Red fluorescence: a novel light emitting mechanism to enhance prey detection in *Tripterygion delaisi*?

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Table of Contents

Short summary ... 5
Zusammenfassung ... 7
List of publications in the thesis ... 9
Introduction ... 11
- Light in the ocean ... 11
- Fluorescence ... 13
- Red fluorescence in fish ... 15

Study species ... 21
Study goals ... 23
Chapter 1 - The consistent difference in red fluorescence in fishes across a 15 m depth gradient is triggered by ambient brightness, not by ambient spectrum
- Summary ... 25
- Discussion ... 28

Chapter 2 - Does red eye fluorescence in marine fish stand out?
In situ and in vivo measurements at two depths.
- Summary ... 35
- Discussion ... 37

Chapter 3 - Red fluorescence allows benthic fish to forage more efficiently in dim light
- Summary ... 31
- Discussion ... 32

Final thoughts ... 35
List of contributors ... 45
Acknowledgments ... 47
References ... 49
Appendix ... 59

Short summary

Since the discovery of red fluorescent fish, scientists argue whether it is of ecologically relevance or merely a side effect of pigment evolution. Red fluorescence could theoretically be involved in many different functions ranging from foraging over species recognition to camouflage. Despite growing evidence supporting the functionality of red fluorescence, we still lack knowledge concerning the type of function and the evolutionary processes influencing it.

Within this thesis, I therefore first focused on identifying the ecological drivers of fluorescence and then assessed whether fluorescence might be used in a context of prey detection facilitation. Using the black-faced triplefin *Tripterygion delaisi* as model species, I conducted empirical and experimental studies to address these points.

In the first chapter, I investigated why fish fluoresce more efficiently when originating from deep water compared with shallow water individuals by identifying the environmental triggers causing this effect. After conducting physiological experiments under controlled ambient light conditions, I confirm that fluorescence increases its efficiency with decreasing brightness and is regulated through phenotypic flexibility (Chapter 1).

In the following chapters, I focused on the question whether red fluorescence is used to enhance prey detection. By illuminating the environment with longer wavelengths, which are nearly absent below 10 m depths, fish capable of emitting red fluorescence could theoretically increase their foraging success by enhancing the visual contrast between prey and natural background. This, however, requires red fluorescence to exceed the ambient light and the emitted substrate radiance in the longer wavelength range (> 600nm).

I tested this by taking spectral measurements of substrate radiance and *in vivo* iris fluorescence in the field. After calculating the brightness contrast between these components, I can confirm that iris fluorescence always exceeds substrate radiance in deeper water (Chapter 2). Contrary to my predictions, however, I also identified several conditions in shallow water within which red fluorescence is likely to generate a visual contrast.

Since a visual contrast at least in deeper water is highly likely, I continued my research by testing if fish are indeed more successful in catching prey under "fluorescence friendly" narrow-spectral, blue-green light conditions compared with broad-spectral, "white" light conditions. I predicted that under the blue-green light typical for deeper water, fish emitting red fluorescence are able to enhance the visual contrast between prey and the blue-green background. This contrast could facilitate prey detection, increasing foraging success. Shallow water environments are characterized by broader, more "white" spectra. Here, I predicted that such contrast cannot be generated and hence, foraging should be less efficient. I tested this under dim light (two levels of shading) to encourage the expression of fluorescence (Chapter 1). The results show that fish were more successful in foraging under heavily shaded, blue-green light conditions, compared with the broad-spectral or brighter treatments (chapter 3).

I conclude that iris fluorescence is likely to be of ecological relevance to *T. delaisi* and might act as a contrast-enhancing mechanism to facilitate visual tasks under dim light.

Zusammenfassung

Seit der Entdeckung von Rotfluoreszenz in Meeresfischen versucht man festzustellen, ob diese von ökologischer Relevanz oder lediglich ein Nebenprodukt evolvierender Pigmente ist. Theoretisch könnte Rotfluoreszenz für zahlreichen Funktionen eingesetzt werden wie zum Beispiel bei der Futtersuche, der Partnerwahl oder auch zur Tarnung. Obwohl sich die Hinweise häufen, dass Rotfluoreszenz tatsächlich ökologische Relevanz besitzt, ist nach wie vor unklar welche Funktion diese erfüllt und welche evolutiven Prozesse die Rotfluoreszenz beeinflussen. Durch empirische und experimentelle Studien am Schwarzkopf-Spitzkopf Schleimfisch *Tripterygion delaisi* versuchte ich diesen Fragen auf den Grund zu gehen.

Im ersten Kapitel versuchte ich zunächst herauszufinden, warum die Rotfluoreszenz bei Fischen im tiefen Wasser effizienter ist als im untiefen Wasser. Hierfür führte ich physiologische Experimente unter kontrollierten Lichtbedingungen durch. Die Ergebnisse zeigen, dass Rotfluoreszenz phänotypisch plastisch ist und durch die Lichtstärke in der Umwelt reguliert wird. Alle Fische erhöhten ihre Fluoreszenz unter dunklen Lichtbedingungen und reduzierten diese wieder unter starker Beleuchtung (Kapitel 1).

In den folgenden Kapiteln versuchte ich herauszufinden ob Rotfluoreszenz zur Detektion von Beute eingesetzt wird. Theoretisch könnten rotfluoreszierende Fische den Kontrast zwischen ihrer Beute und dem Hintergrundsubstrat verstärken in dem sie ihre Umwelt mit einer Wellenlänge ausleuchten die, aufgrund physikalischer Gegebenheiten, in tieferem blaugrünem Wasser fehlt. Sollte dies zutreffen, muss die fluoreszierende Iris stark genug sein um sich vom Umgebungslicht und der Substrat Emission im langwelligem Bereich (> 600 nm) abzuheben.

Um dies zu testen führte ich im zweiten Kapitel dieser Arbeit Helligkeitskontrastberechnungen zwischen Substrat und *in vivo* Fluoreszenzmessungen der Iris durch. Entgegen meiner Erwartungen heb sich die Iris nicht nur im tiefen Wasser vom Hintergrund ab, sondern auch unter verschiedenen Bedingungen im untiefen Wasser (Kapitel 2).

Im nächsten Experiment testete ich den Fangerfolg der Fische unter einem „Fluoreszenz freundlichen", blaugrünen und unter einem breit-weißen Spektrum.

Während Rotfluoreszenz unter blaugrüner Beleuchtung den Kontrast zwischen Rot beleuchteter Beute und dem Substrathintergrund verstärken könnte, sollte solch ein Kontrast unter breit-weißer Beleuchtung mit hohem Rotlicht Anteil nicht generierbar sein. Demnach erwartete ich einen höheren Fangerfolg unter den blaugrünen Lichtbedingungen. Um herauszufinden, ob auch die Helligkeit eine Rolle bei der Kontrasterzeugung durch Rotfluoreszenz spielt, führte ich dieses Experiment jeweils unter dunklen und sehr dunklen Lichtbedingungen durch.

Die Ergebnisse zeigen, dass die Fische unter sehr dunklen, blaugrünen Lichtbedingungen einen höheren Fangerfolg hatten als im breit-weißen Spektrum oder in den helleren Lichtbedingungen (Kapitel 3).

Zusammenfassend kann ich sagen, dass Rotfluoreszenz sehr wahrscheinlich von ökologischer Relevanz für *T. delaisi* ist und vermutlich zur erhöhten Kontrasterzeugung bei der Detektion von Beutetieren und damit zu einem höheren Fangerfolg beiträgt.

List of publications in the thesis

Accepted papers

1.) Harant UK, Michiels NK, Anthes N, Meadows MG: **The consistent difference in red fluorescence in fishes across a 15 m depth gradient is triggered by ambient brightness, not by ambient spectrum**. *BMC Research Notes* 2016, **9**(1):1.

2.) Harant UK, Michiels NK: **Red fluorescent fish forage more efficiently in heavily shaded, blue-green light.** *BMC Ecology* 2017, (17):18.

Submitted papers

1.) Harant UK, Griessler T, Wehrberger F, Meadows MG, Champ CM, Michiels NK: **Does red iris fluorescence stand out on natural substrates? *In situ* measurements under natural light at two depths in a micro-predatory fish.** *Journal of Ecology and Evolution*, under review.

Introduction

The diversity of animal colouration fascinates humans. Apart from pleasing the observer, colour patterns often provide vital cues for con- or heterospecific organisms (Cott 1940, Crook 1997, Endler 1978, Hinton 1976). The most common type of colouration arises through reflective pigments that absorb one part of the ambient spectrum and reflect the non-absorbed part (Breed & Moore 2011). If a spectrum lacks a certain wavelength range, no colour within this range can be reflected. Although this is of minor relevance in terrestrial habitats, these restrictions have far-reaching consequences for organisms living in aquatic systems.

Light in the ocean

The light conditions in the sea differ substantially from terrestrial habitats. First, the overall brightness decreases with increasing depth and second, the spectral quality of the available light is strongly altered by absorption of longer wavelengths (> 580 nm) and scatter in the blue range (430-530nm) (Jerlov 1968, Lythgoe 1979, Mobley 1994, Marshall et al. 2003, Loew & Zhang 2006). Consequently, aquatic environments below 10 m depth are characterized by blue-green light (λ = 450-530 nm) (Figure 1).

At these depths, the downwelling spectrum is narrower than the visual perception limit of most fish. We call this the “stenospectral zone” (Meadows et al. 2014). In contrast, ambient spectra in shallow water are broader than the visual perception range of most fish at both ends of the spectrum and is henceforth called the “euryspectral zone”. While diving at a coral reef, we can easily explore this spectral shift by taking a red coloured fish from 5 m down to 20 m depth. Under stenospectral conditions, the same fish will appear greyish and dull to us, unless we artificially reintroduce the longer wavelengths by using a dive torch for example.

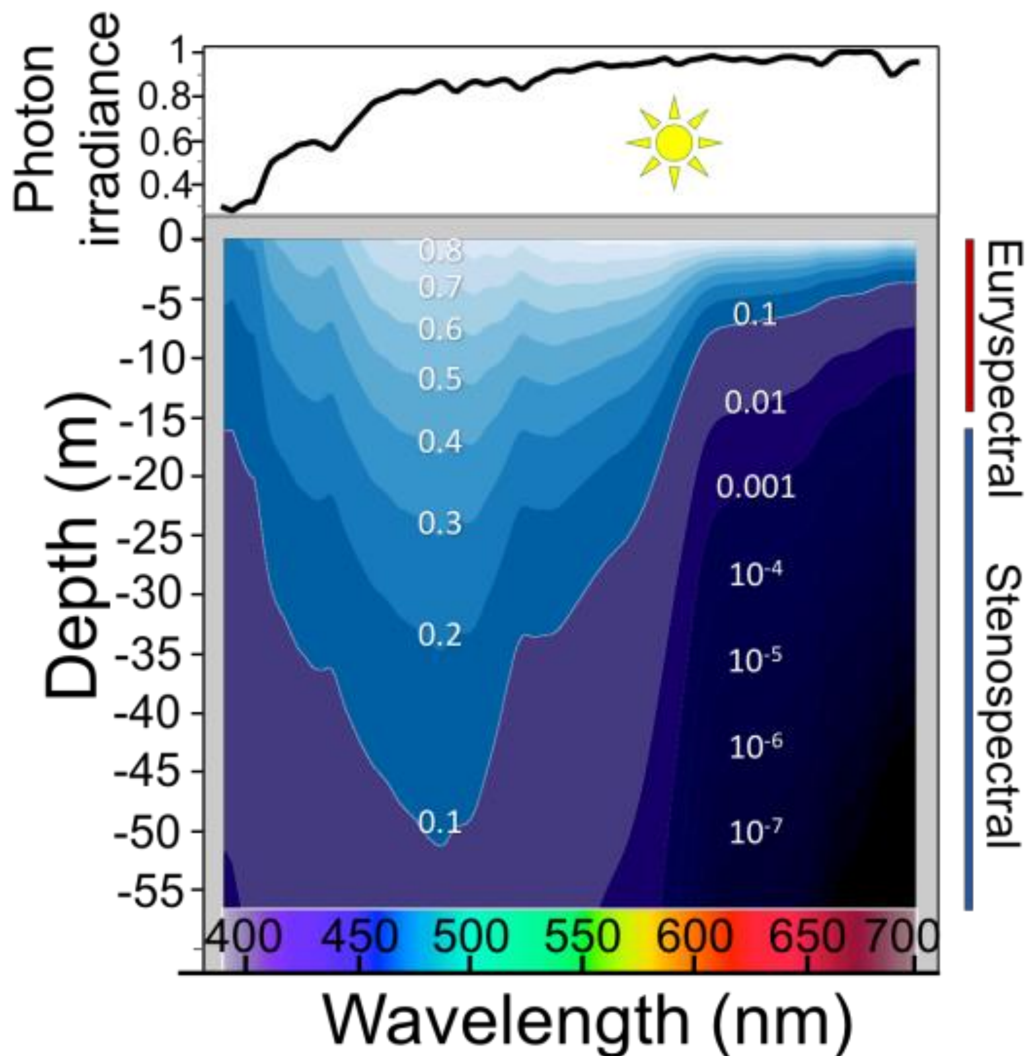

Figure 1: Light absorption in the water column (modified after Anthes et al. 2016).

Due to strong absorption of wavelengths above 600 nm and strong scatter within the blue range, the light in aquatic systems quickly narrows down from a euryspectral (broad) spectrum close to the surface to a stenospectral spectrum (narrow blue-green) below 10 m depth. Shorter UV wavelengths are mostly reflected at the water surface and remaining UV is only slightly more absorbed than blue wavelengths. The upper graph shows the proportion of the ambient spectrum above the water surface relative to the maximum irradiance values per wavelength.

The lower graph shows how the ambient spectrum in the water changes with increasing depth. White lines indicate “iso-brightness” for a given wavelength (Anthes et al. 2016)

Fluorescence

Fluorescent pigments offer a way out of this limitation by absorbing short, high-energy photons and reemitting the energy with a very short delay as photons with longer wavelengths (Johnsen 2012) (Figure 2). Thus, a fluorescent pigment requires ambient light to emit fluorescence. Due to the excitation and emission properties of fluorescence, it mostly comes in tones of green, yellow, orange, and red (Jeffrey & Haxo 1968, Lagorio et al. 2015, Murata et al. 1966, Sparks et al. 2014). However, fluorescence can also be found in the UV and infrared range but is hard to identify as such due to our lack of spectral sensitivity in these ranges. Since nearly all larger molecules fluoresce, most organisms express some degree of fluorescence (Lagorio et al. 2015, Mazel 1997, Mazel & Fuchs 2003, Michiels et al. 2008, Sparks et al. 2014). This ubiquitous occurrence of fluorescence leads to the assumption that fluorescence is merely a side effect of pigment evolution rather than being of adaptive relevance.

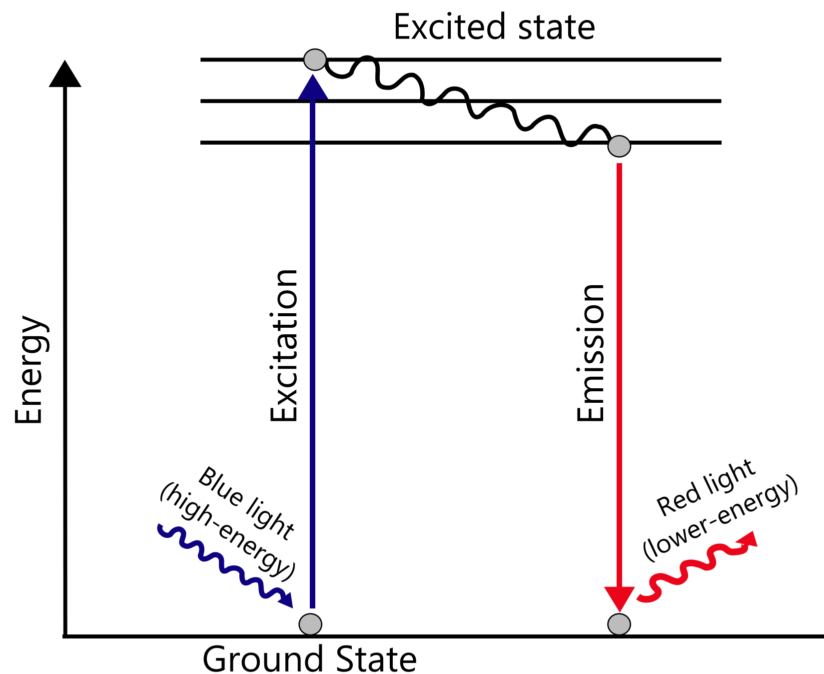

Figure 2: **Simplified Jablonsky energy diagram explaining the process of fluorescence.**
If a high-energy photon excites a fluorescent molecule, the electron is sent to a higher energy level. Instead of jumping immediately back to the ground state, the electron then suffers from energetic loss via non-radiative processes (*e.g.* converted into thermal or kinetic energy). Consequently, when jumping back to the ground state, the electron has a lower energy level than the initial energy input would suggest. This leads to emission of a photon of lower energy and longer wavelength.

While several studies confirmed the importance of fluorescence in terrestrial systems, for instance in courtship displays (Arnold et al. 2002) or sex recognition (Lim et al. 2007), surprisingly little is known about its functionality in aquatic environments. One exception to this are mantis shrimps, which increase the effectiveness of their postural signalling by adding a yellow fluorescent component to their colouration (Mazel et al. 2004). In contrast, the functionality of fluorescence in fish still remains largely unstudied.

Red fluorescence in fish

Since the discovery of red fluorescence within 180 marine fish taxa (Michiels et al. 2008, Sparks et al. 2014, Anthes et al. 2016) the question rises how this common phenomenon remained unnoticed for such a long time. The reason is probably due to the way in which we perceive the aquatic environment. When diving below 10 m depth, the high proportion of blue-green wavelengths leads to a sensory satiation of the visual system and strongly exceeds the relatively small red fluorescent component. By using a long-pass filter for viewing, however, the excitation spectrum is blocked out, allowing us to explore red fluorescence in aquatic environments. We can also see red fluorescence even without using filters. For example, red-brown colouration is common at depth, but seems unimpressive to the untrained eye. Since reflective red colouration does not exist in deeper water, these colours can only emerge from fluorescence.

Possible functions of red fluorescence

Red fluorescence could be functional in many different ways. One of them is UV protection, which has lately been discussed by Meadows et al. (2014). By reemitting radiant energy via red fluorescence, organisms could prevent damage of their photosensitive tissue. This kind of UV protection has been studied in tropical corals, which are known to fluoresce in various colours from green to orange, yellow or red (Salih et al. 2000, Ben-Zvi et al. 2015, Eyal et al. 2015). If fish use red fluorescence in a similar way, one would expect fluorescence to be brightest close to the sea surface where radiation is highest. Surprisingly though, the opposite was found: Fish expressed significantly more red fluorescence at 20 m compared with individuals caught at 5 m depth (Meadows et al. 2014). Hence, we can safely exclude UV protection as the prime function of red fluorescence in marine fish, at least within species tested in this study. These results therefore suggest that red fluorescence is more likely used in a visual rather than photo-protective context.

Does red fluorescence play a role in visual functions?

Recently, a phylogenetic study contributed significantly to the discussion of whether fluorescence plays a role in a visual context by linking the occurrence of red fluorescence in fishes to different ecological niches (Anthes et al. 2016). Within this study, the authors addressed the following questions:

First, is red fluorescence more prevalent in small-sized fish? Given that red wavelengths are quickly absorbed in the water column, a visual function would be limited to short distances only (Anthes et al. 2016).

Second, are fluorescent fins more often found in species displaying specific types of intra-specific communication? Here, the authors distinguished between two different types of intra-specific communication: (*i*) group-living fish, frequently communicating with each other vs. solitary/partner-living species, and (*ii*) sexually dimorphic vs. sexually monomorphic species (Anthes et al. 2016).

Third, do nearly immobile species such as "sit and wait predators" display more often patchy fluorescent patterns than highly mobile fish, suggesting a function in camouflage?

Fourth, do fish display more often red fluorescence when living in deeper environments (Anthes et al. 2016)? Given that stenospectral conditions would favour contrasts generated by red fluorescence (Meadows et al. 2014), species living in deeper, stenospectral environments are expected to be more often fluorescent than fish living in euryspectral environments.

And fifth, is red fluorescence more common in fish foraging on small, eyed prey (Anthes et al. 2016)? Here, the authors follow a previously discussed idea that fish with red fluorescent irides or strongly fluorescent patches close to the eye use this light emitting mechanism to induce retro-reflective eyeshine in prey, aiding in their detection (Bruce 2009, Meadows et al. 2014, Wucherer & Michiels 2014).

Anthes et al. (2016) confirmed that red fluorescence is indeed more common in small-sized fish, sexually dimorphic species and sit and wait predators, suggesting that fluorescence is involved in communication and camouflage. Additionally, they show that red fluorescent irides or fluorescent patches close to the eye are particularly common in small-sized benthic fish that forage on small, eyed prey. Fluorescence might therefore also contribute to prey detection via contrast enhancement (Anthes et al. 2016). However,

the authors could not find any evidence that group-living species or species living in deeper water are more often fluorescent. Hence, intra-specific group communication can safely be excluded to be a prime function of red fluorescence (Anthes et al. 2016).

The visual system of marine fish

Before investigating the applicability and possible limitations of red fluorescence, it is essential to understand the visual system of fish. Except for most nocturnal and deep- sea fish, diurnal fish usually possess two types of photoreceptors in their retina: rods and cones. Rods are typically associated with scotopic vision as they are highly sensitive at low light levels (Munk 1966, Locket 1977, but see the following references for wavelength discrimination within rods: O´Day & Fernandez 1974, Denton et al. 1985, Douglas et al. 2000). In contrast, cones are important under well-lit conditions and can further be divided into single cones, double cones and twin cones. For colour vision, at least two different classes of photoreceptors with reasonably spectrally distinct absorption are required. Within the photoreceptor, it is the visual pigment that defines the maximum absorption of a given receptor.

Under blue-green, stenospectral conditions, fish should be able to gather all relevant chromatic (colour) information with only two types of cones–one with maximum sensitivity matching the background and the other one offset from it. (Barlow 1982, Bowmaker 1983). This type of colour vision where only two types of cones are involved is known as dichromacy. Compatible with this, the maximum sensitivity of most diurnal fish living in the euphotic zone ranges between 360-490 nm in the single cones matching the background light, and 525 nm in the double or twin cones (Lythgoe 1979, McFarland 1991, Levine & MacNichol 1979, Hawryshyn et al. 2003, Losey et al. 2003). The maximum absorption of the long wavelength sensitive cones therefore peaks within the green range. However, the abundance of red fluorescent fish indicates that these long wavelength signals (> 600 nm) might nevertheless be of importance to fish, despite lacking truly red sensitive cones as found for example in the pipefish *Stigmatopora argus* ($\lambda_{max} \sim$ 580 nm) (Mosk et al. 2007).

Are fish able to see red?

Even without dedicated red sensitive cones, fish might still be able to perceive longer wavelengths. In humans for instance, the sensitivity of the long wavelength cone peaks at 564 nm (Bowmaker et al. 1980). However, if we look at an infrared spotlight peaking at 800 nm, we still perceive a fraction of the emitted longer wavelengths. This is possible due to an overlap between the absorption of the visual pigment and the infrared emission. Likewise, fish with an absorption peak of 530 nm could also perceive red signals (> 600 nm) or even near far-red signals (> 650 nm). According to Cuthill (2006), it is also of minor importance how much a single cone is stimulated as long as it receives a higher stimulus compared with other photoreceptive cones.

Male fairy wrasses (genus *Crenilabrus*) for example, were found to show significantly more antagonistic behaviours towards conspecific males if their near-far red fluorescent colouration (> 650 nm) was visible (Gerlach et al. 2014). Despite a maximum sensitivity of 532 nm in the double cones, the overlap between fluorescence emission and cone absorption seems to be sufficient for the fairy wrasse to detect and react to it (Gerlach et al. 2014, 2016).

Similarly, the black-faced triplefin *Tripterygion delaisi* (Cadenat & Blache 1969), possesses single cones with maximum absorption at 468 nm and double cones peaking at 517 and 530 nm (Bitton et al. *2017*). And yet, it responded towards its own red fluorescent signal during behavioural trials (Kalb et al. 2015).

Prerequisite for visual functions: Is red fluorescence strong enough to generate contrast?

According to Anthes et al. (2016) small, benthic predatory fish such as *T. delaisi* often display red fluorescent irides, suggesting that red fluorescence is involved in foraging. However, this implies that the fluorescent iris is strong enough to generate a visible contrast between prey and background. There are two ways of inducing such a contrast. First, fish could compare the brightness within a limited wavelength range (600-650 nm) between the red illuminated object and the background. This contrast principle relies on achromatic information only and is therefore called achromatic contrast. Here, the additive excitation of a single cone type is evaluated by the visual system. Second, a

chromatic contrast arises if the wavelengths between two signals are compared (red object against blue-green background) (Figure 3). In this case, a contrast is generated by exciting two different cone types to a varying degree (Kelber et al. 2003). Although fish might be able to generate both contrast types, these contrasts are more likely to occur under stenospectral conditions where fewer red wavelengths are present.

Figure 3: **Simple scheme visualizing the difference between achromatic and chromatic contrast**. If we compare the squares within each group (A or B), an achromatic (brightness) contrast arises. When we compare the two groups with each other (A and B), a chromatic (colour contrast) is generated.

Does red fluorescence facilitate prey detection?

T. delaisi feeds mainly on small invertebrates such as copepods (Zander 1997). These prey organisms often possess a reflective tapetal layer behind the retina (Fahrenbach 1964, Johnson 1998). This structure allows to double the path length of light through the photoreceptors (Lythgoe 1979) and thereby increases the photon capture efficiency without increasing the size of the eye (Land 1978, Shelton et al. 1992). Simultaneously, it generates a reflection with a high degree of specularity within which more of the light is sent back to the source. This “eyeshine” is common in nocturnal animals or organisms living in dimly lit environments. Additionally, it could also reveal the position of the organism to a predator, provided that the latter has a light source nearly coaxial with its own visual axis (Jack 2014). In other words, induction and detection of eyeshine requires the light source of the observer to be close to its pupil.

We named this mechanism "*active photolocation*" (Anthes et al. 2016) as it is similar to other forms of *active sensing* such as echolocation in dolphins or bats, but uses light rather than sound. Until now, it was only known from nocturnal flashlight fish that produce frequent bioluminescent pulses in their light organ, which is located just underneath the eye (Howland et al. 1992). Here, it is assumed that fish use this light source to detect the reflection of nearby prey and/or predators. Recently it has been shown, that flashlight fish indeed increase the frequency and the longevity of their flashes if prey is nearby (Hellinger et al. 2017), supporting the assumption of active photolocation in this species.

In contrast to bioluminescent signals, red fluorescence has one major advantage: it has a high potential to be invisible to bystanders. Red wavelengths are quickly absorbed over short distances. Thus, fish could use fluorescence to forage more efficiently while remaining cryptic to potential predators. Red fluorescence could therefore represent an effective private light source for fish capable of emitting and perceiving these signals.

Study species

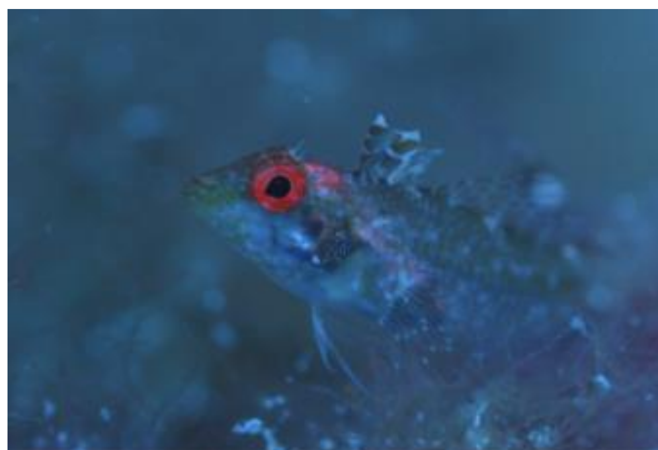

Figure 4: ***Tripterygion delaisi*** in 30 m depth taken in Corsica with Nikon D4 plus LEE Double C. T. Orange filter and manual white balance, without post-processing. Photo courtesy of Michiels NK.

The black-faced triplefin *Tripterygion delaisi* is a small (3-5 cm) crypto-benthic predator frequently found in the Mediterranean and eastern Atlantic Ocean along rocky coastlines between 5 and 50 m depth (Louisy 2002). It usually prefers a solitary lifestyle but inhabits also larger rocks or boulders with several conspecifics. Its diet mainly consists of small invertebrates such as harpacticoid copepods or gammarids (Zander 1997). *T. delaisi* displays a saltatory feeding mode, characterized by small hopping movements interrupted by phases of careful scanning of the substrate (O'Brien et al. 1990).

Apart from a small fluorescent body patch on top of its head, *T. delaisi* also possesses remarkably red fluorescent irides, which can be actively regulated ranging from almost no fluorescence visible to strikingly fluorescent within 10-30 s (Wucherer & Michiels 2014). This active regulation is under nervous control and is possible due to dispersing and aggregating mobile black pigments within the melanophores. In poikilotherm vertebrates, 6 types of chromatophores are responsible for body colouration: Melanophores (black or brown), xanthophores (yellow), erythrophores (red), cyanophores (blue), leucophores (broad-band reflective), and iridophores (metallic or iridescent) (Fujii 1993, 2000). In addition, a red fluorescent chromatophore and a red fluorescent iridophore have lately been described in marine fish (Wucherer & Michiels 2012, 2014). The latter is responsible for the red fluorescent irides observed in *T. delaisi* and the maximum emission

of the red fluorescent pigment peaks at 609 nm with an excitation maximum of 525 nm (Bitton et al. 2017).

While the chemical properties of the fluorescent substance remain unknown, we do know that the fluorescent iridophores are embedded in a layer of dendritic-shaped melanophores. By actively dispersing or aggregating melanosomes (black pigment containing organelles within melanophores), the fluorescent iridophores can be covered or uncovered resulting in more or less fluorescence expressed (Wucherer & Michiels 2014).

Both sexes display a highly cryptic body colouration. However, males develop a conspicuous orange-yellowish body colouration with a prominent black head (Zander & Heymer 1970) during breeding season with strongly decreased iris fluorescence (unpub. data).

T. delaisi is very inquisitive and shows behaviours, which are easy to observe. In addition, individuals are numerous and easy to catch in the field and to keep in the lab. *T. delaisi* is therefore an ideal model organism to test hypotheses concerning red fluorescence.

Study goals

In the first chapter of my thesis, I studied how *T. delaisi* regulates its fluorescence brightness. More specifically, I investigated the depth effect described by Meadows et al. (2014). In this study, several fishes, including *Tripterygion delaisi,* expressed significantly more fluorescence when originating from 20 m compared with 5 m depth and measured under the same laboratory conditions (Meadows et al. 2014). This suggests that in addition to the fast and short-term regulation mechanisms described by Wucherer and Michiels (2014) yet another mechanism must exist to explain the observed depth effect. I therefore conducted two physiological experiments to test if iris fluorescence in *T. delaisi* is phenotypically plastic.

A precondition for active photolocation by means of red fluorescence is that iris fluorescence stands out in front of the natural background. In the second chapter, I therefore collected multiple spectral measurements of preferred *T. delaisi* substrates, ambient downwelling/sidewelling light, as well as *in vivo* iris fluorescence, measured in the field. After calculating the brightness contrast between these components, I then assessed under which conditions an iris contrasts particularly strongly against various natural backgrounds at two depths and different shading levels.

In the third chapter, I then focused on the functionality of red fluorescence. Here, I investigated whether red fluorescence facilitates prey detection in *T. delaisi*, assuming that red fluorescent irides can generate a contrast between red illuminated prey and the blue-green background. Under euryspectral conditions, no such contrast should be generated due to the high content of red wavelengths present in the spectrum. In two separate experiments, I tested whether foraging success indeed increases in the predicted way under "red fluorescent friendly", stenospectral light conditions.

The consistent difference in red fluorescence in fishes across a 15 m depth gradient is triggered by ambient brightness, not by ambient spectrum

Harant UK, Michiels NK, Anthes N, Meadows MG: **The consistent difference in red fluorescence in fishes across a 15 m depth gradient is triggered by ambient brightness, not by ambient spectrum**. *BMC Research Notes* 2016, 9(1):1.

Summary

Searching for potential functions of red fluorescence is certainly of high priority. However, it is equally important to gain a better understanding of the phenomenon itself and the underlying processes driving it. A previous study showed that red fluorescence was more efficient in fish originating from 20 m depth compared with individuals caught at 5 m when measured under the same light conditions (Meadows et al. 2014). This indicates that the previously described short-term regulation mechanism (Wucherer & Michiels 2014) cannot account for the observed depth effect. Two common processes could explain such an observation: Fluorescence brightness could either be genetically determined or phenotypically flexible. While genetic adaptation requires selection over multiple generations, phenotypic plasticity allows individuals to express an ecotype that fits the current environment best. Phenotypic plasticity either occurs during the developmental stage and is then irreversible or can be adjusted multiple times within a lifetime (Bradshaw 1965, Wilson & Franklin 2002, Woods & Harrison 2002, West-Eberhard 2003, Ghalambor et al. 2007). The latter is also referred to as phenotypic flexibility (Piersma & Drent 2003).

Since fish are highly mobile, a genetic basis is less likely to be the prime mechanism given the short physical distance between the 5 m and 20 m depths from which the two extremes were sampled. Frequent local dispersal and the associated gene flow would counteract genetic adaptation.

I tested if red fluorescence is phenotypically flexible and tried to identify the environmental trigger mediating fluorescence brightness. Within these experiments, I

focused on two environmental cues: the ambient spectrum and the ambient brightness. Both factors are suitable candidates for regulating fluorescence brightness as they show a strong transition between depths.

In the first experiment, I illuminated fish in the laboratory with euryspectral and stenospectral light treatments at an identical overall brightness in an alternating order (spectrum experiment). I then assessed iris fluorescence brightness once a week. The results show that the two spectral treatments did not induce any measureable changes in fluorescence brightness (Harant et al. 2016).

In the second experiment, I tested whether the ambient brightness might induce a change in fluorescence brightness. Here, all fish received a bright and a dark light treatment in an alternating order while keeping the ambient spectrum constant among the treatments (brightness experiment). According to the results obtained from the first experiment, spectral shape alone does not influence expressed fluorescence. Hence, to generate the highest possible difference between bright and dark light treatment, I set all available light channels to 100% in the bright treatment, resulting in rather artificial spectral light conditions. Unlike the spectral treatment, the brightness treatment did result in a significant difference in expressed fluorescence brightness. Fish held in the dark treatment significantly increased their fluorescence brightness within a week by 43% on average and reduced it again when exposed to the bright treatment (paired t test comparing initial brightness to brightness after 1 week, 137 $t = 5.4$, df = 8, $p < 0.001$, Harant et al. 2016). While fish increased their fluorescence brightness within 3 to 4 days, decreasing fluorescence took on average 7 to 10 days.

To specify if the brightness changes also occur at the morphological/histological level I sacrificed fish after each experiment and induced maximum and minimum fluorescence physiologically. I achieved this by treating both eyes of 10 individuals with either an elevated potassium chloride solution, leading to aggregation of melanosomes and maximum fluorescence, or a marine ringer solution, resulting in minimum fluorescence expressed due to melanosome dispersal (Wucherer & Michiels 2014).

I repeated this procedure with 20 freshly caught individuals originating from 5 and 20 m depth from the field.

Freshly caught fish did show a clear difference in fluorescence brightness with fish originating from 20 m depth expressing more fluorescence in both chemical treatments than shallow water fish (t test max.: $t = -3.04$, df = 12.6, $p = 0.01$; min.: $t = -3.63$, df = 15.7, $p = 0.002$). Individuals tested after the spectrum experiment did not differ in fluorescence brightness anymore (t test min.: $t = -0.12$, df = 18, $p = 0.9$; max.: $t = -0.13$, df = 18, $p = 0.89$). They could neither be assigned to their original capture depth nor the spectral treatment they received last. All individuals sacrificed after the brightness experiment showed a similar significant difference in fluorescence brightness as individuals processed during the field season (t test max.: $t = 2.3$, df = 17, $p = 0.03$, min.: $t = 2.3$, df = 12.7, $p = 0.03$) Here, individuals held in the dark treatment prior to decapitation expressed more fluorescence in both the maximum and the minimum fluorescence treatment (Harant et al. 2016).

Discussion

In accordance with my general expectations, *Tripterygion delaisi* adjusts its fluorescence brightness to prevailing light conditions via phenotypic flexibility. The observed brightness change in response to the brightness treatments is strong enough to explain the persistent depth effect found in *T. delaisi* and several other fish species (Meadows et al. 2014). Contrary to my first prediction, however, not the spectrum but the overall brightness did result in a significant difference in fluorescence brightness (Harant et al. 2016).

The fact that fish use an achromatic instead of a chromatic proxy to adjust their fluorescence is rather unexpected. While the downwelling/sidewelling spectrum shows a relatively constant and predictable transition between depths, the overall brightness is much more variable as it depends not only on season, time of day and depth, but also on the orientation of the substrate and the habitat complexity. Consequently, fish would have to adapt their fluorescence repeatedly when moving between exposed and shaded areas. At first, this appears needlessly restrictive but it could also allow fish to “fine-tune” their fluorescence brightness even to a microhabitat scale. Timid individuals, often hiding in crevices or cracks, could therefore adjust their fluorescence brightness to match their habitat requirements ideally. Additionally, the relatively short period within which these brightness adjustments occur, also suggests that red fluorescence is of relevance to *T. delaisi* (Harant et al. 2016). However, I cannot entirely exclude that under natural conditions, the ambient spectrum affects fluorescence brightness given that ambient spectrum and brightness are both correlated with depth.

UV radiation as an alternative environmental trigger

Although red fluorescence itself is not associated with photo-protection (Meadows et al. 2014), it could still be affected by UV due to an increase of melanophores in response to higher UV levels. The fluorescent iridophores in *T. delaisi* are embedded in a layer of melanophores. These dendritic-shaped melanophores allow to regulate fluorescence brightness by dispersing or aggregating melanosomes (Wucherer & Michiels 2014). Even

if all melanosomes are aggregated, the dendritic-shaped extensions of the melanophores might partly cover the fluorescent iridophores leading to reduced fluorescence brightness. At high UV radiation, fish often increase the melanophore content in the photo-sensitive tissue as found for example in zebrafish (Mueller et al 2014). The high number of melanophores could thus lead to less fluorescence expressed. Deeper water lacks harmful UV radiation levels. Consequently, fish would not require high levels of melanophores, resulting in more fluorescence expressed. Although this could explain the depth effect in the field (Meadows et al. 2014), it cannot account for the experimental differences in fluorescence brightness as none of the light treatments did contain UV wavelengths. However, I cannot entirely exclude that UV radiation does play a minor role in regulating fluorescence brightness under natural conditions.

Physiological regulation mechanisms of red fluorescence

Given that I observed brightness shifts also in the chemically induced maximum and minimum fluorescence treatments, plasticity clearly occurred at both the immediate and the long-term physiological level (Harant et al. 2016). Since the span between minimum and maximum fluorescence did not differ between treatments, two possible explanations can be given: First, brighter fluorescence of fish held in the dark compared with the bright treatment would indicate an increase of the fluorescent pigment or the number of fluorescent iridophores. Second, a decrease in melanophores or melanosomes may have taken place to explain the brighter fluorescence in the minimum fluorescence treatment. Unfortunately, I cannot say with certainty whether only one or both of the above described processes led to the observed difference in maximum and minimum fluorescence, as this would require more physiological as well as histological data (Harant et al. 2016).

At this point, we know little about the plasticity of the fluorescent iridophore population in adults as most studies on iridophores are focusing on plasticity during fish larval development. In contrast, melanophores were investigated more intensively in the past. Killifish and Mosquitofish, for example, disintegrate their melanophores and then discharge them over time when adapting to a white background (Parker 1943, Waring 1963). Ricefish or Goldfish even perform trans-differentiation, migration or apoptosis of

their melanophores (Sugimoto et al. 2000). The plasticity of the melanophore population in *T. delaisi* remains to be clarified as well, but differentiation from some kind of precursor seems highly probable (Agrawal et al. 1999, Sugimoto 2002). Equally unknown is the rate of degradation and differentiation of melanophores. It is, however, plausible that building-up new melanophores takes more time and resources than to shrink or degrade them. This could explain the delay in decreasing fluorescence when changing to the bright treatment compared with increasing fluorescence when changing to dark conditions.

Conclusion

Summing up, this first chapter shows that red fluorescence is phenotypically flexible in *T. delaisi*. Furthermore, it is mediated by the brightness and not the spectral composition of the ambient light (Harant et al. 2016). Although I cannot exclude that genetic inheritance might have had an additional influence, phenotypic flexibility alone was strong enough to explain the observed depth effect in fluorescence brightness previously reported for *T. delaisi* (Harant et al. 2016).

Does red eye fluorescence in marine fish stand out? In situ and in vivo measurements at two depths.

Harant UK, Griessler T, Wehrberger F, Meadows MG, Champ CM, Michiels NK: Does red eye fluorescence in marine fish stand out? In situ and in vivo measurements at two depths. *Journal of Ecology and Evolution*, under review.

Summary

Active photolocation can only operate effectively if the emitted light is still detectable after traveling from the eye of the fish to the prey and back again. Calculating such a light path requires complex visual modelling as it involves factors such as triplefin photoreceptor sensitivity, reflective eye properties of the prey, and other physical quantities affecting the light signal on its way (*e.g.* attenuation by water). A first step to approach the concept of active photolocation empirically is to test if the fluorescent iris contrasts against its natural background. If this were not the case, then fluorescence is certainly too weak for active photolocation.

In the second chapter, I evaluated whether such a contrast can indeed be demonstrated and under what conditions it is more likely to occur. To this end, I characterized the natural habitat of *T. delaisi* by measuring the ambient light and taking multiple spectral measurements of typical substrates under shallow-exposed, shallow-shaded, deep-exposed or deep-shaded conditions (with shallow being 5 m and deep 20 m). Additionally, I took *in vivo* red fluorescent iris measurements under similar natural conditions. For simplicity, I only calculated the brightness contrast (*Michelson* contrast) between the iris and different substrates under all measured conditions.

In agreement with previous conclusions, I found that iris fluorescence always exceeds substrate radiance at 20 m depth, regardless of exposure and ambient downwelling/sidewelling spectra (Harant et al. *under review*). Moreover, I also identified conditions in shallow water under which red fluorescent irides contrast against their background, depending on substrate type, exposure and time of day (Harant et al. *under review*).

Discussion

In accordance with my general expectations, iris fluorescence always exceeded the ambient longer wavelengths under the stenospectral conditions at 20 m, in the wavelength range between 600 and 650 nm. Additionally, neither substrate type nor exposure influenced the likelihood of generating a brightness contrast at these depths (Harant et al. *under review*). Stenospectral light conditions are therefore more suitable to generate contrast than euryspectral conditions. This could also explain why several strongly fluorescent fish species such as *Bryaninops*, *Ctenogobiops*, or *Crenilabrius* are only found in deeper water. However, no such depth related occurrence of red fluorescent fish could be detected in a recently published phylogenetic study (Anthes et al. 2016).

Surprisingly, an achromatic contrast is also possible under euryspectral conditions. While a contrast at exposed sites is unlikely, iris fluorescence exceeds the ambient longer wavelengths in nearly 50% of all cases under shaded conditions. Furthermore, some substrate types seem to be particularly suitable for contrast enhancement such as *Padina pavonia*, different sponges, or rocky surfaces. These substrates do show strong fluorescence but their displayed spectral radiance differs substantially from the red fluorescent emission of the fish (Harant et al. *under review*). Hence, even in shallow water red fluorescence could potentially be of use to *T. delaisi*.

In shallow water, also the time of the day seems to affect the likelihood of generating a contrast. An exposed iris (fish sitting out in the open) in the morning hours has a higher chance of contrasting against the background compared with the afternoon. Under shaded conditions, the opposite effect was found (Harant et al. *under review*). This outcome is admittedly rather puzzling but could represent an artefact of defining a substrate as exposed even if it did not receive direct sunlight during the measurement.

Limitations and problems of fluorescence measurements

Due to a side effect of anaesthesia, the data presented here are strongly underestimate actual red fluorescence emission. For *in vivo* iris fluorescence measurements in the field, each fish was fixed on a table in front of the radiospectrometer. In order to obtain good measurements, anaesthesia of the fish was inevitably. Apart from tranquilizing the fish, the used clove oil also leads to a relaxation of the eye resulting in a dispersal of the black pigment containing melanosomes. Red fluorescence emission was especially affected in shallow water fish, which nearly halved their fluorescence brightness compared with untreated individuals (Harant et al. under review). This is most likely due to the high-content of iridal melanophores in the eye of shallow water individuals.

Additionally, due to handling limitations of the radiospectrometer, I was not able to measure all important habitat types. Substrate measurements were only feasible if the radiospectrometer remained in a horizontal position. Habitats such as overhangs, crevices or cracks could therefore not be measured. Consequently, I lack information on highly frequented *T. delaisi* habitats, which potentially offer suitable light conditions to generate an achromatic contrast. Hence, red fluorescence might exceed substrate emission in many more cases than the data would suggest.

Conclusion

Summing up, my data reveal that the red fluorescent irides of *T. delaisi* contrast strongly against their natural background under a large range of conditions including deep stenospectral and shallow euryspectral habitats. These results verify that the first crucial precondition for functional active photolocation by means of red fluorescence is fulfilled (Harant et al. *under review*) at least in *T. delaisi*.

Red fluorescent fish forage more efficiently in dim, blue-green light

Harant UK, Michiels NK: **Red fluorescent fish forage more efficiently in dim, blue-green light.** *BMC Ecology* 2017, 17:18

Summary

The aim of the third chapter was to narrow down the conditions under which we can expect to find active photolocation by means of red fluorescence. More precisely, I hypothesized that *T. delaisi* detects copepods more successfully under stenospectral than euryspectral conditions. Under stenospectral conditions, fish might be able to induce a contrast by illuminating prey with red fluorescence and detecting the resulting reflection in front of the blue-green background. Due to the abundance of longer wavelengths, no such enhanced contrast should be possible under euryspectral conditions.

I therefore administered 10 live copepods to 40 fish kept under euryspectral and stenospectral conditions in an alternating order. I recorded the picking behaviour using video recordings and counted how many copepods each fish caught within 5 min. Foraging success did not differ between the spectral treatments under the first shaded treatment (*glmm* shaded spectral treatment: *std-β*: 0.02, $z = 0.14$, $p = 0.81$) but all fish increased their foraging success over time, indicating a learning effect (*glmm* week: *std-β*: 0.34, $z = 5.49$, $p < 0.001$, Harant & Michiels 2017). In a follow-up experiment, I reduced the brightness of the two spectral treatments to a minimum of 3%, mimicking a heavily shaded habitat. Reducing the ambient brightness not only maximizes iris fluorescence efficiency in *T. delaisi* (Harant et al. 2016) but also impedes copepod detection.

In the second, heavily shaded round, fish were more successful in catching copepods under the stenospectral than under the euryspectral treatment (*glmm* spectral treatment: *std-β*: -0.34, $z = -1.98$, $p = 0.047$, Harant & Michiels 2017). Under the first treatment, fish improved their foraging success on average by 7% relative to the second. Individual variation was high: Nearly 44% of the individuals increased their foraging

success by at least 15% while others did not differ in foraging success at all (Harant & Michiels 2017).

Discussion

When tested under heavily shaded, stenospectral conditions, *T. delaisi* showed a higher increase in foraging success than under the euryspectral treatment (Harant & Michiels 2017). Since only the spectral composition varied between treatments, these results indicate that "red fluorescence friendly" conditions are indeed more suitable for hunting than euryspectral conditions.

Since no such difference in foraging success was found between the spectral treatments in the moderately shaded experiment, red fluorescence is most likely of little help under brighter conditions – possibly because regular vision without support by red fluorescence may have been sufficient to detect the copepods. Red fluorescence might therefore be particularly important for hunting in heavily shaded environments. This could also explain the large variation in foraging success between individuals. Such variation could reflect individual preferences for or even adaptations to specific hunting sites and/or preferred daytimes. Although these findings do not directly show that fish *used* red fluorescent irides to detect prey, the results are nevertheless in line with predictions of active photolocation by means of red fluorescence.

Chromatic or achromatic contrast

Assuming that fish indeed use active photolocation, either an achromatic or a chromatic contrast could have led to the difference in detection ability. The achromatic or brightness contrast arises when the absolute photon catch within a single cone photoreceptor is evaluated between signals. A chromatic or colour contrast is generated if the visual system evaluates the ratio of photon catches between different photoreceptor cone classes (Kelber et al. 2003). The latter requires at least two different classes of cones with reasonably varying excitation maxima. Compatible with this, two types of cones with three photoreceptor pigments have recently been described in *T. delaisi* (Bitton et al. 2017). While single cone sensitivity peaks at around 468 nm (short-wavelength-sensitive SWS cone), the two segments of the double cones have two peaks. One segment peaks at 516 nm (medium-wavelength-sensitive MWS), the other at 530 nm (long-wavelength-sensitive LWS) respectively. Although the difference between the two outer segments in the double

cones is small, it could be sufficient to allow for chromatic contrast as found for example in the triggerfish *Rhinecanthus aculeatus* (Pignatelli et al. 2010).

Since a chromatic contrast relies on the ratio of photon catches rather than the actual value it is relatively robust to brightness variations. However, fish still need to correct for spectral fluctuations in the ambient light; a mechanism known as colour constancy (D'Zmura & Lennie 1986). Until now, we know little about colour constancy in fish with only a few studies addressing this topic. Goldfish, for example, are able to distinguish between different colour stimuli under a large range of spectral illumination types. This suggests that goldfish not only possess colour vision but also colour constancy (Dorr & Neumeyer 2000). Colour vision without colour constancy is rather useless, unless foraging takes place exclusively under similar spectral conditions. This implies that most fish capable of colour vision should have some kind of colour constancy mechanism (Dorr & Neumeyer 2000).

An achromatic contrast neither requires colour vision nor colour constancy. It merely assesses the brightness difference between two signals within the visual spectral range of a species. Generally, both contrast principles could help *T. delaisi* to detect prey but usually only one mechanism is used for a specific task (Giurfa et al. 1996, Jones & Osorio 2004, Livingstone & Hubel 1988, Schaerer & Neumeyer 1996, Srinivasan 1985).

Red fluorescence: Yet another tool within the sensory “trick box” of T. delaisi

Foraging success did not differ between the spectral treatments in the first, moderately shaded experiment. Thus, red fluorescence seems to be irrelevant when foraging under relatively bright light conditions. Although *T. delaisi* shows a high preference for heavily shaded overhangs and crevices (where fluorescence may work well), it also possesses two other light re-emitting mechanisms that could assist in localizing prey under bright light. These are called the blue/red ocular spark and the ocular nerve transmitted eyeshine (Michiels et al. *in submitted*, Fritsch et al. 2017).

In contrast to red fluorescence, both mechanisms require downwelling light. An ocular spark appears when downwelling light is focused through the lens onto either a small reflective white patch (blue ocular spark) or a small fluorescent part of the iris (re-emitted as red ocular spark). These ocular sparks appear as small triangular patches at

the lower edge of the iris. Ocular nerve transmitted eyeshine occurs when downwelling light enters through the skull, is guided into the eye by the optic nerve, and exits the eye via the pupil. Similar to red fluorescence, the ecological relevance of these mechanisms is still under investigation. However, growing evidence suggests that at least ocular sparks are involved in foraging (Michiels et al. *in submitted*). Consequently, red fluorescence might be beneficial when foraging in heavily shaded habitats or at dusk and dawn, while ocular sparks could facilitate detecting prey under bright light.

Red fluorescence as a private illumination tool

Since longer wavelengths are quickly absorbed over short distance (Jerlov 1968, Lythgoe 1979, Mobley 1994), red fluorescence might allow *T. delaisi* to increase the visibility of prey while remaining cryptic to potential predators nearby. This also implies that red fluorescence is useful over short distances only. The saltatory foraging strategy of *T. delaisi* is compatible with this, as feeding strikes occur over short distances of about 2 cm. Recently, Anthes et al. (2016) showed that red fluorescent irides are particularly common among small, benthic predators supporting the hypothesis that red fluorescence is used for foraging.

Does red fluorescence attract prey?

Instead of promoting prey detection, red fluorescence could also act as an attractant for prey and increase foraging success indirectly. Small invertebrates are often attracted by light. This phenomenon is called positive phototaxis (Ringelberg 1964, Longhurst 1976). Since red fluorescent irides stand out in front of the blue-green illuminated background (Harant et al. *under review*), prey could mistakenly swim towards this light source. Such aggressive mimicry has recently been documented in a green fluorescent jellyfish *Olindias formosus* (Haddock & Dunn 2015). In this study, juvenile rockfish more often attacked the green fluorescent tentacle tips of the jelly fish when they were visible above the ambient light (Haddock & Dunn 2015). If red fluorescence is used in a similar way, it would require prey organisms to be sensitive to longer wavelengths.

While most marine organisms are sensitive to green wavelengths (Hawryshyn et al. 2003, Levine & MacNichol 1979, Loew & Lythgoe 1978, Losey et al. 2003, McFarland 1991), only a few organisms are known to possess cones with a maximum sensitivity close to red fluorescence emission (but see Cronin & Marshall 2001). Studies on photosensitivity in small benthic crustaceans are additionally rare (but see Martin et al. 2000). In a pilot study, I tested if the used prey species *Tigriopus californicus* (Baker 1912), prefers any of the experimental spectral treatments, including a red-only treatment. The pilot study confirmed that no attraction towards longer wavelengths exists in *T. californicus* (Harant & Michiels 2017, supplementary material). Although red fluorescence might still attract other organisms in the field, it cannot explain the observed increase in foraging success observed in my laboratory experiments.

Conclusion

Summing up, the third chapter shows that *T. delaisi* is more successful in foraging under heavily shaded, stenospectral conditions compare with euryspectral or brighter conditions. These results will help to narrow down the conditions under which active photolocation is likely to be confirmed in the future.

Final thoughts

While the aim of this thesis was to investigate the role of red fluorescence in a context of foraging, it is nevertheless important to discuss further potential functions not addressed so far. Apart from fish, other organisms do also express red fluorescence such as algae, corals, sponges, encrusting organisms or even calcareous rock surfaces (Murata et al. 1966, Jeffrey & Haxo 1968, Marshall et al. 2003, Johnsen 2012, Lagorio et al. 2015). This prominent long wavelength component in the habitat could allow red fluorescent fish to blend in with their background, also referred to as camouflage.

Is red fluorescence used for camouflage?

Camouflage can be achieved in many ways, for example, by adjusting body texture or displaying disruptive colouration to match the substrate complexity (Cott 1940, Endler 1978, 1980, Turner 1977, Crook 1997). Reflecting or emitting wavelengths that mirror the prevailing light conditions could likewise result in camouflage. Pelagic swarm fishes often generate a silvery broadband reflection that allows them to remain camouflaged in the middle of the open water when viewed against the silvery water surface (Denton 1970; McKenzie et al. 1995). A recently published paper gives evidence that red fluorescence might be involved in camouflage as well (Anthes et al. 2016). Sit and wait predators such as scorpionfish, often express patchy red fluorescent body colouration (Anthes et al. 2016, Sparks et al. 2014). Since these fish rely on their camouflage for successful foraging these red fluorescent patches could allow fish to blend in with their background by mimicking irregular patterns of the complex backgrounds. Compatible with this, sit and wait predators mostly display deep red fluorescence peaking at around 680 nm. This coincides well with the maximum fluorescence peak found in marine algae. However, deep red fluorescent body colouration is only useful for camouflage if the predator or the prey of the fluorescing fish from which it tries to hide from, is capable of perceiving these long wavelengths (Anthes et al. 2016). In *T. delaisi*, iris fluorescence peaks at 609 nm, camouflage is therefore unlikely the prime function of red fluorescence in this species (Bitton et al. 2017).

Species recognition or intraspecific communication

Red fluorescence could potentially also facilitate species recognition or intraspecific communication. In coral reefs, diurnal fish often express a variety of colours not only to better blend in with the background but also to simplify species recognition and communication (Marshall 2000a, 2000b). The rocky coasts of the Mediterranean provide a less brilliantly coloured habitat. Most fish therefore also express less vivid colouration (but see Michel et al. 1982). Thus, many fish species sharing a similar life style are considerably alike in their appearance. Here, red fluorescence could help in differentiating between closely related species such as *T. delaisi* outside of breeding colouration and *Tripterygion tripteronotum*. Only a well-trained eye can tell these species apart. If both species are illuminated with blue light, the difference becomes evident as *T. tripteronotum* hardly expresses any iris fluorescence at all (unpubl. data). *T. tripteronotum* inhabits depths between 0.5 m and 5 m while *T. delaisi* occurs from 5 m downwards (Louisy 2002). The overlap between the two species is therefore relatively small. Within overlapping areas iris fluorescence could nevertheless facilitate species recognition, at least under fluorescence favouring conditions. However, since fluorescence efficiency increases with decreasing depth, species recognition alone is hardly the main function of red fluorescence (Harant et al. 2016; Meadows et al. 2014).

Red fluorescent irides contrast particularly well against a blue-green background (Harant et al. *under review)*. Fluorescence could thus also serve as a private communication channel. Although the strong absorption of longer wavelengths would limit such a communication to short distances only, it might still be beneficial to small, group-living fish. Yet, Anthes et al. (2016) revealed that no such trend in small, group-living fish is present. Moreover, male *T. delaisi* lose their iris fluorescence during breeding season. Communication via red fluorescence between males and females would therefore be restricted during this period.

Is red fluorescence an honest signal?

Red fluorescence could also function as an honest signal indicating the state of health in females. Unfortunately, little is known about the chemical properties of the fluorescent pigment in the eye of *T. delaisi* but the turnover rate of GFP (green fluorescing protein) homologous proteins in corals is rather slow. This suggests that the cost of maintaining fluorescent pigments must be relatively small (Leutenegger et al. 2007). Furthermore, females represent the choosy sex within this species, which discriminate between competitive males (Wirtz 1978). This, along with the slow turnover rate of the fluorescent proteins (if comparable with fluorescent pigments mentioned before) argues against red fluorescence being an honest signal.

Concluding remarks

When combining the results of this thesis with previous work on red fluorescence, strong evidence now exists that red fluorescence is of functional importance to fish. Although none of the above mentioned alternative functions can be ruled out, they cannot account for the observed increase in foraging success under heavily shaded conditions. I therefore conclude that red fluorescence in *T. delaisi* is likely a novel light emitting mechanism that is associated with achromatic contrast enhancement. If red fluorescence is functional, it would allow fish to occupy and explore habitats less suitable for other closely related species lacking red fluorescence. However, whether *T. delaisi uses* active photolocation by means of red fluorescence remains to be tested in future studies. The results of my thesis nevertheless provide a solid foundation to narrow down the conditions under which we expect to find active photolocation.

List of contributors

Ulrike K. Harant: conceptualized and planned all studies (except for the spectrum experiment), conducted all experimental work including field spectrometry, processed and analysed the data and authored this dissertation as well as the resulting manuscripts.

Nico K. Michiels: helped planning and conceptualizing the studies, supervised all studies, developed the central research idea, contributed to the manuscripts, provided funding, gave advice on data presentation and on many aspects of conducting solid research.

Melissa G. Meadows: conceptualized the spectrum experiment, participated in data collection within the first experiment, assisted during spectrometric iris fluorescence measurements, edited the first and third manuscript and helped in designing the laboratory light system.

Nils Anthes: provided statistical support, edited the first manuscript (chapter 1) and gave advice whenever needed in scientific discussion.

Connor M. Champ, Thomas Griessler, Florian Wehrberger and Chris Radar: assisted with data collection of spectral measurements during the field season (C. MC., C. R.: field season 2014; T.G. & F. W.: field season 2015).

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Appendix

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BMC Research Notes

RESEARCH ARTICLE

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The consistent difference in red fluorescence in fishes across a 15 m depth gradient is triggered by ambient brightness, not by ambient spectrum

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Abstract

Background: Organisms adapt to fluctuations or gradients in their environment by means of genetic change or phenotypic plasticity. Consistent adaptation across small spatial scales measured in meters, however, has rarely been reported. We recently found significant variation in fluorescence brightness in six benthic marine fish species across a 15 m depth gradient. Here, we investigate whether this can be explained by phenotypic plasticity alone, using the triplefin *Tripterygion delaisi* as a model species. In two separate experiments, we measure change in red fluorescent brightness to spectral composition and ambient brightness, two central parameters of the visual environment that change rapidly with depth.

Results: Changing the ambient spectra simulating light at −5 or −20 m depth generated no detectable changes in mean fluorescence brightness after 4–6 weeks. In contrast, a reduction in ambient brightness generated a significant and reversible increase in mean fluorescence, most of this within the first week. Although individuals can quickly up- and down-regulate their fluorescence around this mean value using melanosome aggregation and dispersal, we demonstrate that this range around the mean remained unaffected by either treatment.

Conclusion: We show that the positive association between fluorescence and depth observed in the field can be fully explained by ambient light brightness, with no detectable additional effect of spectral composition. We propose that this change is achieved by adjusting the ratio of melanophores and fluorescent iridophores in the iris.

Keywords: Phenotypic plasticity, Fluorescence, Visual ecology, Fish colouration, Chromophore, Melanophore, Tripterygiidae

Background

Many organisms adapt to local environmental conditions in a remarkably fine-tuned way. Such adaptation typically occurs across distinct environments or habitats, often over significant spatial scales [1–4] or in situations where migration barriers restrict gene flow [5, 6]. Recent work, however, highlights that persistent adaptive differences in trait expression can also occur over comparably small spatial scales, such as a few kilometers in passerine birds [7, 8], and then usually in habitats characterized by steep environmental gradients as found along e.g. mountain slopes [9]. A recent study in marine fish, however, found persistent differences in red fluorescent color patterns at even smaller spatial scales across a depth gradient of only 15 m: fluorescence was consistently brighter in −20 m than in −5 m [10]. This depth difference coincides with a substantial shift in the spectral composition of the ambient light between the deeper stenospectral (blue–green) zone and the shallow euryspectral (full spectrum) zone [10].

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Small-scale adjustments like these can be generated by genetic local adaptation or phenotypic plasticity. Phenotypic plasticity subsumes environmentally triggered plasticity within genotypes that either occurs during development and then is usually irreversible [11–13] or allows repeated and reversible fine-tuning to changing local conditions [14–16]. It remains difficult, however, to disentangle the degree to which adaptation depends on local adaptation or phenotypic plasticity [17, 18]. We test to what extent the persistent small-scale differentiation in fluorescence brightness in marine fishes can be explained by phenotypic plasticity alone, using the benthic triplefin, *Tripterygion delaisi* [19], as a model species. This species exhibits genetic sub-structure only between distinct habitats separated by kilometers of unsuitable habitat such as sand or deep water [20]. This argues against small-scale local adaptation, as is the case in other fish [21]. Hence, phenotypic flexibility seems the better explanation for short-range variation in fluorescence brightness. *T. delaisi* shows remarkably fluorescent irides, and their brightness can be down- and up-regulated within seconds [22]. The fluorescent structures in the eye of *T. delaisi*, recently described as a special type of fluorescent iridophore [22], can be uncovered or covered by an underlying layer of dendritic melanophores that regulates the fluorescent emission. However, this fast, almost instant regulatory mechanism cannot account for the persistent depth-effect found in Meadows et al. [10] (Fig. 1). Here, all fish, independent of capture depth, were held under identical light conditions for a few hours prior to and during measurement [10]. Hence, we hypothesize that fish plastically adapt the limits within which the instant regulation of fluorescence brightness shown by [22] takes place, and that they do so depending on the conditions at the depth at which they live.

Two environmental cues that are known to decrease with depth could act as stimuli for fluorescence adjustment: ambient spectrum and ambient brightness. In two separate experiments, we tested whether either of them can generate the persistent variation in fluorescence brightness that is consistent with the depth gradient in the field. In both experiments, we allowed fish collected at −20 and −5 m to adapt to controlled light conditions and assessed fluorescence brightness at regular intervals. Light conditions were then reversed to determine whether fluorescence brightness was adjusted. We predicted that fluorescence brightness increases under light conditions that represent the ambient light at depth (narrower spectrum, lower brightness). Using physiological stimulation to induce minimum and maximum fluorescence, we subsequently assessed whether the range within which fluorescence is modulated ad hoc also changed with environmental conditions, e.g. wider under depth-specific light conditions.

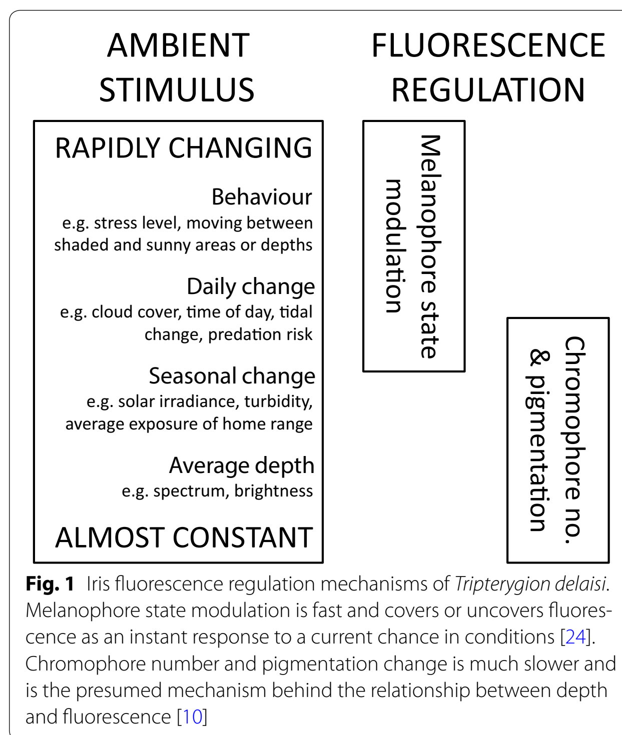

Fig. 1 Iris fluorescence regulation mechanisms of *Tripterygion delaisi*. Melanophore state modulation is fast and covers or uncovers fluorescence as an instant response to a current chance in conditions [24]. Chromophore number and pigmentation change is much slower and is the presumed mechanism behind the relationship between depth and fluorescence [10]

Results

Effects of collection depth

Initially, individuals caught at −20 m (n = 20) showed significantly brighter fluorescence than individuals caught at −5 m depth (n = 20), confirming previous findings (t test adjusted for unequal variances, $t = -4.5$, df = 25.3, $p < 0.001$; Fig. 2). However, after exposure to a single light spectrum in the laboratory for 6 months, this depth effect disappeared ($t = -0.5$, df = 35, $p = 0.61$), confirming the existence and importance of phenotypic plasticity.

Effects of spectral composition on fluorescence

We measured standardized fluorescence brightness in 40 fish initially exposed to euryspectral ($n = 20$) or stenospectral ($n = 20$) light spectra with identical brightness for 6 weeks and then switched each group to the alternative treatment for another 4 weeks (see "Methods").

Contrary to our prediction, spectral composition did not affect fluorescence brightness (see Additional file 1, Fig. 3). Instead, all fish became gradually darker over the experiment, independent of treatment. This generates a pattern where fish showed brighter fluorescence under the shallow spectrum when exposed to this treatment

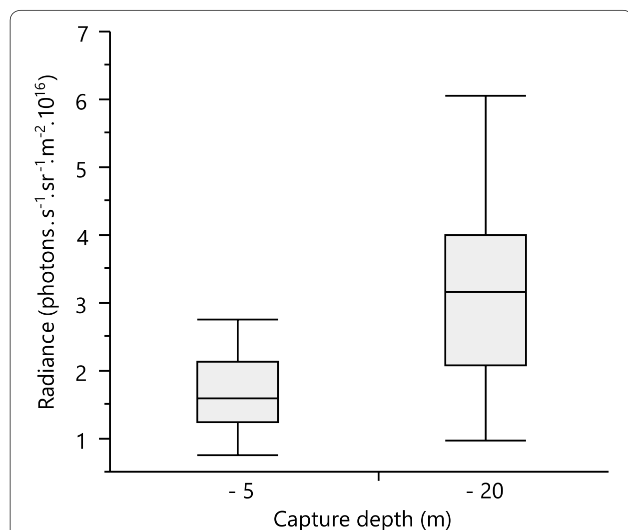

Fig. 2 Iris fluorescence brightness at deep and shallow capture depths. Iris fluorescence brightness of *Tripterygion delaisi* measured as total photon radiance (photons s^{-1} sr^{-1} m^{-2}) ($n = 40$) in the field. *Boxplots* show median (*horizontal line*), upper and lower quartiles (*boxes*) and ranges (*whiskers*)

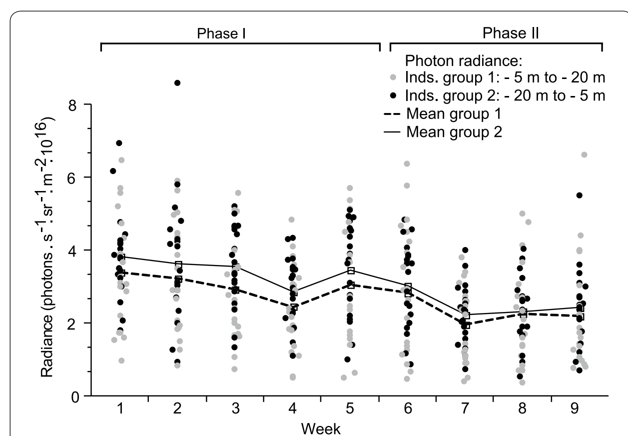

Fig. 3 Iris fluorescence in response to spectral composition. Iris fluorescence brightness of *Tripterygion delaisi* measured as total photon radiance (photons s^{-1} sr^{-1} m^{-2}) in the spectrum experiment. Fish group 1 (n = 20) started with the −20 m spectrum (phase I) and changed to the −5 m spectrum after 6 weeks (phase II), whereas group 2 (n = 20) received the opposite treatment. Fish were checked for another 4 weeks after the light switch. *Lines* represent mean total photon radiance for group 1 (*dashed*) and 2 (*solid*)

first, but the reverse when exposed to this treatment second.

Effects of ambient brightness on fluorescence

In the brightness experiment, fluorescence brightness was measured in fish initially exposed to ambient light of low ($n = 9$) or high ($n = 10$) overall brightness with consistent spectral composition and switched to the alternative treatment after 3 weeks (see "Methods").

In contrast to spectral composition, ambient brightness had a highly significant effect on fluorescence brightness (Table 1; Fig. 4). Within a week, fish moved from relatively bright pre-experimental conditions into a dark light environment increased their fluorescence brightness by 43 % on average (total photon radiance from 1.4×10^{17} to 2.0×10^{17} photons s^{-1} sr^{-1} m^{-2}; paired t test comparing initial brightness to brightness after 1 week, $t = 5.4$, df = 8, $p < 0.001$, Fig. 4), while fish moved into the bright treatment kept their initial low brightness level (1.34×10^{17}–1.35×10^{17} photons s^{-1} sr^{-1} m^{-2}; $t = 0.08$, df = 9, $p = 0.93$). No further change in fluorescence brightness occurred over the remaining 2 weeks the fish were kept under the same treatment (repeated measures ANOVA, bright treatment week 1–3: $F = 0.71$, df = 8, $p = 0.12$; dark treatment week 1–3: $F = 0.2$, df = 7, $p = 0.53$).

The effect was reversed after switching treatments in week 4. Fish switching from bright to dark increased their fluorescence brightness significantly by 39 % within a week (1.24×10^{17}–1.77×10^{17} photons s^{-1} sr^{-1} m^{-2}; paired t test, comparing radiance measurements between week 3 and 4: $t = 4.13$, df = 9, $p = 0.002$) while fish switched from dark to bright showed a decrease of 23 % (1.8×10^{17}–1.41×10^{17} photons s^{-1} sr^{-1} m^{-2}; $t = -3.83$, df = 8, $p = 0.005$). In the course of the remaining 2 weeks, fluorescence brightness remained stable in the bright treatment, but tended to further increase in the dark treatment (repeated measures ANOVA: bright treatment week 4–6: $F = 0.13$, df = 7, $p = 0.64$, dark treatment week 4–6: $F = 0.91$, df = 8, $p = 0.08$).

In order to improve temporal resolution, we subsequently exposed fish to a second switch in light environments at the start of week 7, followed by daily measurements for 7 days. We again found a significant difference between the new treatment and the previous treatment (Table 1 brightness/day; Fig. 5). The increase in fluorescence brightness in the dark treatment occurred faster than the corresponding decrease in the bright treatment. The fish in the dark light environment already had significantly increased their fluorescence the day after the light conditions had been changed (paired t test = 3.032, df = 8, $p = 0.016$) and continued to increase thereafter. In contrast, fish in the bright light treatment showed a comparably small and statistically insignificant change.

Maximum and minimum fluorescence measurements

In order to assess the instantaneous range of fluorescence that a fish can display, fish eyes were treated with either a physiological Ringer solution with elevated potassium

Table 1 Fluorescence brightness in response to ambient spectrum and brightness (adequate minimal linear mixed model)

Experiment	Parameter	Std-beta coefficient estimate	SE	t	p^2	R	Conditional R^2	Marginal R^2
Brightness/week	Intercept	17.1	0.044	384.7	<0.001			
	Brightness	0.14	0.014	9.7	<0.001		0.872	0.107
Repeatability	Bright treatment		0.034		<0.001	0.912		
	Dark treatment		0.053		<0.001	0.856		
Brightness/day	Intercept	17.13	0.061	279.61	<0.001			
	Brightness	0.246	0.056	4.34	<0.001			
	Days	0.086	0.036	2.34	0.02		0.779	0.065
Repeatability	Bright treatment		0.066		<0.001	0.791		
	Dark treatment		0.069		<0.001	0.778		

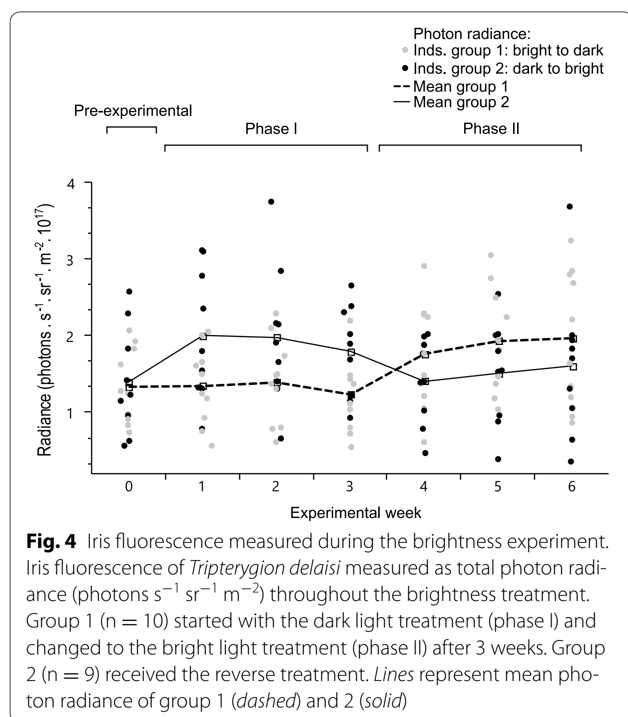

Fig. 4 Iris fluorescence measured during the brightness experiment. Iris fluorescence of *Tripterygion delaisi* measured as total photon radiance (photons s^{-1} sr^{-1} m^{-2}) throughout the brightness treatment. Group 1 (n = 10) started with the dark light treatment (phase I) and changed to the bright light treatment (phase II) after 3 weeks. Group 2 (n = 9) received the reverse treatment. *Lines* represent mean photon radiance of group 1 (*dashed*) and 2 (*solid*)

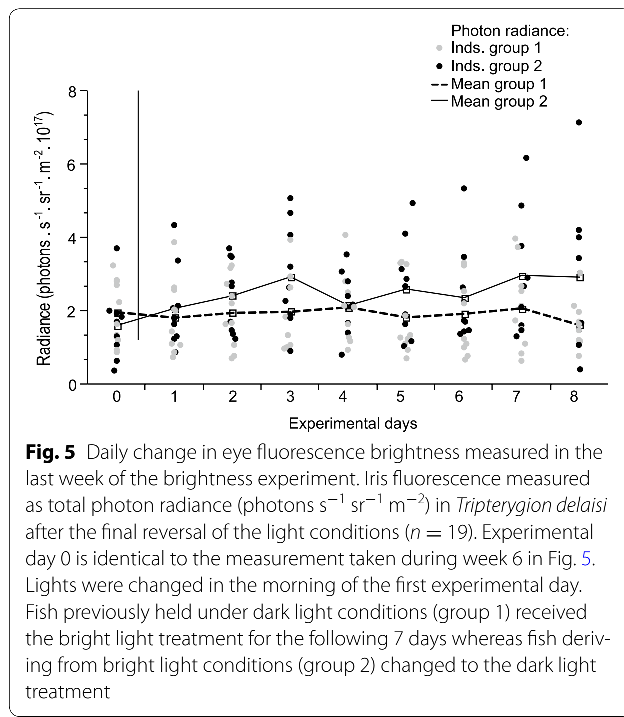

Fig. 5 Daily change in eye fluorescence brightness measured in the last week of the brightness experiment. Iris fluorescence measured as total photon radiance (photons s^{-1} sr^{-1} m^{-2}) in *Tripterygion delaisi* after the final reversal of the light conditions ($n = 19$). Experimental day 0 is identical to the measurement taken during week 6 in Fig. 5. Lights were changed in the morning of the first experimental day. Fish previously held under dark light conditions (group 1) received the bright light treatment for the following 7 days whereas fish deriving from bright light conditions (group 2) changed to the dark light treatment

concentration inducing melanophore contraction and maximal fluorescence exposure, or a regular Ringer solution resulting in melanophore expansion and fluorescence coverage [22]. For each individual fish, one eye underwent the maximum fluorescence treatment and the other the minimum fluorescence treatment.

A second sample of fish freshly caught in the field at −20 m (n = 10) showed significantly brighter fluorescence than those collected at −5 m (n = 10; Fig. 6). This was true for the maximum fluorescence values (t test, $t = -3.04$, df = 12.6, $p = 0.01$) as well as for the minimum fluorescence values (t test, $t = -3.63$, df = 15.7, $p = 0.002$).

After the spectrum experiment, minimum and maximum values did not differ between treatments mimicking euryspectral and stenospectral conditions at −5 and −20 m depth while keeping brightness constant (t test minimum: $t = -0.12$, df = 18, $p = 0.9$; maximum: $t = -0.13$, df = 18, $p = 0.89$). In the brightness experiment, however, both the fluorescence maxima and minima were significantly elevated in fish kept under dark rather than bright conditions in their final treatment (t test maximum: $t = 2.3$, df = 17, $p = 0.03$, minimum: $t = 2.3$, df = 12.7, $p = 0.03$).

We further tested whether the total range between maximum and minimum fluorescence, and thus the range

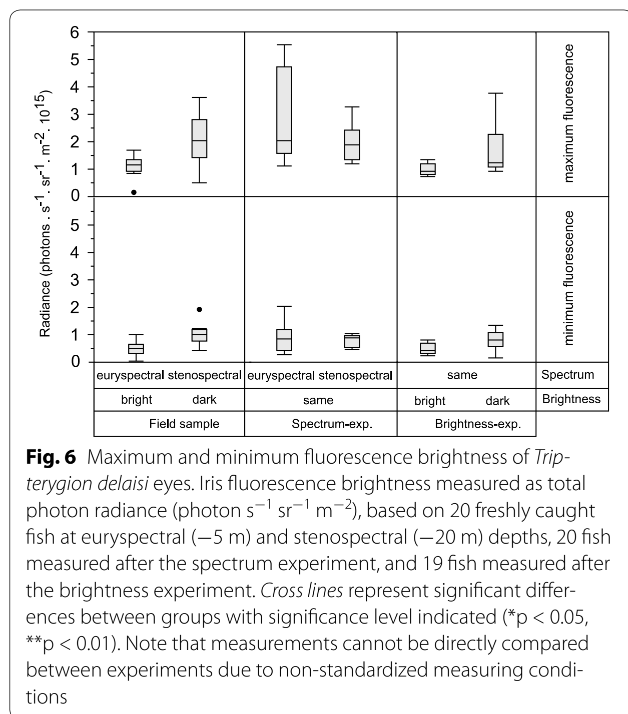

Fig. 6 Maximum and minimum fluorescence brightness of *Tripterygion delaisi* eyes. Iris fluorescence brightness measured as total photon radiance (photon s^{-1} sr^{-1} m^{-2}), based on 20 freshly caught fish at euryspectral (−5 m) and stenospectral (−20 m) depths, 20 fish measured after the spectrum experiment, and 19 fish measured after the brightness experiment. *Cross lines* represent significant differences between groups with significance level indicated (*p < 0.05, **p < 0.01). Note that measurements cannot be directly compared between experiments due to non-standardized measuring conditions

within which individuals can show instant fluorescence modulation, increased or decreased depending on the final treatment. No such difference was present in any of our treatment groups (Wilcoxon test field: $n_{euryspectral} = 10$, $n_{stenospectral} = 10$, $Z = 0.94$, $p = 0.34$; spectrum experiment: $n_{deep} = 10$, $n_{shallow} = 10$, $Z = 1.17$, $p = 0.24$; brightness experiment: $n_{bright} = 10$, $n_{dark} = 9$, $Z = 0.37$, $p = 0.68$).

Discussion

In agreement with our general expectation, *T. delaisi* triplefins adjust their fluorescence brightness to the prevailing light conditions under a scenario of adaptive phenotypic plasticity. This response was directly triggered by the overall brightness of the ambient light environment, but was independent of its spectral composition. Our data thus support brightness-dependent changes in iris fluorescence and refute the hypothesis that previously demonstrated depth-related changes constitute a response to the stenospectral composition of light at depth.

Our observation that *T. delaisi* uses achromatic (brightness) information as an environmental trigger to adjust fluorescence brightness seems initially surprising given that brightness will vary more at a given depth with shading and daytime than spectral shape does. However, the brightness effect alone is strong enough to explain previously observed persistent differences in fluorescence brightness between shallow and deep field sites. It is not unlikely that this effect also applies to the other species for which the depth effect has been observed [10]. A new prediction following from this is that fish living in a shady part of the substrate should fluoresce more brightly than fish living at more exposed sites at the same depth.

Subtle effects of spectrum overlooked?

Although the effect of brightness is strong enough to explain depth-related variation in fluorescence brightness, additional weak effects of spectral composition may still exist, but have gone unnoticed for two reasons. First, UV was not part of the illumination spectra. Although *T. delaisi* is unlikely to see UV (see "Methods"), their skin and iris may passively protect from UV by expressing more melanin ("tanning") in the more UV-exposed shallow water [23]. If this effect was relevant, however, our essentially *UV-free* brightness treatments should not have triggered the observed significant changes in fluorescence brightness. Hence, our experiments indicate a brightness effect *independent* of UV, but a small effect of UV in the field cannot be ruled out.

Second, although differences in brightness seem to serve as the key trigger, it may still represent an adaption to local spectrum too. In the natural environment, depth, brightness, and spectral shape co-vary in a predictable way. Darker environments are more likely to be in deeper, stenospectral sites, where fish fluorescence generates stronger visual contrasts [10].

How is fluorescence regulated?

The fact that a change in the mean fluorescence brightness coincided with a similar change in minimum and maximum values indicates that fluorescence did not just change at the instant melanophore state, but also at the tissue level. This may involve a change in iridophore optical nanostructures or in fluorescent pigment concentration. These chromatophores contain guanine/hypoxanthine crystals [24], but the identity of the fluorescent pigment contained within these crystals remains unknown. More likely, they may modify melanosome density in the melanophores or increase the number of the latter, thereby adjusting the degree to which fluorescent iridophores can be covered. Guppies, killifish, and mosquitofish disintegrate and discharge melanophores when adapting to a white background over time [25, 26]. Rice fish and goldfish even performed transdifferentiation, migration or apoptosis of their melanophores [27], similarly leading to reduced pigmentation. Little is known to date about how fast these changes occur. Nevertheless, it seems plausible that building-up melanosomes and an associated decrease in fluorescence requires more energy and time than reducing or degrading them. This could explain the temporal delay in fluorescence decrease observed in the bright light treatment

during the brightness experiment compared to the relatively fast increase in the dark light treatment.

Room for genes?

Given that phenotypic flexibility seems sufficient to explain the depth effects on fluorescence observed earlier [10], it is questionable that local genetic adaptation through natural selection is involved. However, given that there is substantial variation between individuals, our data do not exclude that a genetic component may be involved in the ability to adapt to a changing environment, irrespective of the depth at which fish are collected. This hypothesis cannot be tested using the data obtained in this study. Indirectly, our results suggest that the spatial separation between −5 and −20 m is indeed too short to result in true local adaptation for this trait.

Conclusion

Fluorescence in *T. delaisi* is phenotypically flexible and regulated by ambient brightness, which tightly co-varies with depth and spectral shape. This finding is a major contribution to understanding the proximate reasons why fish in deeper water fluoresce more. It also offers fish at a single depth to tune their fluorescence range to prevailing light conditions determined by factors that do not change instantly, such as seasonality or degree of shading.

Methods

Study species, collection, and housing

Tripterygion delaisi is a small, cryptic, benthic triplefin from rocky habitats between −3 and −40 m depth in the Mediterranean Sea and the eastern Atlantic [28, 29]. The species possesses a prominent red fluorescent iris in which the interplay between fluorescent iridophores and covering melanophores controls fluorescence brightness [22].

We caught 100 individuals in Elba, Italy, at depths of −5 and −20 m in June 2013. Collection took place under the general permit of the Hydra Institute (Centro Marino Elba, Campo nell´ Elba, Italy). Given that this took place at the end of the breeding season, 28 males still showed breeding coloration. For most individuals, however, sex could not be inferred because both males and females display the same cryptic coloration outside the breeding season. Upon capture, fish were kept individually in perforated 1 L plastic cups placed in a 50 L flow-through tank continuously supplied with fresh seawater at ambient temperature. At the end of fieldwork and initial fluorescence measurements of all specimens (see below), fish were transferred to aquarium facilities at the Eberhard Karls University of Tübingen, Germany on 29 June 2013. They were kept individually in blue LED illuminated 15 L tanks (20 °C, salinity 34 ‰, pH 8.2, 12 h light/dark cycle, fed once per day). In order to ensure that all individuals had adapted well to the laboratory conditions and all males in breeding coloration had fully changed back to their cryptic coloration, fish were allowed to adapt to laboratory conditions for 6 months. Animal husbandry was carried out in accordance with German animal welfare legislation.

Effects of spectral composition on fluorescence

In early December 2013, 20 fish were chosen randomly from each of the −5 and −20 m original capture depths ($n = 40$) and relocated to the experimental room into 40 individual 20 L tanks (20 °C, salinity 34 ‰, pH 8.2, 12 h light/dark cycle, fed once per day) and were allowed to adapt to their new tank for 9 days.

Each aquarium was illuminated by eight LEDs in a single housing with diffusor for homogeneous lighting (custom made Feno Fe s.soft lt 18), controlled by a DMX standalone unit (Feno fc s.dmx 48d). The brightness of each LED (cold white, UV: 395–410 nm, royal blue: 450–465 nm, blue: 465–485 nm, 2× green: 520–535 nm, amber: 585–595 nm and red: 620–630 nm) could be individually controlled in 100 steps from off to maximum to generate custom spectra. We defined two light treatments to mimic the spectral shape of downwelling light in the field at −5 m (euryspectral) or −20 m (stenospectral) depth (Fig. 7). In order to assure that any effect was due to spectral shape only, care was taken to obtain identical total irradiance in the two spectral treatments (total irradiance in photons s^{-1} m^{-2}, euryspectral: 2.55×10^{18}, stenospectral: 2.51×10^{18}). Since the light diffuser of the LED housing blocked most of the UV, the UV channel was switched off for the duration of all the experiments. Given that the eye lens of *T. delaisi* blocks UV, UV is probably irrelevant for vision (unpubl. data N. K. Michiels).

From 16 Dec 2013 onwards, half the fish of each collection depth (−5 and −20 m) received the euryspectral treatment ($n = 10 + 10$), and the other half received the stenospectral treatment ($n = 10 + 10$). Fish were kept under this illumination for 6 weeks. In order to assess changes in iris fluorescence brightness, individuals were measured at the end of each week (details below). Following this first round, the spectral treatments were reversed, exposing each individual to the alternative spectrum for another 4 weeks, again including weekly measurements. Thereafter, 20 randomly chosen fish (10 of each final treatment) were sacrificed to assess the physiological minimum and maximum fluorescence brightness (details below). One individual died during this experiment.

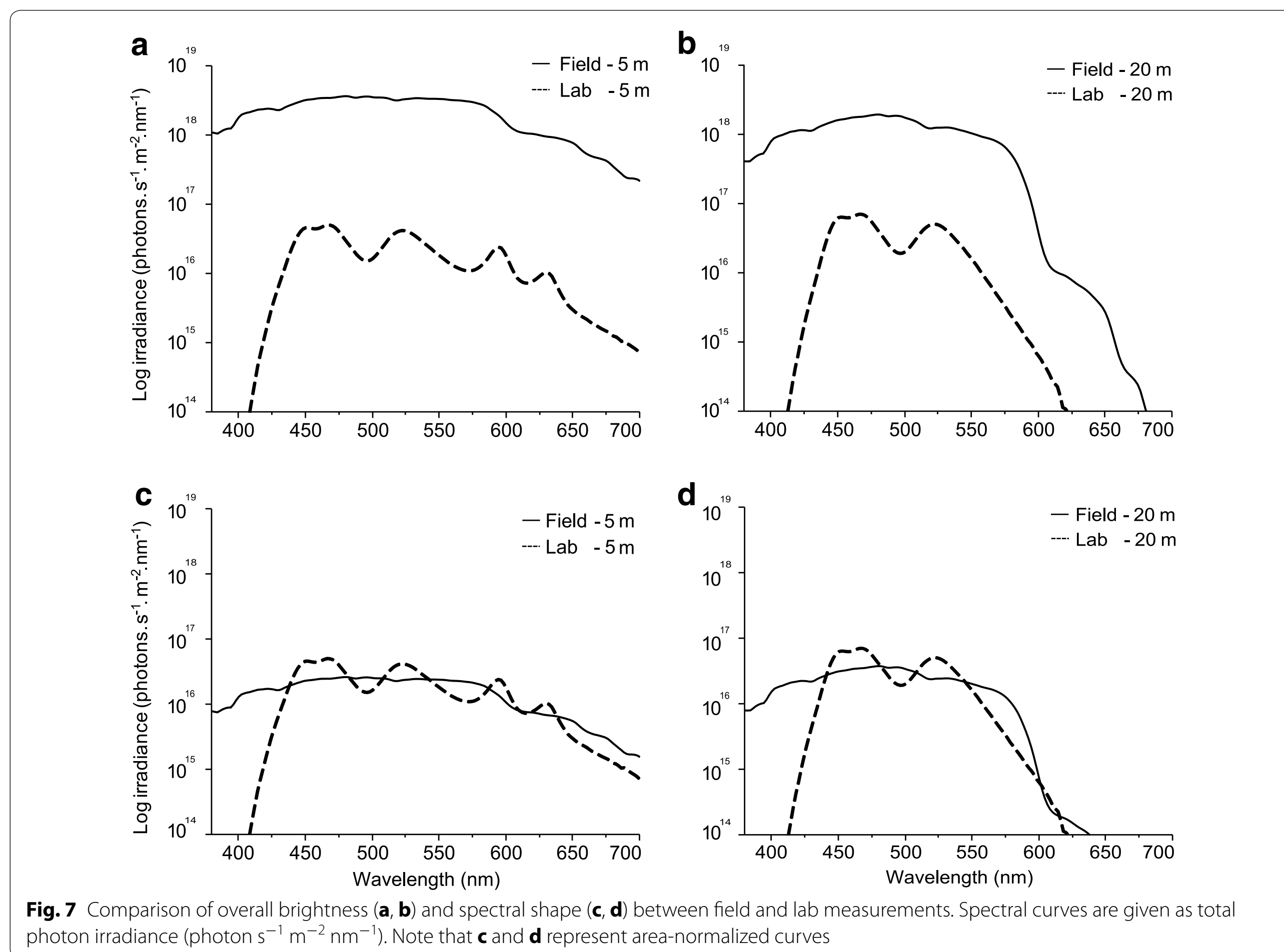

Fig. 7 Comparison of overall brightness (**a**, **b**) and spectral shape (**c**, **d**) between field and lab measurements. Spectral curves are given as total photon irradiance (photon s^{-1} m^{-2} nm^{-1}). Note that **c** and **d** represent area-normalized curves

Effects of ambient brightness on fluorescence

The remaining 19 individuals, each still kept in its own tank, were kept under the same light conditions they experienced at the end of the spectrum experiment for another 11 weeks. On 19 April 2014, the fluorescence of all fish irides was measured again and fish were randomly divided into two groups. One group was exposed to 100 % white light (all light channels on 100 %) whereas the lights in the other group were completely turned off. Since spectral shapes did not affect fluorescence brightness in the spectrum experiment (see "Results") we did not attempt to mimic the spectral shape under natural conditions in the brightness experiment, but instead used the maximum brightness possible with our light system (Fig. 8). Because bright and dark tanks were in alternating positions in the rack but separated by opaque sheets, the only light reaching the dark tanks came from diffuse reflection by the opposing white wall. As a consequence, the irradiance in the dark tanks was 1 % of that in the bright tanks

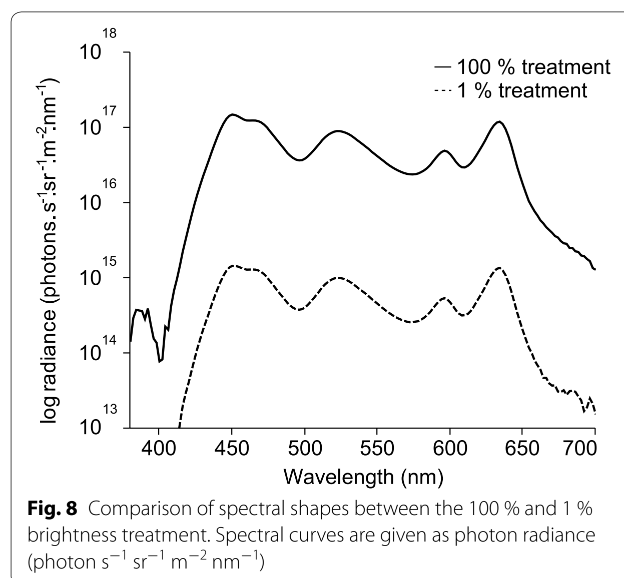

Fig. 8 Comparison of spectral shapes between the 100 % and 1 % brightness treatment. Spectral curves are given as photon radiance (photon s^{-1} sr^{-1} m^{-2} nm^{-1})

(Fig. 2). This procedure was preferred over a solution with the LEDs dimmed in the dark tanks because LEDs flicker when set to lowest brightness. The tanks treated with the bright light treatment received about 70 % of the total radiance measured at a sunny day in the field at −5 m (total irradiance in photons s^{-1} m^{-2}, 1.46 × 10^{19} compared to 2.05 × 10^{19}), whereas the fish in the dark treatment only received about 0.8 % (total irradiance in photons s^{-1} m^{-2}, 1.54 × 10^{17} compared to 2.05 × 10^{19}).

As in the previous experiment, the fluorescence of the irides was measured each week for three subsequent weeks. At the start of week 4, light treatments were reversed in all tanks and measurements continued for another 3 weeks. In order to assess the daily rate of change, a final treatment reversal was performed at the start of week 7, immediately followed by a first measurement on that same day, as well as further daily measurements for another 7 days.

Fish fluorescence measurements

All measurements were taken in a dark room. Two LED-RGB stage lights (LED Par64, 20 × 3/1 PMW, 90–240 V, 50/60 Hz) set to monochromatic blue and supplemented with a short-pass filter (ZILZ direct, Dichroic glass filter, blue) were used for fluorescence excitation in the field and spectrum experiment. In order to shorten measurement duration in individuals with weak fluorescence, which was more common in the brightness experiment, we used a brighter light source during this part of the study: blue Hartenberger Mini Compact LCD divetorch with 7 × 3.5 W 450 nm bulbs with additional short pass filter (Thorlabs FD2C subtractive dichroic color short-pass filter). In all cases, the red fluorescence of the fish iris was measured with a calibrated PR-740 SpectraScan Spectroradiometer (Photo Research Inc., bandwidth: 2 nm, aperture: 0.5, calibrated lens: MS-75, smart dark enabled, speed: normal, exposure time: automatic, extended). SpectraScan spectroradiometers have a camera-like viewfinder and lens, allowing the researcher to point it at the object of interest and to cover the area intended to be measured with a measurement spot that is adjustable in size. Because multichannel spectrometers such as the Photoresearch adjust gain to avoid saturation in the brightest wavelengths, measurements were taken through an orange filter (LEE filters, Double C.T. Orange 287) fitted over the spectroradiometer lens to suppress the blue excitation light. We determined the transmission curve of the filter and used this to calculate the original fluorescence curve of the iris. Measurements were taken at a fixed distance of 27 cm between the front edge of the lens of the spectrometer and the front of the measurement chamber. The person measuring fluorescence brightness was blind to the treatment the fish came from. Prior to the measurement, individual fish were carefully transferred into a small chamber (7 × 10 × 2.5 cm) with a black background and a thin (1.5 mm glass front) filled with seawater and placed in front of the spectrometer. Fish were positioned so that their right eye faced the glass front. The 0.5° measurement spot size covered the complete eye of *T. delaisi*. For each fish we took three measurements of the right eye and one measurement of a non-fluorescent red diffuse reflectance standard (Labsphere SCS-RD-010) to check for stray red light in the room and constancy of the measurement light conditions. The average of the red reflectance standard measurements of 16 December 2013 were used as baseline to adjust all following measurements in the spectrum experiment for variation between measurement sessions. The same procedure was used in the brightness experiment, but we used the average of the red standards from 17 April 2014 as the baseline.

Radiance was measured in W sr^{-1} m^{-2} nm^{-1}. Radiance data were converted into photon radiance (photons s^{-1} sr^{-1} m^{-2} nm^{-1}) and integrated to total photon radiance (photons s^{-1} sr^{-1} m^{-2}) in the 525–700 nm range. The latter value was used as a measure of fluorescence brightness. Since fish reduced their fluorescence in the measurement chamber (the usual stress response to a new environment), only the highest fluorescence brightness measurement of a fish (usually the first) was used for analysis. Note that measurements are slight underestimates because 9.4 % ± 2 SD of the area measured consists of the non-fluorescent pupil.

Maximum and minimum fluorescence measurements

Since *T. delaisi* is able to regulate its fluorescence quickly [22], we also estimated the physiological maximum and minimum fluorescence brightness an individual is able to display in its current light treatment. To this end 20 fish were sacrificed directly after the light spectrum experiment (five randomly taken from each original collection depth and final light treatment group). After the brightness experiment, all 19 remaining fish were sacrificed (10 from the bright treatment, 6 originating from −20 and 4 from −5 m; 9 fish from the dark treatment, 5 from −20 and 4 from −5 m). After decapitation, both eyes were removed. Each eye was placed on top of an eye holder (1.5 ml vial lid glued upside-down in a well of a 12-well culture plate to keep the eye facing upward). One eye was submerged in 3 ml marine physiological ringer solution (mM: NaCl 125.3, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-(+)-Glucose 5.6, Tris–HCl 5.0, pH 7.2) and the other in K^+ elevated saline solution (mM: NaCl 78, KCl 50, $CaCl_2$ 1.8, $MgCl_2$ 1.8, D-(+)-Glucose 5.6, Tris–HCl 5.0, pH 7.2). The total ionic concentration was identical and isotonic in both. Marine physiological ringer induces

melanosome dispersal in melanophores, minimising fluorescence [24]. The elevated K^+ does the opposite, inducing melanosome aggregation and resulting in maximum fluorescence [24]. Each eye was incubated for 15 min and subsequently placed under a fluorescence microscope (Leica DM5000B) with a Leica DualCam excitation filter (480–510 nm), a Leica 550–700 nm emission filter, and a Leica EL6000 as the external light source. Measurements were taken with a c-mounted PR-740 spectroradiometer. Fluorescence brightness was calculated from radiance measurements as described above. We used a measurement spot of 0.5°, covering precisely the whole iris of the fish eye. In June 2014, the same procedure was carried out with 20 freshly caught fish (10 from −5 m and 10 from −20 m) in Calvi, Corsica, France, to provide a field reference. An overall analysis of all treated fish confirms that we obtained the envisaged effect: Elevated K+ and regular Ringer did indeed cause a highly significant gap between minimum and maximum fluorescent brightness (comparison between both eyes for all fish, paired t test, $t = -8.6$, df $= 58$, $p < 0.001$).

Statistical analyses

General and linear mixed models were performed using the lme4 package [30] in R (R x64 3.1.1, [31]), all other analyses were performed using JMP 11 (SAS). All data were checked for normality and homoscedasticity and analysed accordingly. If possible, paired statistical tests were preferred over others to account for differences between individuals.

Backward linear mixed model selection analyses were performed for both experiments to estimate the roles of capture depth, light treatment, week (days for the final week of the brightness experiment), treatment order, the interaction between light treatment × week (days), sex, and body size (not available for the brightness experiment) on iris fluorescence. Since individual fish fluorescence radiances were measured multiple times, fish ID was included as a random factor in every step of the model selection. In the spectrum and brightness experiment, the response variable fluorescence brightness was transformed using $\log_{10}$ to approximate a normal distribution. Due to the experimental design, the time factor (week) could not be separated from a potential treatment effect in the spectrum experiment. Log_{10} iris fluorescence was therefore corrected for the week effect by using studentized residuals from a linear regression with week as predictor, irrespective of treatment or group. Model selection was then performed with the studentized residuals as a response variable. Resulting coefficient parameter estimates were standardized, allowing us to compare the factor influence between the predictors. Starting from a full model containing all fixed factors, the minimal adequate model was selected based on the Bayesian information criterion (BIC) comparing hierarchical models with and without the factor of interest. In the final model, we assessed statistical significance of each parameter using a Kenward–Roger approximation [32]. For each linear mixed model, we provide proxies for the goodness-of-fit of the fixed component (marginal R^2) and the complete model (conditional R^2) [33] as implemented in the piecewiseSEM package for R [34].

We estimated ANOVA-based repeatabilities for the fluorescence measurements within the three experiments (spectrum, brightness and brightness per day) as implemented in the rptR package [35] in R.

Additional file

Additional file 1. R-scripts for all LMM´s reported in the article are provided.

Authors' contributions

UKH carried out the collection and analyses of the data, designed the second experiment and drafted the manuscript. NKM participated in the design of the study and did proof reading of the manuscript. NA performed extensive proof reading and provided statistical guidance. MGM designed the first experiment of the study and participated in its data collection, completed all initial field measurements in Elba, and edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Ethical approval

Fish were sacrificed under Permit No. ZO 1/12 from 23 April 2012. The permit was issued by the Animal Protection Office at the Regierungspräsidium, Referat 35, Konrad-Adenauer-Strasse20, 72072 Tübingen, Germany.

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Does red fluorescence help fish to increase colour contrast? An assessment of applicability and likelihood of creating colour contrast via red fluorescence in the field.

Harant UK, Griessler T, Wehrberger F, Meadows MG, Champ CM, Michiels NK: Does red eye fluorescence in marine fish stand out? In situ and in vivo measurements at two depths. *Journal of Ecology and Evolution*, under review.

Abstract

Since the discovery of red fluorescence in fish, much effort has been made to elucidate its potential functions. A fascinating possibility is that light emission may help to detect prey, as suggested recently in a laboratory experiment using the small, cryptobenthic triplefin *Tripterygion delaisi*. An untested assumption, however, is that red light emission exceeds background radiance and thus potentially generates perceivable contrast. Here, we present spectrometric measurements of natural *in vivo* eye fluorescence in *T. delaisi* against its natural background. We combined background radiance measurements for typical shaded and exposed foraging sites at 5 and 20 m depth with *in situ* iris radiance measurements of live fish. To assess the visual contrast, we then calculated brightness of iris radiance in the 600-650 nm “red” range of the spectrum relative to substrate radiance under ambient light conditions. At 20 m depth, *T. delaisi* iris radiance substantially exceeded substrate radiance in the red spectral range, regardless of exposure, and despite substrate fluorescence. Given that downwelling light in the 600-650 nm range is negligible at this depth, we can attribute this effect to iris fluorescence. As expected, contrasts were much weaker in 5 m, but we identified specific substrates and conditions under which the pooled radiance caused by red reflectance and fluorescence still exceeded the red radiance from the substrate. We conclude that the requirements to create visual contrast are fulfilled for a wide range of conditions in the natural environment of *T. delaisi*, particularly at depth, indicating that our laboratory results may be transferable to the field.

Introduction

The characteristics of downwelling light changes rapidly with depth in the water column, from directional, bright and spectrally broad near the surface to scattered, dim and spectrally narrow at depth [1-4]. The two main underlying processes are light absorption and scattering [1-4]. Light absorption is particularly strong for longer wavelengths, resulting in a skew towards intermediate, blue-green wavelengths in the visible spectrum. The remaining light is increasingly scattered as it penetrates into the water column resulting in soft, homogenous lighting that lacks abrupt illumination boundaries. These effects have profound consequences for animal coloration as well as visual perception. In shallow water, the ambient light usually exceeds the visual perception range of most fish at both ends of the spectrum. We named this zone the euryspectral zone [5]. With increasing depth, the ambient light quickly narrows down leading to a stenospectral zone where the range in visual perception is usually broader than the available ambient light [5]. Most types of coloration originate from the wavelength-specific absorption and reflection properties of pigments or structural color mechanisms. Possible hues and intensities are therefore strictly limited by their availability in the ambient spectrum. Fluorescent pigments do not have this limitation. They transform absorbed photons of a given wavelength (e.g. in the blue-green range) and re-emit light at longer wavelengths (e.g. yellow or red). Although fluorescent pigments are widespread in benthic marine organisms [6-8], their presence in fish has only recently been confirmed [6, 9, 10]. The phylogenetic distribution of red fluorescence in fish was found to correlate with camouflage and sexual signaling [10]. Anthes et al. (2016) also found that the presence of conspicuously red fluorescent irides is associated with a micro-predatory lifestyle [5, 11, 12]. A recent experimental study provided first direct evidence that foraging success increased under dim, “fluorescence-friendly” cyan illumination relative to broad spectral illumination at the same brightness in the micro-predatory triplefin *Tripterygion delaisi* [13].

The underlying assumption, however, is that red fluorescence is strong enough to “illuminate” nearby prey on its natural background. Although emission is weak, it spans a wavelength range that is missing from the downwelling light at depth. Hence, fluorescent fish might indeed be able to create visual contrast between prey items and their backgrounds. Here, we present data demonstrating that red fluorescence makes iris radiance brighter than background radiance in the field. First, we characterize the natural light environment of our model species *T. delaisi* by measuring ambient down- and side-welling light as well as the radiance of typical substrates under euryspectral (5 m) and stenospectral conditions (20 m depth) under naturally shaded and exposed conditions. We also measured iris radiance in anesthetized *T. delaisi in situ*. Direct comparison of substrate and iris radiance allowed us to

identify combinations of substrate, depth and exposure under which the red fluorescent iris of *T. delaisi* contrasts particularly strongly against the background.

Materials & Methods

The black-faced triplefin *Tripterygion delaisi* is a small benthic fish living in rocky habitats between 5 and 50 m depth along the Mediterranean and eastern Atlantic coasts [14]. It feeds mainly on small, benthic invertebrates [15, 16]. Except for the breeding season, where males develop prominent coloration, individuals are highly cryptic against their natural background, with no sexual differentiation. *T. delaisi* displays highly fluorescent irides with an average peak emission (λ_{max}) of 609 nm and a full width at half maximum range of 572 nm to 686 nm [17]. Furthermore, it can perceive fluorescent signals [17, 18], and regulates its fluorescence brightness actively through dispersing and aggregating melanosomes within their melanophores, so that they can switch near-complete absence of fluorescence to maximum brightness within 10-30 sec [19].

Field site

Field data were collected at the Station de Recherches Sous-marines et Océanographiques de Calvi (STARESO) Corsica, France in June-July 2014 and 2015. Data were collected while scuba diving at three sites. The shallow site (1) is located just off STARESO and characterized by rocky slopes, steep walls and granite boulders down to 12 m. Exposed hard substrates are covered with a diverse community of green, red and brown algae. Shaded parts are dominated by coralline red algae and sedentary animals (sponges, cnidarians, bryozoans, ascidians). Flat sandy sediments start at the bottom of the slope and are covered with seagrass (*Posidonia oceanica*), leaving only small patches of rubble and sand. The seagrass meadow slopes gently into deeper water (down to > 30 m). The deep site (2) is located 1 km East of STARESO (“La Bibliothèque”). It features large granite boulders of 1-6 m across from above the surface down to 25 m. A seagrass meadow starts at the bottom of the slope. Areas between the boulders are covered with rubble and sand. The boulders are vegetated mainly by algae including calcareous algae, and some sponges and ascidians, particularly in the permanently shaded parts. A third site (3) was used for a spectrometric depth profile only and is marked with a buoy ca. 200 m NE of STARESO. It has a maximum depth of 36 m with sparse seagrass growth on more or less flat, featureless, sandy ground.

Spectral measurements of the depth profile

Radiance measurements were taken with a calibrated PhotoResearch SpectraScan PR-740 radiospectrometer in a custom-made underwater housing (BS Kinetics). The PR-740 is an all-in-one aim-and-shoot spectrometer with Pritchard optics: It allows to visually focus on a

target from a distance with set acceptance angles between 0.1° and 1°. It produces radiance measurements (watts • sr^{-1} • m^{-2} • nm^{-1}) in the 380–780 nm range with a 1 nm resolution using a bandwidth of 8 nm. Due to its cooled sensor, this spectrometer captures even very weak signals with little noise at short exposure times. Incident light was measured by aiming the radiometer at a Polytetrafluorethylen (PTFE) diffuse white standard (Berghof Fluoroplastic Technology GmbH), fixed 10 cm in front of the port of the UW housing directed towards the surface in a 45° degree angle facing South. This resulted in a mixture of downwelling and sidewelling light. The spectrometer was fitted with a calibrated MS-75 standard lens plus SL-0.5x add-on macro lens. To reduce noise in the long-wavelength range at depth, a LEE 287 Double C.T. Orange filter was used to reduce the abundant blue-green range, allowing longer exposure times to capture better readings in the weak red range of the spectrum. All data were subsequently corrected for the transmission of the port of the underwater housing, the SL-0.5x add-on macro lens, and the LEE 287 filter. Radiance measurements (watts • sr^{-1} • m^{-2} • nm^{-1}) were transformed into photon irradiance (photons • s^{-1}• m^{-2} • nm^{-1}) by multiplication with π • wavelength • 5.05 • 10^{15} at each wavelength [20]. A compass, a level indicator, and an electronic depth gauge were mounted on top of the housing for accurate positioning. The measurements were taken under a blue, sunny sky, around solar noon on 26 June 2015. These values presumably approach the annual maximum at this location. Three initial measurements were taken just below the surface (0.1 m depth), followed by an immediate descent to 36 m depth. While slowly ascending, three measurements were recorded at each 2 m interval and averaged. Buoyancy was controlled by holding on to a fixed buoy line with one hand while operating the neutrally buoyant spectrometer housing with the other. Special care was taken to not swirl up any substrate growing on the rope and to avoid shading by the buoy or the line. Some side-welling light will have been absorbed by the black suit of the diver. The dive buddy followed at a safe distance of 5 m north of the diver operating the device.

Radiance of substrates frequented by T. delaisi

We took spectral measurements throughout the day (07:30 – 18:00) from 29 typical *T. delaisi* sites that were either exposed or shaded at 5 and 20 m depth (Figure 1 A). We defined a substrate to be shaded if it was permanently shaded by e.g. overhanging rocks. Compass direction and surface slope were chosen to cover representative variation. Note that very steep, vertical or overhanging surfaces could not be measured due to handling limitations of the underwater housing, although these areas are also frequented by *T. delaisi*.

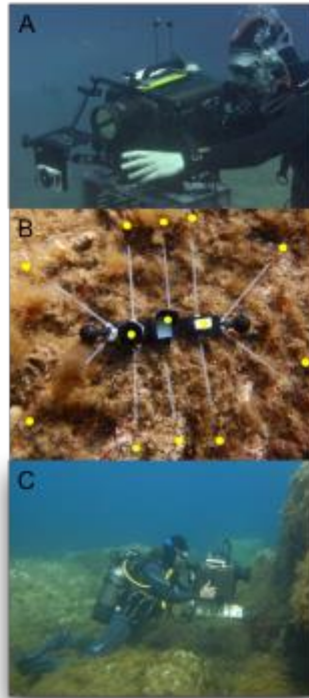

Figure 1 A: Substrate radiance measurements taken at 5 m depth using a calibrated radiospectrometer (PR740) in a custom made underwater housing (BS Kinetics). **B:** Substrate transect device with reflectance standards in the centre (from left to right): black standard, shaded diffuse white standard (PTFE) and non-shaded diffuse white standard (PTFE). Spectral measurements pointing vertically onto the substrate were taken approx. 1 cm beyond each of the ten cable binder tips following the direction of each spine (yellow spot). The length of the central black carrier of the transect device is 22.5 cm. **C:** Iris radiance measurements taken with a radiospectrometer aiming at a laterally oriented and secured fish at 20 m depth.

To standardize measurements and assess small-scale variation of micro-habitat characteristics, a small transect device was created (Figure 1 B). It defined 10 arbitrary measurement points positioned around three centrally positioned colour standards: an exposed PTFE diffuse white standard (DWS) as a combined measure of downwelling and sidewelling light, a shaded DWS to assess sidewelling light only, and a black standard (dark opening of a small vial covered with black cloth inside and outside) as a proxy for the amount of scattered light between spectrometer and substrate. We first measured each standard, then the 10 measurement points (Figure 1 B), followed by a second measurement of each standard. We used the same spectrometer and setup as described above, but omitted the LEE 287 Double C.T. Orange filter and the macro lens (not required). The distance between spectrometer and target was fixed at 80 cm, the minimal focal distance of the spectrometer. Note that radiance summarizes all sources of light coming from the substrate (reflection, transmission and fluorescence). The effect of compass direction was negligible compared to

substrate exposure (shaded/exposed) and time of day. We therefore omitted orientation from the results.

To assess whether substrate radiance exceeds the ambient radiance in the 600–650 nm range, we averaged measurements separately for each specific substrate type within a transect. We then calculated relative radiance as the radiance of that specific substrate type relative to the diffuse white standard of this transect. Values are expected to be smaller than 1, unless substrate fluorescence is strong relative to reflection.

Iris measurements of T. delaisi

Iris radiance was measured at 5 m (site 1, *n* = 16 individuals) and 20 m depth (site 2, *n* = 18 individuals). A collection team first caught fish with hand nets at the target depth and brought them to the nearby measurement spot in 50 ml Falcon tubes. The measurement team then anesthetized fish with diluted clove oil and gently placed them in a transparent plastic holder fixed to a small table attached to the front of the spectrometer port (Figure 1 C). The whole head of the fish was fully exposed to the ambient light. Fish were measured facing South (sun exposed, more directional light) or North (shaded from direct sunlight, more scattered light).

The same spectrometry setup as for the depth profile measurements was used. However, instead of a DWS, a small piece of waterproof paper was taken as a white standard. The measurement series followed a strict order: First, the white standard was measured, followed by 4 fixed positions within the fluorescent iris (position 12, 3, 6, 9 o´clock). Care was taken to assure that the measurement angle was smaller than the width of the iris. Each series ended with an additional measurement of the white standard. Upon completing one eye, the dive buddy turned the fish around to initiate the complementary procedure for the other eye.

All data were transformed to photon radiance and corrected for reflectance (waterproof paper relative to PFTE) and equipment transmission as explained above. The measurements taken at the four positions within each eye were averaged per individual. As for the substrate measurements, we express iris radiance as relative iris radiance.

Using clove oil for anaesthesia leads to a noticeable reduction in iris radiance due to expanding iridal melanophores [19]. This is especially true for fish from 5 m depth, where anaesthesia decreases iris radiance by 56 % on average compared with un-anesthetized fish. Fish caught at 20 m depth reduced their iris radiance by only 10 % on average after being anesthetized (UH, unpubl. data). The depth-dependency can be explained by reduced iridal melanophore densities in individuals at depth [10, 21]. Therefore, and conservatively with respect to our research hypothesis, all measurements presented here underestimate natural iris radiance, particularly in individuals from shallow water.

Data analysis

To assess whether iris radiance is stronger than substrate radiance under the four conditions (shallow exposed, shallow shaded, deep exposed, deep shaded) we averaged relative iris radiance as well as relative substrate radiance in the relevant red range between 600 and 650 nm. We then calculated the brightness contrast using the Michelson brightness contrast formula [22]:

$$C = \frac{(\text{rel. iris rad.} - \text{rel. substrate rad.})}{(\text{rel.iris rad.} + \text{rel. substrate rad.})}$$

C indicates whether iris radiance was stronger ($0 < C \leq 1$) or weaker ($-1 \leq C < 0$) than substrate radiance. For graphical representation, we pooled C values into 10 categories ranging from < 0 (substrate radiance > iris radiance) to > 0.8. The frequency of cases within each category was then compared between different substrates under the four conditions, and displayed in a mosaic plot. In these plots, each rectangle area is proportional to the abundance of substrate measurements with the given Michelson contrast category among all measurements.

Contrast thresholds

Whether a contrast is detectable for fish depends on several factors including the overall brightness in the environment, the size of the stimulus as well as the distance to the stimulus [23]. However, in the euphotic zone, fish with relatively well developed eyes looking at a stimulus roughly matching their size within an ecologically relevant distance have a contrast threshold of 1-2% under bright light conditions [23]. Hence, under optimal daylight conditions, it is assumed that a Michelson contrast between $C = 0.007–0.05$ should be detectable by most fish [24-27].

A recent study by Bitton et al. (2017) shows that the fluorescent iris of *T. delaisi* contributes to the visual contrast in deeper water against the natural substrate [17]. However, the authors only included achromatic backgrounds to calculate the visual contrast. Here we go one step further by providing visual contrast calculations based on real backgrounds which include red fluorescence, an important component of substrate radiance at depth.

Results

Relative radiance of substrates

At 5 m depth, relative substrate radiance was largely below 1, indicating that fluorescent components in the substrate were too weak to compete with the ambient light (Figure 2 A & B). At 20 m depth, however, relative substrate radiance substantially increased at longer wavelengths, starting at 650 nm and peaking at 695 nm. This can be attributed to fluorescence from photosynthetic algae. Depending on type and exposure, substrate radiation exceeded that of the ambient light by a factor 4 in the 600–700 nm range.

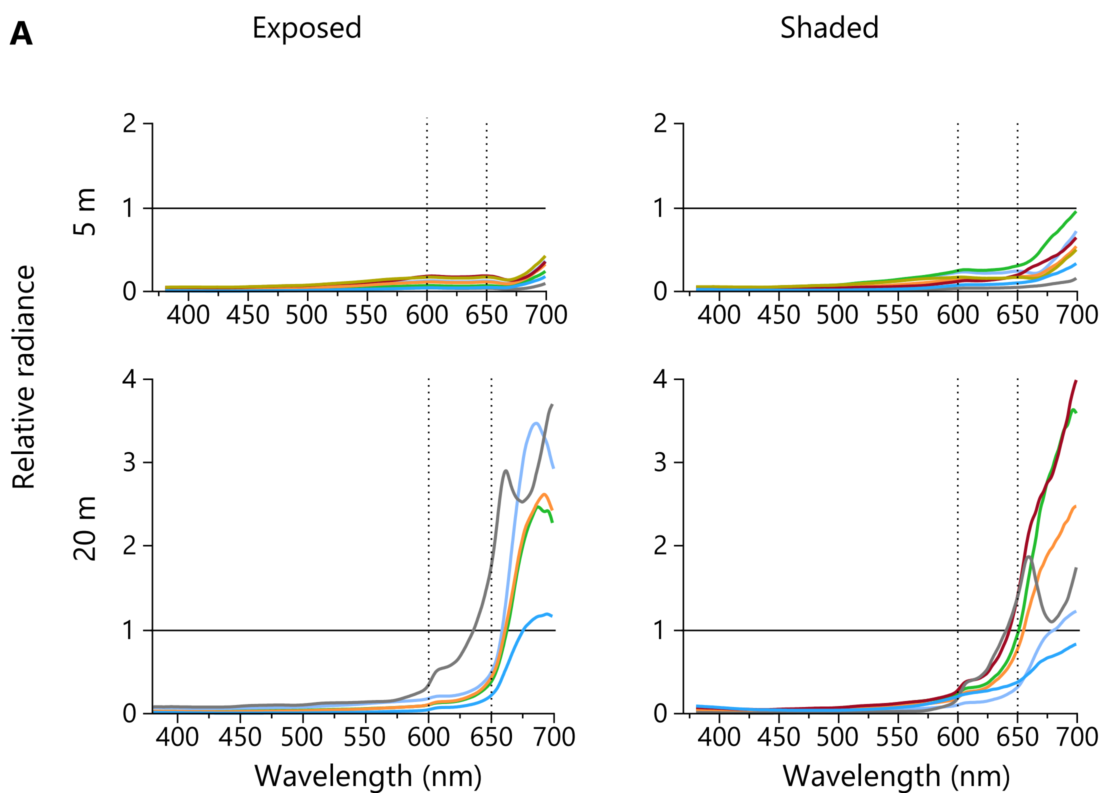

Figure 2: A. Mean relative radiance of typical *T. delaisi* substrate types measured (*n* = 92) at 5 and 20 m depth (rows) under sun-exposed and shaded conditions (columns). Values exceeding 1 (black line) indicate substrates that emitted more light in that spectral range than was available in the side/downwelling spectrum, a typical signature of strong fluorescence. Dashed lines indicate the wavelength range of interest (600–650 nm).

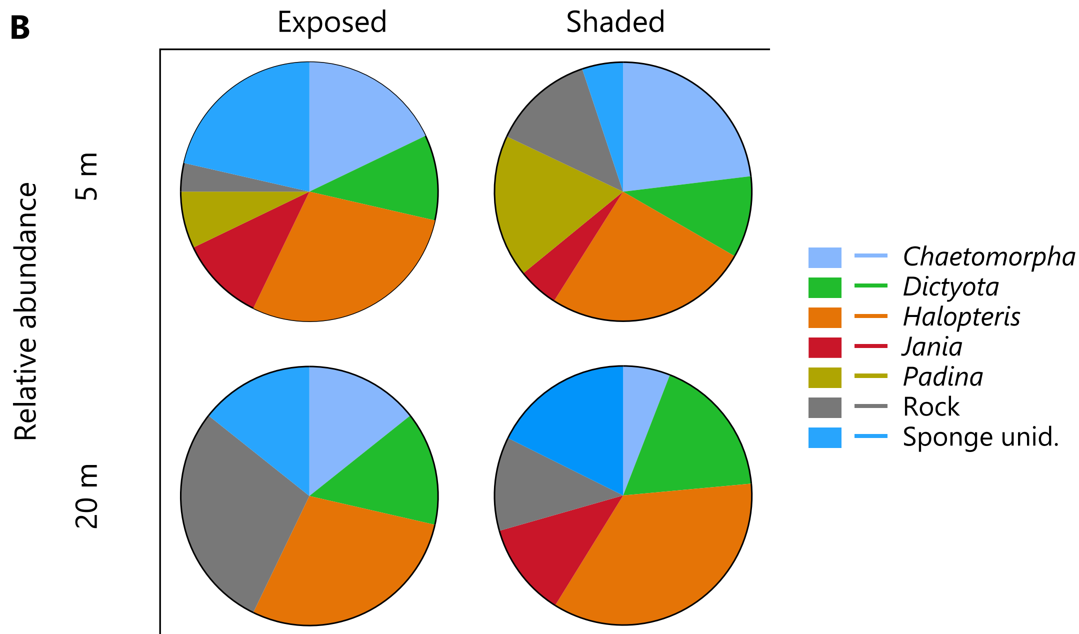

Figure 3 B: Relative abundance of substrates measured at each combination of depth and exposure.

Relative radiance of T. delaisi irides

At 5 m depth, relative radiance of fish irides exceeded 1 in the deep red range (> 680 nm) under shaded conditions (eye facing north) only (Figure 3). This can be explained by the strong red component in the down- and sidewelling light that overrides the fluorescence signal in exposed fish. At 20 m, however, iris radiance exceeded diffuse white standard radiance by up to 9 times (maximum single measurement), irrespective of exposure – an effect that can only be attributed to iris fluorescence.

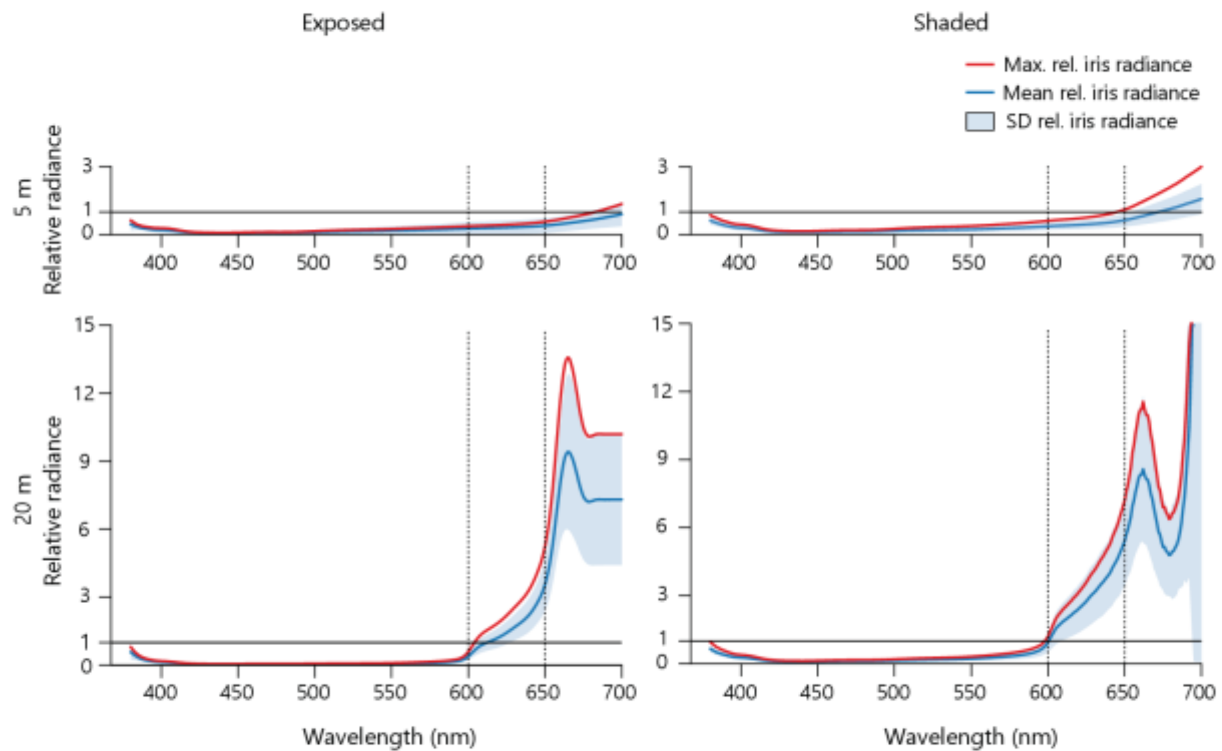

Figure 3: Relative iris radiance of *Tripterygion delaisi* measured under exposed (left column) and shaded (right column) conditions at either 5 m (upper row) or 20 m depth (lower row). Blue lines represent means ± SD (shading) of all fish. Red lines indicate the maximum relative radiance averaged across individuals (*n* = 34). Dashed lines indicate the wavelength range of interest (600–650 nm). Values exceeding 1 (horizontal black line) indicate that more photons were emitted by the fish iris at that wavelength than were available in the ambient spectrum, indicative of red fluorescence (assuming absence of specular reflection).

Comparison between iris and substrate relative radiance

At 5 m depth, substrate type and exposure determined whether iris radiance exceeded substrate radiance. More contrast prevailed under shaded conditions. Under exposed conditions, iris radiances exceeding substrate radiance were limited to bare rock and sponge substrates, as these two exhibit distinct fluorescence compared to others. When comparing iris and substrate relative radiances at 20 m, however, iris radiance was always stronger in the target wavelength range regardless of substrate type and exposure (Figure 4). The time of the day affected iris contrast only at 5 m depth. Under exposed conditions, iris radiance is more likely to exceed substrate radiance in the morning than in the afternoon (Figure 5). Conversely, under shaded conditions, iris radiance always exceeded substrate radiance in the afternoon, but less so in the morning. An effect of the time of the day was absent at 20 m (data not shown).

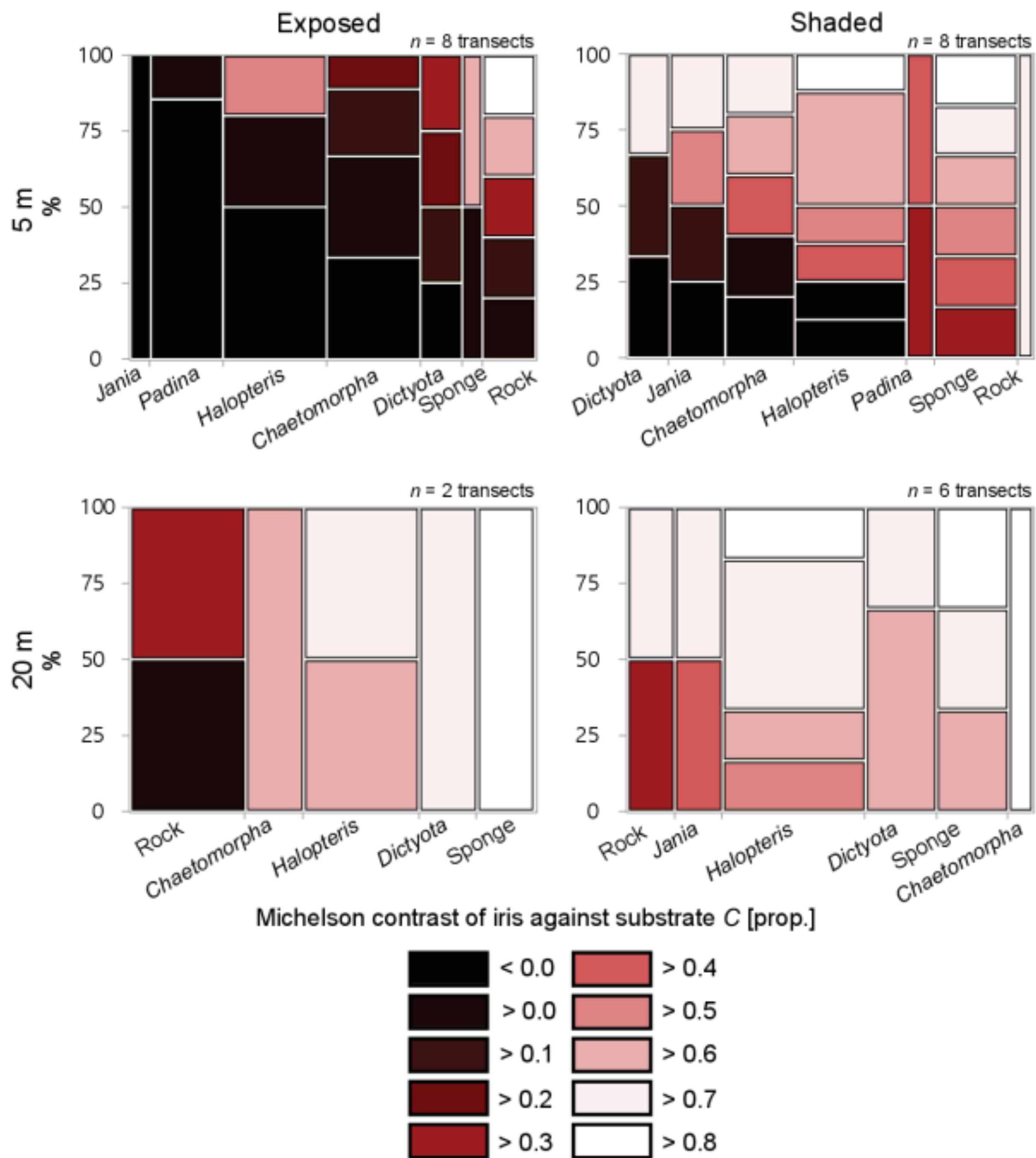

Figure 5: Proportion of calculated Michelson contrasts in the target wavelength range (600–650 nm) (Y-axis) within the 8 commonest substrates (X-axis) at 5 and 20 m depth (rows) under exposed or shaded conditions (columns). We defined 10 Michelson contrast categories for visualization (see legend), where all except the darkest (black) shading indicate iris radiances exceeding substrate radiance, and values exceeding 0.5 indicating strong contrasts. Substrates were ranked from the lowest to the highest brightness contrast. Note that each spine of the transect device was measured 3 times (1 transect: n = 30).

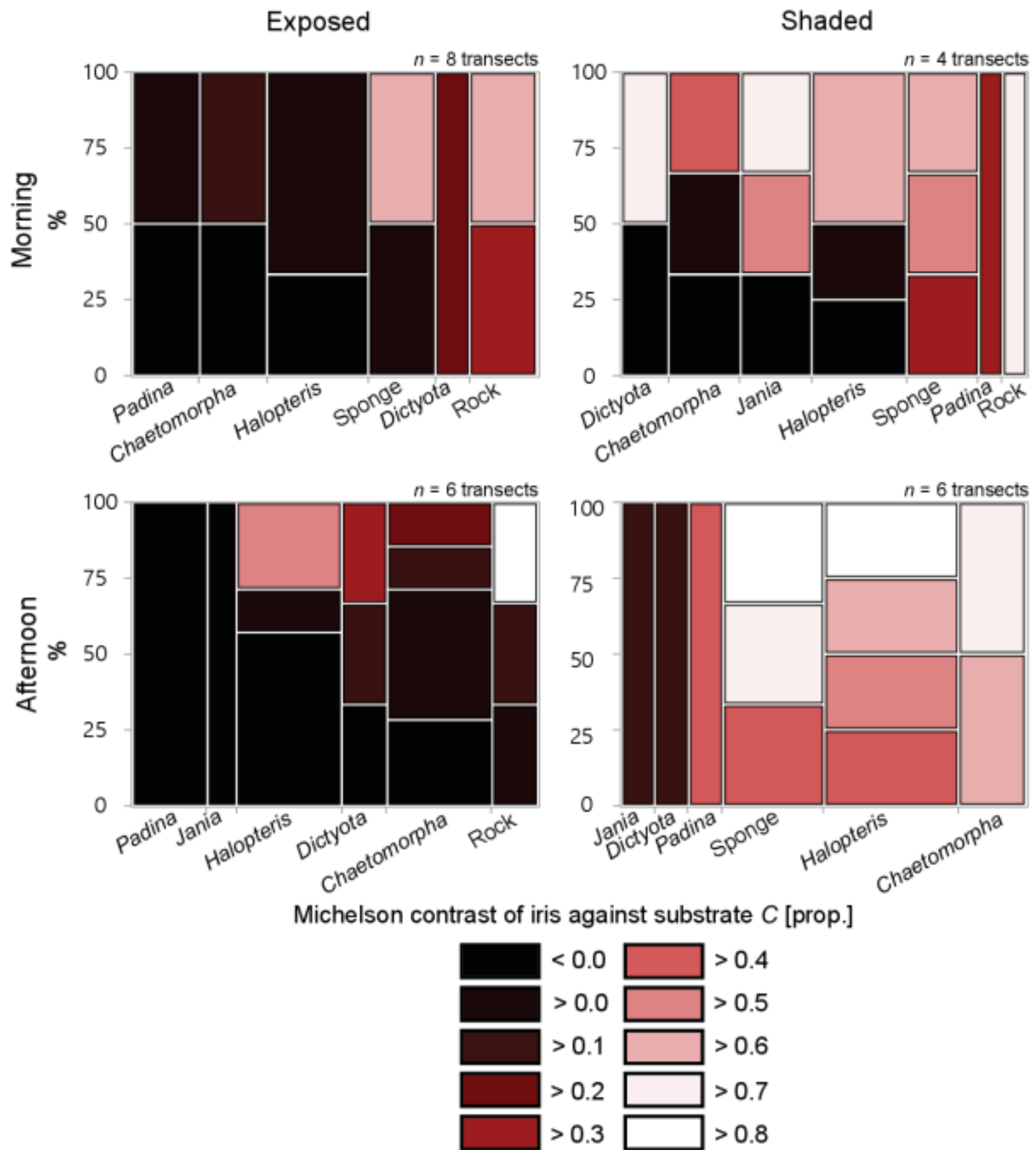

Figure 6: Proportion of calculated Michelson contrasts in the target wavelength range (600–650 nm) (Y-axis) within the 8 commonest substrates (X-axis) at 5 and 20 m depth (rows) in the morning (06:00 – 11:30) or afternoon (12:00 – 18:00; rows) under exposed and shaded conditions (columns). We defined 10 Y-categories for visualization (see legend). All values > 0 (dark red to white) are cases where iris radiance exceeds substrate radiance in the relevant wavelength range. Substrates were ranked from the lowest to the highest brightness contrast. Note that each spine of the transect device was always measured 3 times (1 transect: $n = 30$).

Discussion

Iris radiance of *Tripterygion delaisi* in the 600–650 nm wavelength range exceeded that of the available substrates under stenospectral conditions, irrespective of substrate type, exposure and time of day. Under euryspectral conditions, however, iris radiance was often outcompeted by reflection of the stronger red component in the ambient light. Yet, even at this depth, iris radiance exceeded substrate radiance in shaded sites dominated by side-welling blue-green scatter. Given the suppressing effect of anesthesia on iris fluorescence (see Methods) these estimates are conservative. This is particularly true in shallow water, where *T. delaisi* has more melanophores in the iris [23]. Combined, we confirm previous suggestions that fluorescence in the iris of *T. delaisi* is strong enough to generate visual contrasts in a large part of its natural environment, particularly at depth [14, 27].
This confirms earlier estimates by Bitton et al. 2017, who produced modelling results for the same question, but against achromatic, non-fluorescent backgrounds. Our results suggest that those results may hold against fluorescent backgrounds as well. Future modelling is needed to confirm this.
The lack of longer wavelengths in the ambient light makes stenospectral habitats particularly suitable for the use of fluorescence to generate contrast [10, 21]. This might explain why some particularly strongly fluorescing species are restricted to deeper water such as several species of *Bryaninops*, *Ctenogobiops*, or *Crenilabrius* [10]. Although Anthes et al (2016) did not find a correlation between increasing depth and red fluorescence across species, it is safe to assume that red fluorescence is more likely to contribute to vision in deeper water rather than in shallow water. In fact, when analyzing individuals from two depths within single species (including *T. delaisi.*), Meadows et al 2014 found that fluorescence brightness increased with depth (when measured under the same laboratory conditions).
Hence, although we did not specifically assess whether red fluorescent eyes facilitate prey detection (Harant & Michiels, in press), we confirm that the radiance of *T. delaisi*'s red fluorescent eyes exceeds that of many backgrounds on which it forages under a wide range of relevant conditions.

Limitations of measuring different T. delaisi habitat types

Although we identified several substrate types on which red fluorescence is particularly likely to generate visual contrast, we need to emphasize that certain typical microhabitats could not be measured. Due to handling limitations of the underwater housing, and the need for upward facing substrates to place the transect device (Figure 2 B), we could not take measurements from underneath overhangs or in crevices, important hunting sites for triplefins. However, given that these shaded sites are exclusively illuminated by blue-green,

side-welling light, relative iris radiance in the long-wavelength range should be high, except when encrusting red calcareous algae are common. The latter often cover large areas inside crevices and exhibit strong red fluorescence. For now, we cannot estimate what this implies for red iris fluorescence.

Conclusions

We found that red fluorescence in *T. delaisi* eyes readily exceeds the radiance of natural backgrounds in the 600–650 nm range, particularly in deeper water. Even in shallow water, iris radiance exceeded that of the background for several substrate types, particularly when shaded. Our findings show that iris radiance can generate relevant visual contrasts, a basic prerequisite for active photolocation using red fluorescence. In the future, these data will be used to parameterize visual models that assess whether prey detection using red fluorescent eyes is possible in *T. delaisi*.

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BMC Ecology

RESEARCH ARTICLE

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Fish with red fluorescent eyes forage more efficiently under dim, blue-green light conditions

Ulrike Katharina Harant[1,2*] and Nicolaas Karel Michiels[1,2]

Abstract

Background: Natural red fluorescence is particularly conspicuous in the eyes of some small, benthic, predatory fishes. Fluorescence also increases in relative efficiency with increasing depth, which has generated speculation about its possible function as a "light organ" to detect cryptic organisms under bluish light. Here we investigate whether foraging success is improved under ambient conditions that make red fluorescence stand out more, using the triplefin *Tripterygion delaisi* as a model system. We repeatedly presented 10 copepods to individual fish ($n = 40$) kept under a narrow blue-green spectrum and compared their performance with that under a broad spectrum with the same overall brightness. The experiment was repeated for two levels of brightness, a shaded one representing 0.4% of the light present at the surface and a heavily shaded one with about 0.01% of the surface brightness.

Results: Fish were 7% more successful at catching copepods under the narrow, fluorescence-friendly spectrum than under the broad spectrum. However, this effect was significant under the heavily shaded light treatment only.

Conclusions: This outcome corroborates previous predictions that fluorescence may be an adaptation to blue-green, heavily shaded environments, which coincides with the opportunistic biology of this species that lives in the transition zone between exposed and heavily shaded microhabitats.

Keywords: Foraging success, Visual ecology, *Tripterygion delaisi*

Background

Fluorescence is a common form of luminescence that can be found throughout the entire biotic world [1]. The functionality of fluorescence for intra-specific communication has already been studied in a variety of organisms within terrestrial as well as aquatic habitats [2–4]. Especially in aquatic environments, where long wavelengths are quickly absorbed, fluorescence allows organisms to restore long-wavelength color patterns by absorbing the abundant photons in the blue-green spectral range and reemitting some of that energy as light at longer wavelengths. This situation applies to fairy wrasses for example, where it has been shown experimentally that the fluorescence pattern in males plays a role in sexual interactions [5, 6].

Red fluorescence is present in many reef fishes [7, 8]. In small, benthic, predatory fishes, it is often the eyes that fluoresce and they do so more efficiently in deeper water [9, 10]. This depth effect combined with findings that red fluorescence is also phenotypically flexible [11] and becomes more efficient in fish kept in dim environments [12], suggests an optimization to ambient light conditions. Given that several red fluorescent fish can also perceive their own fluorescence [6, 13], we hypothesize that fish with strongly red fluorescent irides may use fluorescence to illuminate and probe their surrounding environment [14]. More specifically, we argue that this form of fluorescence could theoretically be used to induce reflective eyeshine in small prey such as copepods, aiding in their detection. Such active photolocation where prey is illuminated by some kind of private light source

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has recently been shown in nocturnal flashlight fish [15]. These produce bioluminescent light pulses that might be strong enough to reveal retro-reflection in the eyes of other fish and/or prey nearby. Red fluorescence could be used in a similar way under daylight conditions. This, however, seems more plausible under the heavily shaded, blue-green stenospectral light conditions at depth rather than in shallow, broadly lit euryspectral conditions [9]. We define the euryspectral zone as the depth range close to the surface, with an ambient spectrum that is broader than the visual spectrum of most animals. The stenospectral zone, in contrast, describes the depth range below this, where most of the UV and longer wavelengths have been absorbed by the water column [16, 17], resulting in a spectrum that is narrower than the perception limits of most fish [9, 10]. The transition between the two can be between 5 and 25 m, depending on light conditions and variation in light attenuation by the water column.

Benthic copepods and other micro-crustaceans are a common food source for small fish and their nauplius eyes show strong reflection due to the presence of tapetal cells [18–21]. Own observations and tests (unpubl. data) indicate that copepod eyes reflect incoming light more to the source than elsewhere, similar to a weak retroreflector. Inducing reflective eyeshine in such eyes could therefore be enhanced if the light source (= red fluorescent iris) is close to the pupil, as is also the case for the light organ below the pupil in flashlight fishes [22]. Here, we do not assess whether *Tripterygion delaisi* actually induces and perceives this eyeshine in copepods, but rather examine whether the association between ambient light conditions and foraging behavior is consistent with this hypothesis. More specifically, we test whether fish capture more copepods under bluish light conditions that make fluorescence stand out more compared to broad illumination of the same overall brightness as predicted by our hypothesis. Our model species is the black-faced triplefin *Tripterygion delaisi* (Cadenàt and Blache 1970) [23], a small crypto-benthic fish with strongly red fluorescent irides [12, 24]. Since *T. delaisi* increases the relative efficiency of its fluorescence with decreasing ambient brightness, foraging success was tested under two different brightness levels, mimicking 2° of shading. By doing so, we could assess whether foraging success increases under stenospectral conditions in general, or whether it also requires heavily shaded light conditions.

Methods

We collected *T. delaisi* while SCUBA diving at Stareso (Station de Recherches Sous Marines et Océanographiques) Calvi, Corsica, France in June 2014 and 2015. After transfer to the aquarium facilities at the University Tübingen, Germany, they were held separately in 40, 15 L tanks which were equipped with a living rock as a comfort stone, in a common water recirculation system (20 °C, salinity 34‰, pH 8.2, 12 h light/dark cycle, fed once per day).

Tank illumination

Each aquarium was illuminated with a combined set of 8 LEDs in a single housing covered with a Feno Fe s.soft lt 18 diffuser. The LEDs available were: cold white, UV (395–410 nm), royal blue (450–465 nm), blue (465–485 nm), 2× green (520–535 nm), amber (585–595 nm) and red (620–630 nm). Each LED of each housing could be individually controlled by a DMX standalone unit (Feno fc s.dmx 48d) from off (= 0) to maximum (= 100) allowing spectral shape and brightness to be programmed.

Copepod culture and pilot study on copepod behavior

As a prey model species, we used *Tigriopus californicus* (Baker 1912) a marine harpacticoid copepod that colonizes rock pools from Alaska to Baja California, Mexico [25]. Copepods were cultivated in 1 L tanks (20 °C and 34 ‰ salinity, 12 h light/dark cycle) and fed on a variety of unicellular algae and bacteria. For each of the two experiments, we carried out a pilot experiment in which we tested the preference for the light treatments (stenospectral versus euryspectral) of the copepods. Copepods were inserted into transparent 4 mL cuvettes containing seawater and illuminated each for 2 min with the light treatments used in the main experiment in random order. We then assessed whether the copepods spent significantly more time in the upper or lower half of the cuvette, indicating a preference for a particular light treatment presented. No significant differences were detected (Additional file 1).

First experiment: spectral treatments under shaded conditions

The experimental room in which fish were kept was divided into two benches with 20 aquaria (total $n = 40$). On 10 October 2014 each bench either received a euryspectral or stenospectral treatment with an identical overall irradiance (total irradiance in photons s^{-1} m^{-2}, stenospectral: 2.51×10^{18}, euryspectral: 2.55×10^{18} as in Harant et al. [12]) which represents 3.6% of the total light present just below the water surface on a sunny summer day (Fig. 1). The natural spectrum was measured on the 26 June 2015 at solar noon, close to solar maximum in Corsica, France. These two experimental spectra were designed to mimic the ambient spectra at 5 and 20 m depth, the range in which *T. delaisi* is most abundant. Both benches received a different spectral treatment, alternating every week for 4 weeks. After analyzing the

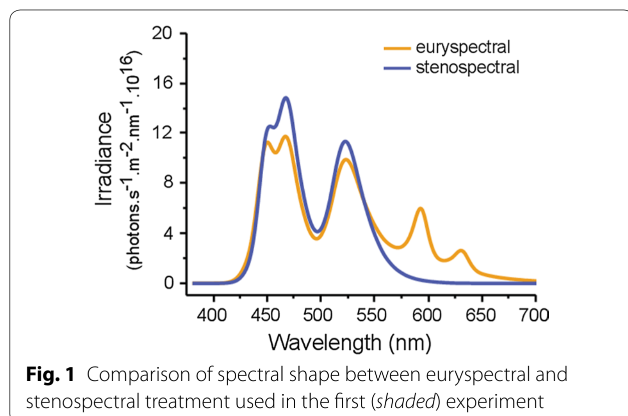

Fig. 1 Comparison of spectral shape between euryspectral and stenospectral treatment used in the first (*shaded*) experiment

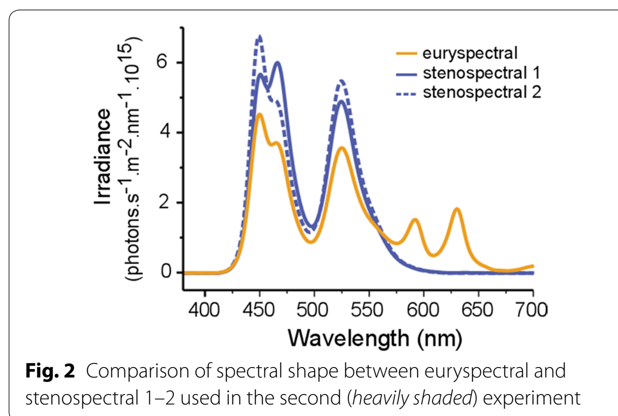

Fig. 2 Comparison of spectral shape between euryspectral and stenospectral 1–2 used in the second (*heavily shaded*) experiment

data, we extended the experimental duration for another 2 weeks to confirm the insignificance of the results (6 weeks total).

Second experiment: same spectra, heavily shaded light conditions

In the second experiment, we used a newly collected set of fish for a test under identical conditions, except that now, brightness was reduced to the lowest level that was manageable to carry out the experiment (about 0.01% of the surface; stenospectral$_1$: 7.05 × 10^{16}, stenospectral$_2$: 7.05 × 10^{16}, euryspectral: 7.04 × 10^{16}). This involved reducing the light produced by the LEDs electronically, but not below a 6% level, where flickering becomes an issue. Since the red LED was already at a low setting in the euryspectral treatment during the first (shaded) experiment, it could not be lowered more. In order to achieve a low light level we therefore added a cap on top of the light diffuser made of 2 layers of neutral density filter (LEE Filters Nr. 210 0.6 ND) which allowed about 6% of the total light intensity to pass.

In *T. delaisi*, brightness perception is mainly mediated by the double cones. According to microspectrophotometric measurements, these peak at 516 and 530 nm. Hence, highest sensitivity in this species lies within the green spectral range [26]. The stenospectral treatments containing more green wavelengths compared to the euryspectral treatment could therefore be perceived as being much brighter regardless of the total brightness. To prevent an increase of foraging success due to this perceived brightness effect, we ran two stenospectral treatments which varied by 10% in the amount of green wavelengths (Fig. 2). However, in order to keep the overall brightness identical, the amount of blue light in the second stenospectral treatment was slightly increased (Fig. 2).

Starting from 26 January 2015, each bench received either a euryspectral or stenospectral light condition, which was swapped after each week for 4 weeks. In the stenospectral treatment the two stenospectral alternatives were changed on a daily basis.

Aquaria experimental setup

The two sides of each aquarium were covered with white non-fluorescing, polypropylene sheets (matt/semi-gloss) sprayed with a fine greyish noise pattern (Hybrid Lack, silver-gray RAL 7001—Additional file 2). This was done to provide a noisy background under the (untested) assumption that it would make it harder for the fish to detect copepods by achromatic contrast alone.

Recording setup

When testing two individual fish in a pair of aquaria, we fitted a GoPro Hero 3+ above each tank, providing a full view of the bottom of the aquarium where the fish move about. In the second experiment, the light was too dim for good recordings, requiring infrared illumination (Versiton SAL-30 IR Illuminator 77 LEDS 30 M (100′) 12VDC 1.5A, peak at 844 nm). For this, the cameras were fitted with a dedicated IR lens (Vision Dimension: 2.97 mm Megapixel M12 × 0.5). In order to minimize the unlikely possibility of sensitivity to strong IR, the IR sources were positioned at ground level in the room, oriented upwards, but not into the tanks. The diffuse reflection from the ceiling and walls was just bright enough to obtain good recordings (Additional file 3).

Fish habituation and testing

Prior to the start of the experiment, fish were familiarized with the pipette that was used to provide copepods: UKH frequently inserted the pipette into the aquaria several times a day during the pre-experimental weeks. In the beginning, the pipettes contained defrosted *Mysis*

shrimp (Aki Frost GmbH) which were released into the aquarium (3–4 *Mysis* per insertion). As soon as no flight response was observed anymore, seawater was delivered instead of food. This procedure made sure that the appearance of a pipette triggered positive anticipation without guaranteeing food.

After starting the light treatments, fish were allowed to adapt for 1 week without any other change in maintenance conditions. On the following day (Monday), 5 randomly chosen aquaria pairs ($n = 20$ individuals) in each treatment were tested by injecting 10 copepods in 1 mL seawater 2 cm from the front glass using an automatic pipette. The next day (Tuesday) the remaining 10 aquarium pairs (n = 20 individuals) followed. Upon completing the testing procedure, the spectral treatments were changed to the opposite treatment. The same procedure was then repeated again (Monday/Tuesday) under the second spectral treatment the week after. Each pair of aquaria was tested twice per testing day with one fish first receiving copepods whereas the other only received water from the copepod culture as a control to check for feeding strikes due to odor only. In the second run of the day, the role of positive treatment and control was reversed in each aquarium pair. Between the first and second run in a single aquarium pair, another aquarium pair in the opposite treatment (and bench) was tested to induce a delay between the two successive trials in a single aquarium pair. This 10 min delay allowed fish to go back to their normal routine.

Pre-experimental trials showed that fish usually stop searching for copepods after about 5 min. In addition to that, copepods started to hide within the comfort stone (living rock) after 5–6 min after injection. Hence, if fish were not able to catch all copepods within the first run, the chance of detecting leftover copepods still left in the tank during the control treatment in the second run was small (but see "Results" Section).

Work flow, copepod preparation and video analysis

To enhance video quality, the water inflow of the aquarium was turned off 10 min prior to testing. The copepods were gently taken up by the pipette and released into the aquarium. After insertion of the pipette, videos were recorded for the following 5 min. To prevent observer bias, recorded videos were randomized and transformed to grayscale before analysis. The inserted copepods were too small to be seen on the video, leaving the observer also blind to the copepod treatment and its control. *T. delaisi* shows a saltatory searching behavior [27, 28] which is characterized by approaching prey with small hopping movements, interrupted by scanning of the substrate and a sudden feeding strike once prey is identified. In a pilot study we found that the number of feeding strikes closely approximated the number of live copepods added to the tank, and never exceeding that number. It confirmed that there are no feeding strikes without copepods, and most or all feeding strikes also resulted in successful prey capture. Only rarely, fish needed two strikes in rapid succession for the same item. Such cases were counted as one strike. Overall, the results show that feeding strikes are a reliable variable for measuring foraging success in *T. delaisi* (Additional file 4). In the main experiments, we used Etholog 2.2.5 [29] to record time (s) for each feeding strike since start of the recording as well as total *n* feeding strikes.

Iris fluorescence of *T. delaisi*

Excitation and emission of iris fluorescence is shown in Fig. 3 with excitation being highest at 550 nm and fluorescence emission peaking at 600 nm [26]. Since *T. delaisi* forages at relatively small distances to prey of a few centimeters only, absorption and scattering is negligible (<1% at 600–650 nm at 4 cm distance, [16, 17]). To calculate the decrease of fluorescence brightness over distance, we fixed an eye of *T. delaisi* on a black stick and illuminated it with a blue Hartenberger Mini Compact LCD divetorch (7 × 3.5 W 450 nm bulbs) from a distance of 24 cm. Two short pass filters (Thorlabs FD2C subtractive dichroic color short-pass) were attached in front of the torch to cut out longer wavelengths. Since the eyes quickly darken after decapitating a fish due to the dispersal of melanosomes, we treated the eyes with potassium chloride solution [24] for 1 h to reverse this process before using it. The eye was then oriented downwards at an angle of approximately 45° looking at a diffuse white standard (PTFE). A ruler was placed in line with the outer edge of the iris to serve as a reference. Consecutive measurements were taken in 0.5 cm steps using a calibrated PR 740 SpectraScan Spectroradiometer (Photo Research Inc.,) pointed at the diffuse white standard and

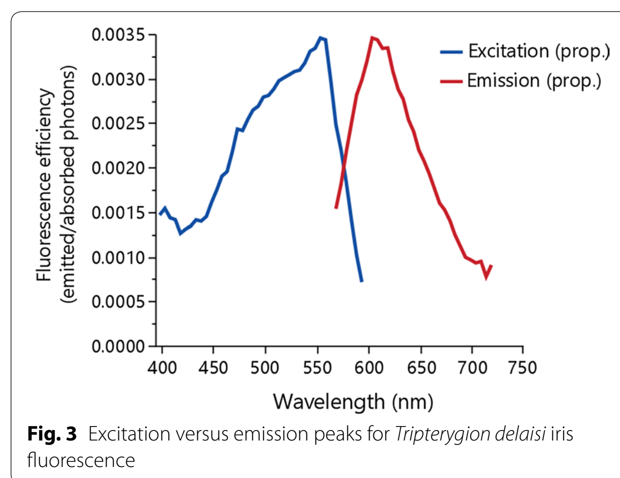

Fig. 3 Excitation versus emission peaks for *Tripterygion delaisi* iris fluorescence

measuring reflected fluorescence starting at 0–2.5 cm distance from the eye. The spectrometer was set to 2 nm bandwidth, an aperture of 0.5, with smart dark enabled at a normal speed with an extended exposure time, and was operated with a calibrated MS-75 lens.

The spectrometer used to take measurements adjusts gain to avoid saturation in the brightest wavelengths resulting in noisy measurements in the longer wavelength range. We therefore used an orange filter (Lee filters, Double C.T. Orange 287) attached in front of the spectrometer lens to suppress the excitation light. Radiance measurements were then corrected for the transmission of the used filter and converted to photon radiance by multiplying the measurements with wavelength*5.05*10^{15} [30]. Photon radiance was then summarized between 600 and 650 nm and averaged among the two measured eyes. Figure 4 displays the exponential loss of iris fluorescence with distance. Note that measurements were taken from a diffuse white standard reflecting all wavelengths equally in a 180° angle. These measurements are therefore very conservative compared to a reflector such as a copepod nauplius eye.

For easier comprehensibility, we provide a demonstration of how red fluorescence behaves with increasing/decreasing proportion of longer wavelengths present in the light environment. Since *T. delaisi* is able to quickly regulate its fluorescence we did not conduct this demonstration with live triplefins but used a special mixture of fluorescent paint which resembles different intensities of fluorescence emission of *T. delaisi* (Additional file 5). We then illuminated the fluorescent patches along with a non-fluorescent red diffuse reflectance standard (Labsphere SCS-RD-010) from a distance of 24 cm with the euryspectral and stenospectral light treatment used during the second experiment. The demonstration shows that with decreasing proportion within the longer wavelength range fluorescence appears more intense while in direct comparison the non-fluorescent red standard becomes grey.

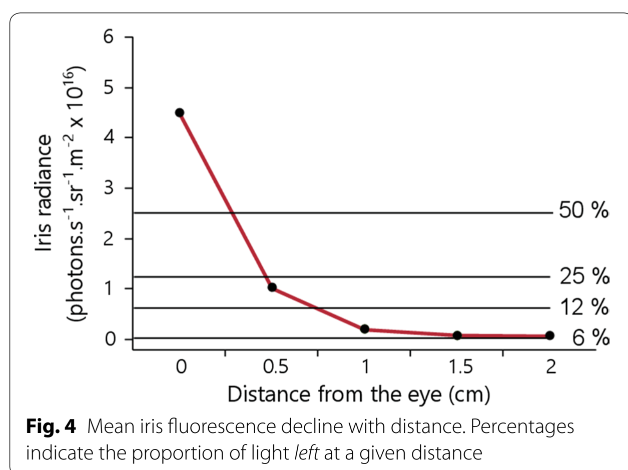

Fig. 4 Mean iris fluorescence decline with distance. Percentages indicate the proportion of light *left* at a given distance

Statistical analysis of fish behaviour

Data were analysed using a generalized linear mixed model using the lme4 package [31] of R [32]. The response variable *n copepods caught*, was modelled as a binomial (*n copepods caught*/*n copepods missed*) response variable with logit link. In both experiments all initial models contained *light treatment*, *bench*, and *week* as well as all biologically relevant interactions as fixed components. To account for the repeated measurements per fish, fish ID was integrated as a random factor with random slopes. An observation-level random factor (random effect that models extra-Poisson variation of count data, [33]) was added as well to account for overdispersion. By using the Bayesian information criterion (BIC), a backward model selection was performed on random (excluding fish ID) and fixed factors to identify the best fitting model with the fewest predictors. Here, we only present the final models including proxies for the goodness-of-fit of the complete model (conditional R^2) as well as the fixed component (marginal R^2) [34]. Proxies were calculated using the pairwise SEM package for R [35]. Wald *z* tests were performed to assess the overall significance of fixed effects. All other statistical tests as given, two-way ANOVAs and paired *t* tests were carried out using JMP 11 (SAS) after confirming normality and homoscedasticity.

All data necessary to reproduce our conclusions are provided in the supplementary files section (Additional files 6, 7).

Pre-results: participation and exclusion criterion

In the first experiment, 31 out of 40 fish participated throughout the entire study whereas the remaining 9 showed no interest and were therefore excluded from the analysis. In the second experiment, 37 of 39 fish successfully participated throughout the whole duration of the experiment. However, 3 males changed to male breeding coloration during the experiment and were excluded from further analysis. Males in breeding coloration increase the content of melanophores in the iris which reduces expressed red fluorescence (unpubl. data). We therefore only considered adults in our analyses that showed their cryptic coloration throughout the entire study.

Pre-results: spectral treatments

In the second experiment there was no detectable difference between the two stenospectral treatments, that differed only slightly in the spectral range (blue-green), which is why these data were pooled together (paired *t* test: $dF = 33$ $t = 0.25$, $p = 0.81$).

Pre-results: control treatments

Out of 322 recorded control videos (no copepods), only 12 feeding strikes were observed, eleven of which occurred during the second run where fish had previously received copepods. It is therefore likely that these fish caught copepods that were still around from the earlier runs on that day.

Results

Experiment 1: feeding success under shaded conditions

There was no difference in the number of copepods caught under euryspectral versus stenospectral conditions under shaded illumination (Fig. 5; Table 1). Fish caught on average 4.83 ± 1.98 SD copepods in the euryspectral treatment and 4.88 ± 1.82 SD in the stenospectral treatment. Similarly, the time it took until 5 out of 10 copepods were caught ("copepod half-time"), did also not differ between light treatments (Additional file 8).

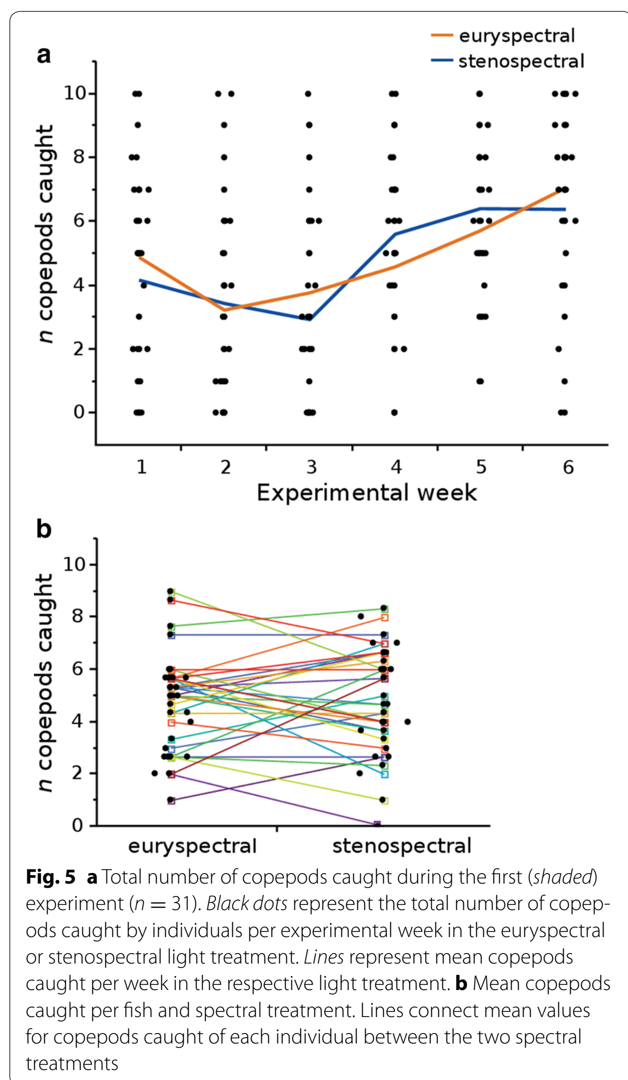

Fig. 5 **a** Total number of copepods caught during the first (*shaded*) experiment ($n = 31$). *Black dots* represent the total number of copepods caught by individuals per experimental week in the euryspectral or stenospectral light treatment. *Lines* represent mean copepods caught per week in the respective light treatment. **b** Mean copepods caught per fish and spectral treatment. Lines connect mean values for copepods caught of each individual between the two spectral treatments

Interestingly, however, fish significantly increased their foraging success irrespective of the light treatment from 4.5 ± 2.9 SD copepods in week 1 to 6.7 ± 2.8 SD in week 6 (Fig. 3; Table 1), indicating a learning effect.

Experiment 2: foraging success under heavily shaded light conditions

Fish held in the stenospectral treatment caught on average 6.5 ± 1.97 SD copepods while fish tested under euryspectral conditions only caught 5.8 ± 1.63 SD copepods, a significant difference (Table 1; Fig. 6). Hence, the use of red fluorescence under blue-green illumination in deeper *and* more shaded habitats, allows *T. delaisi* to increase its foraging success by an average of 7%. However, fish showed quite some variation. More than one third (44.4%) of the fish for example, increased their foraging success by 15% on average and 29% of all fish increased their efficiency by at least 20% under stenospectral conditions relative to euryspectral conditions. The highest mean increase for any individual was twofold (9 copepods caught compared to 4.5). In contrast, 8 out of 34 fish showed a higher foraging success in the euryspectral treatment compared with the stenospectral treatment (7.3 ± 1 SD compared with 4.9 ± 2 SD copepods). Similar to the first (shaded) experiment, copepod half-time was not affected by treatment (Additional file 9). Improved performance over the course of the experiment could not be confirmed (no effect of week, Table 1), but experiment 1 ran for 6 weeks, experiment 2 for 4 weeks.

When comparing both experiments (shaded and heavily shaded), fish were generally more successful at catching copepods under heavily shaded conditions (average shaded experiment: 4.86 ± 1.68 SD; heavily shaded experiment: 6.29 ± 1.48 SD). This difference, however, needs to be interpreted with care since the two experiments did not run in parallel, but in successive years, with 2 different fish cohorts.

Discussion

Tripterygion delaisi showed on average a 7% higher foraging success under heavily shaded, blue-green light favoring fluorescence compared with broad-spectral or shaded conditions. Although these results do not represent direct evidence that fish use red fluorescence to enhance their prey detection under stenospectral conditions, they are nevertheless consistent with the active photolocation hypothesis. Assuming that red fluorescent irides indeed facilitate prey detection, they probably do so under heavily shaded conditions only, either under rocks or overhangs where the light is dominated by side-welling blue-green scatter from the open water, or at depths, times of day or degrees of cloud cover, where bright and broad-spectral light is lacking.

Table 1 Foraging success and copepod half-time in response to light treatments

Experiment	Parameter	Std-beta coefficient estimate	SE	z	p	Conditional R^2	Marginal R^2
1: Shaded conditions: foraging success	Intercept	−1.323	0.29	−4.47	<0.001		
	Week	0.34	0.06	5.49	<0.001	0.157	0.058
	Light treatment	0.02	0.21	0.14	0.89		
2: Heavily shaded conditions: foraging success	Intercept	0.73	0.15	5.01	<0.001		
	Light treatment	−0.34	0.17	−1.98	0.047	0.063	0.007

Hence, when fish are hunting in sunlit sites, fluorescence is likely to be of little help for foraging. However, it coincides well with *T. delaisi*'s preference for rocky substrates with crevices and overhangs where brightness transitions are frequent and strong, regardless of depth. Under these conditions, red fluorescence might offer a significant advantage when foraging in the shade.

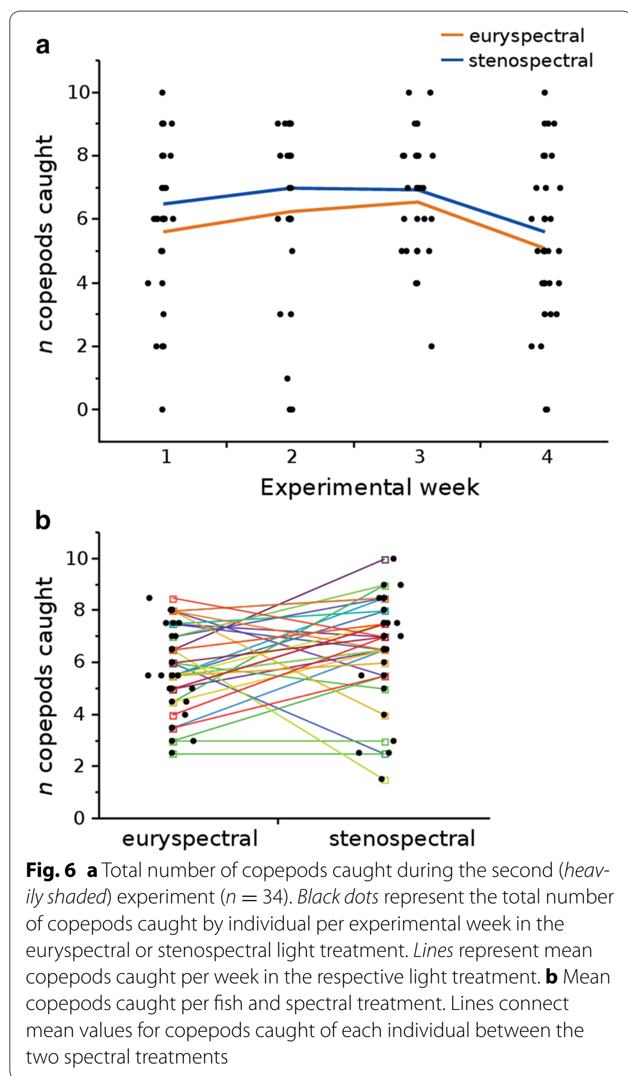

Fig. 6 **a** Total number of copepods caught during the second (*heavily shaded*) experiment ($n = 34$). *Black dots* represent the total number of copepods caught by individual per experimental week in the euryspectral or stenospectral light treatment. *Lines* represent mean copepods caught per week in the respective light treatment. **b** Mean copepods caught per fish and spectral treatment. Lines connect mean values for copepods caught of each individual between the two spectral treatments

Similar benefits would exist when foraging during dusk and dawn.

Predator–prey interaction

While fish could have theoretically generated a contrast between red illuminated prey and background, explaining the observed increase in foraging success, they could have also used their red fluorescent irides to attract prey. Similar to phototaxis in diurnal vertical migrating invertebrates [36, 37], *T. californicus* could be attracted by certain light cues. This, however, would require copepods to be sensitive to longer wavelengths. Although studies on visual sensitivity of *T. californicus* are rare (but see [20]), our own results obtained from the first pre-experimental study on light preferences of *T. californicus* suggest that such differentiation is absent for the light conditions used here (Additional file 1, red light treatment). However, longer wavelengths are quickly absorbed, implying that red fluorescence can only be effective over very short distances. This is compatible with the saltatory foraging and short-distance strikes typical for *T. delaisi* [27, 28]. Over such short distances, fluorescence could be strong enough to create the proposed contrast. A recent study by Anthes et al. [10] strengthens this hypothesis by showing that red fluorescent irides are a common feature among small benthic predatory fish that predominantly hunt for small invertebrates.

Additionally, wavelengths above 570 nm are rapidly absorbed over larger distances [16, 17, 38]. *T. delaisi* could therefore use red fluorescence to forage more efficiently while remaining hidden from predators nearby.

Individual variation

Fish tested under heavily shaded light showed substantial individual differences despite the fact that fish were given sufficient time to adjust. Such differences were also present in a previous study of a phenotypic response to different light environments [12]. We propose that this degree of variability may represent a form of microhabitat specialization in this very cryptic species. Fish predominantly foraging in exposed sites may show weaker fluorescence because it is less functional and its absence prevents attracting (red-sensitive) visual predators.

Whereas fish that predominantly forage in the shade face the opposite situation. Individual variability also explains the small size of the effect found in the heavily shaded experiment, despite the very strong effects in some individuals. Future work could specifically compare fish collected from exposed sites versus fish collected from overhangs to confirm this view.

Does brightness perception influence foraging success?

In order to keep the overall brightness similar in all spectral treatments, we increased the abundance of blue and green wavelengths within the stenospectral treatment. *T. delaisi* shows highest sensitivity in the green wavelength range [26]. The stenospectral treatment might therefore have been perceived brighter by the fish, regardless of the real overall brightness. We attempted to take care of this by including a second even "greener" stenospectral treatment and comparing foraging success between the two stenospectral treatments. Since foraging success did not differ between these two treatments, a difference in brightness perception between stenospectral and euryspectral treatment alone cannot explain the observed increase in foraging success in the second, heavily shaded experiment. Furthermore, if perceived brightness indeed affected foraging success, a similar effect would have been present in the first, brighter experiment. Although no such effect could be found, we cannot entirely exclude that perceived brightness might still have had a small effect on foraging success in *T. delaisi*.

Conclusions

Summarizing, this study shows that *T. delaisi* forages more efficiently under heavily shaded, blue-green light conditions compared with broad light. Assuming that fish may be using red fluorescent emission to enhance prey detection, this result suggests that the functionality of such a mechanism is more plausible over short distances, under stenospectral, shaded conditions. This offers important clues for the design of future experiments to test active photolocation in this system.

Additional files

Additional file 1. Pilot study: behavior of *Tigriopus californicus* under two different spectra.

Additional file 2. Distraction pattern on polypropylene foil used to cover the walls of the aquaria.

Additional file 3. Video sequence of typical foraging behavior in *T. delaisi* recorded during the experiment.

Additional file 4. Pilot study: feeding strikes in *Tripterygion delaisi.*

Additional file 5. Animated gif illustrating the contrast generated by red fluorescence under the two spectral treatments.

Additional file 6. Foraging success data of the first (shaded) experiment.

Additional file 7. Foraging success data of the second (heavily shaded) experiment.

Additional file 8. R script used to analyze foraging success in the first (shaded) experiment.

Additional file 9. R script used to analyze foraging success in the second (heavily shaded) experiment.

Authors' contributions

UKH and NKM designed the experiments. UKH carried out the collection and analyses of the data, and drafted the manuscript. Both UKH and NKM edited the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data generated or analysed during this study are included in this published article (and its Additional files).

Ethical approval

We followed all institutional, national, and international standards for keeping and using the animals in the experiments. All experiments were performed in such a way that no ethical approval was required from any ethics review board.

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Behavior of *Tigriopus californicus* under two different spectra

Differences in feeding strikes in *T. delaisi* due to spectral treatment may be caused by a different behavior of the live copepods used as prey. Hence, to assess whether *Tigriopus californicus* reacts differently to the two spectral treatments used in the experiments we conducted a pilot study to assess to what extent their movement changed between the euryspectral and stenospectral conditions. To this end, 18 transparent 4 ml cuvettes filled with seawater containing 10-15 copepods were placed on a dark underground in front of a black background, illuminated with an infrared light source (Versiton SAL-30 IR Illuminator 77 LEDS 30M (100') 12VDC 1.5A, peak at 844 nm). Swimming behavior was then recorded using an infrared sensitive camera (uEye UI-1440 M/C USB2.0) with a macro-lens (MLH-10X, 1/2" 13-130 mm Computar) and an IR pass-filter attached to the lens. This setup visualized copepods as white moving dots on a dark background. The position of the cuvette as well as the distance between the cuvette and the camera was 21 cm. The copepods received the same two spectral treatments used in experiment one and an additional red-light-only treatment (red channel = 100 %, all other channels = 0 %) in a randomized order. Before a treatment started, the lights were turned off for one minute to allow the copepods to calm down. Afterwards, the first light treatment started and the copepods were given one minute to adapt to the new light conditions. The swimming behavior was recorded for 2 min, followed by another 1 min darkness and the next light treatment. The same procedure was carried out for the spectral treatments used in the second experiment (*n* = 18 cuvettes). Videos were analyzed using Image J 1.47v with

a self-written macro plugin. All videos were transformed into black and white picture stacks in which the movements of the copepods were visualized as white spots. The picture stacks were then summarized leading to a final picture were the cumulative presence of all copepods in the cuvette could be expressed as a raw intensity value per pixel. The cuvette area in the picture was then divided into an upper and lower part. Using the proportion of copepod presence between the two produced a ratio that indicated in which half the copepods frequented more during the different light treatments.

Results and conclusion

Statistical analyses of copepod movement were carried out using JMP 11 (SAS). The proportions of copepod presence were transformed using a square-root ArcSin correction. A nested two-way ANOVA was performed using the corrected *proportion* as response variable, the *spectral treatment* and the *order of treatments* as fixed factors and the *cuvette ID* as random factor. The two-way ANOVA showed that neither the order in which the treatments were presented (two-way ANOVA $_{\text{bright experiment}}$: $F = 2.02$, $dF = 5$, $p = 0.12$; two-way ANOVA $_{\text{dark experiment}}$: $F = 2.65$, $dF = 5$, $p = 0.08$) nor the spectral treatments had a significant effect on the time the copepods spent in one of the two half´s of the cuvette (two-way ANOVA $_{\text{bright experiment}}$: $F = 0.0005$, $dF = 2$, $p = 0.99$; two-way ANOVA $_{\text{dark experiment}}$: $F = 1.6$, $dF = 2$, $p = 0.21$). We conclude that *Tigriopus californicus* does not respond behaviorally to the difference between the two light treatments. It therefore seems unlikely that this may explain the treatment effect in the feeding strikes of *T. delaisi.*

Feeding strikes in *Tripterygion delaisi*

Goal and methodology

Does *T. delaisi* only strike at something when it sees prey? In a pilot study we tested how strongly picking behavior by *T. delaisi* is linked to copepod presence. After familiarizing fish with pipette tips (see section fish habituation and testing – methods section main text) 15 fish received six different copepod treatments in which either 1, 2, 3, 4, 6 or 10 live *T. californicus* copepods were administered to their tank in 1 ml salt water. All fish received only one treatment per day in a randomized order across 6 different days to ensure that no copepods were left in the aquarium from prior trials. The picking behavior was then directly observed by the experimenter for a total of 10 min. Since copepods were prepared in advance, stored in 1 ml Eppendorfer tubes, and labeled with the fish ID only, the observer was blind to the actual number of copepods added to the aquarium.

Results and conclusions

Out of 15 fish only 12 participated. The remaining three individuals were excluded from analysis. The results show that the number of picking could be very precisely explained by the number of copepods administered (Figure). We never saw more picks than the number of copepods provided. Due to the repeated measures design we only used the mean number of *copepods caught between the individuals per treatment (n copepods administered)* to calculate

the linear regression: R^2 = 0.94, df_{effect} = 1, df_{error}= 4 F = 74.78, p = 0.001). We conclude that feeding strikes are indeed a good measure of feeding success in *T. delaisi*.

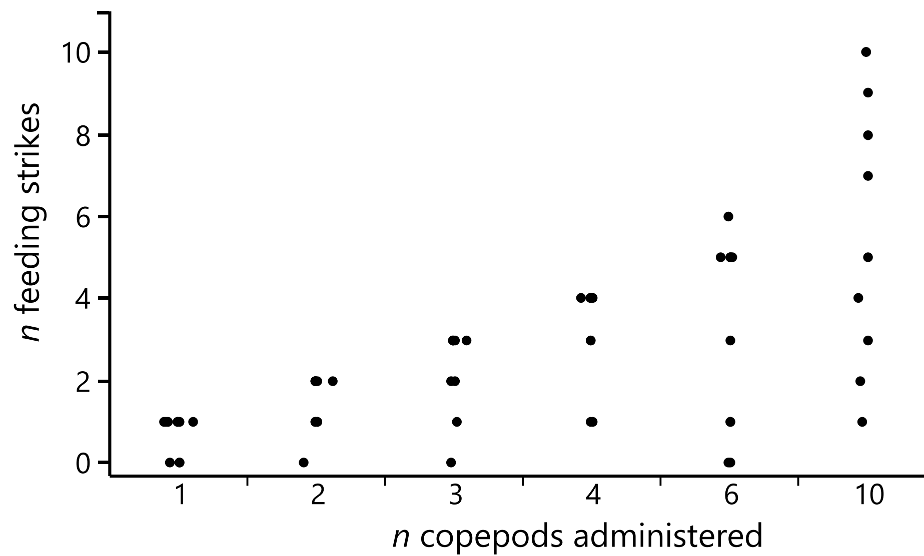

Figure: Relationship between number of copepods administered and the number of feeding strikes of *Tripterygion delaisi* (n = 12).