Red fluorescent irises reveal retroreflective pupils in cryptic predators

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

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Tübingen

2026

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	20.07.2026
Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter:	Prof. Dr. Nico Michiels
2. Berichterstatterin:	Prof. Dr. Katharina Foerster

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Summary

In marine environments, active sensing using light is rare and has mainly been described in nocturnal and deep-sea fishes. Previous work showed that triplefins, a diurnal fish, can redirect downwelling light to improve the detection of scorpionfish. However, this mechanism is expected to operate mainly in shallow water (3–10 m), where sufficient downwelling light is available. With increasing depth, or in shaded microhabitats, the underwater light field becomes dimmer, more scattered, and increasingly shifted towards blue-green wavelengths. Under these conditions, redirecting downwelling light is likely to become less effective, whereas red fluorescence may provide an alternative light source.

In this dissertation, I test whether red iris fluorescence in the yellow black-faced triplefin, *Tripterygion delaisi*, can enhance the detection of the black scorpionfish, *Scorpaena porcus*, by specifically making their retroreflective pupils light up. This hypothesis is tested in four linked chapters that combine behavioural experiments with visual modelling.

Chapter 1 shows that triplefins respond more strongly to live scorpionfish when red fluorescence can contribute to illumination. This suggests that fluorescence can improve predator detection, but it does not establish the underlying mechanism.

Chapter 2 tests whether this effect can be explained by interaction with the retroreflective scorpionfish pupil, using 3D-printed scorpionfish models with either retroreflective or non-retroreflective eye inserts. The results provide partial support for the retroreflective-pupil hypothesis, but also show that simplified models do not fully reproduce the response elicited by live scorpionfish.

Chapter 3 uses visual modelling to test whether red fluorescence can increase pupil-iris contrast in scorpionfish. The model predicts that fluorescence should extend the distance over which the pupil remains distinguishable from the surrounding iris.

Chapter 4 then tests this prediction directly by manipulating whether the scorpionfish eye is visible. Triplefins detect scorpionfish at greater distances when fluorescence is available, but only when the eye remains visible. When the eye is hidden, the fluorescence effect disappears.

Taken together, these findings support the conclusion that red iris fluorescence can function as a short-range sensory mechanism for detecting scorpionfish. Rather than generally enhancing vision, fluorescence appears to exploit a specific optical vulnerability in predator camouflage, namely the retroreflective pupil.

This dissertation provides experimental evidence that fluorescence can serve an active sensory function. More broadly, it identifies a previously unrecognized mechanism by which prey may exploit the optical properties of predator eyes, and it suggests that red fluorescence in small micropredatory fishes may have evolved to enhance survival by improving the detection of cryptic predators.

List of publications included in this dissertation

Published or accepted publications

1) van der Schoot, B. K. M., & Michiels, N. K. (2025). Improved predator detection through illumination with red fluorescence by a small benthic fish. Does it work? *Frontiers in Ecology and Evolution*, 13, 1728992.

Author	**Author position**	**Scientific ideas %**	**Data generation %**	**Analysis & interpretation %**	**Paper writing %**
Bram K. M. van der Schoot	1	90	100	100	90
Nico K. Michiels	2	10	0	0	10
Title of paper:	Improved predator detection through illumination with red fluorescence by a small benthic fish. Does it work?				
Status in publication process:	Published in *Frontiers in Ecology and Evolution*. https://doi.org/10.3389/fevo.2025.1728992				

Manuscripts in preparation

1) van der Schoot, B. K. M., Vetter, P., & Michiels, N. K. (2026*).* Active sensing with light: Fish use fluorescence to visually detect predators. Manuscript in preparation for submission to a high impact journal.

Author	**Author position**	**Scientific ideas %**	**Data generation %**	**Analysis & interpretation %**	**Paper writing %**
Bram K. M. van der Schoot	1	90	80	90	90
Patrick Vetter	2	0	20	0	0
Nico K. Michiels	3	10	0	10	10
Title of paper:	Active sensing with light: Fish use fluorescence to visually detect predators.				
Status in publication process:	Manuscript in preparation for submission to a high impact journal.				

Introduction

In this dissertation I test whether red iris fluorescence can improve detection of a cryptic predator in a marine predator-prey system. Since this question links underwater optics, predator camouflage, active sensing, and fluorescence, the introduction first outlines the optical properties of the underwater light field, then considers the problem of detecting camouflaged predators and finally describes the potential role of active sensing with light to improve predator detection.

Light in the ocean

Before we dive into visual interactions in marine environments, we first have to consider the optical conditions under which these visual interactions occur.

Underwater vision is fundamentally shaped by the way light is transformed as it travels through water (Mobley, 1994; Johnsen, 2011; Kirk, 2011). Unlike in air, light underwater is rapidly altered by both absorption and scattering. Absorption removes photons from the light field, thus reducing the overall amount of light available with depth and distance. Scattering redirects photons into new directions, thus removing photons from the path between the object and observer while adding scattered photons from other directions to this path. Together, these processes weaken the light signal travelling from an object to an observer and add scattered light from the water column into the line of sight (Mobley, 1994; Zaneveld and Pegau, 2003). As a result, object-background contrast declines with distance, and fine spatial details become harder to distinguish.

The underwater light field also spectrally transforms with depth (Figure 1). Since long wavelengths are attenuated more strongly than shorter blue-green wavelengths, the ambient spectrum generally becomes narrower and increasingly blue-green shifted with depth (Johnsen, 2011; Kirk, 2011). As a consequence, in clear coastal water, shallow habitats may have a relatively broad-spectrum, whereas deeper habitats are often dominated by a narrower blue-green spectrum (Marshall *et al.*, 2003; Johnsen, 2011). Here, it is useful to distinguish between two spectral zones: the euryspectral zone and the stenospectral zone. In the euryspectral zone, the ambient spectrum remains broad relative to the visual range of colour vision of most reef fishes. In a stenospectral zone, the ambient spectrum is narrower than what many fish could potentially perceive and mostly concentrated in the blue-green range. The boundary between these zones is not fixed at a single depth, but depends on several factors, including water clarity, solar angle, cloud cover, and local habitat structure. In clear coastal settings the loss of reds typically occurs in the top 10 to 20 meters. However, similar stenospectral conditions can also arise in shallow shaded microhabitats where there is no direct downwelling light (Meadows *et al.*, 2014; Harant *et al.*, 2016, 2018).

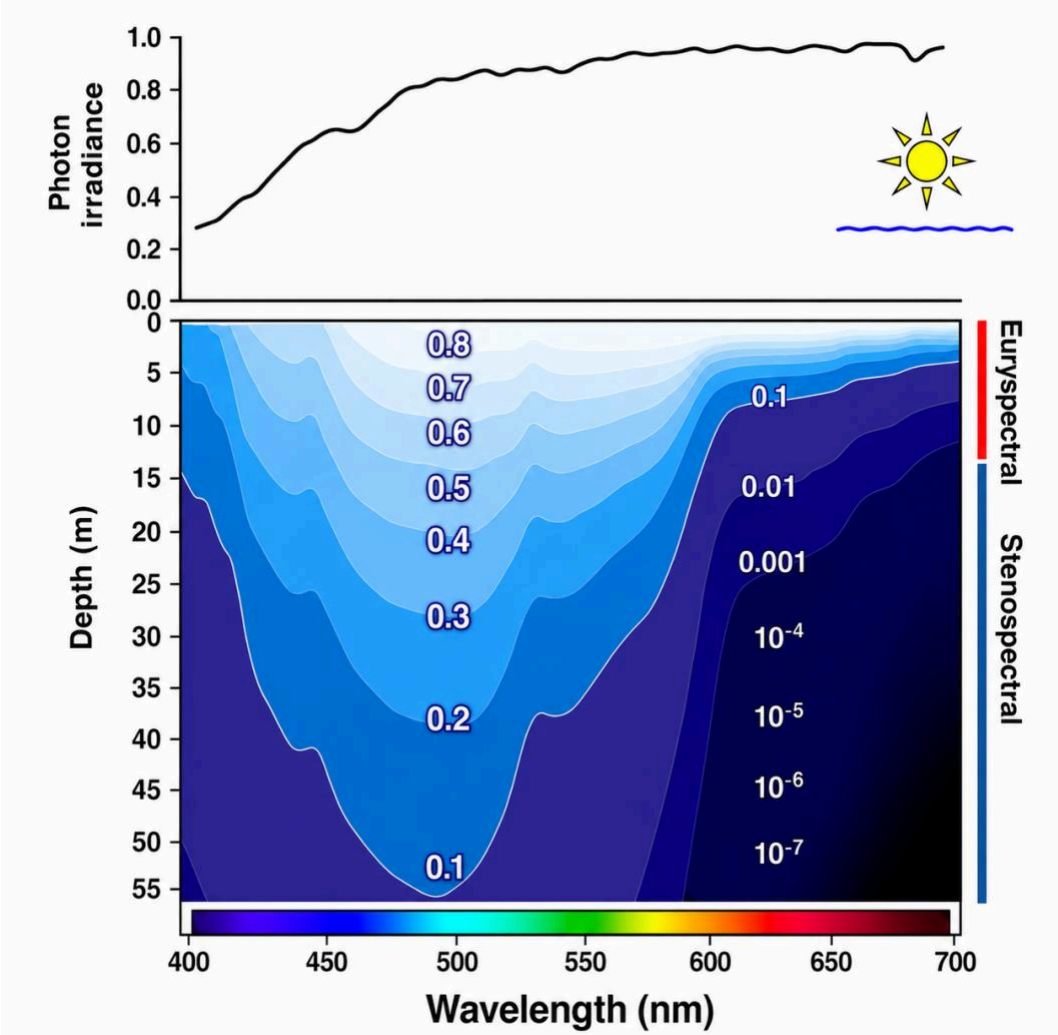

Figure 1: Light absorption in seawater. The upper graph shows the relative photon irradiance of the ambient light field measured above the water surface. The lower graph shows how the underwater light spectrum changes with increasing depth. Near the surface, in the euryspectral zone, a broad range of wavelengths are available. In the stenospectral zone, longer wavelengths, especially those above 600 nm, are rapidly absorbed, resulting in the light field narrowing towards the blue-green range. White contour lines indicate iso-brightness values for individual wavelengths, showing the relative proportion of light remaining at each depth. The colour bar below the graph indicates wavelength in nm, and the side bars distinguish the euryspectral and stenospectral depth ranges. Modified after Anthes et al. (2016).

This spectral filtering has direct consequences for colour perception. This is especially true for reflective surfaces, because a reflective surface can only reflect wavelengths that are present in the light spectrum (Marshall *et al.*, 2003; Johnsen, 2011). When long-wavelength photons become scarce, reflective surfaces that appear red in air or in shallow water can no longer appear red in the same way at depth (Figure 2). This is simply because only few long-wavelength photons are available for reflection (Johnsen, 2011). Underwater colour therefore has to be understood in relation to local illumination rather than as a fixed property of the object alone (Marshall *et al.*, 2003; Johnsen, 2011).

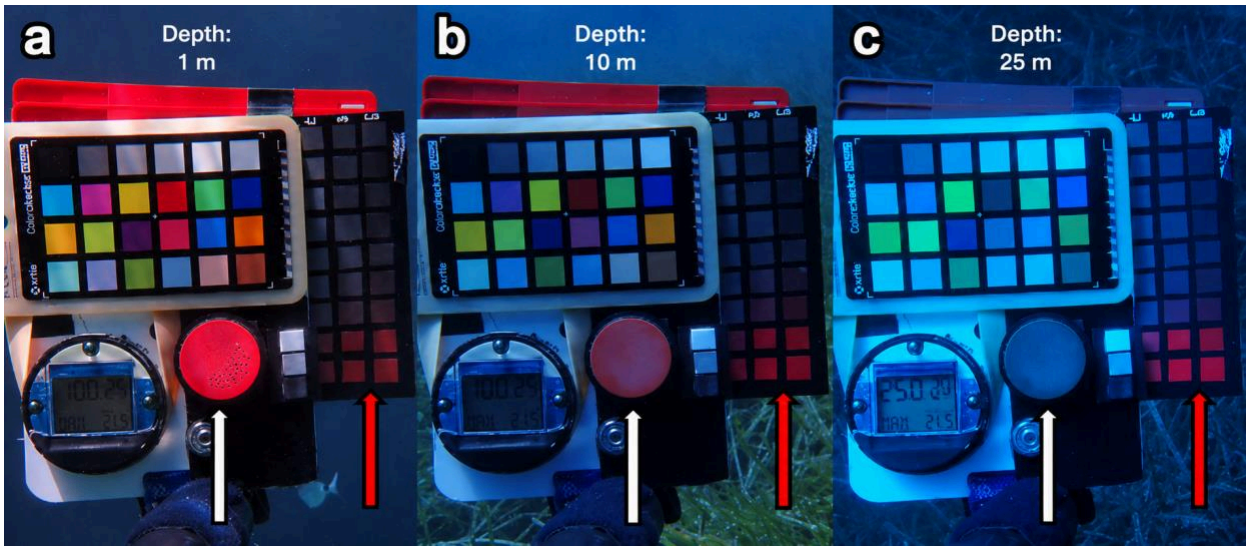

Figure 2: The effect of light absorption on colour perception in the first 25 meters. Photo showing a ColourChecker board, a red reflective standard made of material that only reflects red light that is present in the ambient light spectrum (white arrow), and red fluorescent paint (red arrow) photographed at increasing depths. **a)** At 1 m depth, the broad-spectrum ambient light is still available, including long-wavelength red light. Under these conditions, the red reflective standard and the red fluorescent paint appear similar in colouration. **b)** At 10 m depth, the ambient light spectrum has narrowed towards blue-green wavelengths. The red reflective standard has lost much of its red appearance, whereas the red fluorescent paint remains visibly red. **c)** At 25 m depth, the longer-wavelength red light is largely absent from the ambient spectrum, causing the red reflective standard to appear greyish. However, the red fluorescent paint is still perceived as red because it absorbs the remaining shorter-wavelength light and re-emits it at longer (red) wavelengths. Photo credits: Nico Michiels.

For vision, it is more important to focus on radiance rather than irradiance. Irradiance is used to describe the total light arriving at a point or surface from all directions, whereas radiance is the light emitted or reflected by an object, as seen by an observer from a particular direction. Since image formation depends mostly on directional light, radiance is often the most relevant measure that is used to determine what an animal sees (Mobley, 1994; Johnsen, 2011). Therefore, a habitat may be broadly illuminated (irradiance) while still offering poor visual conditions for the viewing path of an observer (radiance).

Together, absorption, scattering, and spectral filtering impose strong limitations on underwater vision. They reduce contrast, alter the colour appearance, and constrain the distance over which visual information can be transmitted and received. These constraints are especially relevant for benthic predator-prey interactions, where many encounters occur at short range and where predator camouflage can further reduce the ability of prey to detect predators.

Predator camouflage and the importance of the eye

For a prey species, early detection of a predator is essential for survival. This is especially difficult when predators rely on camouflage to avoid detection, as many sit-and-wait predators do (Corrigan *et al.*, 2008; Coulson *et al.*, 2015). In complex benthic environments, predator camouflage is often achieved through multiple interacting strategies, including background matching, disruptive colouration, colour change, morphological complexity, and background choice (Cott, 1940; John, Santon and Michiels, 2023, 2024, 2025). These strategies can make cryptic predators difficult to detect, especially under the constrained visual conditions described above.

Yet even highly cryptic predators face a particular problem, namely the eye. Pupils are often dark, circular, and sharply contrasted against the surrounding iris, making them potentially conspicuous even when the rest of the body is well camouflaged (Cott, 1940; Marshall *et al.*, 2019). Concealing the pupil, without compromising their vision, is therefore a recurring challenge in cryptic predator evolution. A variety of eye-camouflage strategies have evolved (Figure 3). These include pupil shape modifications (Cott, 1940), iris patterning (Karplus and Algom, 1981), skin flaps (Cott, 1940), and retroreflective ocular structures that produce daytime eyeshine (Lythgoe, 1975; Santon *et al.*, 2018).

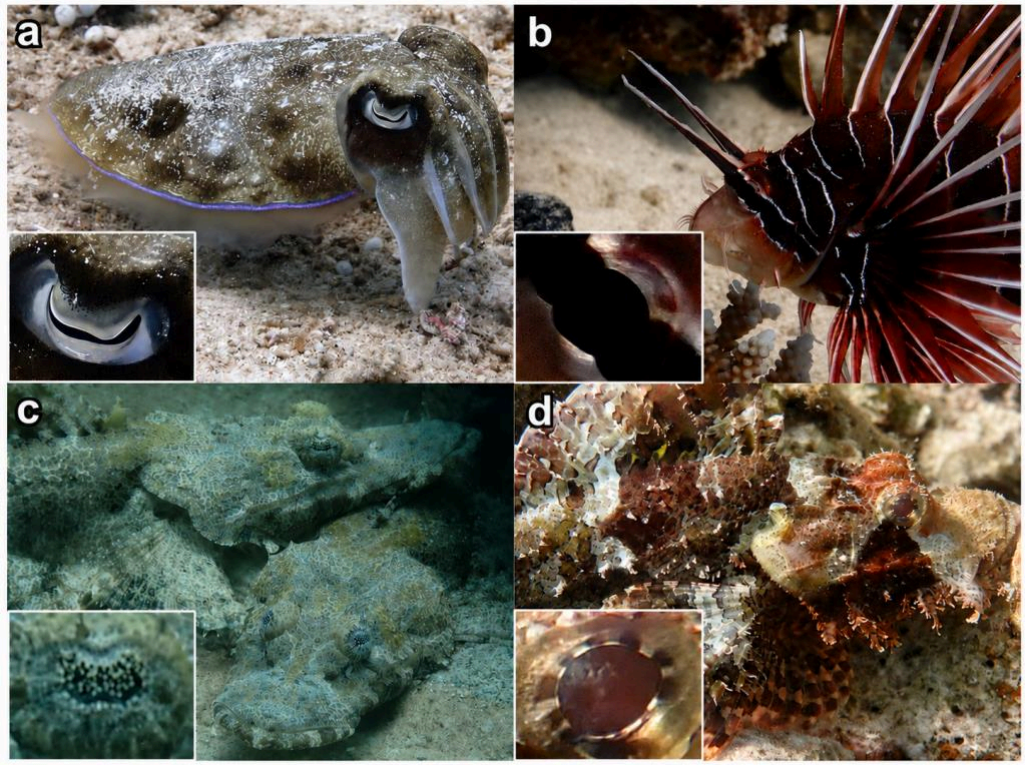

Figure 3: Eye camouflage strategies in predators, bottom left inserts show an enlargement of the eye. a) *Sepia pharaonis* showing a w-shaped pupil modification, **b)** *Pteropterus cinctus* showing an eye mask with a dark vertical stripe making the pupil less conspicuous, **c)** *Papilloculiceps longiceps* showing skin flaps that cover part of the pupil, **d)** *Scorpaenopsis oxycephala* showing daytime eyeshine resulting in a bright pupil. Photo credits: Madeleine Parnot & Bram van der Schoot.

Eyeshine is commonly associated with visual sensitivity under dim-light conditions and is often suppressed under bright light. However, cryptic sit-and-wait predators such as scorpionfish and toadfish represent important exceptions. In these species, eyeshine can be present during the day and is often reduced at night. In this context, daytime eyeshine may contribute to eye camouflage. Here, reflective or retroreflective ocular structures can brighten the pupil, thereby reducing pupil–iris contrast and making the pupil less conspicuous against the surrounding iris (Nicol, 1980; Santon *et al.*, 2018).

However, this same strategy also creates a potential vulnerability. Retroreflective structures return incoming light in a narrow angle back towards the light source. For daytime eyeshine, this means that light reaching the predator pupil from near an observer's visual axis can be reflected back towards that observer. From the observer's perspective, the predator pupil may then appear brighter than it would under normal ambient conditions, increasing the pupil-iris contrast and potentially revealing the cryptic predator. This makes the predator pupil a potential optical target for prey that possess a light source close to their own pupil (Santon *et al.*, 2018, 2020).

Active sensing to detect predator eyes

Using a light source to reveal a predator pupil would represent a form of active sensing using light. In general, active sensing involves the generation or redirection of energy and the interpretation of the returning signal. This is comparable to echolocation or electrolocation (Nelson and MacIver, 2006). While the use of sound or electric fields is well described, the use of light for active sensing in the animal kingdom is considered rare. It is best known from nocturnal and deep-sea fish that possess light organs that are positioned close to the eye and are thought to induce detectable reflections in nearby prey, predators, or conspecifics (Howland, Murphy and McCosker, 1992; Hellinger et al., 2017). This form of light-based active sensing is termed active photolocation because it relies on the observer inducing and perceiving reflections in a target.

Although this principle is well established for chemiluminescent fish, recent research has pointed out that it may also apply to diurnal fish that do not generate their own light. Instead, these diurnal fish redirect ambient downwelling light from structures near the pupil. In triplefins (*Tripterygion delaisi*), the protruding spherical lens can focus downwelling light onto a spot on the iris below the pupil, generating a bright local spot called an "ocular spark". This focussed light is then redirected sideways and can induce detectable reflections in nearby retroreflective targets like the pupil of a scorpionfish (Figure 4). This proposed mechanism is termed "Diurnal Active Photolocation (DAP)" (Santon *et al.*, 2020). Experimental work has shown that triplefins turn ocular sparks on or off at will. This suggests that triplefins can actively use it to improve the detection of retroreflective targets and is therefore not an accidental by-product (Michiels *et al.*, 2018).

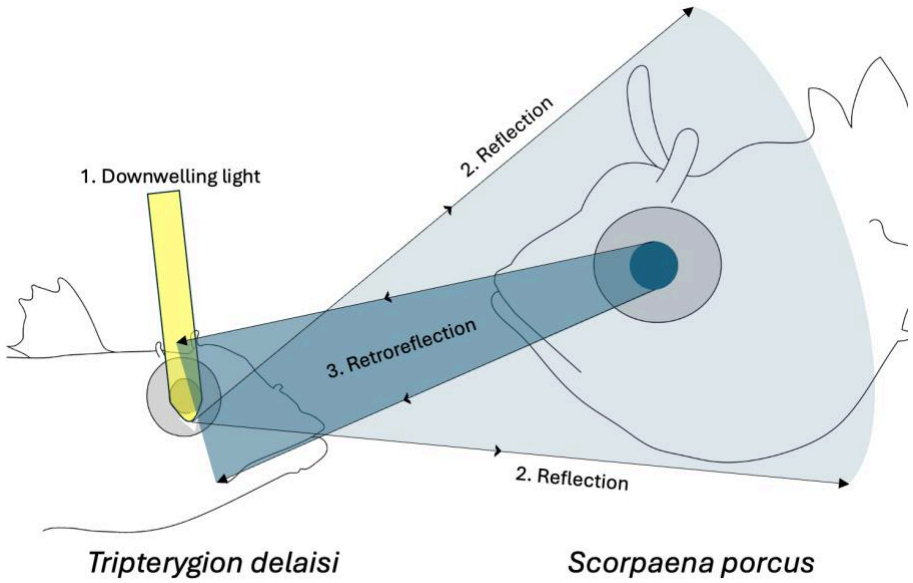

Figure 4: Diurnal active photolocation by means of reflection of downwelling light focussed on the lower iris. The figure illustrates the three main steps involved in diurnal active photolocation. **1)** Downwelling light is focussed by the triplefin's protruding lens onto a bright spot of the iris below the pupil. **2)** This focussed light is then reflected sideways towards the surroundings. **3)** Part of the reflected light reaches the scorpionfish pupil. Since the pupil is retroreflective, this light is returned towards the source, i.e. towards the triplefin's eye. From the triplefin's perspective, this makes the scorpionfish pupil appear brighter, thereby improving predator detection (Modified from a scheme by N. Michiels).

The key mechanistic principle of Diurnal Active Photolocation is near-coaxial illumination. When a light source is positioned close to the observer's pupil, as an ocular spark is in triplefins, illumination is approximately aligned with the viewing axis. Under these conditions, retroreflective structures return incident light back towards its source. Because the light source is positioned close to the observer's pupil, some of this returned light can enter the observer's pupil and may therefore generate a detectable signal. Since eyes with retroreflective properties are widespread, this is a realistic scenario (Kjernsmo, Grönholm and Merilaita, 2016; Satoh, Tanaka and Kohda, 2016).

However, this mechanism is expected to become less effective as the underwater light field changes with increasing depth or in shaded habitats. Ocular spark based Diurnal Active Photolocation depends on downwelling light that is sufficiently strong. As described under "light in the ocean", in deeper water and shaded microhabitats, direct downwelling light becomes less dominant. Here the light field becomes not only weaker and blue-green shifted, but also more diffuse through scattering. Under these conditions, ocular sparks may no longer function as an effective light source. This raises the question of whether another form of ocular radiance could take over the role of ocular sparks under these conditions. One promising alternative is red fluorescence.

Red fluorescence in marine fish

Fluorescence can be described as a three-step physical process (Figure 5). First, a molecule absorbs short-wavelength light (excitation light), causing the molecule to move from its ground state to an (unstable) excited state. The second part consists of energy loss through the collision with surrounding molecules while in the excited state. Finally, the molecule emits longer wavelength light when returning to its ground state (Johnsen, 2011). Fluorescence is especially relevant in deeper or shaded marine habitats, where the ambient light field is increasingly restricted to these shorter blue-green wavelengths. Under such conditions, pigment-based red colouration becomes less effective since hardly any long-wavelength light remains for reflection. Fluorescence, however, can generate long-wavelength radiance, provided that sufficient shorter-wavelength light is available for excitation (Michiels *et al.*, 2008; Harant *et al.*, 2016; Bitton *et al.*, 2017; Harant and Michiels, 2017).

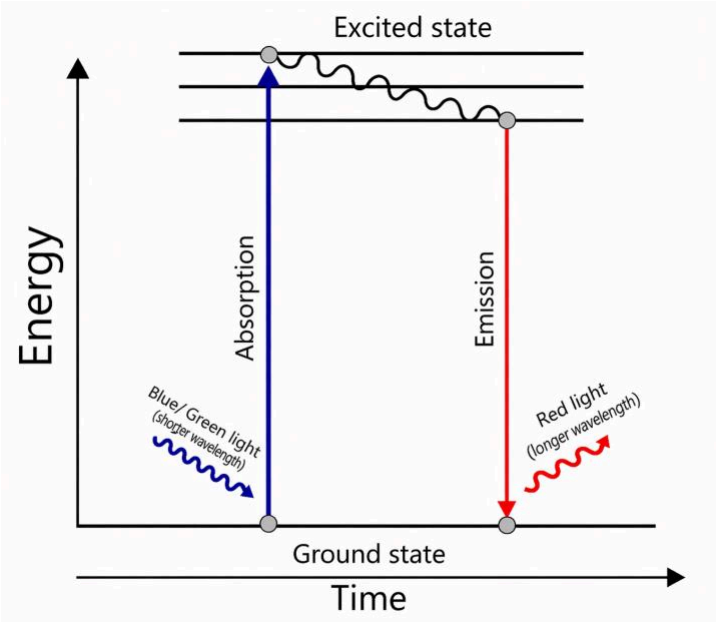

Figure 5: Simplified Jablonski diagram illustrating the principle of fluorescence. Absorption of short-wavelength light excites a molecule, moving it from the ground state to an excited state. In this excited state, part of the absorbed energy is dissipated through non-radiative processes. The emission of longer wavelength light results in the molecule moving back to its ground state. The horizontal axis indicates the temporal sequence from absorption to emission (adapted from Jablonski, 1933).

Red fluorescent structures are widespread in marine fish, occurring in more than 40% of surveyed reef fish, yet their ecological function remains poorly understood (Sparks *et al.*, 2014; Anthes *et al.*, 2016; Carr *et al.*, 2025). Fluorescence in fish follows one striking pattern. Namely, In small micropredatory fish, red fluorescence is especially common around the head and eye region possibly suggesting a visual function (Figure 6) (Anthes *et al.*, 2016). Since red light is strongly attenuated in water, any visual function of red fluorescence is expected to operate only over short

distances. This is in line with the widespread occurrence of red fluorescence around the head and eye region of small micropredatory fish, whose ecologically relevant interactions typically occur over very short distances (Neiße *et al.*, 2020).

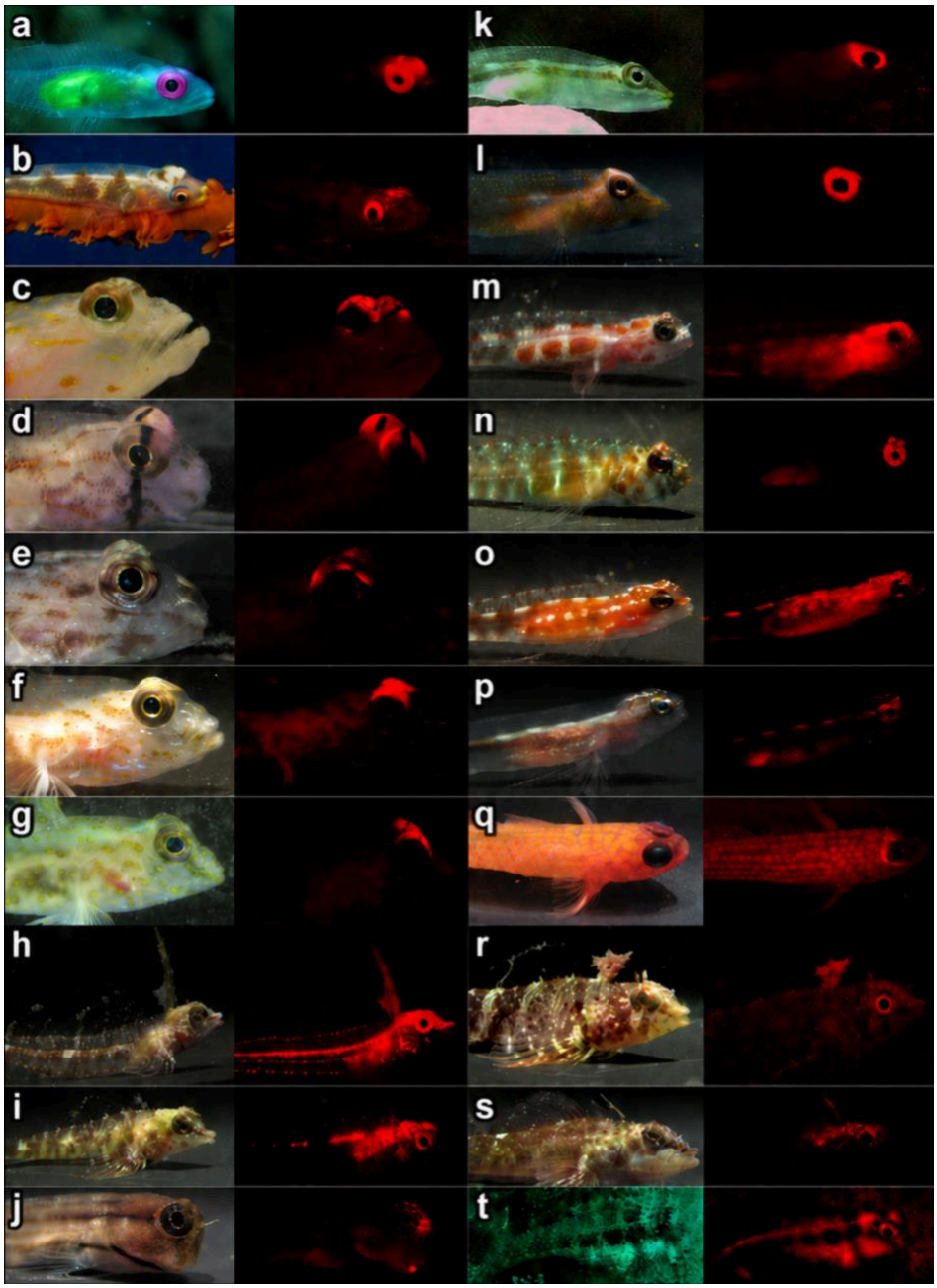

Figure 6: Red fluorescence around the head and eye region of small micropredatory fish. a) *Bryaninops natans*, **b)** *Bryaninops yongei*, **c)** *Ctenogobiops tangaroai*, **d)** *Gnatholepis anjerensis*, **e)** *Istigobius decoratus*, **f)** *Fusigobius duospilus*, **g)** *Fusigobius longispinus*, **h)** *Enneapterygius pusillus*, **i)** *Enneapterygius abeli*, **j)** *Ecsenius dentex*, **k)** *Pleurosicya micheli*, **l)** *Pleurosicya prognatha*, **m)** *Eviota guttata*, **n)** *Eviota prasina*, **o)** *Eviota zebrina*, **p)** *Eviota sebreei*, **q)** *Trimma avidori*, **r)** *Enneapterygius destai*, **s)** *Helcogramma steinitzi*, **t)** *Crossosalarias macrospilus*. Left: broad spectrum illumination, right: red fluorescence under blue (laboratory) or natural (field) illumination (adapted from Michiels et al., 2008).

Visual system of fish and seeing red

Before we ask the question if red fluorescence can actually serve a visual function in small micropredatory fish, it is necessary to consider the visual system of the observer that possesses red fluorescence. Visual signals must be interpreted from the observer's perspective rather than from a human perspective, because animals differ strongly in the wavelengths they detect, the spatial detail they can resolve, and the contrast differences they can discriminate (Land and Nilsson, 2012; Caves, Brandley and Johnsen, 2018). Here, three visual properties are particularly important for predator detection: spectral sensitivity, spatial resolution, and contrast sensitivity. Spectral sensitivity determines which wavelengths can stimulate the photoreceptors and therefore which parts of the spectrum contribute to the vision of an observer (Cronin *et al.*, 2014). Spatial resolution determines how much spatial detail can be resolved, including small features such as a predator pupil (Caves, Brandley and Johnsen, 2018). Contrast sensitivity defines the smallest contrast difference that can be detected between an object and its background or between two adjacent visual targets (Pateras and Karioti, 2020). A contrast sensitivity function (CSF) describes how contrast sensitivity changes with spatial frequency and is therefore useful for estimating whether visual details of different sizes and contrasts can be detected (De Valois and De Valois, 1980).

The retina of fish typically contains two main types of photoreceptors: rods and cones (Land and Nilsson, 2012). Rods are highly sensitive and are mainly associated with vision under dim light conditions, whereas cones are used primarily under brighter conditions and provide the basis for colour vision. Colour vision requires at least two photoreceptor classes with different spectral sensitivities. This is because colour is generated by comparing the relative stimulation of different photoreceptor classes. Many diurnal marine fishes possess single and double cones, and their longest-wavelength-sensitive cones often peak in the green rather than in the red part of the spectrum (Figure 7) (Lythgoe, 1979; McFarland, 1991; Losey, 2003). From an evolutionary perspective, this coincides well with the blue-green dominated light field found in many marine habitats. However, this also raises an important question for red fluorescence: can long-wavelength fluorescence still be detected by fishes that lack a dedicated red-sensitive cone?

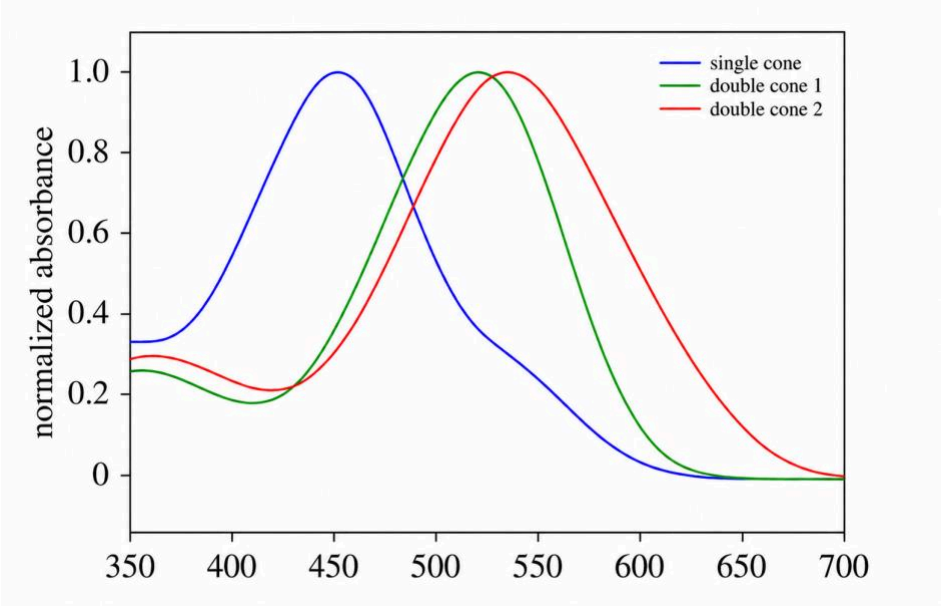

Figure 7: Photoreceptors of *T. delaisi*. Predicted normalized absorbance curves of the single cone (blue), and double cones (green, red) in *T. delaisi*. Fish possessing cones with a peak in the green range might still be able to perceive red (adapted from Bitton et al., 2017).

The absence of a "true" red-sensitive cone in fish does not necessarily mean that fish cannot perceive red. Photoreceptor sensitivities are broad, and the long-wavelength part of green-sensitive photoreceptors can still be stimulated by sufficiently intense red light (Mosk *et al.*, 2007). The important question is therefore not if fish possess red-sensitive cones but whether red fluorescence produces enough receptor excitation to actually generate a detectable contrast against the surrounding visual scene.

This generated contrast can be either chromatic or achromatic (Kelber, Vorobyev and Osorio, 2003). Chromatic contrast occurs when different photoreceptor classes are stimulated in different proportions, and thus generate a colour contrast. Achromatic contrast depends mainly on differences in overall photon catch and can thus be interpreted as a brightness contrast. Both forms of contrast may be relevant for perceiving red fluorescence but this depends on the spectral background, the intensity of the fluorescent emission, and the specific visual system of the observer.

Study species

This dissertation focuses on the interaction between the yellow black-faced triplefin (*Tripterygion delaisi)* and one of its predator species, the black scorpionfish (*Scorpaena porcus)*. These species are referred to below as *T. delaisi* and *S. porcus*. This study system brings together the key elements required to test a fluorescence based Diurnal Active Photolocation in a realistic predator-prey context.

Yellow black-faced triplefin (*Tripterygion delaisi)*

T. delaisi is a small cryptobenthic fish that inhabits rocky substrates in the Mediterranean Sea and Eastern Atlantic Ocean, typically between 5 and 50 m depth. It possesses a conspicuous red fluorescent iris that forms a ring around the pupil (Figure 8). This position places the fluorescent light source extremely close to the visual axis, creating an almost perfect optical geometry for the near-coaxial illumination of nearby targets. The fluorescence of *T. delaisi* peaks around 609 nm and has a broad excitation spectrum with a peak in the green range around 530 nm (Bitton *et al.*, 2017). They can also regulate their iris fluorescence in response to ambient light conditions, and individuals from dimmer or deeper environments show stronger fluorescence (Michiels *et al.*, 2008, 2018; Wucherer and Michiels, 2012, 2014; Kalb *et al.*, 2015; Harant *et al.*, 2016).

Figure 8: *Tripterygion delaisi*. Photo taken in a blue-green environment at 29 m depth as seen by a human SCUBA diver (Nikon D500, natural light, manual white balance, LEE Sunset-Red colour correction filter). Top right insert: *T. delaisi* showing an ocular spark, photographed at shallow depth (Photo credits: Nico Michiels).

Several key properties of *T. delaisi's* visual system have already been described, including spectral sensitivities, spatial resolution, and contrast sensitivity (Bitton *et al.*, 2017, 2019; Fritsch, Collin and Michiels, 2017; Santon, Münch and Michiels, 2019). This makes it possible to estimate whether changes in predator pupil-iris contrast are theoretically detectable from the perspective of a triplefin. In addition, *T. delaisi* shows a characteristic bobbing behaviour, a repeated raising and lowering of the anterior body, which increases in the presence of a scorpionfish (Wirtz, 1978; Santon *et al.*, 2021; van der Schoot and Michiels, 2025). This behaviour can therefore be used as an accurate behavioural indicator of perceiving something dangerous, such as a scorpionfish.

Black scorpionfish (*Scorpaena porcus)*

S. porcus is a cryptobenthic, sit-and-wait predator common on rocky coastal substrates and seagrass habitats in the Mediterranean Sea and northeast Atlantic (Figure 9). It preys on small benthic fish, including blennies (Compaire *et al.*, 2018), and is highly cryptic to both human and prey-fish visual systems. This crypsis is achieved through multiple interacting camouflage strategies, including rapid adjustment of body colouration, modulation of skin-pattern contrast, background choice, and eye camouflage (Santon *et al.*, 2018; John, Santon and Michiels, 2023, 2024, 2025). *S. porcus* possesses large pupils with strong retroreflective properties, especially in the red spectrum (Figure 9c) (Santon *et al.*, 2018). These retroreflective pupils improve camouflage through daytime eyeshine but, as a trade-off, may make the eyes vulnerable to detection by observers possessing a near-coaxial light source, especially a red one (Figure 9d,e).

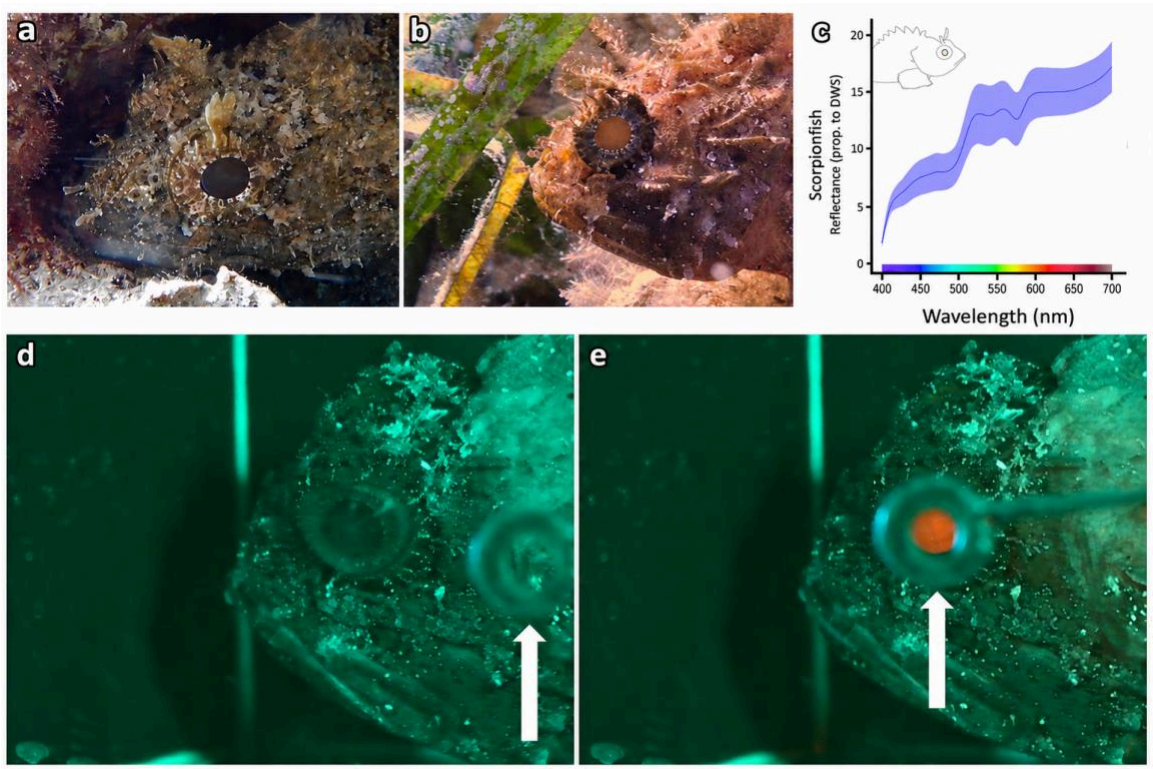

Figure 9: *Scorpaena porcus*. a) In the field, shaded at 3 m depth, with dark pupil (weak eyeshine). **b)** In the field, exposed in 7 m depth, with strong eyeshine, caused by a mix of transmission-based and retroreflective eyeshine. **c)** Pupil reflectance expressed as a proportion of the pupil photon radiance relative to a PTFE white diffuse reflectance standard (y-axis). A value of 15 means that pupil radiance is 15 times brighter than a 99% white reflectance standard when measured under the same conditions. Blue area: SE of mean curve (adapted from Santon et al., 2018). **d)** In an experimental set-up, fluorescent ring (white arrow) positioned non-coaxially with the scorpionfish pupil and camera (no retroreflection), photo taken through a yellow spectral filter. **e)** In an experimental set-up, fluorescent ring (white arrow) positioned coaxially with the scorpionfish pupil and camera (strong retroreflection), photo taken through a yellow spectral filter.

Objectives and dissertation structure

My research aims to test whether red iris fluorescence in triplefins improves the detection of a cryptic scorpionfish. The proposed mechanism is that the fluorescent iris of the triplefin acts as a short-range light source. Under suitable geometry, red fluorescent light from the triplefin can illuminate the scorpionfish pupil. Since the scorpionfish pupil is strongly retroreflective, especially in the red range, part of this light is returned towards the triplefin. Given that the fluorescent iris forms a ring around the triplefin's own pupil, some of the returned light from the scorpionfish is expected to enter the triplefin's eye. From the triplefin's perspective, this should make the scorpionfish pupil appear brighter, increase pupil-iris contrast, and thereby improve predator detection.

To test this hypothesis, I combined behavioural experiments with visual modelling. Together, these approaches provide both an empirical and a theoretical basis for evaluating whether red fluorescence can be used to improve the detection of a scorpionfish. The dissertation is structured around four chapters.

1. In Chapter 1 I tested whether triplefins can use their red fluorescence to improve detection of a live scorpionfish under controlled conditions. This experiment establishes the general behavioural phenomenon and provides the basis for the follow-up studies.
2. In Chapter 2, I used 3D-printed scorpionfish models to isolate the role of the retroreflective pupil. This behavioural study tests whether the red fluorescent iris of a triplefin can improve detection of a retroreflective target.
3. In Chapter 3 I used visual modelling to provide a theoretical basis for the proposed mechanism. The visual model estimates whether, and over which distances, red fluorescence can generate a detectable chromatic contrast between the pupil and iris of a scorpionfish as perceived by a triplefin under these specific experimental conditions.
4. In Chapter 4, I performed a behavioural experiment where I manipulated the visibility of the scorpionfish pupil, to test if triplefins use red fluorescence to specifically improve the detection of the predator's pupil.

Together, these experiments comprehensively test whether red iris fluorescence can improve predator detection by revealing the otherwise camouflaged pupil of a cryptic scorpionfish.

Chapter 1 - Fluorescence-enabled predator detection in live scorpionfish

Associated publication

van der Schoot, B. K. M., & Michiels, N. K. (2025). Improved predator detection through illumination with red fluorescence by a small benthic fish. Does it work? *Frontiers in Ecology and Evolution, 13*, 1728992.

Extended summary

In this chapter we performed an experiment that tests whether triplefins can use their red fluorescent irises to improve detection of a scorpionfish. The experiment was conducted under light conditions that simulated the deeper, blue-green spectrum found at 20 m depth.

Triplefins were released in an arena featuring a central lane of glued-on black sand (11.5 x 30 cm) that encouraged linear movement (Figure 10). At the end of the lane, a stimulus box with a glass front presented either a live scorpionfish (predator) or a size-matched stone (control). We predicted that triplefins would move towards the stimulus box because they prefer shaded microhabitats over the brightly lit open arena. Triplefins were tested under two fluorescence conditions: one that allowed red iris fluorescence to contribute to illumination of the stimulus (**Fluo-ON**) and one that prevented it (**Fluo-OFF**). This was achieved by placing different spectral filters on the glass front of the stimulus box. Each triplefin encountered either a scorpionfish or a stone under both fluorescence conditions, resulting in two runs per individual.

During each run, triplefins could explore the arena and gradually approach the stimulus box for closer inspection. We hypothesised that they would respond more cautiously to a scorpionfish than to a stone, either by keeping a greater distance, approaching more slowly, or bobbing more frequently. We expected this effect to be stronger when fluorescence was allowed to illuminate the scorpionfish.

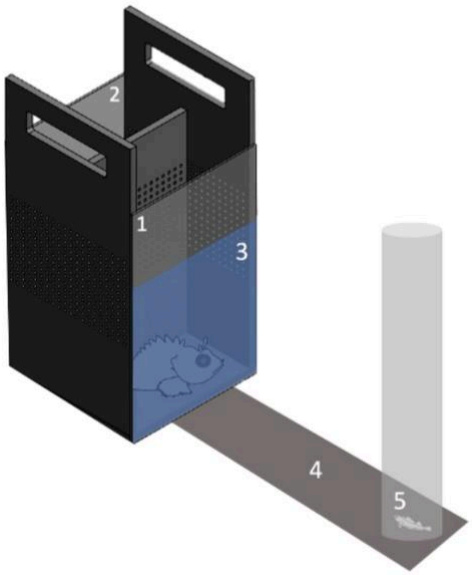

Figure 10: Schematic overview of the general experimental set-up. Triplefins are released from a plexiglass cylinder (5) on a strip of glued black sand (4), which they prefer over the bright surface of the arena. Within 10 minutes, most individuals will gradually hop towards the stimulus box for close inspection. The box contains two compartments for two visual stimuli. Only one is visible to a triplefin through glass (1) and a filter (3) at a time.

We found that, upon release, triplefins rapidly approached the display compartment regardless of the visual stimulus or fluorescence condition. As a result, we failed to detect clear treatment effects on approach distance or latency. However, we did find that triplefins bobbed more when confronted with a scorpionfish than with the stone control, and they bobbed significantly more when fluorescence was allowed to illuminate the scorpionfish (Figure 11). No corresponding fluorescence effect was found in the stone treatment, indicating that the difference observed in the scorpionfish treatment was not caused by an unintended filter effect.

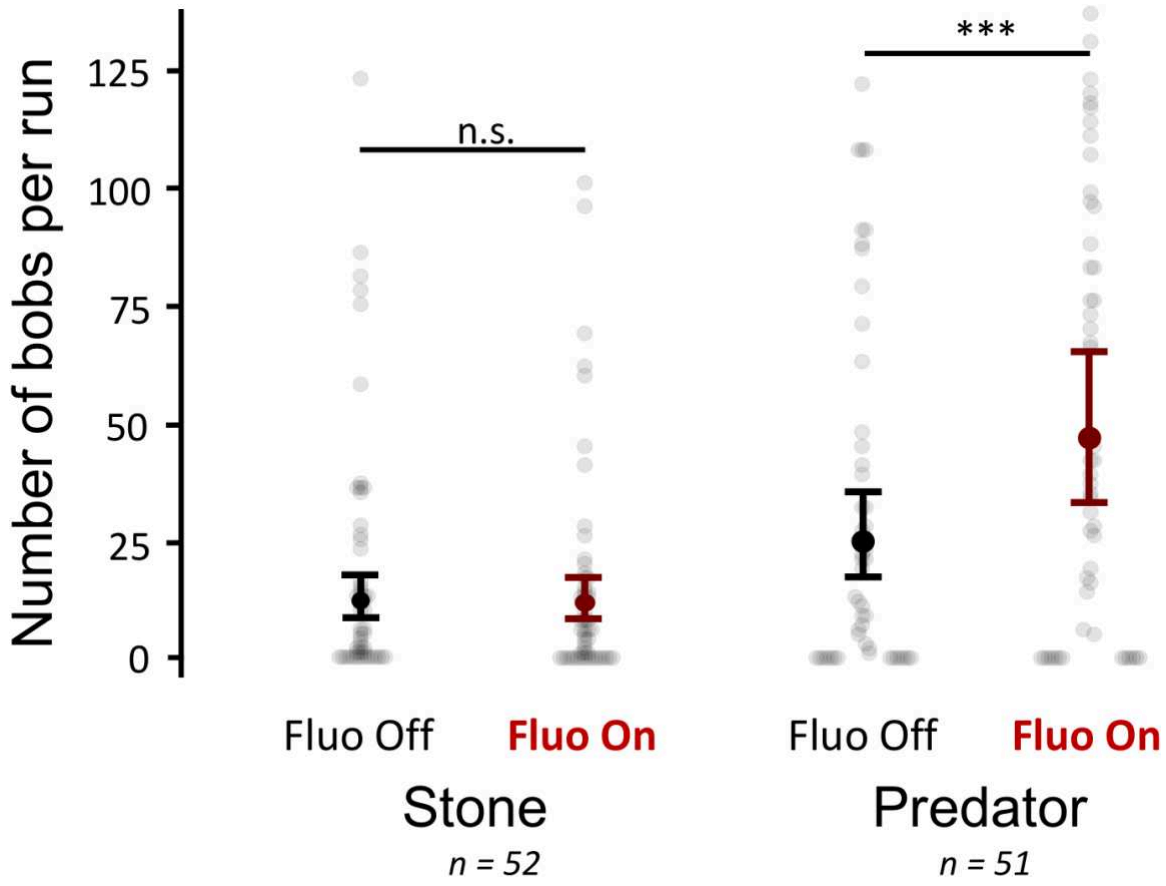

Figure 11: **Results of the experiment: Fluorescence-enabled predator detection.** Absolute bobbing frequency in a 10-minute run as a function of visual stimulus (Stone or Predator) and filter treatment (**Fluo-ON** or **Fluo-OFF**). *T. delaisi* individuals were tested in a paired design per visual stimulus, and therefore either saw a stone or a Predator, under both **Fluo-ON** and **Fluo-OFF** treatments in varying and balanced order. Predicted means and 95% compatibility intervals (CI). **n.s.** denotes a non-significant difference, ******* denotes a significant difference.

Limitations

Although this experiment provides the first suggestive evidence that fluorescence may improve the detection of a scorpionfish, two important limitations should be discussed. First, the experiment tested whether fluorescence improves overall scorpionfish detection, but it did not establish if the improved detection was caused by fluorescence-induced eyeshine in the scorpionfish pupil.

Second, although no fluorescence effect was found in the stone treatment (control), the experiment cannot fully exclude the possibility that the filters caused a change in the general scene visibility inside the stimulus box or that the filters did not have an influence on the behaviour of the live scorpionfish. If the filters influenced the behaviour of the scorpionfish this could in turn have affected the triplefin's response to the scorpionfish. The control treatment makes a simple filter effect on the visual scene less likely, but it cannot fully exclude a subtle filter effect on the overall appearance or behaviour of the live scorpionfish.

Conclusion and next step

This experiment provides the first behavioural evidence that red iris fluorescence can improve predator detection. Triplefins showed stronger bobbing responses to a scorpionfish than to a stone control, and this response increased when fluorescence was allowed to illuminate the scorpionfish. However, the experiment did not isolate the optical target responsible for this effect. We therefore do not know whether the increased response was caused specifically by fluorescence-induced retroreflection in the scorpionfish pupil.

Chapter 2 therefore tests the retroreflective-pupil prediction more directly by replacing the live predator with 3D-printed scorpionfish models fitted with either retroreflective or non-retroreflective eye inserts.

Chapter 2 - Testing the retroreflective-pupil prediction with 3D-printed scorpionfish models

Associated publication

van der Schoot, B. K. M., & Michiels, N. K. (2025). Improved predator detection through illumination with red fluorescence by a small benthic fish. Does it work? *Frontiers in Ecology and Evolution, 13*, 1728992.

Extended summary

This experiment was designed to address the main limitation raised by the experiment of Chapter 1. Here we used the same general set-up and testing procedure as in the experiment of Chapter 1, but replaced the live scorpionfish stimulus with 3D-printed scorpionfish models fitted with either retroreflective or non-retroreflective eyes. This allowed us to isolate the retroreflective pupil component without (surgically) manipulating the pupil of a live scorpionfish.

In this experiment, triplefins were exposed to one of three different visual stimuli: a retroreflective scorpionfish model, a non-retroreflective scorpionfish model, or a live scorpionfish used as control (Figure 12). For the live scorpionfish treatment, GoPro cameras were mounted above the stimulus box to assess whether the filters used to create the **Fluo-ON** and **Fluo-OFF** conditions altered its behaviour. Each stimulus was presented under both fluorescence conditions. For the live scorpionfish, we predicted the same behavioural pattern as in the experiment of Chapter 1. In addition, we expected triplefins to show stronger bobbing responses to the retroreflective model than to the non-retroreflective model, but only when fluorescence was allowed to illuminate the stimulus.

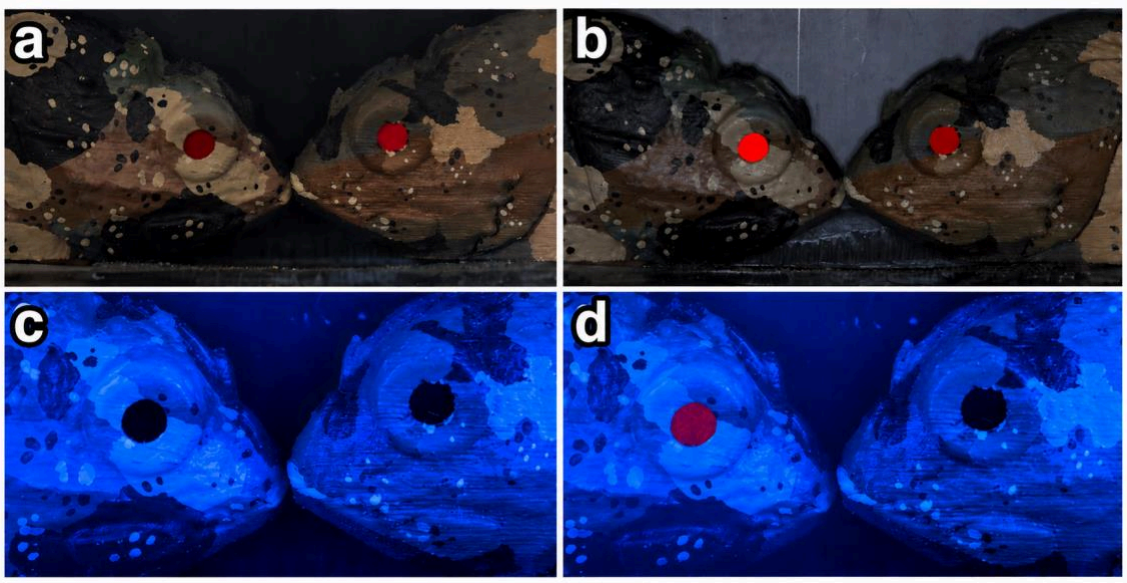

Figure 12: 3D-printed scorpionfish models used in the experiment: Isolating eye-retroreflection. Photos of 3D-printed scorpionfish models used in Experiment 2. In all photos the left model is a model with retroreflective eye-insert and the right model is a model with non-retroreflective eye-insert. Both models are fitted with a Sunset Red filter in the eye to simulate the natural reddish retroreflection of a scorpionfish's eye. **a)** Photo taken under ambient white light, no coaxial light. **b)** Photo taken under ambient white light, with added coaxial white light. **c)** Photo taken under the blue-green light conditions of the set-up, no coaxial light. **d)** Photo taken under the blue-green light conditions of the set-up, with added coaxial red light.

The results partly supported these predictions (Figure 13). As in the experiment of Chapter 1, triplefins bobbed more to live scorpionfish when fluorescence was allowed to illuminate the scorpionfish, confirming the main behavioural result of the first experiment. The model treatments produced a weaker and less consistent pattern. No clear fluorescence effect was detected within the model treatments themselves. However, triplefins bobbed more to the retroreflective model under **Fluo-ON** conditions than to the non-retroreflective model under either fluorescence condition. This difference disappeared when the retroreflective model was presented under **Fluo-OFF** conditions.

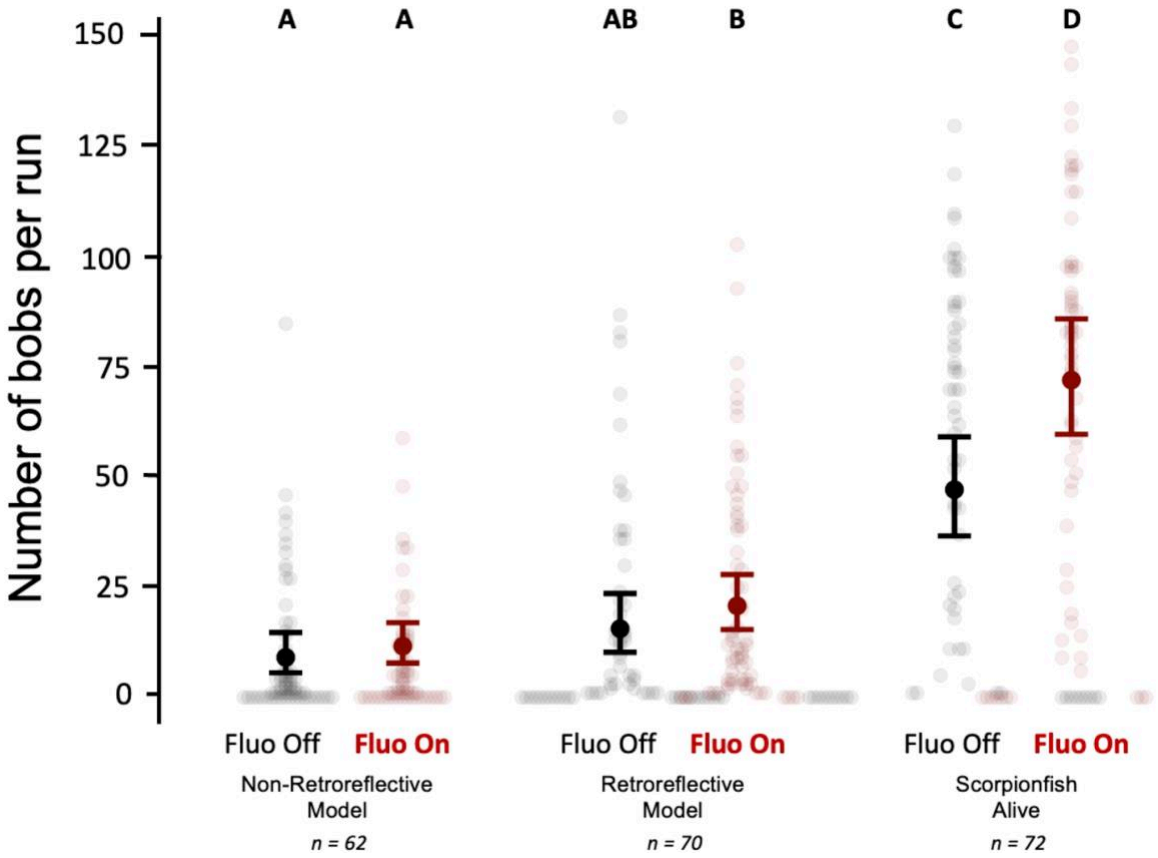

Figure 13: Results of the experiment: Isolating eye-retroreflection. Absolute bobbing frequency in a 10-minute run as a function of visual stimulus (Non-retroreflective model, Retroreflective model or Predator) and filter treatment (**Fluo-ON** or **Fluo-OFF**). *T. delaisi* individuals were tested in a paired design per visual stimulus, and therefore either saw a Retroreflective model, a Non-retroreflective model, or a Scorpionfish, under both **Fluo-ON** and **Fluo-OFF** treatments in varying and balanced order. Predicted means and 95% compatibility intervals (CI). Different letters indicate a significant difference between the treatments.

Furthermore, we found that scorpionfish did not behave differently when placed behind the filters used to generate the **Fluo-ON** and **Fluo-OFF** conditions, ruling out the important alternative explanation for the observed effects mentioned in the limitations of Chapter 1.

Limitations

Although this experiment was designed to isolate the retroreflective pupil component, its main limitation is that the 3D-printed scorpionfish models did not elicit the same behavioural response as a live scorpionfish did. This indicates that the models did not fully capture the visual appearance, or behavioural relevance, of a real predator, and therefore may not have been recognised by triplefins as a real threat. For now we have to conclude that the models used in this experiment are not yet at a stage where they can be used as a replacement for a real scorpionfish.

Conclusion and next step

This experiment provides partial support for the retroreflective pupil target prediction. Retroreflective models elicited stronger bobbing responses than non-retroreflective models under **Fluo-ON** conditions, which is consistent with the idea that fluorescence can interact with a retroreflective pupil. However, the models did not reproduce the full response elicited by live scorpionfish in Chapter 1. This suggests that although retroreflection may contribute to the detection process, the simplified models lacked important visual or behavioural cues that make a real scorpionfish appear dangerous to triplefins.

Because we learned that at this stage, artificial models could not fully replace a live scorpionfish, the next step was to test the optical feasibility of the mechanism independently of the limitations found in the behavioural experiments. Chapter 3 therefore uses visual modelling to test whether red iris fluorescence can theoretically increase pupil–iris contrast over distances that are relevant to the behavioural experiments.

Chapter 3 - Visual modelling of fluorescence-induced pupil–iris contrast

Associated manuscript

van der Schoot, B. K. M., Vetter, P., & Michiels, N. K. Active sensing with light: Fish use fluorescence to visually detect predators.
Manuscript in preparation for submission to a high impact journal.

Extended summary

In the previous chapters, we showed that triplefins detect live scorpionfish better when their red fluorescence contributes to the illumination of a predator, but we did not test whether this effect was specifically caused by interaction with the retroreflective scorpionfish pupil. In this chapter, we use a visual model to examine whether the red fluorescence from a triplefin can, theoretically, generate a perceivable pupil-iris contrast in a scorpionfish.

The visual model was designed to test whether fluorescence could increase the visibility of the scorpionfish pupil from the perspective of a triplefin. To do this, we first estimated the total photon flux that reaches the triplefin's pupil from the scorpionfish pupil and iris. This photon flux consisted of two components, the baseline radiance of the scorpionfish eye under the ambient light conditions of the experimental set-up, and the additional radiance generated when triplefin fluorescence was reflected back from the scorpionfish eye.

These photon-flux estimates were then used in a visual model that was informed about the triplefin visual system. The model calculated the quantum catch of each photoreceptor type and allowed us to estimate the chromatic contrast between the scorpionfish pupil and iris under **Fluo-ON** and **Fluo-OFF** conditions from the perspective of the triplefin. Contrast was expressed in just noticeable differences (JNDs). Values below one JND indicate that two stimuli are expected to be indistinguishable under optimal viewing conditions, whereas values above one JND indicate an increasing probability of discrimination. Because a threshold of one JND is unlikely under natural viewing conditions, three JNDs are often used as a more conservative criterion.

The visual model indicated that triplefins should be able to discriminate the scorpionfish pupil from the surrounding iris at close range under both fluorescence conditions (Figure 14). However, fluorescence increased the distance over which pupil-iris contrast was predicted to be perceivable by a triplefin. At a conservative threshold of three JNDs, the scorpionfish pupil was predicted to be detectable from 7.5 cm under **Fluo-ON** conditions, compared with 5.5 cm under **Fluo-OFF** conditions. This corresponds to a fluorescence-dependent increase in the detection distance of 2.0 cm. Across different thresholds, the estimated increase in

detection distance ranged from more than 5 cm at 1.5 JNDs to 1.6 cm at 4 JNDs, depending on the threshold used (Figure 14).

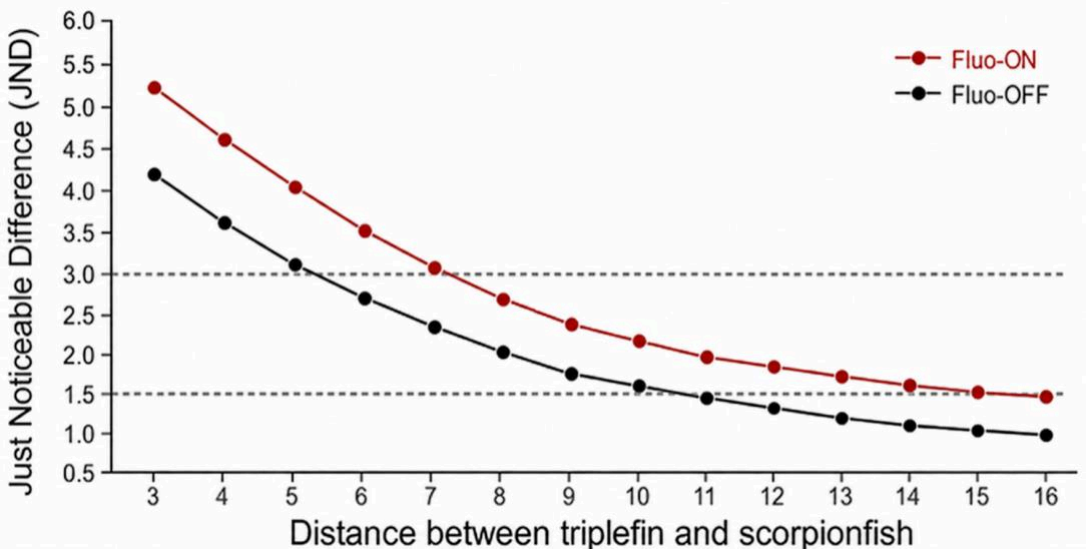

Figure 14: Visual-model prediction of fluorescence-enhanced scorpionfish pupil detection. Predicted chromatic contrast between the scorpionfish pupil and iris, expressed in just noticeable differences (JNDs), as perceived by a triplefin at increasing distances. When red iris fluorescence is allowed to contribute to illumination (**Fluo-ON**), pupil–iris contrast remained higher across all modelled distances than when red fluorescence was blocked (**Fluo-OFF**). The dashed horizontal lines indicate different JND thresholds, with values above 1 JND theoretically detectable under optimal conditions and values around or above 3 JND representing a more conservative detection criterion. Fluorescence extends the distance over which the retroreflective scorpionfish pupil should be discriminable from the surrounding iris.

Limitations

The visual model provides a theoretical estimate of whether red fluorescence can increase scorpionfish pupil-iris contrast as perceived by a triplefin, but it necessarily simplifies several aspects of the real predator-prey interaction.

First, the model assumes a simplified viewing geometry. In the behavioural experiment, the position, orientation, and gaze direction of both the triplefin and the scorpionfish can vary, whereas the model treats the optical path between the triplefin and the scorpionfish in a standardised way.

Second, the model uses average values for several biological parameters that can vary strongly among individuals. These include the brightness of triplefin iris fluorescence, the size of the triplefin pupil and iris, and the reflectance of the scorpionfish pupil and iris. Individual variation in any of these parameters could make the fluorescence effect stronger or weaker than predicted by the model.

Finally, the model relies on several optical parameters that are not specific to the exact experimental set-up. For example, light attenuation was based on general values for marine water, whereas the behavioural experiment used filtered flow-through seawater. The actual attenuation and scattering conditions in the set-up may therefore have differed from those assumed in the model.

Conclusion and next step

The visual model predicts that fluorescence should increase pupil-iris contrast over relevant distances inside the experimental set-up. This supports the prediction that it is indeed the retroreflective scorpionfish pupil that causes the response. However, visual modelling alone cannot show whether triplefins actually use this contrast to improve scorpionfish detection.

Chapter 4 therefore returns to a final behavioural test with live scorpionfish and directly manipulates whether the scorpionfish pupil is visible from the triplefin's perspective.

Chapter 4 - Pupil visibility determines fluorescence-enhanced predator detection

Associated manuscript

van der Schoot, B. K. M., Vetter, P., & Michiels, N. K. Active sensing with light: Fish use fluorescence to visually detect predators.
Manuscript in preparation for submission to a high impact journal

Extended summary

The visual model in Chapter 3 predicted that red iris fluorescence should increase the distance over which a triplefin can discriminate the scorpionfish pupil from the surrounding iris. However, the model alone cannot show whether triplefins actually use this pupil-specific contrast for predator detection. To test this directly, we performed a behavioural experiment based on the same general set-up as in Chapters 1 and 2, but with two important modifications. First, we manipulated whether the scorpionfish pupil was visible from the triplefin's perspective. Second, we added a 1 cm wide and 15 cm long ridge to the central lane in the arena. The ridge started at 5 cm from the stimulus compartment and increased in height from 0.5 cm at the stimulus end to 1 cm at the release end, creating a gradient of exposure. This ridge slowed the triplefin's approach towards the stimulus compartment, allowing detection distance to be quantified more precisely.

As before, triplefins were exposed to a live scorpionfish under two fluorescence conditions, **Fluo-ON** and **Fluo-OFF**. In contrast to the previous behavioural experiments, however, we used three scorpionfish stimulus treatments: a lightly sedated scorpionfish with the eye visible, a lightly sedated scorpionfish with the eye hidden, and an untreated scorpionfish (control). The lightly sedated scorpionfish treatments allowed us to manipulate pupil visibility while keeping the position of the scorpionfish fixed during the trials. The untreated scorpionfish treatment was included as a control to compare the responses with a natural, unmanipulated predator.

Pupil visibility in the sedated treatments was manipulated using a natural, non-fluorescent stone suspended in front of the scorpionfish with transparent fishing line. In the eye-visible treatment, the stone was positioned in front of the body while leaving the eye unobstructed. In the eye-hidden treatment, the stone was positioned directly in front of the eye, removing the pupil as a visible optical target (Figure 15).

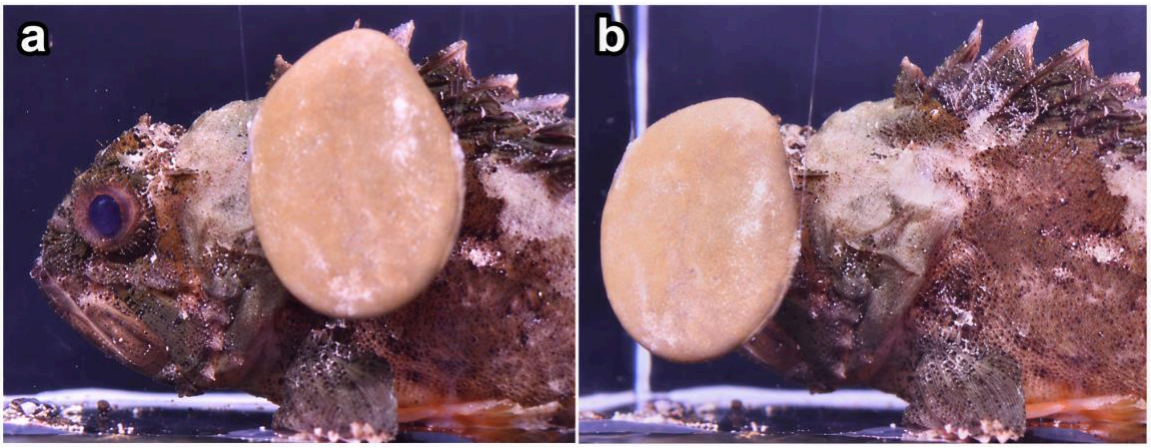

Figure 15: Sedated scorpionfish treatments. Photos taken under white overhead light, not representative of experimental conditions. **a)** Eye-visible treatment, here the stone is positioned in front of the scorpionfish body. **b)** Eye-hidden treatment, here the stone is positioned in front of the eye of the scorpionfish, hiding the eye from the triplefin's perspective.

We predicted that, if red fluorescence really improves detection by interacting with the retroreflective pupil, the fluorescence effect should be present when the eye is visible but disappear when the eye is hidden. If fluorescence instead improves detection by generally increasing scene visibility, the fluorescence effect should remain even when the pupil is obscured.

The testing procedure was identical to the one used in the experiments of Chapters 1 and 2. However, with the addition of the ridge we recorded the distance of the triplefin from the predator at each bobbing event. This allowed us to use mean bobbing distance as the response variable. This was defined as the average distance from the predator across all bobbing events within a run.

We found that triplefins bobbed at greater distances under **Fluo-ON** than under **Fluo-OFF** conditions but only when the scorpionfish pupil was visible (Figure 16). The mean bobbing distance was 14.8 cm under **Fluo-ON** and 10.2 cm under **Fluo-OFF** conditions, resulting in an increase of 4.6 cm. This increase in detection distance corresponds well with the visual model, which predicted a fluorescence-dependent increase of approximately 1.6 cm to more than 5 cm, depending on the JND threshold used (Figure 14).

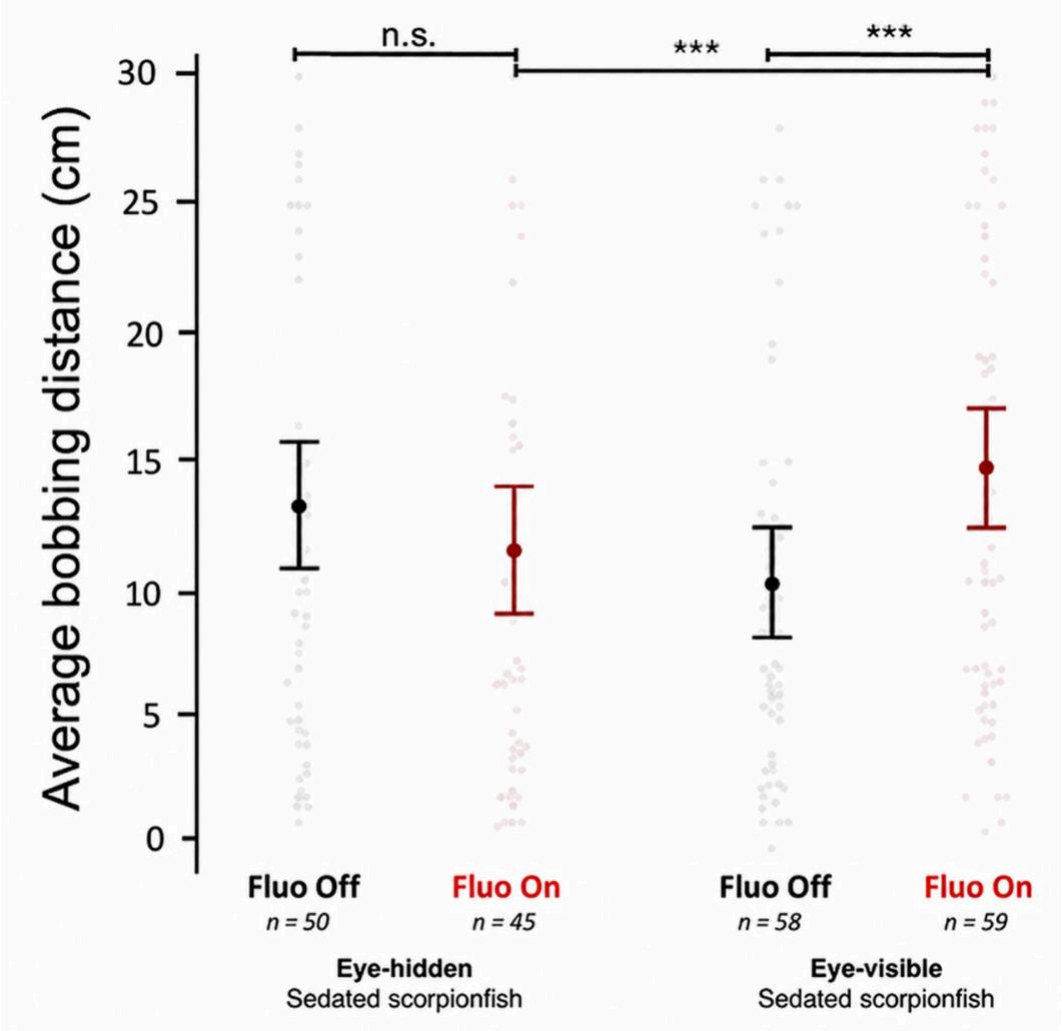

Figure 16: Results of the experiment: Manipulating scorpionfish pupil visibility. Average bobbing distance (cm) of *T. delaisi* in a 10-minute run as a function of pupil visibility (eye-hidden or eye-visible) and filter treatment (**Fluo-ON** or **Fluo-OFF**). Individuals were exposed to a lightly sedated *S. porcus* in which the eye was either hidden by a natural, non-fluorescent stone or visible, under both fluorescent treatments in a balanced paired design. **Fluo-OFF** blocked the red part of the spectrum, whereas **Fluo-ON** allowed red fluorescence to contribute to the illumination of the scorpionfish. Error bars show predicted means and 95% compatibility intervals (CI). **n.s.** denotes a non-significant difference, ******* denotes a significant difference.

Limitations

Although this experiment directly tested whether the fluorescence effect depends on the visibility of the scorpionfish eye, several limitations should be considered.

First, the experiment manipulated the visibility of the scorpionfish eye region rather than the pupil alone. The eye-hidden treatment removed the pupil as a visible optical target, but it may also have altered the visibility of nearby eye or head features. Therefore, although the results strongly suggest that the fluorescence effect depends on interaction with the retroreflective pupil, we cannot state with absolute certainty that the behavioural response was caused by an increase in

pupil-iris contrast. A more specific test would require surgically manipulating the scorpionfish pupil itself. However, such an intervention would be highly invasive and difficult to justify ethically, especially given the converging evidence from the visual model and the behavioural experiments.

Second, the experiment was conducted under controlled laboratory conditions. This was necessary to isolate the role of pupil visibility and fluorescence, but it also means that the results cannot be transferred directly to natural field conditions. In the field, light intensity, spectral composition, water clarity, background structure, predator orientation, and the viewing/gaze angle of the triplefin and scorpionfish all vary continuously. These factors could influence both the amount of red fluorescence produced by the triplefin and the strength of the light reflected from the scorpionfish eye. The experiment therefore shows that the mechanism can operate under controlled conditions, but further work would be needed to determine how often and under which conditions it operates under realistic field conditions.

Conclusion

This final experiment shows that the behavioural effect of red fluorescence is not explained by a general increase in scene visibility. If fluorescence simply made the predator easier to see, similar increases in bobbing distance should have occurred in both sedated scorpionfish treatments. Instead, the fluorescence-dependent increase in detection distance occurred only when the retroreflective scorpionfish pupil remained visible from the triplefin's perspective. This indicates that red fluorescence improves predator detection through interaction with the visible scorpionfish eye, supporting the hypothesis that fluorescence increases pupil–iris contrast by illuminating the retroreflective pupil. Together with the visual model, this experiment links the initial behavioural effect observed in Chapter 1 to the optical prediction tested in Chapter 3 to provide further proof that fluorescence can be used to increase the pupil-iris contrast in scorpionfish, thereby facilitating detection.

General conclusion

Main findings

In this dissertation I tested if *T. delaisi* can use its red fluorescent irises to improve the detection of *S. porcus,* a cryptic predator. More specifically, it tests whether red fluorescence can induce eyeshine in the scorpionfish pupil, thereby increasing pupil brightness and pupil-iris contrast from the triplefin's perspective and, in turn, improving predator detection.

Across the four chapters, my results provide stepwise support for this hypothesis and place it within a clear mechanistic framework. Below is a short summary of the main results.

In Chapter 1, I found that red iris fluorescence can improve the detection of a live scorpionfish under controlled conditions. Triplefins showed stronger predator-related behaviour when fluorescence could contribute to the illumination of a scorpionfish than when it could not, and this effect was observed only with live scorpionfish, not in the control treatment.

In Chapter 2, I aimed to isolate the contribution of a retroreflective scorpionfish pupil. For this I used 3D-printed scorpionfish models fitted with either retroreflective or non-retroreflective pupil inserts. Unfortunately, triplefins did not perceive these models as a threat and therefore showed an overall low response to the model. Although the results showed a weak trend in the predicted direction, they did not provide strong evidence that retroreflection alone can account for the observed behavioural effect found in Chapter 1.

In Chapter 3, I used a visual model to test whether red fluorescence could extend the distance over which the scorpionfish pupil can be discriminated from the surrounding iris. The model predicted that red fluorescence could increase this distance by approximately 1.6 to more than 5 cm, depending on the discrimination threshold used.

In Chapter 4, I then tested these model predictions in a final behavioural experiment. Here I used lightly sedated live scorpionfish and manipulated whether the eye was visible while controlling whether fluorescence could contribute to the illumination of the scorpionfish. Fluorescence improved predator detection by approximately 4.6 cm, but only when the scorpionfish eye was visible. When the eye was obscured, the fluorescence effect disappeared. This result coincides with the results of the visual model and rules out the alternative explanation that fluorescence merely improves general scene visibility. Instead, it shows that the behavioural effect depends on a specific optical target, namely the eye of a scorpionfish with its retroreflective pupil.

Interpretation and broader implications

The results support the interpretation that, in this study system and under these experimental conditions, red iris fluorescence can improve predator detection. Rather than generally enhancing vision, fluorescence appears to improve detection by exploiting a specific optical vulnerability created by the retroreflective scorpionfish pupil.

Evolution of red fluorescence structures in small micropredatory fish

From an evolutionary perspective, these findings highlight an advanced stage in a predator-prey sensory arms race centred on the eyes. Retroreflective pupils reduce detectability under ambient illumination but inherently return light towards their source (Howland, Murphy and McCosker, 1992; Benson and Suter, 2013; Feller and Cronin, 2014; Santon *et al*., 2018). By evolving a light source positioned close to the pupil, prey can exploit this vulnerability and compromise the predator's eye camouflage (Howland, Murphy and McCosker, 1992; Santon *et al*., 2020; van der Schoot and Michiels, 2025). I propose that red iris fluorescence represents a counteradaptation that targets a specific eye-camouflage strategy rather than enhancing general visual sensitivity. Because long-wavelength light is rapidly attenuated in water (Maritorena and Guillocheau, 1996; Woźniak and Dera, 2007), fluorescence-based illumination is effective only over short distances. This physical constraint potentially explains why red fluorescent structures are particularly common around the head and eye region in small micropredatory fish (Anthes *et al.*, 2016), where predator-prey interactions typically occur at close range and where even modest increases in detection distance can yield large survival benefits.

Could red fluorescence function as a semi-private sensory channel?

Since longer wavelengths are rapidly absorbed in water, red fluorescence is expected to function only at very short range (Johnsen, 2011; Marshall *et al.*, 2019). This could potentially mean that triplefins use their red fluorescence as a semi-private sensory channel. It is known that triplefins can perceive their own fluorescent signal (Kalb *et al.*, 2015). However, potential predators or more distant observers may detect it only weakly, or not at all, due to the increased distance.

One speculative possibility arises from the scorpionfish's eye reflectance and visual sensitivity. The fact that the scorpionfish pupil retroreflects particularly strongly in the red part of the spectrum (Santon *et al.*, 2018), suggests that longer wavelengths are not strongly absorbed by the scorpionfish eye but are instead reflected back out. If so, red fluorescence may be particularly useful to triplefins since they could use it to reveal the predator's pupil without necessarily making themselves highly visible to the predator.

However, this interpretation remains speculative and untested. Strong red retroreflection does not prove that scorpionfish cannot perceive red light, and as mentioned before fish without true red-sensitive cones may still detect longer wavelengths as increased luminance if the signal is strong enough. This could potentially be true for scorpionfish since their visual system has single and double cones, peaking at 455 and 530 nm (Schweikert *et al.*, 2018). Nevertheless, strong red retroreflection could mean that only a limited proportion of long-wavelength light is absorbed within the scorpionfish eye, potentially reducing, but not necessarily eliminating, the visibility of red fluorescence to the predator.

The role of fluorescence

This study provides the first clear experimental evidence for a mechanism in which fluorescence is used actively to enhance survival. Fluorescence has been proposed to mediate predator-prey interactions across a wide range of taxa, but direct experimental evidence of an active sensory role remains rare. In marine systems, fluorescence has been suggested to lure prey in corals (Ben-Zvi *et al.*, 2022) and gelatinous zooplankton (Haddock and Dunn, 2015). In terrestrial systems, fluorescence has been proposed to attract prey in carnivorous plants (Kurup *et al.*, 2013) and to modify predator behaviour in insects and amphibians (Lamb and Davis, 2020). In other cases, such as scorpions and caterpillars, fluorescence has been hypothesised to influence visibility to predators or to function in predator avoidance, but experimental support has so far been limited (Kloock, Kubli and Reynolds, 2010; Gaffin *et al.*, 2012; Gálvez, Nieto and Samaniego, 2020). Notably, studies using artificial models have frequently failed to detect effects of fluorescence on predator-prey interactions, including experiments with frogs (Whitcher, Beaver and Lemmon, 2024), fireflies (Wilcox and Lewis, 2019), and my experiment using scorpionfish models (van der Schoot and Michiels, 2025). Together, these mixed results suggest that fluorescence is unlikely to function broadly but instead plays a role only in specific ecological contexts.

Limitations

The first major limitation is that all experiments were conducted under controlled laboratory conditions. This was necessary to isolate the proposed mechanism and to manipulate pupil visibility in a controlled way so it could be thoroughly tested. However, natural predator-prey encounters are more variable. In the field, light intensity, spectral composition, water clarity, background structure, and gaze direction all change continuously. The experiments conducted for this dissertation therefore show that the mechanism can operate under controlled conditions, but they do not yet show under which ecological conditions it operates in the field.

A second limitation concerns the visual model. The model includes the most important optical components, including fluorescence emission, scorpionfish pupil and iris reflectance, light attenuation, and the spectral properties of the triplefin visual system. Nevertheless, it necessarily simplifies the interaction. In real encounters, fluorescence intensity, orientation, gaze direction, distance, and local light conditions are likely to vary among individuals and between situations.

Finally, this dissertation focuses on a single predator-prey interaction, between *T. delaisi* and *S. porcus*. The focus on a single system allowed the proposed mechanism to be tested thoroughly, but it also limits how broadly the findings can be generalized. The findings therefore apply mostly to this specific system, and further work is needed to assess whether comparable mechanisms occur in visual interactions between other species and/or under different optical conditions.

Although these limitations reduce the extent to which the results can be generalized, they also provide interesting directions for future research.

Future directions

The limitations outlined above point to two main future directions: testing the mechanism under realistic field conditions in the same study system, and assessing whether similar mechanisms occur in other predator-prey systems.

The first future direction is to test whether red fluorescent irises improve scorpionfish detection under natural field conditions. A useful first step would be to adapt the current visual model to field conditions. Much of the necessary data is already available, including measurements of ambient light and fluorescence emission from 50 triplefins at 22 m depth. These data could be used to estimate whether red fluorescence is predicted to increase scorpionfish pupil-iris contrast under natural light conditions.

If the field-based visual model supports this prediction, the next step would be a behavioural field experiment. This could be done using an adapted version of the submerged tank set-up developed by Santon et al. (2020), which was used to test whether triplefins use ocular sparks to detect scorpionfish. A similar set-up could be modified to test whether triplefins use red fluorescence to improve scorpionfish detection under real-life field conditions.

The second future direction is to ask how taxonomically widespread this mechanism is. In particular, it would be valuable to test whether other small micropredatory fish with fluorescent structures near the pupil also use them for predator detection. Promising candidates would include blennies (*Blenniidae*), gobies (*Gobiidae*), and dragonets (*Callionymidae*), which often face similar visual challenges in benthic habitats. On the predator (detection target) side, comparable systems may involve cryptic ambush predators with retroreflective pupils, such as scorpionfish (*Scorpaenidae*), stonefish (*Synanceiidae*), and toadfish (*Batrachoididae*). Such comparisons would help determine whether the mechanism described here is specific to the *Tripterygion delaisi-Scorpaena porcus* system or reflects a more broadly recurring visual solution.

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Appendix

frontiers | Frontiers in Ecology and Evolution

TYPE Original Research
PUBLISHED 03 December 2025
DOI 10.3389/fevo.2025.1728992

OPEN ACCESS

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RECEIVED 20 October 2025
REVISED 14 November 2025
ACCEPTED 17 November 2025
PUBLISHED 03 December 2025

CITATION
van der Schoot BKM and Michiels NK (2025) Improved predator detection through illumination with red fluorescence by a small benthic fish. Does it work?.
Front. Ecol. Evol. 13:1728992.
doi: 10.3389/fevo.2025.1728992

Improved predator detection through illumination with red fluorescence by a small benthic fish. Does it work?

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Background: Using controlled illumination to improve vision was believed to be limited to bioluminescent organisms in dark environments. Recent findings suggest that in shallow water, triplefins redirect sunlight to induce luminance contrast in the retroreflective pupils of nearby predators. At greater depths this mechanism becomes less effective. Triplefins, however, also possess red fluorescent irises. These may serve as an alternative light source to detect predator pupils in deeper waters by inducing a colour contrast. We tested whether triplefin iris fluorescence enhances the detection of predators.
Methods: In the first experiment, triplefins were exposed to a scorpionfish (predator) or a stone (control) under conditions that either allowed or prevented their iris fluorescence from illuminating the target. In the second experiment, triplefins were exposed to dummy scorpionfish with (1) retroreflective eyes, (2) non-retroreflective eyes, or (3) a live scorpionfish (control). In both experiments head bobbing was quantified as a known measure of caution.
Results: Triplefins bobbed more to live scorpionfish under all conditions, but significantly more when fluorescence could be used to illuminate the scorpionfish. Neither was the case for stones or (non-)retroreflective models. A weak fluorescence effect was found when comparing retroreflective versus non-retroreflective models.
Conclusions: Fluorescent irises may facilitate detection of retroreflective scorpionfish pupils. However, the weak response to the models suggests more than just retroreflection plays a role.

KEYWORDS

fluorescence, behavioural ecology, evolutionary ecology, predatory-preydynamics, active sensing

1 Introduction

Predation exerts one of strongest selection pressures on prey populations, as failure to evade predators can result in mortality, physical harm or stress-related fitness costs (Kerfoot and Sih, 1987; Preisser et al., 2005). Successful evasion requires prey to detect predators reliably and assess the risk they pose. To achieve this, prey integrate information from a variety of sensory modalities, including visual (Coates, 1980; Gerlai, 1993; Wisenden and Harter, 2001), chemical (Kats and Dill, 1998; Bronmark and Hansson, 2000; Hettyey et al., 2015), acoustic (Blumstein et al., 2008; Radford et al., 2012; Hettena et al., 2014), and mechanical cues (Janssen, 2004; Yack, 2016).

Aquatic vertebrates mostly rely on chemical (Chivers and Smith, 1998; Wisenden, 2000; Brown, 2003; McCormick and Manassa, 2008) and visual cues (Helfman, 1989; Smith and Belk, 2001; Murphy and Pitcher, 2005; McCormick and Manassa, 2008) for predator detection. Recent research revealed that fish mostly use chemical cues to indicate the presence of a predator and use visual cues to modify their response to the predator (Hartman and Abrahams, 2000; Chivers et al., 2001). Visual cues are often considered to be part of passive sensing systems since they rely on external conditions that are not controllable by the organism. One exception to this are cases where fish improve their vision by means of controlled illumination (Nelson and MacIver, 2006). Controlled illumination was previously believed to be limited to bioluminescent organisms (Hellinger et al., 2017; Howland et al., 1992; Kenaley et al., 2014; Sutton, 2005). However, recent findings suggest that diurnal fish such as the triplefin, *Tripterygion delaisi*, are also capable of using active illumination. As is the case for many fishes, triplefins use the spherical lens that protrudes from their pupil to focus downwelling light on a bright patch located on the iris below the pupil. This is called an "ocular spark" and can be turned on or off at will (Michiels et al., 2018). It is proposed that they can use their ocular sparks to generate perceptible reflections in the retroreflective eyes of nearby prey or cryptic predators, and defined this process as 'Diurnal Active Photolocation (DAP)' (Bitton et al., 2019; Santon et al., 2020). For DAP to be effective, three conditions must be met:

1. The target must possess strong retroreflective eyes, and the observer must possess a light source located nearly coaxial to its gaze, e.g. very close to the pupil (Greene and Filko, 2010; Jack, 2014; Michiels et al., 2018; Santon et al., 2018). The latter is required because retroreflectors reflect the incoming light in a narrow angle back to the light source. If the observer's light source is in line with its gaze, the light that is reflected back from the target's retroreflective eye will enter the observer's pupil.
2. The distance between observer and target is less than 10 cm (Bitton et al., 2019; Santon et al., 2020), making it more likely in small fishes that detect their predators (or prey) over short distances.
3. The ocular-spark-based variant of DAP requires strong directional downwelling sunlight, conditions usually found in waters shallower than 10 meters.

At increasing depths, clear marine light fields undergo a fundamental shift: Illumination changes from direct downwelling to bluish, scattered light (Warrant and Johnsen, 2013). This change in both the angular distribution and spectral quality significantly reduces the efficacy of ocular-spark-based light redirection. Clear deep water, however, provides excellent conditions for red fluorescence to stand out (Neumeyer et al., 2002; Harant et al., 2016, 2018; Wilkins et al., 2016; Bitton et al., 2017). This is not the case in shallow water: Although there is more excitation light available here, any red fluorescence is flooded by the overwhelmingly strong red component of the shallow light spectrum.

Triplefins, along with many other small benthic fish species (2–5 cm), possess strong red fluorescent irises (Figure 1) (Anthes et al., 2016; Michiels et al., 2018). In *T. delaisi*, fluorescence peaks at 609 nm with a broad excitation spectrum peaking in the green range (530 nm) (Bitton et al., 2017). Triplefins regulate their fluorescence in response to ambient light conditions and show significantly stronger fluorescence in waters deeper than 20 meters. This, combined with the fact that they can perceive their own fluorescence, suggest that it serves a visual function (Michiels et al., 2008, 2018; Wucherer and Michiels, 2012, 2014; Kalb et al., 2015; Harant et al., 2016). Because of this we hypothesize that a red fluorescent iris can function as a light source for DAP in deeper water, e.g. below 20 m.

In this study, we test whether the red fluorescent iris of *T. delaisi* improves its ability to visually detect a nearby black scorpionfish (*Scorpaena porcus*), a cryptic sit-and-wait predator with strong retroreflective eyes (Schwab et al., 2002; Fritsch et al., 2017; Santon et al., 2018, 2020). To test this, we performed two experiments. First, we tested if red fluorescence affected the behavioural response of a triplefin to a nearby scorpionfish. To do so we placed different filters that blocked specific wavelengths between the triplefin and the scorpionfish to control whether the triplefin's red fluorescence was allowed to contribute to the illumination of the scorpionfish or not. In the second, triplefins were confronted with a 3D-printed model of a scorpionfish with either a retroreflective or a non-retroreflective eye. A third group was tested with live scorpionfish as a positive control.

2 Methods

2.1 Location

Data collection took place at STARESO (Station de Recherches Sous Marines et Océanographiques, Calvi, France). Fish were collected and returned to the field under the general research permit of the station.

FIGURE 1
The triplefin *Tripterygion delaisi* in a blue-green environment at 29 m depth as seen by a human SCUBA diver (Nikon D500, natural light, manual white balance, LEE Sunset-Red colour correction filter) (N.K. Michiels). Inset: A triplefin under a broad light spectrum in 5 m depth, where fluorescence is overwhelmed by the strong red component of sunlight.

2.2 Model species

2.2.1 Triplefin (*Tripterygion delaisi*)

Triplefins (3–6 cm, Figure 1) are cryptobenthic micro-predators inhabiting rocky substrates between 5 and 50 m depth in the Mediterranean Sea and Eastern Atlantic Ocean. They use a saltatory movement pattern, consisting of short forward hops interspersed with pauses, during which they assess their surroundings with independently moving eyes. While exploring an environment, a triplefin often shows bobbing, which involves raising and lowering its anterior body in a "push-up" motion which takes one second to complete (Wirtz, 1978). Bobbing increases in frequency in the presence of a scorpionfish (Wirtz, 1978; Santon et al., 2021). Bobbing is not unique to triplefins, it has also been linked to threat perception in other taxa including: different gobies (Smith, 1989; McCormick and Manassa, 2008), harvestmen (Calhoun et al., 2025), jumping spiders (Geiger et al., 2025) and meerkats (Graw and Manser, 2007). We collected triplefins using hand nets at 20–30 m depth while SCUBA diving. Individuals from these depths fluoresce more strongly than those from shallower waters (Michiels et al., 2008; Wucherer and Michiels, 2012, 2014; Harant et al., 2016). After collection, fish were transported in perforated Falcon tubes and housed in flow-through tanks with natural substrates. To prevent downregulation of fluorescence, tanks were lit with dim blue-green light simulating 25 m depth, using RGB-LEDs (Cameo Q-SPOT 40 RGBW WH) with setting: red = 0, green and blue = 255 and covered with a double layer of "LEE 172 Lagoon Blue" filters. Spectrometry confirmed that no light > 570 nm was emitted under these settings (see electronic Supplementary Material, Supplementary Figures S1, S2).

2.2.2 Scorpionfish (*Scorpaena porcus*)

Scorpionfish are cryptobenthic, sit-and-wait macropredators common on hard substrates and seagrass beds across the Mediterranean Sea and Northeast Atlantic Ocean (Louisy, 2015). They prey on small cryptobenthic fishes, including triplefins (Compaire et al., 2018; Santon et al., 2018). Interestingly scorpionfish can produce acoustic signals that may be perceivable by triplefins (Kobayashi et al., 2004; Radford et al., 2012). Scorpionfish have large pupils with strong retroreflective properties, especially in the red spectrum (Figure 2) (Santon et al., 2018). This improves their camouflage but, as a trade-off, makes them prone to detection by observers possessing a light source, especially a red one, near their pupils. The rest of their body only fluoresces very weakly in the red range of the visual spectrum (John et al., 2023) (see electronic Supplementary Material, Supplementary Figure S3). We collected scorpionfish using hand nets at 2–10 m depth after sunset and housed them in large, shaded flow-through tanks with rocky shelters.

2.3 Experiment 1: Scorpionfish detection with and without fluorescence (Summer 2022)

We tested each triplefin individually in a white polyethylene tray (52 x 31 x 21 cm, Figure 3) featuring a central lane with glued on black sand (11.5 x 30 cm) that encouraged linear movement. At the end of the track, we placed a stimulus box with two compartments, both with a glass front. One compartment contained a scorpionfish (predator), the other a size-matched stone (control). Only one compartment was visible to the triplefins at any time. The box's perforated opaque side panels allowed olfactory cues to pass.

To control the contribution of red fluorescence to the visual perception of the stimulus by the triplefin we attached different filters to the glass fronts of the stimulus box. A "LEE 172 Lagoon Blue" (LB) filter, blocking the red part of the spectrum, was used in runs in which the contribution of fluorescence to visual assessment of the stimulus was blocked. A "LEE 209 0.3 Neutral Density" (ND)

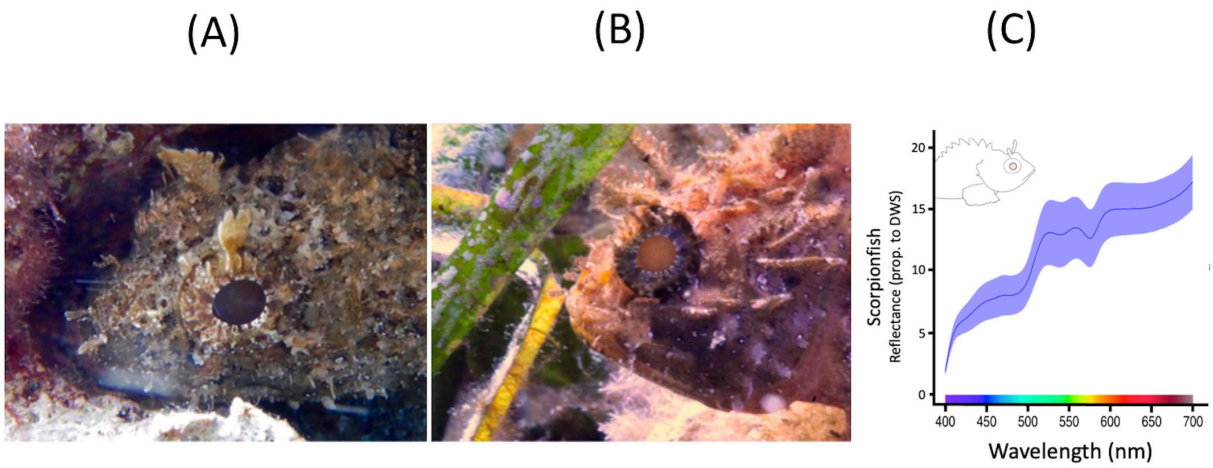

FIGURE 2
Scorpaena porcus in the field. **(A)** Shaded at 3 m depth, with dark pupil (weak eyeshine). **(B)** Exposed in 7 m depth, with strong eyeshine, caused by a mix of transmission-based and retroreflective eyeshine. **(C)** Pupil reflectance expressed as a proportion of the pupil photon radiance relative to a PTFE white diffuse reflectance standard (*y*-axis). A value of 15 means that pupil radiance is 15 times brighter than a 99% white reflectance standard when measured under the same conditions. Blue area: SE of mean curve (Santon et al., 2018).

filter was used in control runs to correct for the reduced brightness caused by the LB filter, while leaving the spectral shape of the light illuminating the stimulus unaffected. The Neutral Density filter allowed triplefins to also perceive longer wavelengths, including their own (reflected) red fluorescence. Spectrometry confirmed that both filters allowed about the same amount of light to pass under the experimental light conditions (LB: 1.451*1017 photons/s/sr/m/nm; ND: 1.276*1017 photons/s/sr/m/nm – measured by aiming at a diffuse white PTFE reflectance standard). Filters were replaced every 20 runs. This resulted in four treatments in a 2 x 2 design (Figure 4).

We exposed each triplefin to all four treatments (4 "runs") in one of four fixed treatment sequences. Since our main interest was

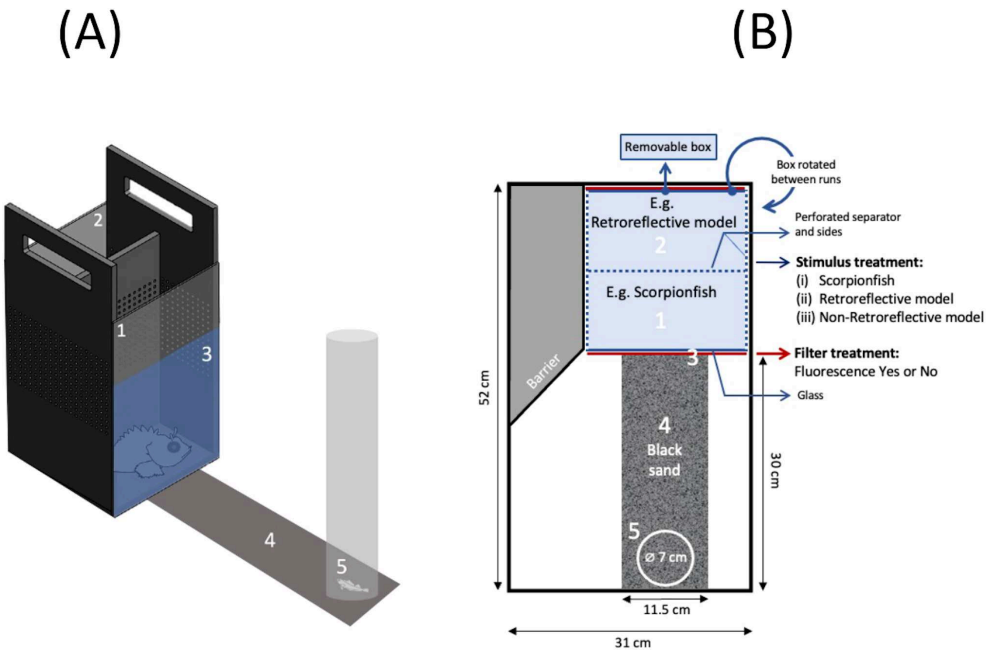

FIGURE 3
Schematic of the setup used in Experiment 1 and 2, built into a rectangular PE tray and filmed from above. **(A)** 3D view showing key elements. **(B)** Detailed top view. Triplefins are set free from a plexiglass cylinder (5) on a strip of glued black sand (4), which they prefer over the bright surface of the arena. Within 10 minutes, most individuals will gradually hop towards the stimulus box for close inspection. The box contains two compartments for two visual stimuli. Only one is visible to a triplefin through glass and a filter (3) at any time. The box can be rotated 180° or replaced by another box between runs. The top part of the black panels of the box are perforated for water exchange, allowing the transfer of chemical and acoustic cues between the scorpionfish and triplefin. They also prevent that the scorpionfish falls dry when taking the box out.

		Filter	
		Neutral-Density *transmits full spectrum*	Lagoon-Blue *blocks reds*
Stimulus	Stone (control)	Stone & **Fluo-ON**	Stone & Fluo-OFF
	Scorpionfish	**Predator** & **Fluo-ON**	**Predator** & Fluo-OFF

FIGURE 4
Table of possible treatment combinations of Experiment 1. Two stimuli, a stone and a scorpionfish, together with two different filters resulted in four different treatments. Fluo-ON refers to treatments with a Neutral-Density filter and Fluo-OFF refers to treatments with a Lagoon-Blue filter.

to reveal the difference between the scorpionfish with a Neutral-Density filter (Fluo-ON) versus the scorpionfish with a Lagoon-Blue filter (Fluo-OFF), a scorpionfish treatment always followed a scorpionfish treatment, and a stone treatment always followed a stone treatment (Figure 5).

Before each run, we placed the triplefin in an opaque holding container outside the setup for 2 minutes. Each run started with a 2-minute acclimation in a see-through plexiglass cylinder at one end of the setup (Figure 3, object 5) after which fish were released and recorded from above for 10 minutes using a GoPro (Hero black 7). To minimize stress and effects caused by individual differences between scorpionfish, we replaced scorpionfish repeatedly during the whole experiment (n = 35 *S. porcus*).

2.3.1 Video analyses experiment 1

During video analysis, we counted triplefin bobbing frequency, a measure of recognition of potential danger (Santon et al., 2021), using BORIS (Friard and Gamba, 2016). We also measured the minimum distance to the stimulus box (± 1 cm), the latency until triplefins reached this minimum distance (in seconds) and the average distance to the stimulus box during the 10-minute run. The average minimum distance of the triplefins to the display compartment across all treatments was 7 cm. However, with a median of 0 cm this indicates a heavily skewed distribution (Figure 6A). This also applies to the average distance (Figure 6B) and latency (not shown). Since only bobbing frequency showed significant treatment effects, the other measurements are not addressed in detail.

We tested a total of 127 triplefins, balanced across all treatment sequences, but excluded five triplefins due to technical problems. The final analyses include 122 triplefins. Data exploration indicated that bobbing became less frequent during the four consecutive runs, indicating strong habituation to the set-up. Some triplefins did not perform a single "bob" during all runs. We therefore chose to only include the first two runs of each triplefin that bobbed at least once during one of these two runs. This still allowed us to compare a visual stimulus with both fluorescent treatments within each triplefin (n = 52 and n = 51 for stone and scorpionfish respectively).

2.4 Experiment 2: Isolating eye-retroreflection (Summer 2023)

The first experiment did not allow us to distinguish whether triplefins responded specifically to the retroreflective eyes of the scorpionfish or to other visual features. To address this, we conducted a second experiment that isolated the retroreflective-eye effect using 3D printed scorpionfish models. We created realistic scorpionfish models by 3D-printing them with matt grey PLA filament (1.75 mm, FormFutura, Nijmegen, the Netherlands) using a Prusa i3 MK3s printer (Prusa Research, Czech Republic). After hand-painting the models with commercial acrylic paints and a matte varnish, we soaked them in saltwater for several days before use (Figure 7).

The models had a 15° tilt to orient their gaze to a point on the substrate at 10–12 cm from the model's eye. All models shared an

	Subsequent runs per individual within one treatment sequence			
	1st run	2nd run	3rd run	4th run
Four treatment sequences (1 sequence per tested individual) 1	Stone & Fluo-ON	Stone & Fluo-OFF	**Predator** & Fluo-ON	**Predator** & Fluo-OFF
2	Stone & Fluo-OFF	Stone & Fluo-ON	**Predator** & Fluo-OFF	**Predator** & Fluo-ON
3	**Predator** & Fluo-ON	**Predator** & Fluo-OFF	Stone & Fluo-ON	Stone & Fluo-OFF
4	**Predator** & Fluo-OFF	**Predator** & Fluo-ON	Stone & Fluo-OFF	Stone & Fluo-ON

FIGURE 5
Table of each possible treatment sequences that a triplefin could encounter during Experiment 1. Each triplefin was tested 4 times (4 runs) and represents a single "treatment sequence". A scorpionfish treatment always followed a scorpionfish treatment, and a stone treatment always followed a stone treatment to ensure a balanced design.

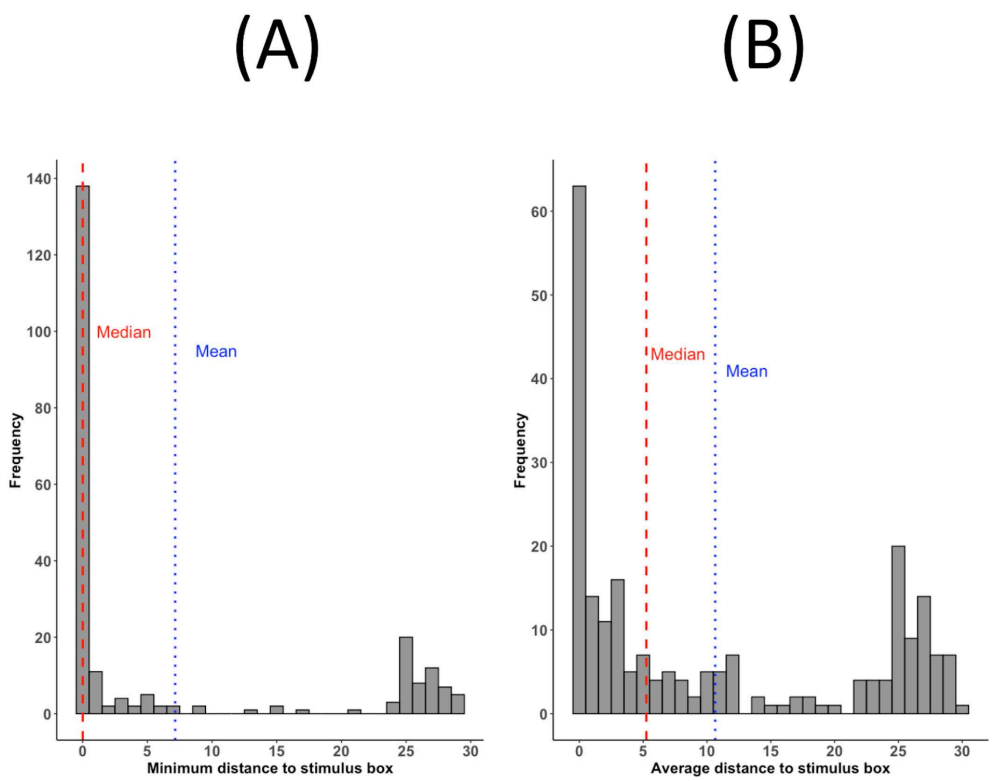

FIGURE 6
Heavily skewed distributions of the **(A)** Minimum distance (mean:7, median:0) and **(B)** Average Distance (mean:11, median:5) of Experiment 1.

identical pupil area of 50.27 mm². We produced the pupil inserts separately from PVC cylinders, allowing us to fit either a retroreflective or a non-retroreflective artificial eye.

For the retroreflective eye treatment, we used Orafol ("ORALITE Commercial Grade 5410") which generates strong white retroreflection. We overlaid the retroreflector with a LEE 025 Sunset Red filter (Lee Filters) to mimic the warm reddish glow observed in natural scorpionfish eyes under coaxial illumination with white light. The filter also suppressed any bluish reflection caused by environmental blue-green light (< 550 nm). Spectrometry confirmed that the artificial eye's retroreflection was 5–10 times stronger than that of a real scorpionfish's eye in the long-wavelength range (Figure 8). It was a deliberate choice to use a "super-stimulus". For the non-retroreflective eye treatment, we inserted a piece of diffuse white underwater paper (Weatherproof transparencies, Avery Zwerckform GmbH), also covered with a LEE 025 Sunset Red filter (Lee Filters). It ensured that both eye types appeared similar under ambient light without coaxial illumination. We refrained from using a glass lens in the models since previous trials showed that they produce reflection artifacts on their outer surface which are virtually absent in the cornea of real scorpionfish. As a third visual stimulus we included a live scorpionfish in the experiment to validate the findings from the first experiment.

In all three treatments, the contribution of red fluorescence was controlled by using filters attached to the glass front of the stimulus box as in experiment 1 (LEE 172 Lagoon Blue and LEE 209 0.3 Neutral Density for Fluo-OFF and Fluo-ON), resulting in 6 treatments (Figure 9).

We presented a single stimulus (retroreflective model, non-retroreflective model or scorpionfish) to each triplefin under both fluorescent treatments (Fluo-ON and Fluo-OFF). This design limited the number of runs per triplefin to two. To address potential effects of olfactory, chemical and acoustic cues, a live scorpionfish was always present in a perforated opaque box within the arena while running the model treatments. This opaque box was empty in runs with a live scorpionfish stimulus. The testing procedure and data collection were identical to experiment 1.

2.4.1 Video analyses experiment 2

We filmed all runs using GoPros (Hero 7 Black) and quantified bobbing frequency, using BORIS (Friard and Gamba, 2016). To ensure that scorpionfish behavior did not confound the results, we also recorded their movement during each run. We quantified total movement duration per run using BORIS and used these data to test whether Fluo-ON and Fluo-OFF treatments differentially affected scorpionfish activity, potentially offering an alternative explanation for the patterns observed in Experiment 1.

2.5 Statistical analyses

We conducted all statistical analyses in R (R Core Team, 2024), using the glmmTMB package (Brooks et al., 2017), following the framework established in Santon et al. (2023). In the following, we explain our data analyses for each experiment in detail.

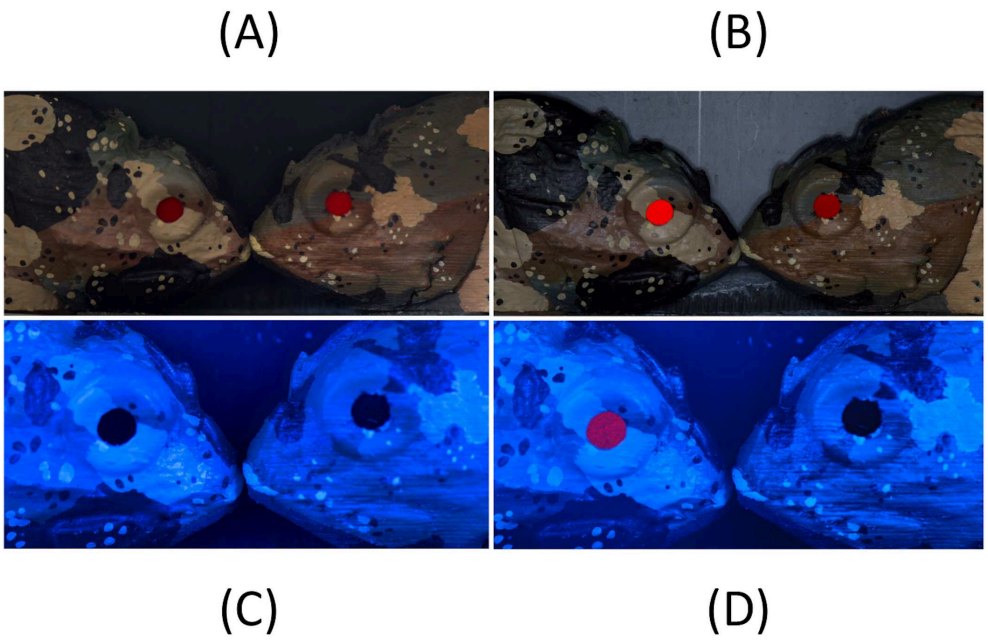

FIGURE 7
Photos of 3D-printed scorpionfish models used in Experiment 2. In all photos the left model is a model with retroreflective eye-insert and the right model is a model with non-retroreflective eye-insert. Both models are fitted with a Sunset Red filter in the eye to simulate the natural reddish retroreflection of a scorpionfish's eye. **(A)** Photo taken under ambient white light, no-coaxial light. **(B)** Photo taken under ambient white light, with added coaxial white light. **(C)** Photo taken under the blue-green light conditions of the set-up, no-coaxial light. **(D)** Photo taken under the blue-green light conditions of the set-up, with added coaxial red light.

2.5.1 Experiment 1

2.5.1.1 Scorpionfish detection with and without fluorescence

To evaluate whether red fluorescence influenced scorpionfish detection, we analyzed bobbing frequency across treatments during the first two runs for each triplefin, which were either exposed to a stone under Fluo-ON and Fluo-OFF conditions or a live scorpionfish under Fluo-ON and Fluo-OFF.

The model used a negative binomial distribution (linear parameterization with log-link) (Hardin and Hilbe, 2012) with

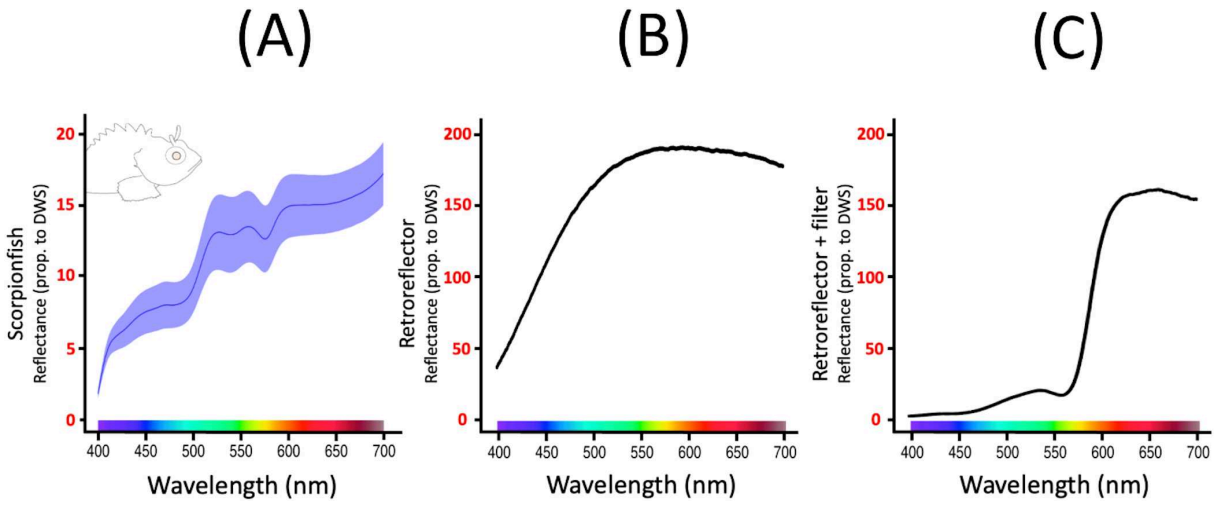

FIGURE 8
Wavelength-dependent retroreflective eyeshine in *Scorpaena porcus*. **(A)** Pupil reflectance expressed as a *proportion* of the pupil photon radiance relative to a PTFE white diffuse reflectance standard (y axis). A value of 15 means that pupil radiance is 15 times brighter than a 99% white reflectance standard when measured under the same conditions. Blue area: SE of mean curve (modified from: Santon et al., 2018). **(B)** Retroreflector reflectance expressed as a proportion of the pupil photon radiance relative to a PTFE white diffuse reflectance standard (y axis). **(C)** Retroreflector covered by a LEE 025 Sunset Red filter, as used in Experiment 2, expressed relatively to a scorpionfish pupil.

		Filter / fluorescence treatment	
		Neutral-Density *transmits full spectrum*	Lagoon-Blue *blocks reds*
Visual stimulus	Model (control)	Retro-OFF & **Fluo-ON**	Retro-OFF & Fluo-OFF
	Model (retroreflective)	**Retro-ON** & **Fluo-ON**	**Retro-ON** & Fluo-OFF
	Scorpionfish (reference)	**Predator** & **Fluo-ON**	**Predator** & Fluo-OFF

FIGURE 9
Table showing the possible treatment combination of Experiment 2. Three different visual stimuli (Model without retroreflective eyes, Model with retroreflective eyes and a Scorpionfish) together with two fluorescent treatments (Fluo-ON and Fluo-OFF) result in the six different treatments.

three fixed components: (1) stimulus (stone or scorpionfish), (2) filter (Fluo-ON or Fluo-OFF), and (3) the interaction term between stimulus and filter. To account for repeated measures, we included Triplefin ID as a random effect (Schielzeth and Forstmeier, 2009). Additionally, we incorporated a zero-inflation formula following a constant (= 1) to address potential excess zeros in the count data (Campbell, 2021). To verify that the model did not violate key assumptions, we checked residual distribution, residuals against predictors (split by random factor levels), dispersion, zero inflation, data distribution and autocorrelation. We also confirmed that there were no order, time, or date effects.

2.5.2 Experiment 2

2.5.2.1 Scorpionfish movement

To check for possible correlation between the movement of a scorpionfish and the bobbing frequency of triplefins, a model was made based on a negative binomial distribution with a linear parameterization and a log-link function (Hardin and Hilbe, 2012). The model contained the bobbing frequency of triplefins as response variable and scorpionfish movement as a fixed component. A zero-inflation formula following a constant (= 1), was added (Campbell, 2021). To verify that the model did not violate key assumptions, we checked residual distribution, residuals against predictors (split by random factor levels), dispersion, zero inflation, data distribution and autocorrelation. We also confirmed that there were no order, time, or date effects.

2.5.2.2 Isolating eye-retroreflection

To examine whether eye retroreflection influenced bobbing behaviour, we constructed a model using a negative binomial distribution with log-link function and linear parameterization (Hardin and Hilbe, 2012) with three fixed components: (1) stimulus (retroreflective model, non-retroreflective model, or scorpionfish), (2) filter (Fluo-ON or Fluo-OFF), and (3) the interaction between stimulus and filter. Triplefin-ID was added as a random term to account for the two repeated measurements of each triplefin (Schielzeth and Forstmeier, 2009). A zero-inflation formula following the filter component and a dispersion formula following the stimulus and filter components was added (Campbell, 2021). To verify that the model did not violate key assumptions, we checked residual distribution, residuals against predictors (split by random factor levels), dispersion, zero inflation, data distribution and autocorrelation. We also confirmed that there were no order, time, or date effects.

3 Results

3.1 Experiment 1: Scorpionfish detection with and without fluorescence

In experiment 1, we confronted a triplefin with either a scorpionfish or a stone under both Fluo-ON and Fluo-OFF conditions. The absolute number of bobs per run differed across the treatments (Figure 10, Table 1): Triplefins bobbed 50 times (95% compatibility interval: 35.4-69.8) when confronted with a scorpionfish under the Fluo-ON treatment. This was significantly decreased when confronted with a scorpionfish with Fluo-OFF (26.3 times, 95% CI: 18.2-37.7). In contrast, they only bobbed 12.7 times (95% CI: 8.8-18.0) when seeing a stone under Fluo-ON, which was similar to seeing a stone under Fluo-OFF (12.9 times, 95% CI: 8.9-18.9).

3.2 Experiment 2: Scorpionfish movement

When comparing the movements of the scorpionfish between the Fluo-OFF and Fluo-ON treatments, we found no effect of filter type on the movement of the scorpionfish (see electronic Supplementary Material, Supplementary Figure S4). We further analyzed if scorpionfish movement, expressed as movement in seconds during a run, was related to the bobbing frequency of a triplefin. We found no correlation between the movement durations of a scorpionfish and the bobbing frequency of a triplefin (see electronic Supplementary Material, Supplementary Figure S5). Since we can assume that

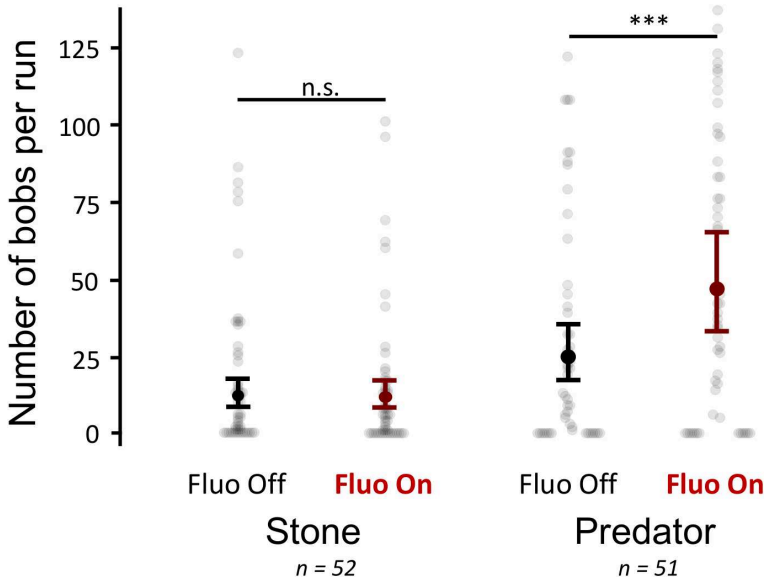

FIGURE 10
Experiment 1, Stone vs Scorpionfish. Absolute bobbing frequency in a 10-minute run as a function of visual stimulus (Stone or Predator) and filter treatment (Fluo-ON or Fluo-OFF). *T. delaisi* individuals were tested in a paired design per visual stimulus, and therefore either saw a stone or a Predator, under both Fluo-ON and Fluo-OFF treatments in varying and balanced order. Predicted means and 95% compatibility intervals (CI), based on a negative binomial distribution with a log-link. n.s. denotes a non-significant difference, *** denotes a significant difference.

scorpionfish do not behave differently depending on the treatment, and that scorpionfish movement does not influence the bobbing frequency of a triplefin, it is safe to assume that the main results cannot be explained by a confounding effect caused by scorpionfish movement.

3.3 Experiment 2: 3D printed scorpionfish model retroreflection present or absent

In experiment 2, we confronted a triplefin with either a non-retroreflective model, a retroreflective model or a live scorpionfish (predator) under both Fluo-ON and Fluo-OFF conditions. Triplefins showed more bobbing when confronted with a predator under Fluo-ON conditions 71.8 times (95% compatibility interval: 59.8-86.1) compared to a predator under Fluo-OFF conditions 46.9 times (95% compatibility interval: 36.7.8-59.3). This confirms the results from experiment 1. When comparing fluorescent conditions (Fluo-ON and Fluo-OFF) within the two model treatments, we found no difference in bobbing frequency (Figure 11, Table 2). However, triplefins bobbed more often when confronted with the retroreflective model under Fluo-ON conditions 20.8 times (95% compatibility interval: 15.4-27.9) in comparison with the non-retroreflective model under both the Fluo-ON 11.6 times (95% compatibility interval: 7.8-17.0) and Fluo-OFF 9.0 times (95% compatibility interval: 5.6-14.7) conditions. This difference is absent when comparing the Fluo-OFF condition of the retroreflective model with the Fluo-ON and the Fluo-OFF condition of the non-retroreflective model (Figure 11, Table 2).

TABLE 1 Pairwise comparisons between the different treatments of Experiment 1.

Contrast	Ratio	SE	Lower-CI	Upper-CI
Predator Fluo-ON/ Predator Fluo-OFF *	1.904	0.379	1.288	2.807
Stone Fluo-ON/ Stone Fluo-OFF	0.977	0.204	0.649	1.464

Contrast, Ratio, Standard Error and 95% compatibility intervals, * denotes significantly different comparisons.

4 Discussion

We tested whether the red light emitted by a triplefin's fluorescent iris enhances its ability to detect a nearby scorpionfish (Experiment 1), and whether this can be explained by inducing and detecting fluorescence-induced eyeshine in the scorpionfish (Experiment 2).

Experiment 1 shows that bobbing, an indicator of seeing something "potentially dangerous" in triplefins (Santon et al., 2021), was particularly high in response to a scorpionfish relative to a stone, regardless of the FLUO treatment. This confirmed that bobbing in triplefins can be considered a reliable indicator of perceived danger, whereas a lack of it is associated with a safe visual scene (Smith, 1989; Graw and Manser, 2007; McCormick and Manassa, 2008; Calhoun et al., 2025; Geiger et al., 2025). Moreover, in the presence of a scorpionfish, bobbing was observed significantly more when fluorescence was "ON" relative to the "OFF" situation. This suggests that red fluorescent irises may indeed contribute to early detection of a scorpionfish.

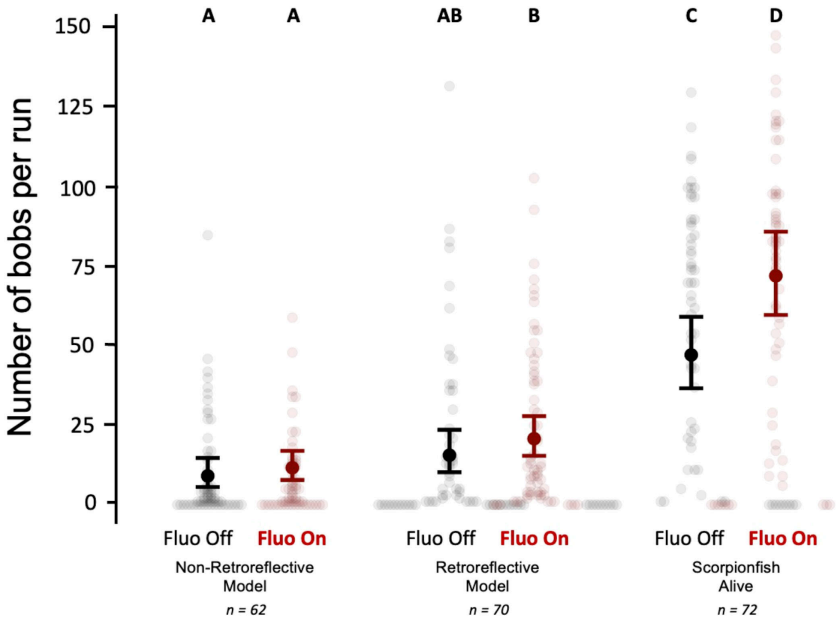

FIGURE 11
Experiment 2, 3D models vs Predator. Absolute bobbing frequency in a 10-minute run as a function of visual stimulus (Non-retroreflective model, Retroreflective model or Predator) and filter treatment (Fluo-ON or Fluo-OFF). *T. delaisi* individuals were tested in a paired design per visual stimulus, and therefore either saw a Retroreflective model, a Non-retroreflective model, or a Scorpionfish, under both Fluo-ON and Fluo-OFF treatments in varying and balanced order. Predicted means and 95% compatibility intervals (CI), based on a negative binomial distribution with a log-link. Different letters indicate a significant difference between the treatments.

However, these results cannot demonstrate that fluorescence was used to induce and detect retroreflective eyeshine in scorpionfish. Moreover, we did not check whether the scorpionfish moved more under the Fluo-ON than under the Fluo-OFF treatment. These issues were addressed during experiment 2. Here we showed that live scorpionfish did not move more in the Fluo-ON treatment and that there was no correlation between scorpionfish movement and triplefin bobbing frequency. Bobbing in triplefins is not exclusively associated with perceived threats, as triplefins also exhibited this behaviour when confronted with a stone. The comparatively low number of bobs observed in this context may be explained by motion parallax, a depth perception mechanism in which objects at varying distances appear to move at different speeds as the observer moves its head (Gibson et al., 1959; Kral, 2003; Bruckstein et al., 2005).

Experiment 2 confirmed a strong bobbing response toward live scorpionfish, with a significant difference between the fluorescence ON and OFF conditions, consistent with the results of Experiment 1. In contrast, responses to the retroreflective models were considerably weaker. Although the fluorescence ON/OFF comparison for these models showed only a subtle trend in the predicted direction, triplefins displayed more frequent bobbing toward retroreflective models with fluorescence ON than toward both non-retroreflective model treatments. This difference was not present when comparing the retroreflective model with fluorescence OFF against both non-retroreflective models. This suggests that models lacking retroreflective eyes are perceived as neutral objects, similar to stones, whereas retroreflective models elicit a response indicative of fluorescence-based retroreflection. The weak trend observed here might become more pronounced if the models

TABLE 2 Pairwise comparisons between the different treatments of Experiment 2.

Contrast	Ratio	SE	Lower-CI	Upper-CI
Predator Fluo-OFF/Predator Fluo-ON *	0.715	0.102	0.540	0.946
Retroreflective Fluo-OFF/Retroreflective Fluo-ON	0.820	0.210	0.499	1.348
Non-retroreflective Fluo-OFF/Non-retroreflective Fluo-ON	0.851	0.262	0.466	1.556
Retroreflective Fluo-ON/Non-retroreflective Fluo-ON *	0.560	0.136	0.348	0.901
Retroreflective Fluo-ON/Non-retroreflective Fluo-OFF *	0.477	0.134	0.275	0.827
Retroreflective Fluo-OFF/Non-retroreflective Fluo-ON	0.683	0.195	0.391	1.194
Retroreflective Fluo-OFF/Non-retroreflective Fluo-OFF	0.582	0.182	0.314	1.076

Contrast, Ratio, Standard Error and 95% compatibility intervals, * denotes significantly different comparisons.

were more lifelike (Brown and Warburton, 1997). Overall, these findings reinforce the idea that fish integrate multiple sensory modalities when detecting potential predators (Chivers and Smith, 1998; Blumstein et al., 2008; Radford et al., 2012; Hettena et al., 2014; Hettyey et al., 2015).

Research on active photolocation has mainly focused on two different light sources: redirected downwelling light (Bitton et al., 2019; Neiße et al., 2020; Santon et al., 2020) and bioluminescent organs (Howland et al., 1992; Hellinger et al., 2017) as the main light sources. Although it has been proposed that fluorescence could function as a light source for active photolocation (Jack, 2014), most of the studies on fluorescence focused on alternative function such as camouflage, photo-enhancement, intraspecific communication, prey attraction, photoprotection, or stress mitigation (Haddock and Dunn, 2015; Ben-Zvi et al., 2022; Poding et al., 2024). The use of red fluorescence for active photolocation can be expected to be limited to short-ranged interactions due to the low amounts of light involved. This aligns well with the observation that small benthic fishes in particular evolved red fluorescent irises, which may facilitate short-range visual predator detection (Anthes et al., 2016; Michiels et al., 2018).

Our findings highlight the potential for red fluorescence to serve as an alternative light source for diurnal active photolocation in blue-green environments at depth or in permanent shade, where bluish, scattered light is the dominant illuminant.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements. The general scientific permit of the STARESO Marine Station includes a general permission to collect animals for scientific purposes.

Author contributions

BS: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. NM: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research and/or publication of this article. Nico K. Michiels was supported by the Deutsche Forschungsgemeinschaft (grant number: MI 482/18-1).

Acknowledgments

We thank STARESO and its staff for their kind support and hosting us, and Leonie John, Mario Schädel, Lena Wesenberg, and Patrick Weygoldt for assistance in the field.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

1. Light field in the room in which the triplefins were housed

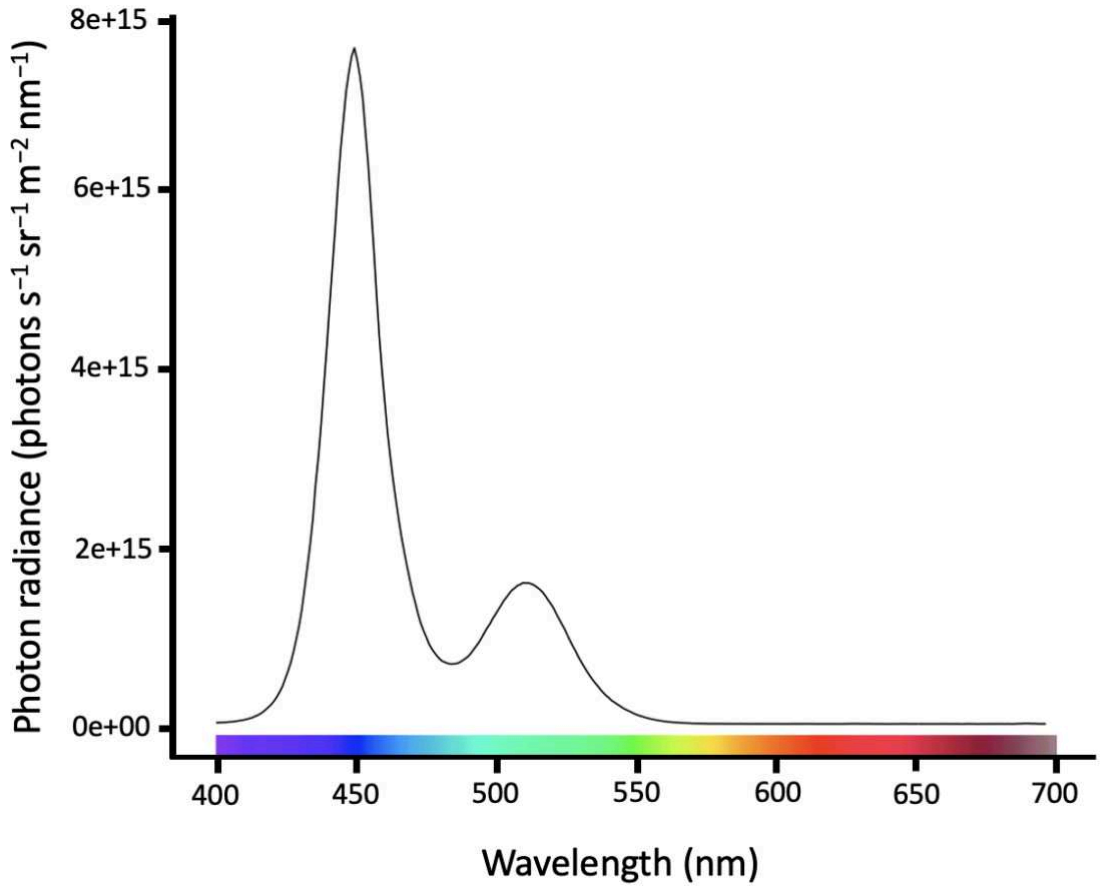

Figure S1: Light field in the room in which triplefins were housed (Y-axis linear). Photon radiance of a diffuse white standard (PTFE) taken with a SpectraScan® PR-740 spectroradiometer.

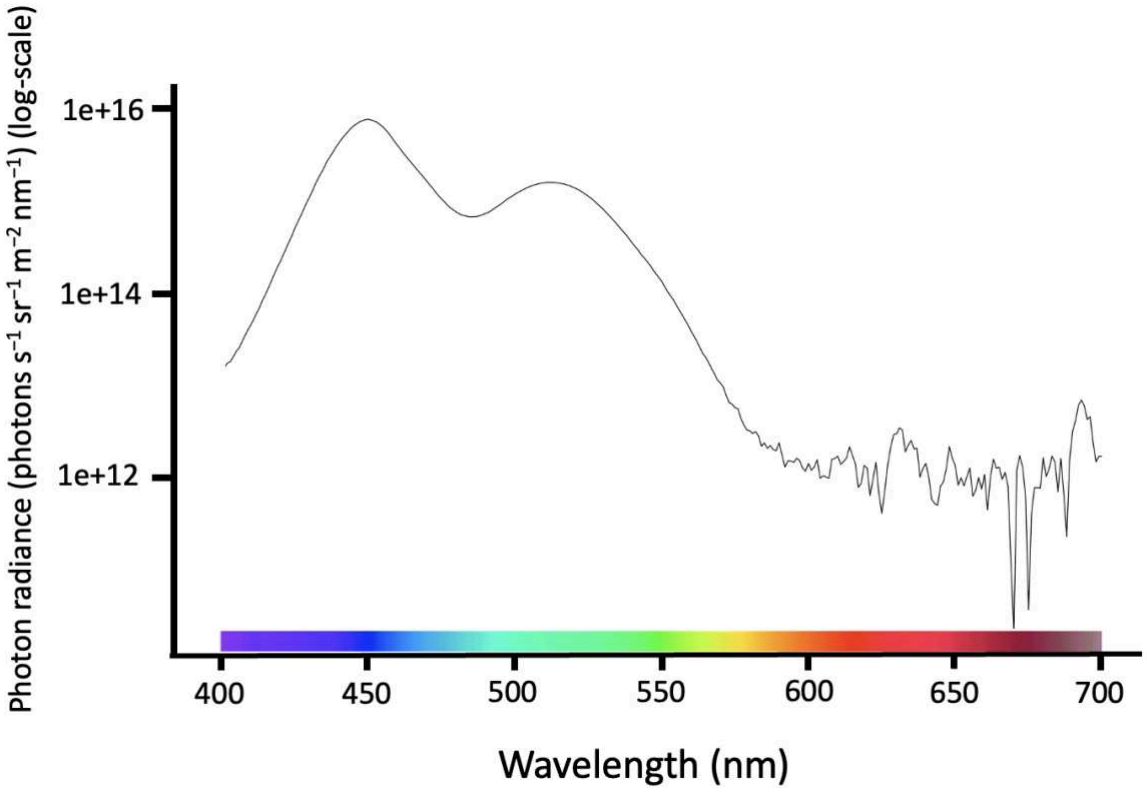

Figure S2: Light field in the room in which triplefins were housed (Y-axis $\log_{10}$ transformed). Photon radiance of a diffuse white standard (PTFE) taken with a SpectraScan® PR-740 spectroradiometer. Same data as in Figure S1, log transformed.

2. *S. porcus* red fluorescence

Figure S3: Photo of *S. Porcus* photographed in the lab under strong blue LED light as seen through a yellow filter to block the excitation light. The red fluorescence of a *S. porcus* is limited and weak in comparison to other scorpionfish species (Anthes et al. 2026) (picture by Jenny Theobald)

3. *S. porcus* movement

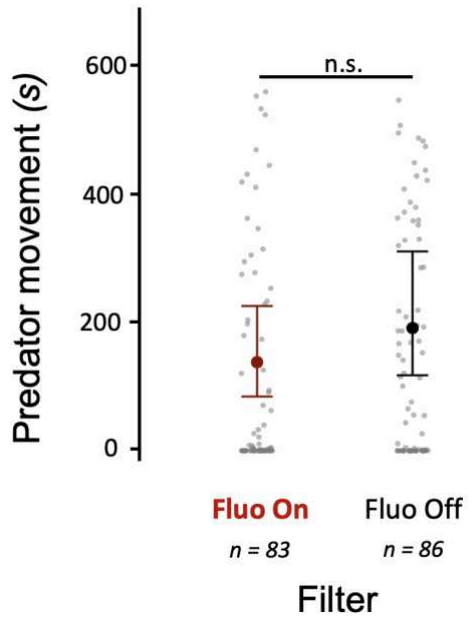

Figure S4: Effect of the different filters on the movement of a *S. porcus*.
There is no correlation between the different filters and the total time (seconds) that a black scorpionfish (*S. porcus*) was moving during a run.

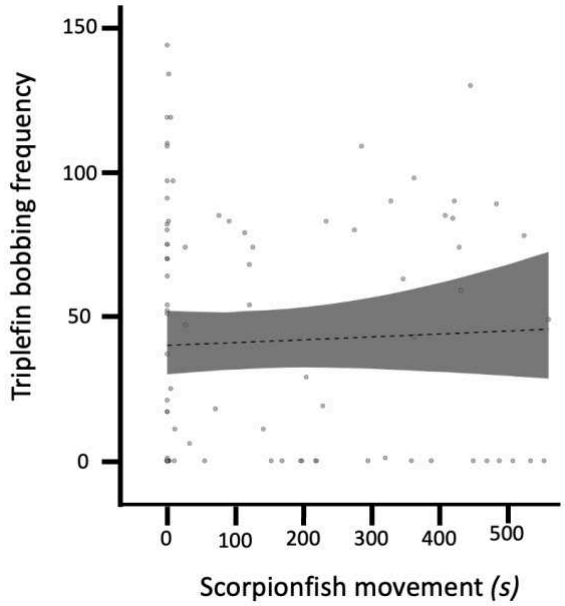

Figure S5: Effect of scorpionfish movement on the total number of bobs of a triplefin during a run.
There is no correlation between scorpionfish movement and the number of bobs a triplefin performs. Dashed line indicates the model predictions, and the grey error zone represents the 95% compatibility intervals (CI) (see also Fig. S4)

Active sensing with light: Fish use fluorescence to visually detect predators

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Keywords: Fluorescence, Behavioural ecology, Evolutionary ecology, Predator-prey dynamics, Active sensing, Visual ecology

Abstract

Retroreflective eyeshine enhances eye camouflage in cryptic predators but creates an optical vulnerability by returning light towards its source. Here, we show that small benthic reef fish exploit this weakness by using red iris fluorescence to actively enhance predator eye detection. Using controlled behavioural experiments with lightly sedated live scorpionfish, we demonstrate that triplefins detect predators at significantly greater distances when red fluorescence contributes to illumination, but only when the scorpionfish's pupil is visible. This effect disappeared when the pupil was obscured, indicating that detection depends on interaction with the retroreflective pupil rather than a general increase in scene visibility. A visual model confirms that fluorescence increases pupil-iris contrast over distances that closely match the behavioural response. Together, these results provide causal evidence that red fluorescence can function as an active sensory tool, revealing fluorescence-induced diurnal active photolocation in predator-prey interactions.

Introduction

Cryptic sit-and-wait predators create a particularly strong selective challenge for their prey. By combining visual concealment with rapid close-range strikes, they reduce the time available for prey to detect and respond to an attack. This should favour the evolution of sensory mechanisms that allow prey to detect cryptic predators before an attack is initiated (Stevens and Merilaita, 2009; Moore and Biewener, 2015).

Sit-and-wait predators minimise visual detectability not only of their body, but specifically also their eyes (Feller and Cronin, 2014; Santon *et al.*, 2018). Vertebrate pupils are typically circular, black and sharply defined, making them easy detection targets even when the surrounding body is well camouflaged (Cuthill and Székely, 2009; Douglas, 2018). Many aquatic predators therefore reduce eye conspicuousness through pupil closure, reduced eye size, modified pupil shape, iris patterning or skin flaps that partly cover the pupil (Cott, 1940). An alternative strategy is to brighten the pupil rather than to hide or reshape it. In several cryptic fishes, retroreflective structures behind the retina produce *daytime eyeshine*, causing the pupil to reflect ambient light and thereby reducing pupil-iris contrast (Lythgoe, 1975; Santon *et al.*, 2018). This mechanism is especially pronounced in scorpionfishes (Fam. *Scorpaenidae*), common cryptic sit-and-wait predators whose pupils can closely match the hue and brightness of the surrounding iris and skin (Santon *et al.*, 2018).

However, pupil camouflage based on retroreflection creates an optical vulnerability. Since retroreflectors return incident light back towards their source, an observer with a light source close to its pupil can make a retroreflective predator pupil light up (Santon *et al.*, 2018, 2020). Triplefins (*Tripterygion delaisi)*, a small benthic fish, potentially exploits this mechanism to detect a predator, the scorpionfish (*Scorpaena porcus)*. At depths of 3-10 m, triplefins reflect downwelling sunlight sideways using their irises which improves scorpionfish detection (Santon et al., 2020). This mechanism is expected to function best in shallow water where a large part of the downwelling sunlight remains sufficiently directional and intense.

Yet, *T. delaisi* also occurs well below 20 m. At these depths, the underwater light field is dim, diffuse and limited to the blue-green parts of the visual spectrum. These conditions may reduce the effectiveness of ocular-spark-based illumination, but they also provide suitable excitation light for the red fluorescence strongly expressed in the irides of *T. delaisi* (Michiels et al., 2008; Harant et al., 2016, 2018; Bitton et al., 2017; Figure 1a). A red, near-coaxial light source in a bluish environment suggests an ideal configuration to detect retroreflective eyes (Jack, 2014; van der Schoot and Michiels, 2025).

Previous experiments showed that triplefins displayed more predator-related bobbing when their red iris fluorescence was allowed to contribute to the illumination of a scorpionfish (van der Schoot and Michiels, 2025). This response was consistent with improved predator detection, but the experiment did not establish whether it was caused by triplefins perceiving fluorescence-induced reflections from the scorpionfish pupil (Figure 1b–c). It therefore remained unclear whether fluorescence improved

detection specifically by revealing the retroreflective pupil or through a more general change in the appearance of the predator or visual scene.

Here, we tested whether improved detection with fluorescence is specifically linked to the visibility of the scorpionfish's pupil. We presented triplefins with a lightly sedated scorpionfish, whose eye facing the triplefin was either visible or hidden, while controlling whether iris fluorescence illuminated the scorpionfish eye (**Fluo-ON**) or not (**Fluo-OFF**). If iris fluorescence improves predator detection, the fluorescence effect should be present only when the **eye** of the scorpionfish is visible. By contrast, if fluorescence simply improves general scene visibility, the effect should persist even when the eye is hidden. To validate the results, we add the results of a visual model that assesses the chromatic pupil-iris contrast of a scorpionfish from the perspective of an approaching triplefin with and without fluorescence.

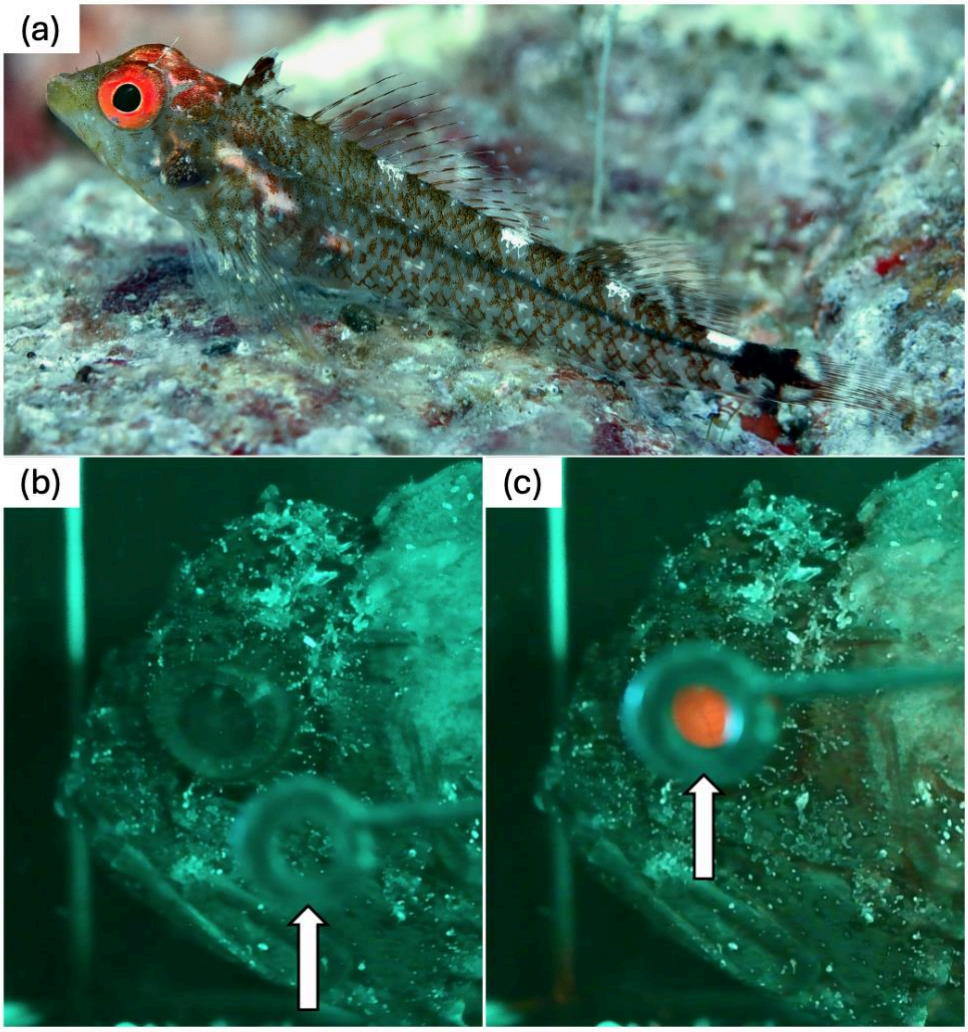

Figure 1: Red iris fluorescence in triplefins and fluorescence-induced retroreflection in scorpionfish pupils. **a)** *Tripterygion delaisi* triplefin at 30 m depth under natural light showing its red fluorescent iris (with colour-correcting filter). **b)** *Scorpaena porcus* scorpionfish under blue LED light in the lab with colour correcting yellow filter, showing no retroreflection in response to non-coaxial position of an artificial red fluorescent iris (white arrow, facing away from the camera). **c)** red fluorescent retroreflection when artificial red fluorescent iris is positioned coaxially**.**

Results

Behavioural experiment

The average distance at which triplefins performed bobbing when approaching a scorpionfish differed significantly among treatments. When exposed to a scorpionfish with its eye visible to the triplefin, the distance increased from 10.2 cm under **Fluo-OFF** conditions (95% CI: 8.2-12.5 cm) to 14.8 cm under **Fluo-ON** conditions (95% CI: 12.5-17.0 cm). Hence, showed a response from 4.6 cm further away when red fluorescence was allowed to contribute to detection of the illumination of the scorpionfish eye.

This effect depended on eye visibility. When the scorpionfish eye was obscured, mean bobbing distances were 3.3 cm closer to the scorpionfish under **Fluo-ON** conditions (11.5 cm, 95% CI: 9.1-14.1 cm), which was indistinguishable from the **Fluo-OFF** conditions. Together, these results confirm our prediction that the detection advantage associated with fluorescence relies on perceiving the scorpionfish's pupil. This is the strongest evidence to date that red fluorescent irides in a prey species induces perceivable reflections in the eye of a predator.

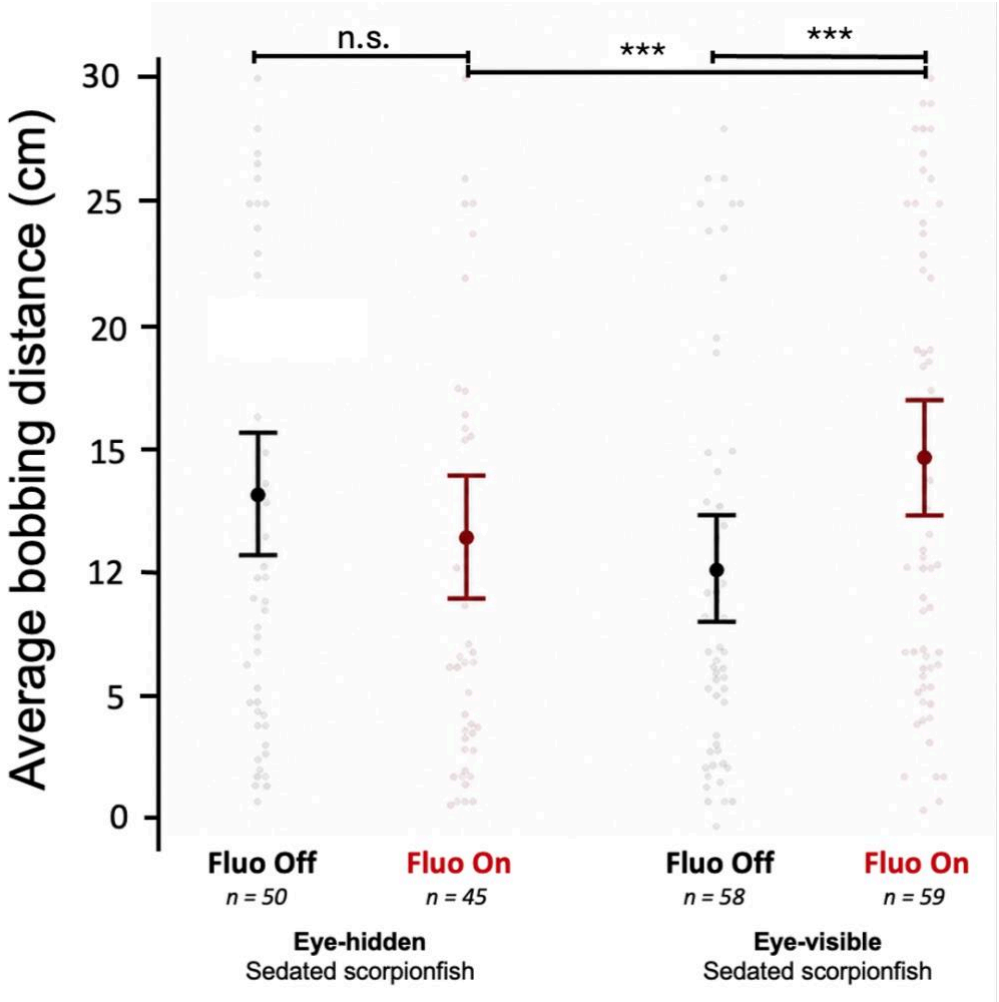

Figure 2: Results of the experiment: Manipulating scorpionfish pupil visibility. Average bobbing distance (cm) of *T. delaisi* in a 10-minute run as a function of pupil visibility (eye-hidden or eye-visible) and filter treatment (**Fluo-ON** or **Fluo-OFF**). Individuals were exposed to a lightly sedated *S. porcus* in which the eye was either hidden by a natural,

non-fluorescent stone or visible, under both fluorescent treatments in a balanced paired design. **Fluo-OFF** blocked the red part of the spectrum, whereas **Fluo-ON** allowed red fluorescence to contribute to the illumination of the scorpionfish. Error bars show predicted means and 95% compatibility intervals (CI), based on a Beta distribution with a logit-link. **n.s.** denotes a non-significant difference, *** denotes a significant difference.

Visual model

A visual model was designed to test whether fluorescence could increase the chromatic contrast between a scorpionfish iris and pupil and thereby improving detection distance from the perspective of a triplefin. The visual model indicated that triplefins can discriminate the scorpionfish pupil from the surrounding iris under both **Fluo-ON** and **Fluo-OFF** conditions. However, there is a clear distance effect: Red fluorescence increased the distance over which the pupil-iris contrast was predicted to be perceptible by a triplefin (Figure 3). At a conservative discrimination threshold of 3 just noticeable differences (JNDs), the scorpionfish pupil was predicted to be detectable from 7.5 cm under **Fluo-ON** conditions, compared with 5.5 cm under **Fluo-OFF** conditions. This corresponds to a fluorescence-dependent increase of 2.0 cm in predicted detection distance. Across thresholds, the estimated fluorescence-dependent increase in detection distance ranged from more than 5 cm at 1.5 JND to 1.6 cm at 4 JNDs.

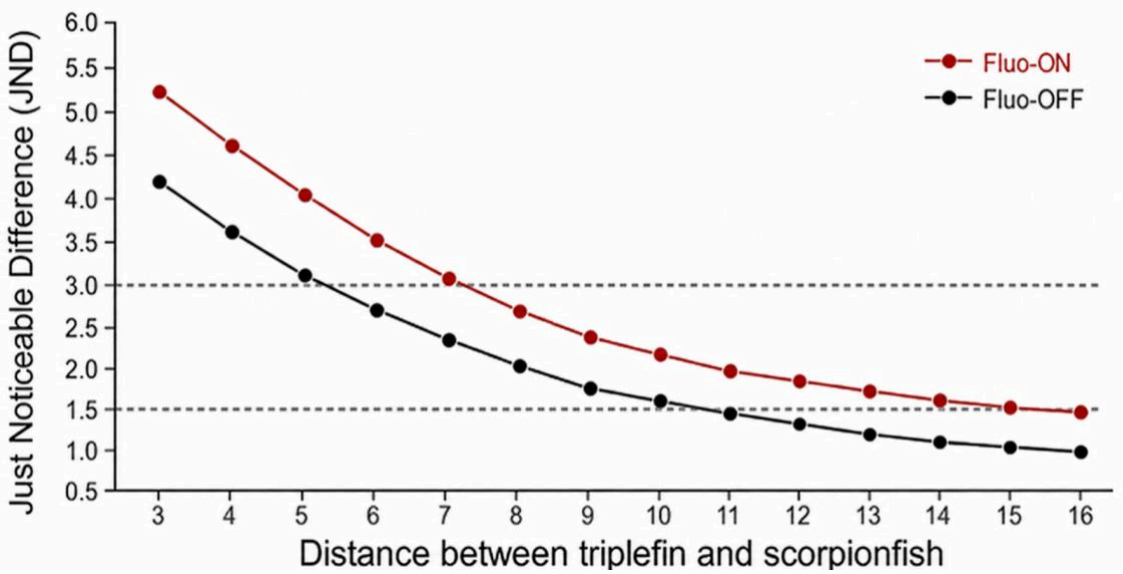

Figure 3: Visual-model prediction of fluorescence-enhanced scorpionfish pupil detection. Predicted chromatic contrast between the scorpionfish pupil and iris, expressed in just noticeable differences (JNDs), as perceived by a triplefin at increasing distances. When red iris fluorescence is allowed to contribute to illumination (**Fluo-ON**), pupil-iris contrast remained higher across all modelled distances than when red fluorescence was blocked (**Fluo-OFF**). The dashed horizontal lines indicate different JND thresholds, with values above 1 JND theoretically detectable under optimal conditions and values around or above 3 JND representing a more conservative detection criterion. Fluorescence extends the distance over which the retroreflective scorpionfish pupil should be discriminable from the surrounding iris.

<Discussion>

The results show that red iris fluorescence enhances predator detection through a specific visual interaction with the scorpionfish eye. Triplefins detected scorpionfish at greater distances when red fluorescence was allowed to contribute to the illumination, but only when the scorpionfish eye was visible. When obscured, the effect disappeared. This identifies the retroreflective pupil as a critical vulnerability in the scorpionfish. This interpretation is supported by the visual model, which predicted that fluorescence increased the chromatic pupil-iris contrast over distances that matched the observed increase in average bobbing distance under **Fluo-ON** conditions.

These findings extend the concept of diurnal active photolocation. Previous work showed that triplefins redirect downwelling light sideways to improve scorpionfish detection in shallow water and suggested (but did not prove) that the retroreflective scorpionfish pupil is the optical target. Given the results of this study, it seems very plausible that light redirection in shallow water is also specifically aimed at revealing the retroreflective pupil of a predator.

From an evolutionary perspective, these findings suggest an advanced stage in a predator-prey sensory arms race centred around the eyes. Retroreflective pupils reduce detectability but can be hacked by design, return light towards anyone with a coaxial light source (Howland, Murphy and McCosker, 1992; Benson and Suter, 2013; Feller and Cronin, 2014; Santon *et al.*, 2018). By evolving a light source positioned close to the pupil, prey can exploit this vulnerability and compromise the predator's eye camouflage (Howland, Murphy and McCosker, 1992; Santon *et al.*, 2020; van der Schoot and Michiels, 2025). Since long-wavelength light is rapidly attenuated in water, red fluorescence can only be effective over short distances (Maritorena and Guillocheau, 1996; Wo'zniak and Dera, 2007) and can be described as a "private signal". This physical constraint potentially explains why red fluorescent structures are particularly common around the head and eye region in small (< 4 cm) benthic fishes (Anthes *et al.*, 2016), where predator-prey interactions typically occur at close range and where even modest increases in detection distance can yield large survival benefits.

More broadly, our results provide experimental evidence that fluorescence can function as an active sensory tool in predator detection. Fluorescence has been proposed to mediate ecological interactions across many taxa, including prey attraction (Ben-Zvi et al., 2022; Kurup et al., 2013), predator avoidance and signalling (Gaffin *et al.*, 2012; Gálvez, Nieto and Samaniego, 2020) (Lamb and Davis, 2020) but direct behavioural evidence for such functions remains limited. The present study focuses on an example in which fluorescence is effective in a unique context of active sensing.

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Methods

Model species and location

Prey: Yellow Black-Headed Triplefin (*Tripterygion delaisi*)

The yellow black-faced triplefin is a small (2-5 cm) cryptobenthic fish, that inhabits rocky substrates between approximately 3 and 50 m depth in the Mediterranean Sea and eastern Atlantic Ocean (Louisy, 2015). While exploring an environment, triplefins often show bobbing, a repeated raising and lowering of the anterior body in a push-up like motion lasting approximately one second (Wirtz, 1978). Bobbing increases in frequency in the presence of a scorpionfish (Wirtz, 1978; Santon et al., 2021; van der Schoot and Michiels, 2025). Triplefins possess strong red fluorescent irises with a peak emission at 609 nm and a broad excitation spectrum peaking in the green range (530 nm). They can regulate their fluorescence in response to ambient light conditions and show significantly stronger fluorescence in waters deeper than 20 meters (Michiels *et al.*, 2008; Wucherer and Michiels, 2012, 2014; Bitton *et al.*, 2017; Harant *et al.*, 2018).

Predator: Black Scorpionfish (*Scorpaena porcus*)

The black scorpionfish (15-20 cm) is a cryptobenthic sit-and-wait macropredator, living on hard substrates throughout the Mediterranean Sea and Northeast Atlantic Ocean where it preys on invertebrates as well as small fishes, including triplefins (Compaire *et al.*, 2018) (Louisy, 2015). They possess strong retroreflective pupils that reduce eye conspicuousness by matching the brightness and colour of the surrounding iris and skin. This retroreflection is particularly strong in the long-wavelength, red part of the visual spectrum, making the pupil a suitable optical target for red, near-coaxial, illumination (Santon *et al.*, 2018). (Compaire *et al.*, 2018)

Data collection

Data collection took place at STARESO (Station de Recherches Sous Marines et Océanographiques, Calvi, France). Fish were collected, housed, and returned to the field under the station's general research permit. Further details are provided in van der Schoot and Michiels (2025).

Behavioural experiment

Experimental setup

The experimental setup consisted of an arena in which the triplefin was released at one end, and a stimulus compartment was positioned at the other end. The stimulus compartment functioned as a positive attractor for the triplefin and served as the

predator presentation compartment. The arena and stimulus compartment were separated by a transparent barrier, allowing visual interaction while preventing physical or chemical contact. The setup followed van der Schoot and Michiels (2025), including the use of a separate scorpionfish in an opaque perforated box to provide non-visual predator cues. This experiment included one modification. In previous experiments, triplefins often moved rapidly from the release point towards the stimulus compartment. To slow this approach and allow detection distance to be quantified more precisely, we added a 1 cm wide and 15 cm long ridge to the central lane of the arena. The ridge started 5 cm from the stimulus compartment and increased in height from 0.5 cm at the stimulus end to 1 cm at the release end, creating a gradient of exposure. This ridge also provided an edge that triplefins could follow while moving through the unfamiliar arena, thereby promoting movement along the central lane towards the stimulus compartment.

Experimental design

In all treatments, a live scorpionfish was present in the stimulus compartment, positioned perpendicular to the triplefin with one eye (left or right) facing a triplefin. Each tested triplefin was exposed to one of three stimulus treatments:

1. Lightly sedated scorpionfish with **eye-visible**
2. Lightly sedated scorpionfish with **eye-hidden**
3. Untreated scorpionfish (**control**)

Within each stimulus treatment, triplefins were tested twice: once under conditions in which red iris fluorescence could contribute to illumination of the scorpionfish (**Fluo-ON**), and once under conditions in which red fluorescence was blocked (**Fluo-OFF**). Details of the scorpionfish sedation protocol and eye manipulation are provided in the esm. In the **Fluo-OFF** condition, a LEE 172 Lagoon Blue filter was placed on the glass panel separating the triplefin from the scorpionfish, blocking the red part of the spectrum. In the **Fluo-ON** condition, red wavelengths were allowed to pass, and a LEE 209 0.3 Neutral Density filter was used to match the reduced brightness caused by the Lagoon Blue filter under the experimental light conditions (van der Schoot and Michiels, 2025). The order of **Fluo-ON** and **Fluo-OFF** runs was balanced across individuals.

Behavioural recordings and scoring

All experimental runs were recorded from above using GoPro Hero7 Black cameras, and the recordings were analysed using a custom MATLAB script (The MathWorks Inc., 2025). Scoring was performed blind to treatment. Each time the triplefin moved, its distance from the stimulus compartment was recorded, and all bobbing events were scored. Mean bobbing distance, defined as the average distance from the stimulus compartment across all bobbing events within a run, was used as the primary measure of predator detection.

Statistical analyses

Statistical analyses were conducted in R (R Core Team, 2025), using the glmmTMB package (Brooks *et al.*, 2017), following the framework established in Santon et al. (2023). Mean bobbing distance was transformed to a proportion bounded between 0 and 1 and analysed using a beta regression with a logit link function.

The model included fluorescence treatment (**Fluo-ON/Fluo-OFF**), eye visibility (**eye-visible/eye-hidden**), and their interaction as fixed effects. Triplefin identity was included as a random effect to account for paired measurements (Schielzeth and Forstmeier, 2009). Model assumptions were assessed by inspecting residual distributions, residuals plotted against predictors and fitted values, dispersion, zero inflation, data distribution, and temporal autocorrelation. Before the final analyses, we tested for effects of treatment order, time, and date, but found no evidence that these variables influenced the response.

In total, 100 triplefins were tested in each stimulus treatment. Runs were excluded when the triplefin did not perform any bobbing in that run, when the lightly sedated scorpionfish moved during the trial, or due to technical recording problems.

Visual modelling

To validate the experimental results, we estimated the chromatic contrast between a scorpionfish pupil and its iris as perceived by a triplefin under the **Fluo-ON** and **Fluo-OFF** conditions. We first calculated the total photon flux reaching the triplefin eye from the scorpionfish pupil and iris. This total photon flux consisted of two components: (1) the baseline radiance of the scorpionfish pupil or iris under the ambient light conditions of the experimental setup, and (2) the additional photon flux generated by the red fluorescence from a triplefin iris that was reflected back from the scorpionfish eye. Full details of the photon-flux estimates are provided in the esm.

Using these photon-flux estimates, we constructed a visual model in R (R Core Team, 2025) using the *pavo* package (Maia *et al.*, 2019). The model estimated the quantum catch for each photoreceptor of a triplefin. We then calculated the chromatic contrast between scorpionfish pupil and iris under both **Fluo-ON** and **Fluo-OFF** conditions using the '*coldist*' function in *pavo*. Chromatic contrast was expressed in Just Noticeable Differences (JNDs), where values above 1 JND indicate an increasing probability of discrimination. A threshold of 3 JNDs is often used as a conservative discrimination criterion (Siddiqi *et al.*, 2004; Stevens, Lown and Denton, 2014; da Silva *et al.*, 2020).

Supplementary Material

Behavioural experiment

Scorpionfish sedation

Scorpionfish were lightly sedated inside the stimulus compartment to standardise body position and prevent movement during the **eye-visible** and **eye-hidden** treatments. Sedation was induced using MS-222 (tricaine methanesulfonate; 50 mg L^{-1}), buffered 1:1 with sodium bicarbonate to achieve neutral pH. Fish typically reached stage-1 anaesthesia within 1-5 min, characterised by loss of body movement while ventilation was maintained. Following the experimental runs, scorpionfish were transferred to fresh seawater and recovered fully within approximately 5 min.

Each scorpionfish was sedated for a maximum of 60 min per session, corresponding to four experimental runs involving two different triplefins. Individual scorpionfish were sedated no more than three times, with at least one washout day between sedation events.

Scorpionfish eye-visibility

To manipulate scorpionfish pupil visibility, a natural, non-fluorescent pebble was suspended in front of the lightly sedated scorpionfish using transparent fishing line. In the eye-visible treatment, the pebble was positioned in front of the body while leaving the eye unobstructed from the triplefin's perspective (Figure 1a). In the eye-hidden treatment, the pebble was positioned directly in front of the eye facing the triplefin, thereby obscuring the pupil (Figure 1b).
In the untreated scorpionfish control, no pebble was present, allowing comparison with previous experiments in which triplefins detected scorpionfish more effectively under **Fluo-ON** conditions (van der Schoot and Michiels, 2025).

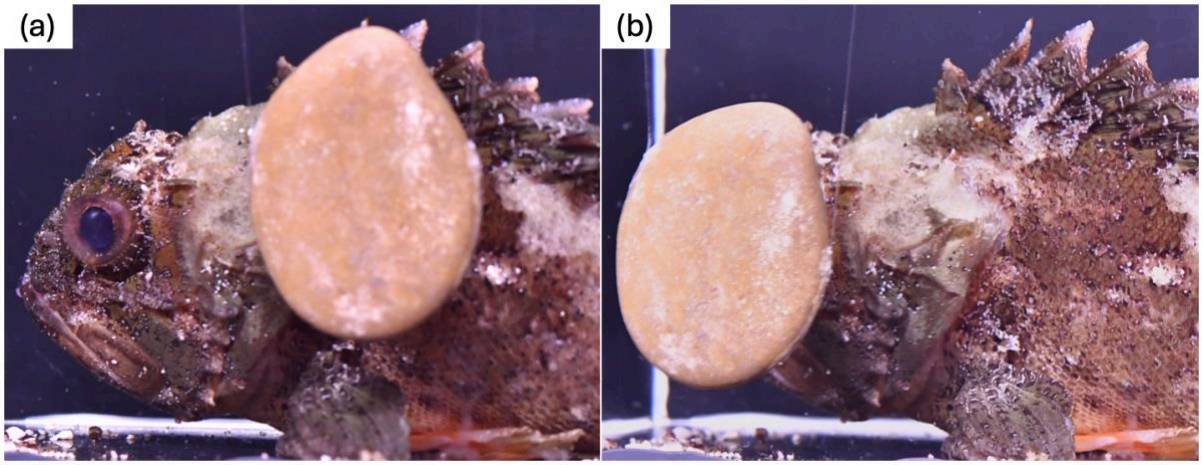

Figure 1: Manipulation of scorpionfish eye visibility. Photographs of a lightly sedated *Scorpaena porcus* under white overhead illumination. These photographs are not representative of the experimental light conditions. (a) **Eye-visible** treatment, with a natural, non-fluorescent pebble positioned in front of the body while leaving the eye unobstructed. (b) **Eye-hidden** treatment, with the pebble positioned directly in front of the eye facing the triplefin, thereby obscuring the eye and the retroreflective pupil.

Comparison of triplefin bobbing responses across years

To verify that the response of triplefins to scorpionfish in the present experiment was comparable to responses observed in previous experiments, we compared bobbing behaviour across experimental years (2022, 2023 and 2025). This comparison allowed us to assess whether the use of lightly sedated scorpionfish and the modified arena design altered the general predator-elicited response of triplefins relative to earlier experiments.

Across all three experimental years, triplefins showed the same pattern. Namely, bobbing responses were significantly higher under **Fluo-ON** than under **Fluo-OFF** conditions (Figure 2). This indicates that the fluorescence-dependent response to scorpionfish was consistent across experiments. However, the overall bobbing frequency was substantially lower in 2025 than in 2022 and 2023. This lower response may be related to the addition of the ridge, which slowed approach time and may have reduced the total number of bobbing events per run.

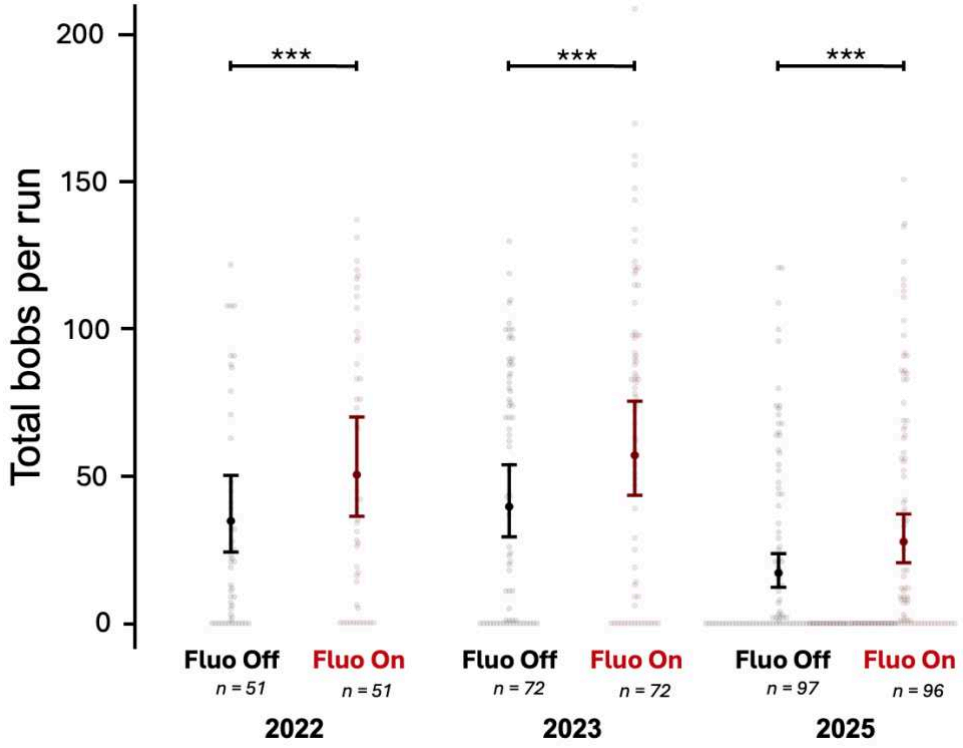

Figure 2. Comparison of scorpionfish-elicited bobbing responses across years. Total bobbing frequency per run in response to scorpionfish under **Fluo-OFF** and **Fluo-ON** conditions across experiments conducted in 2022, 2023 and 2025. Points show individual runs; violins show the distribution of responses; error bars show predicted means and 95% compatibility intervals. Asterisks indicate significant differences between **Fluo-OFF** and **Fluo-ON** within each year.

Visual Model

Fluorescence measurements

Triplefins were euthanised by severing the spinal cord. The corneas were removed, and fish were stored overnight for 18 h in potassium-enhanced Ringer solution to facilitate melanophore retraction. After melanophore retraction, each fish was transferred to an optically neutral cell-culture bottle filled with the same potassium-enhanced Ringer solution. To allow direct use of the fluorescence measurements in the visual model, fish were illuminated using the same light sources and spectral configuration as in the behavioural experiment. Illumination was provided by two RGB LEDs (Cameo Q-SPOT 40 RGBW WH; red = 0, green = 255, blue = 255), each covered with two layers of LEE 172 Lagoon Blue to suppress residual long-wavelength light.

Iris fluorescence was measured using a SpectraScan PR-740 spectroradiometer (Photo Research, Syracuse, USA). For each individual, a bright fluorescent region of the left iris was measured three times, with slight repositioning between measurements, and the resulting spectra were averaged for the three measurements. To improve sensitivity in the orange-red range, the spectroradiometer was fitted with two layers of LEE 287 Double CT-Orange colour-correction filter. The transmission of this filter was accounted for during spectral correction. Fluorescence radiance was expressed relative to a diffuse white polytetrafluoroethylene reflectance standard (DWS; Berghof Fluoroplastic Technology GmbH, Germany). The DWS was positioned at 45° to the incident light to measure the illumination spectrum used for normalisation.

Ambient light measurements of the behavioural test setup

To correct for differences in light intensity between the behavioural experiment and the laboratory fluorescence measurements, we quantified the illumination spectrum of the behavioural arena. A DWS, angled at 45°, was positioned on the arena centreline, facing the display compartment. Spectra were recorded at 1 cm intervals from 4 to 15 cm from the display compartment using the same SpectraScan PR-740 spectroradiometer. These measurements were used to estimate the photon flux travelling from the triplefin iris towards the scorpionfish eye and from the scorpionfish eye back towards the triplefin.

We also quantified the light field inside the display compartment, where the scorpionfish was positioned during the behavioural experiment. A DWS was placed inside the display compartment at a 45° angle and oriented towards the arena, corresponding to the direction of the triplefin. Spectra were recorded through each of the two filter conditions used in the experiment: the LEE 172 Lagoon Blue filter used for the **Fluo-OFF** condition and the LEE 209 0.3 Neutral Density filter used for the **Fluo-ON** condition. These measurements were used to estimate the baseline photon radiance of the scorpionfish pupil and iris under both filter treatments.

Photon flux estimation

We modelled the contribution of fluorescence-induced retroreflection to scorpionfish detection under the laboratory conditions used in the behavioural experiment. The model was adapted from Santon et al. (2020), but differed in the visual contrast being estimated. Santon et al. modelled a temporal achromatic contrast between a scorpionfish pupil illuminated by ambient light alone and the same pupil illuminated by ambient light plus redirected light from the triplefin. Here, we instead modelled the spatial chromatic contrast between the scorpionfish pupil and the surrounding iris, separately under **Fluo-ON** and **Fluo-OFF** conditions.

For each distance between a triplefin and scorpionfish, we estimated the total photon flux reaching the triplefin pupil from either the scorpionfish pupil or iris. This total photon flux consisted of two components: (1) baseline photon flux generated by ambient illumination of the scorpionfish eye, and (2) additional photon flux generated when red fluorescence from the triplefin iris travelled to the scorpionfish eye and was reflected back towards the triplefin. The baseline and fluorescence-induced photon flux were calculated separately for the scorpionfish pupil and iris and then summed to obtain the total photon flux used in the visual model.

Baseline photon flux

Baseline photon flux was calculated in six steps corresponding to Figure 3. First, we used the photon radiance of a PTFE standard measured at 45° at the position of the scorpionfish (1). This spectrum was multiplied by the reflectance of either the scorpionfish pupil or iris (2), yielding the photon radiance of each eye structure. The light was then propagated over distance *d* using wavelength-specific attenuation coefficients *k* (3), corrected for transmission through the relevant filter treatment, *LB* or *ND* (4), and converted to photon flux using the solid angle subtended by the pupil or iris (5) and the area of the triplefin pupil (6).

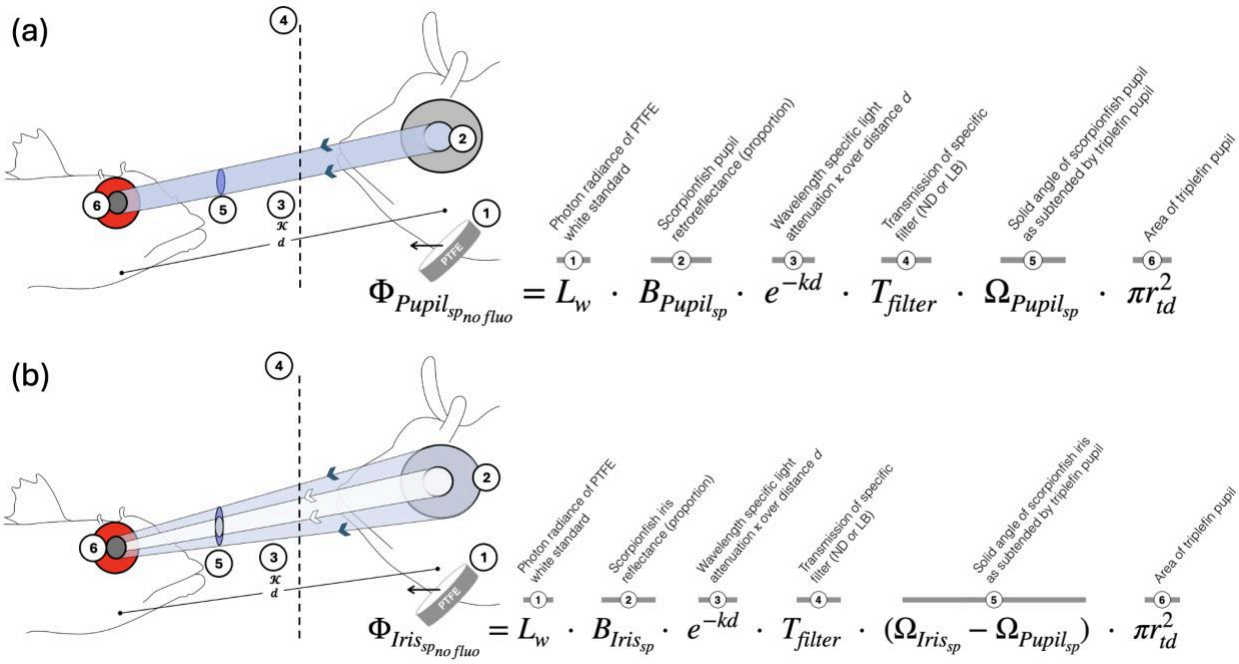

Figure 3. Baseline photon flux from the scorpionfish eye to the triplefin pupil.
Schematic representation of the photon flux **Φ** originating from baseline scorpionfish eyeshine under the ambient light conditions of the behavioural experiment. Calculations were performed separately for (**a**) the scorpionfish pupil and (**b**) the surrounding iris. This baseline component excludes any contribution from triplefin fluorescence, which is shown in Figure 4. Calculations were performed per wavelength between 400 and 700 nm.

Fluorescence-induced photon flux

To estimate the additional photon flux generated by triplefin iris fluorescence, we modelled the photon flux from the fluorescent triplefin iris to either the scorpionfish pupil (Figure 4a) or iris (Figure 4b), and then back from the scorpionfish eye to the triplefin pupil (Figure 4c,d). First, we multiplied the photon radiance of a PTFE standard measured at the position of the triplefin (1) by the relative radiance of the fluorescent triplefin iris (2). The resulting fluorescent radiance was propagated over distance *d* using wavelength-specific attenuation coefficient *k* (3) and corrected for transmission through the relevant filter treatment, *LB* or *ND* (4).

The photon flux arriving at the scorpionfish eye was then calculated from the solid angle subtended by the fluorescent iris, defined as the solid angle of the triplefin iris minus the solid angle of the triplefin pupil (5). At the scorpionfish eye, the incident photon irradiance was converted into outgoing photon radiance according to the reflectance properties of either the scorpionfish pupil or iris (6). This outgoing light then travelled back towards the triplefin over the same distance *d*, again corrected for wavelength-specific attenuation *k* (7) and transmission through the filter a second time (8). Finally, the solid angle of the scorpionfish pupil or iris as perceived by the triplefin pupil was calculated (9), and this was used to estimate the photon flux entering the triplefin pupil (10). The solid angle of the scorpionfish pupil was calculated directly, whereas the solid angle of the iris was obtained by subtracting the solid angle of the pupil from that of the full iris.

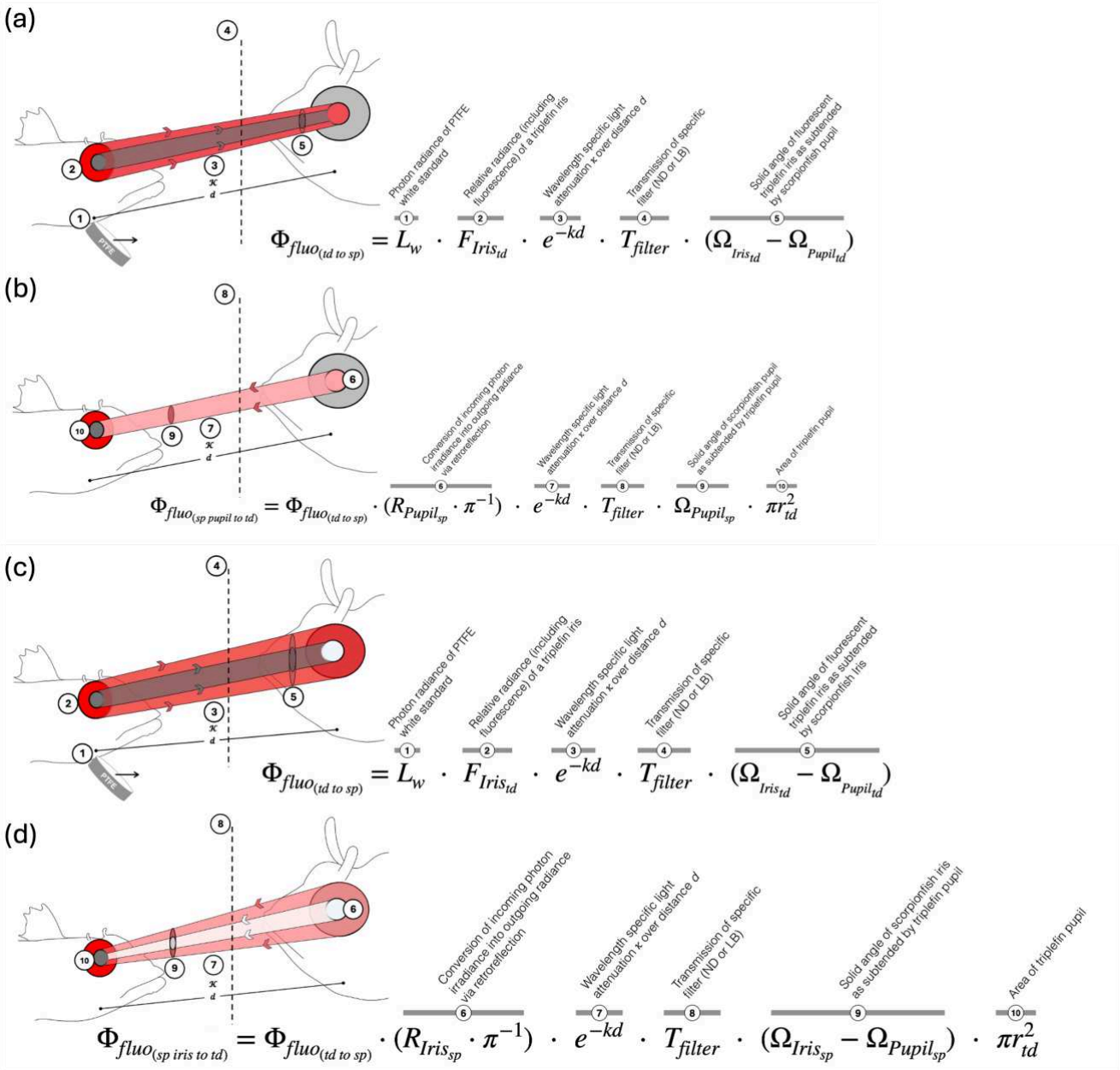

Figure 4: Fluorescence-induced photon flux from the triplefin iris to the scorpionfish eye and back. Schematic representation of the photon flux Φ generated by red fluorescence from the triplefin iris and reflected by the scorpionfish eye. Calculations were performed separately for the optical path from the triplefin iris to (**a**) the scorpionfish **pupil** and (**b**) the scorpionfish **iris**, and for the returning photon flux from (**c**) the scorpionfish **pupil** and (**d**) the scorpionfish **iris** back to the triplefin pupil. This fluorescence-induced component was added to the baseline scorpionfish eyeshine shown in Figure 3 to obtain the total photon flux reaching the triplefin eye. Calculations were performed per wavelength between 400 and 700 nm.

Total photon flux calculation

The total photon flux reaching the triplefin pupil was calculated separately for the scorpionfish pupil and iris. For each structure, total photon flux was defined as the sum of the baseline photon flux generated by ambient illumination of the scorpionfish eye (Figure 3) and the additional photon flux generated by red fluorescence from the triplefin iris (Figure 4).

Most parameters used in the photon-flux calculations were available from previous work [2,7–15]. Two parameters were estimated specifically for the present model: (1) the relative radiance of the fluorescent triplefin iris and (2) the reflectance of the scorpionfish pupil and iris.

To calculate the relative radiance of the triplefin iris, we used the fluorescence measurements described above. The average photon radiance from the fluorescent triplefin iris was divided by the average photon radiance from the DWS under the same illumination conditions. This yielded the relative radiance of the fluorescent triplefin iris, which was then used to estimate the fluorescence-induced photon flux (Figure 4a,c, number 2).

To calculate scorpionfish pupil and iris reflectance, we used the spectral measurements reported by Santon et al. (2018) [7]. For each structure, photon radiance was divided by the photon radiance of the DWS measured under the same conditions, yielding proportional reflectance spectra for the scorpionfish pupil and iris. These reflectance spectra were then used to estimate both the baseline scorpionfish eyeshine (Figure 3a,b, number 2) and the reflected fluorescence component (Figure 4b,d, number 6).