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Research report

Routine handling methods affect behaviour of three-spined sticklebacks in a novel test of anxiety

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HIGHLIGHTS

- We develop a new combined diving and scototaxis test of anxiety in fish.
- We compare box (in water) and net (out of water) transfer between tanks.
- Net transfer results in less anxiety like-behaviour. Explanations are considered.
- Novel-object and open-field tests fail to detect these differences.
- The combined diving and scototaxis test is a promising biologically-meaningful test.

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ABSTRACT

Fish are increasingly popular subjects in behavioural and neurobiological research. It is therefore important that they are housed and handled appropriately to ensure good welfare and reliable scientific findings, and that species-appropriate behavioural tests (e.g. of cognitive/affective states) are developed. Routine handling of captive animals may cause physiological stress responses that lead to anxiety-like states (e.g. increased perception of danger). In fish, these may be particularly pronounced when handling during tank-to-tank transfer involves removal from water into air. Here we develop and use a new combined scototaxis (preference for dark over light areas) and novel-tank-diving test, alongside conventional open-field and novel-object tests, to measure the effects of transferring three-spined sticklebacks (*Gasterosteus aculeatus*) between tanks using a box or net (in and out of water respectively). Preference tests for dark over light areas confirmed the presence of scototaxis in this species. Open-field and novel-object tests failed to detect any significant differences between net and box-handled fish. However, the combined diving and scototaxis detected consistent differences between the treatments. Net-handled fish spent less time on the dark side of the tank, less time in the bottom third, and kept a greater distance from the 'safe' bottom dark area than box-handled fish. Possible explanations for this reduction in anxiety-like behaviour in net-handled fish are discussed. The combined diving and scototaxis test may be a sensitive and taxon-appropriate method for measuring anxiety-like states in fish.

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1. Introduction

Recent studies have shown that the way in which laboratory rodents are handled may profoundly influence anxiety-like behaviour with potential knock-on effects for welfare and experimental outcomes [1]. Fish are increasingly important laboratory animals [2–5] and are frequently 'handled' by transferring them between tanks using nets that remove them from water for the duration of transport. This handling method may have welfare and behavioural implications, particularly in laboratories where fish are moved regularly for behavioural testing [6], and has been used as a manipulation in experiments investigating the time-course of physiological stress responses in fish [e.g. 7–9].

Whilst fear is hypothesised to occur in response to immediate and present threats, anxiety may occur when an animal perceives increased uncertainty about a potential future threat, or enhanced probability of danger [e.g. 10], for example in the presence of cues

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that predict danger or uncertain outcomes, or following recent dangerous events (e.g. being caught and removed from the water in the case of an aquatic species). Consequently, tests of exploratory behaviour are often used as measures of animal anxiety, with the prediction that increased anxiety should result in decreased risk-taking and exploration and an increased tendency to remain in 'safe' areas—so-called 'anxiety-like' behaviour. In fish, examples of such tests include measures of thigmotaxis (the tendency to remain close to walls or other solid objects [e.g. 11,12]), open-field use [e.g. 2], novel object inspection [e.g. 13], novel-tank-diving [e.g. 14,15], and scototaxis (the preferential movement of an organism to a dark area, as distinct from negative phototaxis [e.g. 16–18]).

In their review of the validity of fish models of anxiety, Maximino et al. [19] identified the novel-tank-diving paradigm as having the best-established predictive validity. When fish are transferred to a new tank they usually swim to the bottom, a response that may be adaptive in helping them to avoid any predator lurking above. Fish taking a longer time to move back up towards the surface are assumed to be more anxious, and this is supported by studies showing that this behaviour changes as predicted in response to pharmacological agents that are known to have anxiolytic or anxiogenic effects in humans and other species [e.g. 20].

Maximino et al. [19] also argued that scototaxis has good construct validity (i.e. it 'makes sense' in light of fish ecology and evolutionary theory). Scototaxis-based exploratory tests are seen as appropriate measures of anxiety because they present the fish with a motivational conflict between staying in the perceived safest area (the dark area) and exploring the perceived more risky (light) area in order to discover potential resources (food/mates/passage to an even safer place). The light areas of tanks are thought to be more dangerous because they reduce the background-matching camouflage of fish, particularly when seen from above as they would be by an aerial predator.

Pharmacological tests indicate that particular drugs (e.g. chlordiazepoxide; citalopram) may not induce consistently anxiogenic or anxiolytic effects in both the novel-tank-diving and scototaxis tests, suggesting that these tests may reflect slightly different motivational or affective processes [19]. A combination of the novel-tank-diving and scototaxis methods may therefore be a sensitive, novel, and biologically meaningful way of identifying anxiety-like states in fish, and one aim of the current study was to develop such a combined test.

The three-spined stickleback (*Gasterosteus aculeatus*) is an important study species in behavioural and neurobiological research, including in recent investigations into the influence of anthropogenic disturbance on animal behaviour [21–26]. Brydges et al. [27] investigated the effects of tank-transfer methods in this species and found higher physiological stress responses (as measured by increases in opercular gill beat rate and cortisol) in individuals that had been transferred using a net rather than using a 'scoop' which kept the fish in the water. Surprisingly, however, the apparent stress of the net transfer procedure did not translate into subsequent increased anxiety-like behaviour as measured by conventional emergence-latency and novel-object tests. Here, we develop and use a new combined diving and scototaxis test of anxiety-like behaviour to explore further the implications of handling using a net vs. scoop/box method in this species.

The stickleback populations from which our subjects were drawn live in habitats with sharply contrasting dark and light areas due to shadows cast by vegetation on a light toned substrate in clear water (unpublished field observation). Like many British sticklebacks, they are likely to have been under high predation pressure from birds [e.g. 28,29], many of which have been recorded in the area from which the fish were sourced [e.g. 30]. Sticklebacks, in common with many fish, have a counter-shaded body [31] with a dark upper and a lighter, reflective lower body, making them appear dark when seen from above thus camouflaging them against dark backgrounds. They also actively adapt their dorsal colouration to improve background matching [32], attesting to the importance of this camouflage. There is thus a good case for expecting scototaxis in sticklebacks. The tendency to stay in relatively safe areas of a tank will, however, be traded-off against motivation to forage and explore [33] and there appears to be no published scientific evidence to support the idea that sticklebacks will prefer to be in an area with a dark background. Thus, one aim of the current study was to establish that such a preference exists in the three-spined stickleback.

Here, we first establish the suitability of using a scototaxis test of anxiety in the three-spined stickleback by investigating whether the species has the predicted preference for dark over light areas, and whether this is maintained even when fish have had prior experience of living in a light-coloured tank (Experiment 1). We then carry out standard open-field and novel-object tests [cf. 27] to investigate the effects of net and scoop/box handling, with the prediction that more anxious individuals will spend more time away from the central area and close to the tank walls, and will be slower to approach the novel object (Experiment 2). In addition, we use both black- and white-walled test tanks to investigate whether any effects of handling are more readily revealed in an apparently more dangerous (white) or less dangerous (black) environment, and also whether anxiety-like behaviour is more clearly observed in the lighter tank. Finally, we combine scototaxis and novel-tank-diving paradigms to create a new test incorporating both principles, and we investigate whether this approach can detect any differential effects of net and scoop/box handling on anxiety-like behaviour (Experiment 3) and, if so, whether it is more sensitive than traditional open-field and novel-object tests. Based on our discussion above, the more time a fish spends in the darker side of the tank, near the bottom, and close to the dark half of the bottom area (putatively the area that is perceived as 'safest'), the more anxious it is deemed to be.

2. Materials and methods

For all experiments, animal husbandry was performed by University animal care staff and researchers at the School of Biological Sciences, University of Bristol. Ethical permission was granted by the University of Bristol Animal Welfare and Ethical Review Body (University Investigation Number: UB/10/020), and procedures complied with the Association for the Study of Animal Behaviour/Animal Behavior Society guidelines for the treatment of animals in behavioural research and teaching [34].

2.1. Experiment 1: testing for scototaxis in sticklebacks

2.1.1. Animals and husbandry

Fifty adult three-spined sticklebacks (of mixed, unknown age and sex) were caught using hand-held nets from a freshwater pond in southwest U.K. (51°30′44″N, 2°38′13″W; online still-water associated with Hazel Brook/River Trym) with appropriate Environmental Agency permission. They were transported to the University of Bristol Aquarium Facility and acclimatised to holding tanks (see Ref. [35] for full details). Groups of up to 20 sticklebacks were held in 100-l, white-bottomed, glass tanks (90 × 36.5 cm; water depth: 30 cm) containing artificial plants for shelter, an external power filter and an airstone. Fish were kept in non-breeding condition at 17 °C on a 12:12 h light:dark cycle, and were fed three times weekly with frozen bloodworms (chironomid larvae) and flakes (Aquarian Goldfish Flake, Masterfoods, Batley, U.K.). Tanks were cleaned and water was changed through regular siphon cleaning which was performed slowly and gently, allowing fish to swim out of the way in

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