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ORIGINAL ARTICLE

The role of the visual information in the fright reaction of *Dawkinsia filamentosa* (Valenciennes, 1844) (Teleostei: Cypriniformes: Cyprinidae)

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Abstract

The Blackspot Barb, *Dawkinsia filamentosa* (Valenciennes, 1844), is a shoaling fish of India. As in many ostariophysians, alarm cues released after skin injuring elicit striking anti-predator behaviour in conspecifics. The alarm substance necessary for tests was obtained from a single donor specimen (female). Each fish was tested individually (in visible light or with IR illuminators) in an aquarium with size: 105×8×20cm; water height: 8cm. The tank was subdivided in 9 parts and the stimulation (water or alarm substance) was released just under the water surface at the higher posterior right edge: the elongated shape of aquarium allowed evidencing a possible avoidance response. Fish were tested in light or dark conditions both in absence or presence of a chemical alarm cue. In both test conditions, fish behavior was monitored with a camera, which was sensitive to both infrared and visible light. In each of two experimental conditions, 12 tests were conducted. In each test, 3 consecutive registrations were made: 15 minutes in absence of stimulus, 15 minutes after administration of 50 ml of pure water, and 15 minutes after administration of a solution of 48 ml of water and 2 ml of alarm substance. Each sector of tank was correlated with a number in crescent order from 1, where administration was being carried out, to 9, in the opposite side. In each 15 minutes part of a test, fish position and associated number were relived every 30 seconds and the medium score of each fish obtained in absence of stimulus, in presence of water, and in presence of alarm substance were calculated. The medium scores obtained in presence of water and in presence of alarm substance were subtracted from the medium score obtained in absence of stimulus, obtaining a Changing Spatial Use Index. Indexes obtained for each fish (in presence of alarm substance and in presence of water) were compared with Wilcoxon test. As in others cyprinids, Blackspot Barb fright reaction differs in light or darkness. In particular, its alarm reaction in darkness seems to consist simply in moving to the bottom without any swimming rapid movements and without any avoidance component of the alarm substance.

Keywords: Alarm reaction, Spatial distribution, Visual communication, Predator-prey recognition.

INTRODUCTION

The Blackspot Barb, *Dawkinsia filamentosa* (Valenciennes, 1844), is an Indian cyprinid (Dahanukar et al. 2004) of the *Puntius* complex (Ren et al. 2020), recently accommodated in the genus *Dawkinsia* restricted to Sri Lanka and southern India (Pethiyagoda et al. 2012) and currently including 12 species (Pethiyagoda et al. 2012; Katwate et al. 2020). This shoaling species has specialised epidermal club cells, which produce and store a chemical alarm cue, as occur in many ostariophysan fish (Smith 1992). Chemical alarm cues were first noted by von Frisch (1938, 1942); they are released when the skin is ripped, often during a predation event (Pfeiffer 1967; Wisenden 2015). A vast literatures show that when released in water after skin injury, these alarm substances elicit striking, short-term anti-predator behavior in nearby conspecifics (e.g. Pfeiffer 1963; Magurran 1989; Smith 1992; Jachner 1996; Brown & Smith 1997; Chivers & Smith 1998; Mirza & Chivers 2003; Wisenden & Barbour 2005; Lautala & Hirvonen 2007; Speedie & Gerlai 2008; Ferrari et al. 2010; Wisenden 2015; Canzian et al. 2017; Carina et al. 2017). The effect of the alarm substance is so pronounced that a fish can show the fright reaction just hearing a sound previously heard in the presence of the alarm substance (Wisenden et al. 2008). Fright reaction can have a broad multi-level impact, including, in addition to changes in swimming

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behaviors, cortisol levels and gene expression patterns (Yang et al. 2019). The alarm substance perception occurs by sense of smell (Ide et al. 2003; Giaquinto & Hoffmann 2010; Maximino et al. 2018) and fish can learn to recognize novel predators by associating a predator's odor with the release of alarm cue (Brown et al. 2011). If smell allow the perception of alarm substance, vision is important for the alarm reaction (Verheijen 1956; Magurran & Higham 1988) which can be a visual signal to allow diffusion of the information that there is a predator in the shoal (von Frish 1942; Verheijen 1956; Schutz 1956; Magurran & Higham 1988; Suboski et al. 1990).

In shoaling species, alarm reaction is often characterized by behavioural patterns as: an avoidance of the place in which alarm substance is been perceived (Brown et al. 2000; 2001; Wisenden et al. 2004), an approach to the bottom (Thinès &Vandenbussche 1966; Alessio & Gandolfi 1975; Wisenden et al. 2004), an increment of the shoal cohesion (Thinès & Vandenbussche 1966; Alessio & Gandolfi 1975; Brown et al. 2000), and a swimming with zig-zag trajectories and quick changes of direction (Magurran & Pitcher 1987; Krause 1993). Moreover, environmental conditions may affect the fish response (Magurran et al. 1996; Smith 1997). The light is a particularly important condition that can influence the alarm response, since it may vary greatly in aquatic habitats, not only between night and day, but also according to depth, turbidity, season, lunar stage, surface level movements, cloud cover, and shading (Loew and McFarland 1990). If optical conditions are investigated in various works about the predator-prey interaction in fish (Miner & Stein 1996; Utne 1997; Vogel & Beauchamp 1999; Beauchamp et al. 1999), few studies have been conducted to investigate the role of visual information in alarm reaction and mostly considering the effect of turbidity of water, but not the effect of complete darkness (Hartman and Abrahams 2000; Ranåker et al. 2012; Swanbrow Becker & Gabor 2012). Paglianti et al. (2010) reported that the fright reaction of *Pethia conchonius* (Hamilton, 1822) (= *Barbus conchonius*) is different in light and dark as in dark the alarm reaction is simplified, lacking the zig-zag swimming typical in light condition, even if in both conditions the distances from the bottom and from the barycentre of the group decreased. In this study, we investigated how visual information availability influenced the avoidance component of fright reaction in *D. filamentosa*, which shows a similar alarm reaction to that of *P. conchonius* (personal observation). In particular, besides observing if eventually the zig-zag swimming and the distance from the bottom and from the barycentre of the group are modulated, the principal focus of the current study is analyzing if in darkness the avoidance component of alarm reaction is maintained or not.

MATERIALS AND METHODS

Adult specimens of *Dawkinsia filamentosa* were purchased from a commercial supplier. In standard conditions, they were held in 50-litre aquaria at 24°C on 12-12 h light/dark cycle and fed 6 days a week with commercial flake food and frozen chironomid larvae. Fish were subdivided in groups of 20 and each aquarium contained a 100-W heater and an active coal filter.

Preparation of alarm substance: Fish were treated in accordance with the guidelines of the local ethics committee and one single donor specimen was sacrificed to obtain all the alarm cues necessary for tests. The fish was taken out of its breeding aquarium and rinsed to eliminate the characteristic odour of the aquarium, like food and catabolites odour. The rinsing procedure consisted of three consecutive washes, of a few minutes each, in buckets filled with dechlorinated tap water (Berti & Zorn 2001). The fish was killed by the crushing of its head. For the preparation of the alarm cues, the skin sample was extracted with scalpel and the area was then measured from its digital image by the software ImageJ. Skin fragment was grinded and water was added to standardise the solution on 40ml of water per 1cm^2 of skin. The solution was filtered on coffee paper, then it was divided in microvials of 2ml. Microvials were immediately put in freezer (-20°C), until their utilization. After this procedure, length and volume of the specimen were measured (total length 8.5 cm; body mass 11 ml). The specimen used for the skin extraction was a female, because males of some cyprinids show a regression of club cells in the mating period (Smith & Smith 1983).

Experimental apparatus: The experimental aquarium was a long parallelepiped (size: 105 x 8 x 20 cm; water height: 8cm). Each fish was tested individually (in light or dark condition) and the specimen to be tested was taken from its breeding tank, "rinsed", and then put in the experimental tank for 18 h before the beginning of test. During the acclimatation, test tanks were illuminated on a 12h light: 12h dark cycle with 25-W Sunglow™ fluorescent lamps. Stimulation (water or alarm substance) was released just under the water surface at the higher posterior right edge of the aquarium via a silicon hose 2 m long sustained by a guide passing through the wall, thus the operators during stimulus preparation and administration were in a neighbouring room (in order to avoid any disturbance to the test fish). The elongated shape of aquarium allowed evidencing a possible avoidance response. Fish were tested in two experimental conditions: in presence and in absence of visible light. For the experiments in visible light, a 100W lamp illuminated the test tank, the light was reflected toward the aquarium by a white surface. The lamp was located above and frontally to the aquarium, at a distance of about one meter from the front wall of the tank. The photoperiod in experimental room was in phase with that of the holding room. Vertical lines were drawn on the rear wall of the tank, so that aquarium was subdivided in 9 parts.

For the experiments in the absence of visible light (IR illumination), the back side of the aquarium was illuminated by two IR illuminators (FR117 Everlight Electronics). Each illuminator included 96 IR light emitting diodes with peck wavelength at 875 nm and a cut off at zero below 815 nm. Behind the back of aquarium there was a tracing paper, in which vertical lines were drawn, so that aquarium was subdivided in 9 parts, like in the experiments in the presence of visible light. IR light Illuminators were positioned far enough to obtain diffused and uniform retroillumination.

In both test conditions, fish behaviour was monitored with a camera, mounted 8 m in front of the apparatus, which transferred to a monitor in a neighbouring room. The camera was sensitive to both infrared and visible light and trials were recorded on VHS tape.

In each of two experimental conditions, 12 tests were conducted. Tests were carried out at 24°C in a thermostatic room. Fish were always tested during the light phase of their circadian rhythm, between 9:00 and 10:00. In test series in dark, the light was turned off five seconds before the first stimulus (water) administration. The choice of carrying out even tests in the dark during the light phase based on two different remarks. On the one hand this was aimed to avoid fish activity rhythm interfered with the obtained results, on the other hand preliminary observations evidenced that the fish behaviour was unaffected by the sudden change from light to dark.

Data collection and statistics: Each test consisted of 3 consecutive registrations: the test fish was filmed for 15min in absence of stimulus, for 15min after administration of 50ml of pure water, and for 15min after administration of a solution of 48ml of water and 2ml of alarm substance. From preliminary test, we had verified that 15min was the time necessary for a coloured solution of water to diffuse from an end to the other in the aquarium.

Thus, during the test, in each moment the fish could choose a portion of water without alarm substance. At the end of every test, experimental aquarium was washed with abundant warm water, and the test fish was transferred in an aquarium, different to breeding one. When movies were examined, each sector of aquarium was correlated with a number in crescent order from 1, where

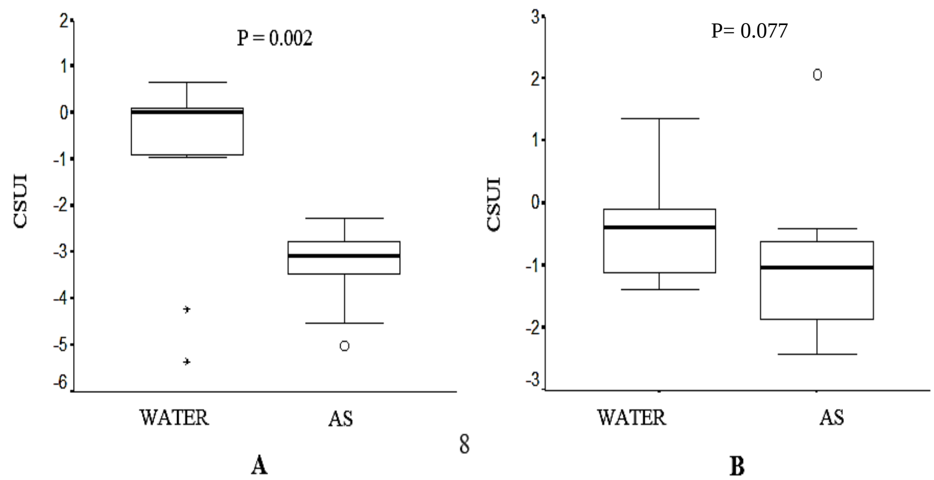

Fig.1. Values of Changing Spatial Use Index (CSUI) calculated in light (A) and dark (B) condition and in presence of water and then of alarm substance (AS). Wilcoxon Test, n = 12.

administration was been carried out, to 9, in the opposite side. In each 15 minutes part of a test, fish position and associated number were recorded every 30 seconds. If fish was coming from a sector to another in the moment of the record, we assumed the sector in which was the head (with pectoral fins) of the animal. Then, the medium score of each fish obtained in absence of stimulus, in presence of water, and in presence of alarm substance were calculated. So, the medium scores obtained in presence of water and in presence of alarm substance were subtracted from the medium score obtained in absence of stimulus. This differences were a Changing Spatial Use Index (CSUI). If the CSUI was positive, there was an attraction toward the stimulus, if it was negative, there was an avoidance. The indexes relating to the totality observation of 15min after administration were calculated. Indexes obtained for each fish (in presence of alarm substance and in presence of water) were compared with Wilcoxon test.

RESULTS

In presence of visible light, Changing Spatial Use Index for water and alarm substance are significantly different from each other. In presence of alarm substance, fish show an avoidance of the part of the aquarium in which stimulus was administered (Fig. 1a).

Specimens observed without stimulus swam from an end to the other in the tank with constant speed. After water stimulus nothing changed, unless in two test, in which fish reduced their movements and showed an avoidance of the end in which stimulus was administrated.

In all test with alarm substance, when fish came the first time to the end in which stimulus was been administered, they suddenly changed swimming: an evident speed increment, with zig-zag trajectories and quick changes of direction, was observed. At the same time the body axis assumed an angle of about 45° to the substrate with head against the bottom. The medium time of this behavioural pattern was 25 seconds. Then fish moved on the opposite side from that in which the stimulus was administered. Here they remained stationary on the bottom (in 50% of cases) or swam slowly in the 3 last sectors of the tank.

In absence of visible light, CSUI for water and alarm substance did not differ significantly (Fig. 1b) and fish never showed changes in their standard patterns of swimming. In dark condition alarm substance does not determine an avoidance of the part of the aquarium in which stimulus was administered.

Moreover, in this condition it is evident that alarm reaction is simplified, lacking the rapid zig-zag swimming typical in light condition. All tested fish evidently slowed down their swimming speed and often completely stopped at the bottom of the tank; in all tests, fish assumed a position of the body parallel to the bottom, touching it. At the end of each test, light was turned on and fish behaviour was observed for 5 minutes. All fish exhibited the zig-zag swim, thus we can exclude that the alarm substance used was inactive.

DISCUSSION

The alarm response of *Dawkinsia filamentosa* is different in light or dark condition. This confirms that the availability of visual information has a role in modulation of the exhibition of the response to chemical alarm cues (Paglianti et al. 2010) and that different patterns of this antipredator behaviour can be influenced by it in different ways.

In light, *D. filamentosa* shows the avoidance of alarm substance with a zig-zag swimming. In natural habitat, the perception of alarm substance means that a predator is near. The avoidance of alarm substance is advantageous, because the prey can avoid the risk of incurring into a predator. The following activity reduction can be a strategy to become less detectable after the exit from visual range of the predator. A fish, which normally lives in shoal, initially shows a zig-zag swimming even when it is alone. This behaviour is particularly advantageous when an individual is in a group, because the "confusion effect" is incremented (Ohguchi 1981; Milinski 1979, 1990), but it is advantageous also when fish is alone because dashing swimming can head off the predator, that need to preview the trajectory of prey (Guthrie 1980).

The situation changes if fish cannot use the vision. Some patterns occur only in light: zig-zag swimming lacks if fish cannot use visual information (Paglianti et al. 2010). In darkness, fish (including a lot of species that are predators to other fish) can obtain accurate information about surroundings using lateral line system. Thus, they have "hydrodynamic images" about position, size, and shape of nearby objects, based on distortions of waves created by swimming of fish self (Coombs 2001). In this situation a little dynamic alarmed behavior is advantageous; more movements are made by a specimen, more easily it can become a target for a predator. For the same reason, in darkness *D. filamentosa* does not move away from the zone in which it has perceived alarm substance, reducing movements that create mechanical traces. In this way not only the predator cannot use vision to see the prey, but neither lateral line to follow it. We can suppose that alarm reaction of *D. filamentosa* in darkness consists simply in moving to the bottom, where usually in nature there are more chances to find a refuge, and probably, like in *Pethia conchonius* (Paglianti et al. 2010), in increasing shoal cohesion to raise "dilution effect" *sensu* Bertram (1978). Anyway, the case of *D. filamentosa* shows how the component of avoidance of alarm substance can be influenced by visual information availability and how the vision has an important role in modulating the alarm reaction. Further similar research in other species of Teleostei showing an alarm reaction could be useful to increase information on the role of visual stimuli in the expression of the fright reaction and to verify if the patterns observed in cyprinids are present also in not relative and not shoaling species.

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نقش اطلاعات بصری در واکنش ترس *Dawkinsia filamentosa* (Valenciennes, 1844) (ماهیان استخوانی عالی: کپورماهی شکلان، کپورماهیان)

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چکیده: کپورماهی خال سیاه، *Dawkinsia filamentosa* (Valenciennes 1844)، یک ماهی هندی است که به‌صورت گله‌ایی زندگی می‌کند. این ماهی مانند بسیاری از استخوانچه‌داران، علائم هشداری که پس از آسیب پوستی آزاد می‌شود، رفتار ضد شکارچی قابل توجهی را در هم‌نوعان خود برمی‌انگیزد. ماده هشدار دهنده لازم برای آزمایشات از یک نمونه ماده به دست آمد. هر ماهی به صورت جداگانه (در نور مرئی یا با روشن کننده‌های IR) در یک آکواریوم با اندازه: ۲۰×۸×۱۰۵سانتی متر و ارتفاع آب ۸ سانتی‌متر آزمایش شد. مخزن به ۹ قسمت تقسیم شد و تحریک کننده (آب یا ماده هشداردهنده) درست در زیر سطح آب در لبه خلفی بالاتر سمت راست منتشر شد: شکل کشیده آکواریوم امکان ثبات پاسخ مانعی احتمالی را فراهم می‌کرد. ماهی‌ها در شرایط روشنایی یا تاریک، هم در غیاب و هم در حضور یک علایم هشدار شیمیایی آزمایش شدند. در هر دو شرایط مورد بررسی، رفتار ماهی با دوربینی که هم به نور مادون قرمز و هم به نور مرئی حساس بود، بررسی شدند. در هر یک از دو شرایط آزمایشی (روشنایی و تاریکی)، ۱۲ تست انجام شد. در هر آزمایش، ۳ ثبت متوالی شامل ۱۵ دقیقه در صورت عدم وجود محرک، ۱۵ دقیقه پس از حضور ۵۰ میلی‌لیتر آب خالص، و ۱۵ دقیقه پس از تجویز محلول ۴۸ میلی‌لیتر آب و ۲ میلی‌لیتر ماده هشداردهنده انجام شد. هر بخش از تانک با تعدادی ۹-۱ دسته از اشکال هلالی که در سمت مخالف هم همبستگی داشتتند، مدیریت می‌شد. در هر بخش ۱۵ دقیقه‌ای آزمایش، موقعیت ماهی و تعداد مربوطه هر ۳۰ ثانیه بررسی می شد و میانگین امتیاز هر ماهی در صورت عدم وجود محرک، در حضور آب و در حضور ماده هشداردهنده محاسبه می‌شد. میانگین امتیازات به‌دست‌آمده در حضور آب و در حضور ماده هشداردهنده از امتیاز متوسط به‌دست‌آمده در غیاب محرک کم شد و یک شاخص کاربری فضایی در حال تغییر به‌دست آمد. شاخص‌های به دست آمده برای هر ماهی (در حضور ماده هشداردهنده و در حضور آب) با آزمون Wilcoxon مقایسه شدند. مانند سایر کپورماهیان، واکنش ترس گونه خال سیاه در روشنایی یا تاریکی متفاوت است. خصوصاً، به نظر می‌رسد که واکنش هشداری آن در تاریکی صرفاً شامل حرکت به سمت پایین بدون هیچ گونه حرکت سریع شنا و بدون هیچ ترکیب اجتنابی از ماده هشداردهنده است.

کلمات‌کلیدی: واکنش هشداری، توزیع فضایی، ارتباط بصری، تشخیص شکارچی.