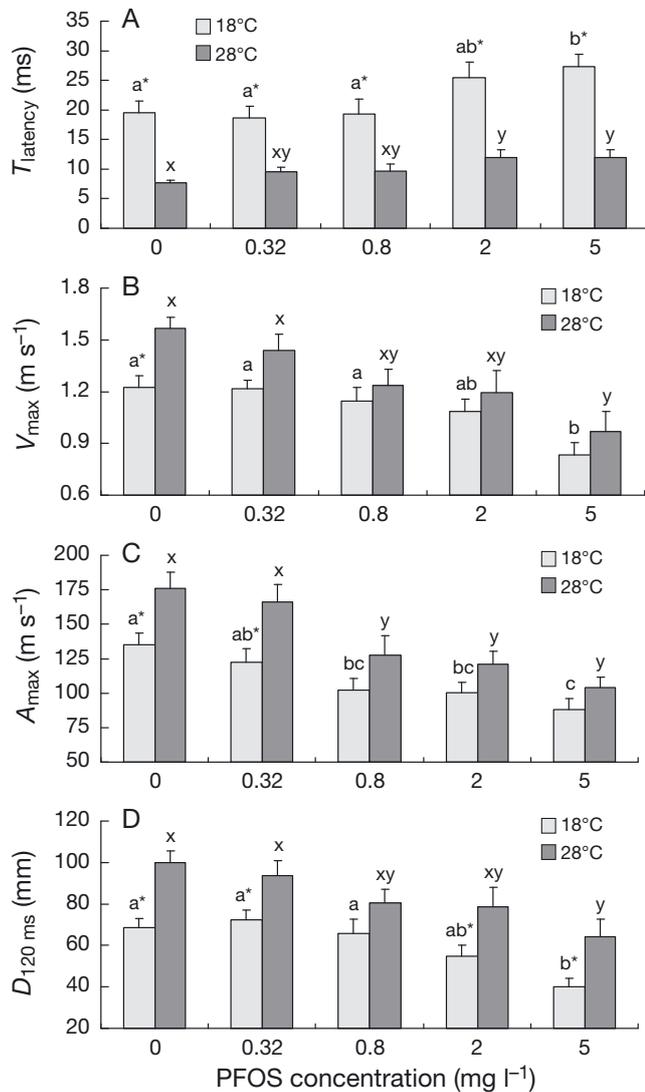
Fig. 3. Effects of perfluorooctane sulfonate (PFOS) on fast-start performance of juvenile qingbo *Spinibarbus sinensis* at different temperatures, showing (A) latency time ($T_{latency}$), (B) maximum linear velocity (V_{max}), (C) maximum linear acceleration (A_{max}) and (D) distance covered within 120 ms after the stimulus onset ($D_{120 ms}$). Data are mean ± SE. Values in 18°C groups (n = 12) without a common lowercase letter (a,b,c) are significantly different (p < 0.05); values in 28°C groups (n = 12) without a common lowercase letter (x,y) are significantly different (p < 0.05); *significant difference between the 18 and 28°C groups (p < 0.05)

degeneration, thereby affecting cognitive function. Chen et al. (2014) reported that in *Caenorhabditis elegans*, PFOS induced obvious behavioral defects (e.g. the decline of chemotaxis learning ability) and neurotoxicity via oxidative stress damages. They proposed that the neurotoxicity of PFOS was mediated, at least in part, by an oxygen radical mechanism involving overproduction of reactive oxygen species (ROS) and downregulation of certain key antioxidant enzymes such as glutathione peroxidase (GPX). Additionally, the glucocorticoid hormones cortisol and corticosterone are considered to facilitate components of antipredator behavior in animals because they prepare the body to perform behaviors that address stressors (Barreto et al. 2014). PFOS exposure induced a global inhibition of the hypothalamic-pituitary-adrenal (HPA) axis activity, resulting in decreased corticosterone secretion and release (Pereiro et al. 2014), and ultimately affecting the antipredator response.

Fast-start swimming is typically controlled by the Mauthner cells, a pair of large reticulospinal neurons that receive various sensory inputs and are powered by intracellular stores of adenosine triphosphate (ATP) and creatine phosphate (Reidy et al. 2000, Marras et al. 2011). From an ecological point of view, fast-start swimming is of great importance for both predator avoidance and prey capture. To our knowledge, however, there is little information available on the effects of pollutants on fast-start swimming. Our previous study indicated that fast-start performance in juvenile goldfish *Carassius auratus* was not affected by short-term (48 h) exposure to PFOS (Xia et al. 2013). However, the present study demonstrated that longer-term (4 wk) exposure to PFOS potently inhibited the fast-start performance of juvenile qingbo (manifested as increased $T_{latency}$, and decreased V_{max}, A_{max} and $D_{120 ms}$). This indicated that the effect of PFOS on fast-start performance is probably related to the length of exposure. Given that exposure to PFCs (e.g. perfluorooctanoic acid) can result in an impairment of aerobic ATP production, depletion of liver glycogen stores and altered expression levels of transcripts involved in carbohydrate metabolism (Hagenaars et al. 2013), we postulate that the reduced fast-start performance caused by chronic PFOS exposure was due to the disturbance of the Mauthner system and a constraint in muscle power output. According to Walker et al. (2005), faster fast-starts increase the probability of evading predators. Therefore, the reduced V_{max}, A_{max} and $D_{120 ms}$ in this study indicate that fish exposure to PFOS would be subjected to a higher risk of being captured by a predator.

28°C resulted in a reduced antipredator response by the fish, manifested as a higher frequency of use of the risky area, an increased percentage of time that the fish spent in the risky area, and a strong decrease in the average distance to the predator. Potentially, a variety of mechanisms might be responsible for the alterations in antipredator behavior caused by PFOS. Johansson et al. (2008, 2009) speculated that PFOS exposure could affect processes linked to neuro-

Temperature is one of the most important abiotic factors in the habitats of ectothermic animals and has been coined the 'ecological master factor' for fish (Brett 1971). Our results demonstrate that the effects of PFOS on risk recognition and fast-start performance was temperature-dependent, i.e. higher temperatures magnified the toxic effects of PFOS. Of note, PFOS treatment in our experiment resulted in a remarkable decrease in the average distance the qingbo maintained from the predator, which was reduced by 20.5 and 29.0% after exposure to the highest concentration of PFOS (5 mg l^{-1}) at 18 and 28°C, respectively. In general, fish exhibit decreased fast-start performance at low temperatures compared to high temperatures (Domenici & Blake 1997). However, significant differences in V_{max} and A_{max} were not observed between 18 and 28°C in cases where the PFOS concentration was >0.32 mg l^{-1}. This was mainly because PFOS had a more profound effect on the experimental fish at the higher temperature. For example, after exposure to 0.8, 2 and 5 mg l^{-1} PFOS, the values of V_{max} decreased by 6.5, 11.5 and 31.9%, respectively at 18°C, in comparison to a decrease of 21.1, 23.8, and 38.1%, respectively at 28°C. The increased toxicity of PFOS at the higher temperature was probably due to a faster uptake, absorption and bioaccumulation (Del Piero et al. 2012). The observed temperature-dependent PFOS effects on risk recognition and fast-start performance of the fish provide further support to the appeal by Bednarska et al. (2013) to include a range of realistic temperatures in standard ecotoxicity tests. Our study indicates that PFOS as an environmental pollutant could have ecological consequences for wild fish by decreasing risk recognition and escape behaviors.

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