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RESEARCH ARTICLE

# Retinal temporal resolution and contrast sensitivity in the parasitic lamprey *Mordacia mordax* and its non-parasitic derivative *Mordacia praecox*

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## ABSTRACT

Lampreys and hagfishes are the sole extant representatives of the early agnathan (jawless) vertebrates. We compared retinal function of fully metamorphosed, immature *Mordacia mordax* (which are about to commence parasitic feeding) with those of sexually mature individuals of its non-parasitic derivative *M. praecox*. We focused on elucidating the retinal adaptations to dim-light environments in these nocturnally active lampreys, using electroretinography to determine the temporal resolution (flicker fusion frequency, FFF) and temporal contrast sensitivity of enucleated eyecups at different temperatures and light intensities. FFF was significantly affected by temperature and light intensity. Critical flicker fusion frequency (cFFF, the highest FFF recorded) of *M. praecox* and *M. mordax* increased from 15.1 and 21.8 Hz at 9°C to 31.1 and 36.9 Hz at 24°C, respectively. Contrast sensitivity of both species increased by an order of magnitude between 9 and 24°C, but remained comparatively constant across all light intensities. Although FFF values for *Mordacia* spp. are relatively low, retinal responses showed a particularly high contrast sensitivity of 625 in *M. praecox* and 710 in *M. mordax* at 24°C. This suggests selective pressures favour low temporal resolution and high contrast sensitivity in both species, thereby enhancing the capture of photons and increasing sensitivity in their light-limited environments. FFF indicated all retinal photoreceptors exhibit the same temporal response. Although the slow response kinetics (i.e. low FFF) and saturation of the response at bright light intensities characterise the photoreceptors of both species as rod-like, it is unusual for such a photoreceptor to be functional under scotopic and photopic conditions.

**KEY WORDS: Flicker fusion frequency, Electroretinography, Retinal adaptations, Dim-light vision**

## INTRODUCTION

The ability of animals to detect objects and conspecifics, and to perform visually guided tasks depends on the resolution (spatial and temporal) and sensitivity of their visual systems (Land and Nilsson, 2012). In dim-light environments, light sensitivity must be adjusted such that the visual system can form an image, and this can be accomplished either optically or neurally, or both (Land and Nilsson, 2012; Warrant, 1999). Sensitivity can be enhanced neurally by extending the photoreceptor integration time, which increases the capture of photons, the signal to noise ratio and contrast discrimination (van Hateren, 1993; Warrant, 1999). Increasing integration time, however, comes at the expense of an ability to resolve fast-moving objects clearly (i.e. requires a concomitant low temporal resolution, or low flicker fusion frequency, FFF). As a consequence, fast-moving objects appear blurred (Cohen and Frank, 2006; Fritsches et al., 2005; Warrant, 1999). In bright light, the integration time of the photoreceptors can be shorter as the signal to noise ratio improves. There is, however, an inevitable trade-off between resolution and sensitivity (Fleishman et al., 1995; Matsumoto et al., 2009; McComb et al., 2010; Warrant, 1999). Greater temporal resolution (i.e. high FFF) inevitably comes at the cost of reduced light sensitivity (Kalinoski et al., 2014; Warrant, 1999).

Visual function can be assessed using electroretinography (ERG). Measuring temporal resolution of the retina provides an indication of the ability to identify and track moving objects (Fleishman et al., 1995; McComb et al., 2010). Temporal resolution is determined by exposing the retina to flickering light, increasing the frequency of stimulation until the retina is unable to respond to individual stimuli, and the response appears to be that with a constant light source. This is referred to as the FFF (Fritsches et al., 2005; Lisney et al., 2012), with the maximum FFF known as critical FFF (cFFF). The visual systems of animals that feed on slow-moving prey in dim-light environments generally have low temporal resolution (low cFFF), whereas those that attack fast-moving prey in clear bright-light environments typically have higher temporal resolution (high cFFF) (Autrum, 1958; Frank, 1999; Fritsches et al., 2005; Healy et al., 2013; Horodysky et al., 2008, 2010, 2013; Jenssen and Swenson, 1974; Johnson et al., 2000; Landgren et al., 2014; McComb et al., 2010, 2013).

ERG can also be used to assess contrast sensitivity (CS), which is the ability to discriminate between stimuli based on differences in relative luminance (Wandell, 1995). CS can be assessed by adjusting the contrast levels of a flickering light stimulus (at a fixed mean light intensity) until the difference between two brightness levels becomes indistinguishable by the retina.

The visual system of lampreys (Petromyzontiformes) has been of particular interest because this group is one of the two surviving representatives of the early agnathan (jawless) stage in vertebrate evolution (Janvier, 2007). The life cycle of all lamprey species comprises a protracted microphagous and burrowing larval phase spent in freshwater, which is followed by a radical metamorphosis (Dawson et al., 2015; Hardisty and Potter, 1971a,b). Several species then embark on a parasitic phase at sea, during which they feed mainly on teleost fishes and, when fully grown, migrate back into rivers to spawn (Hardisty and Potter, 1971b; Moser et al., 2015). In contrast, other (non-parasitic) species do not feed as adults and remain in

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**List of symbols and abbreviations**

| | |
|---|---|
| cFFF | critical flicker fusion frequency |
| CS | contrast sensitivity |
| ERG | electroretinogram/electroretinography |
| FFF | flicker fusion frequency |
| LED | light-emitting diode |
| sFFF | flicker fusion frequency defined by significance |
| tFFF | flicker fusion frequency defined by threshold |
| VEP | visual evoked potential |

freshwater, reaching maturity soon after the completion of metamorphosis (Hardisty and Potter, 1971c). Each non-parasitic species is considered to have evolved from a particular parasitic species (Docker, 2009; Potter, 1980a), thus constituting a species pair. Such pairs include the parasitic *Mordacia mordax* and non-parasitic *Mordacia praecox* (Potter et al., 1968), which co-occur in rivers.

The fully metamorphosed individuals of parasitic species remain burrowed during the day and emerge at night, when they are transported downstream. In contrast, after completing metamorphosis, the non-parasitic species undergo a short nocturnal migration to their spawning areas (Hardisty, 1979; Potter, 1980b; Potter et al., 1968). Thus, fully metamorphosed *M. mordax* and *M. praecox* are both active in dim-light conditions.

The retina of *M. mordax* possesses adaptations that increase optical sensitivity including: (1) a reflective tapetum within the retinal pigment epithelium (Collin and Potter, 2000), (2) a large single rod-like photoreceptor with a long outer segment (Collin and Potter, 2000; Collin et al., 2004), and (3) a large ellipsosome within the inner segment of the photoreceptor that would focus (refract) light onto the outer segment (Collin and Potter, 2000). In spite of the uniqueness of the visual system of this lamprey species, and despite the detailed knowledge of retinal morphology in *M. mordax*, there have been no studies on the physiology of their visual system. The only physiological study to assess visual function in any species of lampreys is that on the FFF of the parasitic species *Lampetra fluviatilis*, which has a cFFF of 24 Hz at 10°C (Dreyfert et al., 1979); to the best of our knowledge no CS estimates exist.

Because FFF and CS increase with temperature (Cohen and Frank, 2006; Fritsches et al., 2005; Hanyu and Ali, 1963; Landgren et al., 2014; Tatler et al., 2000), the environmental temperature has profound effects on the visual function of ectotherms, such as lampreys (Saad et al., 1959). As temperature in the rivers in which *M. mordax* and *M. praecox* occur ranges from ∼6 to 30°C (Potter, 1970), the temporal resolution and CS of their visual system will change seasonally, with visual function probably becoming limited at low temperatures.

We compared physiological aspects of the visual system of immature individuals of the parasitic *M. mordax*, which are about to embark on a marine trophic phase, with those of sexually maturing individuals of the non-parasitic derivative *M. praecox*. We employed ERG to determine whether the visual systems of these species have adapted to increase the capture of photons in their dim-light environments, i.e. by possessing relatively low FFFs, which would enhance the ability of the photoreceptors to capture light, and high CS, which would facilitate the discrimination of small differences in luminance. We determined the FFF and temporal CS at a wide range of temperatures and light intensities to elucidate the extent to which the visual function of these species is influenced by environmental conditions. We also focused on testing the hypothesis that temporal resolution (i.e. FFF) is greater in the fully metamorphosed *M. mordax*, as these individuals are parasitic and require vision to detect prey, whereas the mature non-parasitic derivative *M. praecox* requires no such ability as this species does not feed after completing the larval phase. As there is morphological evidence to suggest that the retina of *M. mordax* contains only one photoreceptor type (Collin and Potter, 2000; Collin et al., 2004), we hypothesise that all retinal photoreceptors will exhibit the same temporal response characteristics. Our results provide only the second recorded cFFF values and the first quantification of CS for agnathan fishes.

## MATERIALS AND METHODS

### Source of animals

All capture, holding and experimental procedures followed the guidelines of the National Health and Medical Research Council – Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, in accordance with The University of Western Australia Animal Ethics protocol (approval number: RA/3/100/917 and RA/3/100/1220). *Mordacia mordax* (Richardson 1846) and *Mordacia praecox* Potter 1968 were caught using an electro-fish shocker in the Wonboyn and Wallagaraugh Rivers in New South Wales, Australia (NSW collection permit: P10/0043-1.0) and transported to The University of Western Australia (Department of Fisheries translocation permit: 871/11). They were kept in aquaria in a controlled-temperature room maintained at 10–14°C with a 12 h light:12 h dark cycle (lights on 06:00 h to 18:00 h). The aquaria contained soft sediment into which the lampreys burrowed. We used five sexually mature female *M. praecox* (mean±s.d. length 122±8.6 mm) and three fully metamorphosed immature *M. mordax* (123±5.9 mm).

### Eyecup preparation

Animals were killed by immersion in 500 mg $l^{-1}$ tricaine methanesulfonate salt (MS-222, Sigma-Aldrich, USA) solution buffered with an equal concentration of sodium hydrogen carbonate (Ajax Finechem, Australia). The eyes were excised and the cornea, lens and vitreous removed under room light using a dissecting microscope (Nikon SMZ745T, USA). Eyecups were placed on moist filter paper upon a stage in a Petri dish (diameter: 20 mm) containing 6 ml of Ringer solution (in mmol $l^{-1}$: 115 NaCl, 2.1 KCl, 2.6 $CaCl_2$, 2 $MgCl_2$, 6 $NaHCO_3$ and 3 glucose, pH 7.4; Buchanan and Cohen, 1982), which had been bubbled with carbogen (95% $O_2$ and 5% $CO_2$) for at least 15 min. Ringer solution was also pipetted onto the retina. Because of the small size of eyecups (∼2 mm) and the avascular retina of lampreys (Collin and Potter, 2000), the carbogenated Ringer solution provided enough oxygen to keep the retina viable for the duration of the experiment.

We utilised enucleated eyecups because lampreys possess two corneas (dermal and scleral), which prevented us from obtaining a retinal response from live, anaesthetised animals. A study that compared ERGs recorded from live, anaesthetised animals with enucleated eyecup preparations demonstrated that temporal FFF did not differ significantly between the two methods (Ryan et al., 2017). Temporal CS, however, was significantly different at bright light intensities ($1.8\times10^{-5}$ to $1.2\times10^{-2}$ W $cm^{-2}$), with CS lower in enucleated eyecup preparations (Ryan et al., 2017). Therefore, FFF estimates presented in the current study may resemble those of *in vivo* preparations, while temporal CS estimates may be more conservative than responses obtained from anaesthetised animals.

### Experimental regime

Electroretinograms (ERGs) were recorded using platinum electrodes inside a light-proof Faraday cage. The tip of the recording electrode (diameter 0.125 mm) was shaped into a loop



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and positioned on the retina using a micromanipulator, while the reference electrode (diameter 0.25 mm), with a ring at the terminal end, was placed under the moist filter paper in the Ringer solution. The electrodes were connected to a differential AC amplifier (DAM-50, World Precision Instruments, USA), where the responses were amplified either 1000 times (*M. mordax*) or 10,000 times (*M. praecox*) and bandpass filtered between 0.1 and 1 kHz. The signal was visualised on a digital storage oscilloscope (Tektronix 2211, USA) and digitized with a 5 kHz sampling frequency using a multifunction data acquisition (DAQ) board (USB-6353 X series, National Instruments, USA). Custom-written software (J.M.H.) in MATLAB (R2012a, The MathWorks, USA) was used for data acquisition and analysis of the signals.

### Optical apparatus

White light stimuli were produced using a light-emitting diode (LED; 5 mm, C503D-WAN, Cree, USA), located 50 mm from the eyecup so that the output cast a circular patch of light over the entire preparation. The LED produced an irradiance of $4.5\times10^{-4}$ W cm$^{-2}$ (49,000 lx) at the level of the retina (diameter ∼2 mm), which was measured using a research radiometer (ILT1700, International Light Technologies, USA). Intensity was controlled with pulse-width modulation at 1 kHz by a custom-made LED controller under computer control.

### Temperature regulation

We determined the effect of temperature on the response characteristics of the retina by embedding the eyecup holder (isolating the eyecup and Ringer solution) in a water bath, in which the temperature was gradually increased and stabilised (over an average of 48 min) between recordings by gravity feeding ice-cooled water through an in-line solution heater (SH-27A, Warner Instruments, USA) connected to a temperature controller (TC-324B, Warner Instruments, USA). Each series started at the lowest temperature, because it was closest to the temperature of aquaria. Four temperatures were employed (9, 14, 19 and 24°C), covering most of the temperature range typically experienced by *Mordacia* spp. in their riverine environment (Potter, 1970). The temperature was measured by a calibrated thermistor placed in the Ringer solution surrounding the eyecup and maintained within 0.5±1.2°C (mean±s.d.) of the target temperature.

### FFF

We determined FFF by presenting the retina of enucleated eyecups of five *M. praecox* and three *M. mordax* with a flickering square-wave white light stimulus over a range of stimulation frequencies from 2 to 55 Hz. Each frequency was presented for 30 s. FFF was measured over a ∼6 log unit intensity range ($7.9\times10^{-9}$ to $4.5\times10^{-4}$ W cm$^{-2}$), increasing in 1 log unit steps apart from the lowest stimulus intensity (0.7 log unit). Prior to each series of intensity measurements, the eyecup was dark-adapted for a minimum of 20 min, a period which was also used for temperature adaptation (average of 48 min). Because of time constraints, each intensity series was recorded at sequential temperatures of 9, 14, 19 and 24°C with *M. praecox* and at 9 and 24°C with *M. mordax*, for which there were fewer animals. We combined FFF (and CS) responses for both eyes of an individual in two out of three *M. mordax* subjects because responses from the first eye declined in unrelated experiments (after conducting experiments at 9°C). Therefore, the second eyecup, which had been stored in the dark at 4°C in Ringer solution, was used for recordings at 24°C the following day.

The signal output from the retina to flickering lights was Fourier transformed to calculate the frequency composition of the signal. We then determined FFF in two ways. First, we defined FFF as the stimulation frequency that produced a significant response from the retina. Significance was calculated by comparing the signal frequency against noise frequencies, following Maddess et al. (2000). Second, FFF was defined as the stimulation frequency at which the response power ($\log_{10}$ of the response amplitude squared) crossed a predetermined threshold for each species. The threshold power was based on the highest noise level at the highest stimulation frequency that produced a significant response, for each individual and each temperature. We then calculated the average threshold for each species. The threshold was fixed at a response power of $10^{-6.5}$ mV$^2$ for *M. praecox* (Fig. 1A) and $10^{-7}$ mV$^2$ for *M. mordax* (Fig. 1B). This second measure has the advantage that it is not affected by the general decrease in noise level of the signal output from the retina at higher stimulus frequencies. The defined

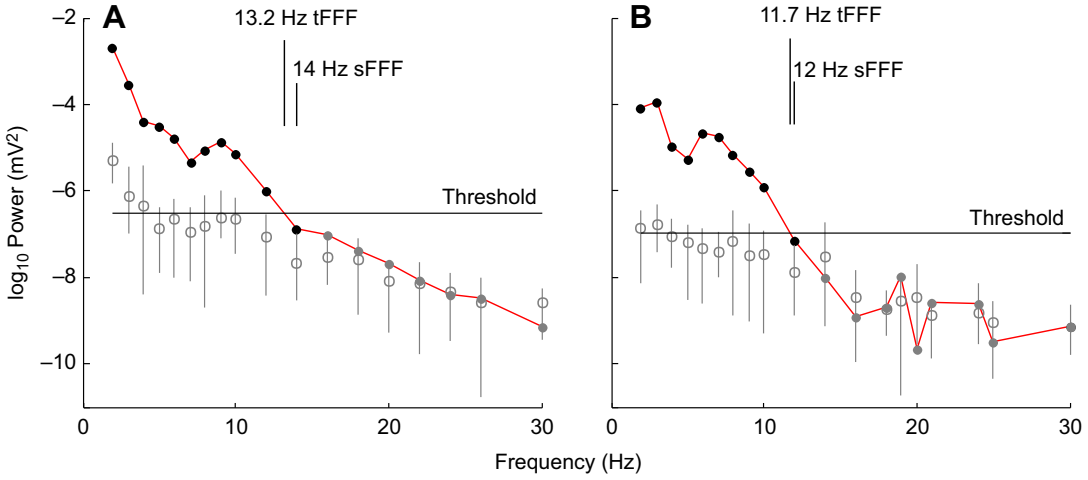


**Fig. 1. Example of temporal response characteristics from one animal to a low light intensity stimulus ($7.9\times10^{-9}$ W cm$^{-2}$), across a range of frequencies.** Flicker fusion frequencies (FFF) were defined using both significance (sFFF) and threshold (tFFF). The threshold was fixed at a response power of (A) $10^{-6.5}$ mV$^2$ for *Mordacia praecox* and (B) $10^{-7}$ mV$^2$ for *Mordacia mordax*. The signal (red line) and noise (grey open circles, minimum and maximum noise represented by grey vertical lines) are shown, as are responses that were significantly above the noise (black circles) and those that could no longer be differentiated from the noise (grey filled circles).

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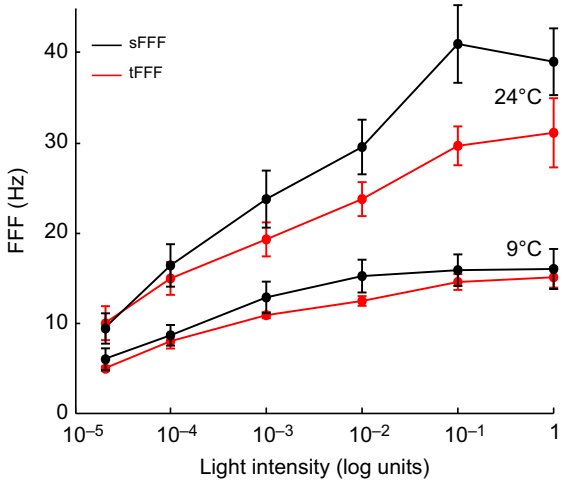


**Fig. 2. Difference between average threshold and significance FFF in *M. praecox* at 9 and 24°C.** FFF defined by threshold (tFFF) and significance (sFFF) follow the same trend. At brighter light intensities, however, the sFFF is more likely to be significant as a result of the reduced environmental noise, particularly at higher temperatures. tFFF is used to standardise the responses across different conditions as it compares how the power of the response changes under different conditions. Light intensity, $4.5\times10^{-4}$ W cm$^{-2}$. $n$=5.

thresholds were 4.6±0.8 and 4.4±0.8 log units (mean±s.d.) lower than the maximum response power at full intensity for *M. praecox* and *M. mordax*, respectively.

FFF defined by threshold proved to be a reliable method to standardise FFF across conditions and animals, as FFF estimated by threshold was close to FFF using response significance alone (Fig. 1A,B). Overall, FFF defined by threshold was slightly lower than significance FFF, with the greatest difference occurring at the brightest intensities at 24°C (Fig. 2). The significance measure potentially suffers from electrical artefacts at high temporal frequencies and high stimulus contrasts as electrical switching noise increases and independent electrical noise level decreases under these conditions (Fig. 2). In comparison, FFF defined by threshold compares how the power of the response varies under different conditions, which are independent of environmental noise, and thresholds can be set above the level of electrical artefacts. Therefore, FFF values are presented based on threshold.

Each intensity series took up to 1.5 h to complete at a particular temperature, while each temperature series took up to 8 h to complete (recorded between 12:00 h and 01:00 h for *M. praecox* and 09:30 h and 17:00 h for *M. mordax*). In order to check that the responses were stable over time, we repeated the recordings at the highest temperature up to 6 h after completing the temperature series. Repeated recordings were consistent with the initial recordings with only minor variation in response over time. FFF varied on average 2.1 Hz (range: 0.2–9.3 Hz), confirming that the eye remained viable throughout the duration of an experiment.

We did not formally assess circadian rhythms of FFF and CS, although our data suggest no obvious correlation between responses and time of recording.

## CS

We assessed temporal CS in one *M. praecox* and three *M. mordax* by stimulating the retina with a flickering square-wave light stimulus at a frequency of 5 Hz (as lower stimulation frequencies produced the highest response power). Because of time constraints, we tested 10 descending contrast levels (each halving the previous contrast level) at a fixed average intensity with a maximum contrast of 100% and a minimum contrast of 0.2%. We controlled contrast using pulse-width modulation in an equivalent manner to stimulus intensity, and each contrast level was presented for 30 s. CS was measured at a range of mean intensity levels over 3 log units (mean light intensity: $2.5\times10^{-6}$ to $2.3\times10^{-4}$ W cm$^{-2}$), increasing in 1 log unit steps, at temperatures of 9 and 24°C. We interleaved CS measurements with the relevant FFF measurements to avoid changing the adaptation state of the retina.

We estimated response power from a Fourier transform of the retinal response signal (as in FFF experiments). Contrast was calculated using the formula presented by Michelson (1927):

$$\frac{L_{\max}-L_{\min}}{L_{\max}+L_{\min}}, \quad (1)$$

where $L_{\max}$ and $L_{\min}$ are the maximum and minimum stimulus intensity, respectively. We determined the CS at different mean

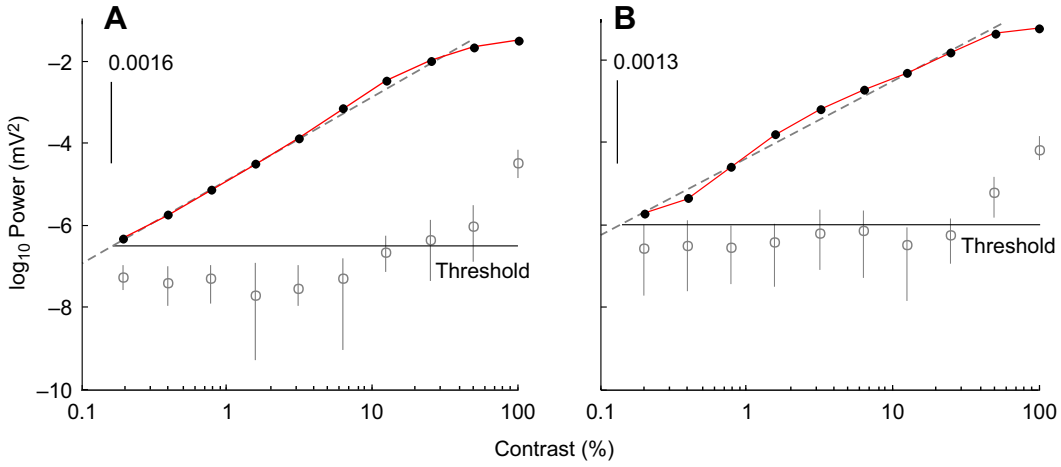


**Fig. 3. Example of threshold contrast for 5 Hz flicker of one animal.** Contrast was defined using the threshold measure. The threshold was fixed at a response power of (A) $10^{-6.5}$ mV$^2$ for *M. praecox* and (B) $10^{-6}$ mV$^2$ for *M. mordax*. The signal (red line) and noise (grey open circles, minimum and maximum noise represented by grey vertical lines) are shown, along with responses that were significantly above noise (black circles). At the lowest contrast levels, threshold contrast responses did not reach the noise level. Threshold contrast was, therefore, extrapolated to the predetermined threshold as the relationship of the response appears as a straight line on logarithmic axes, after omitting the highest contrast level. Highest mean intensity, $2.3\times10^{-4}$ W cm$^{-2}$.



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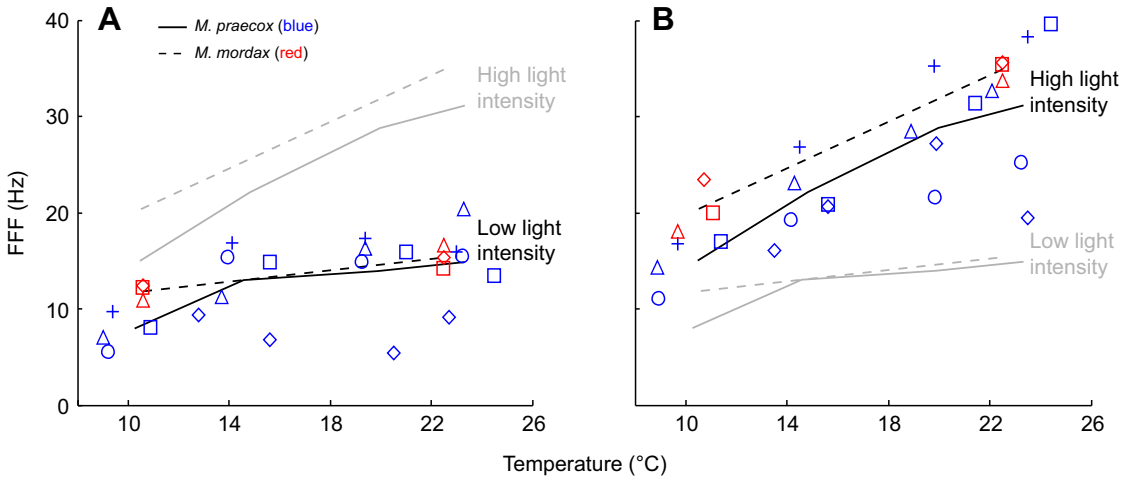


**Fig. 4. Effect of temperature and light intensity on FFF.** Average FFF values for *M. praecox* (*n*=5) and *M. mordax* (*n*=3) along with the responses from each animal (symbols represent different animals, and colours represent different species). FFF was recorded at four temperatures in *M. praecox* and two in *M. mordax*. (A) Low light intensity ($8.7\times10^{-8}$ W cm$^{-2}$). (B) High light intensity ($4.5\times10^{-4}$ W cm$^{-2}$). Grey lines are for comparison between the two light intensities.

intensities based on measured thresholds, as justified previously. The threshold power for contrast was determined from the lowest contrast level tested at the brightest mean intensity (because there was no electrical artefact produced from the LED under these conditions) that produced a significant response, for each temperature and individual assessed. For each species, we then calculated the average threshold, which was fixed at a response power of $10^{-6.5}$ mV$^2$ for *M. praecox* (Fig. 3A) and $10^{-6}$ mV$^2$ for *M. mordax* (Fig. 3B). The defined thresholds were 4.3±1.1 and 4.4±0.5 log units (mean±s.d.) lower than the maximum response in each animal at the brightest mean intensity for *M. praecox* and *M. mordax*, respectively. We calculated the average CS for each individual by taking the inverse of the threshold contrast and plot the results on a logarithmic scale.

At the brightest mean intensities, the minimum contrast level tested (0.2%) was not sufficiently low to reach threshold contrast in some cases (*M. praecox*: 2 of 6 recordings; *M. mordax*: 3 of 18 recordings). For these recordings, threshold contrast was therefore extrapolated from the responses to the predetermined threshold (Fig. 3), as the relationship between the contrast levels examined and sensitivity appears to be a straight line on logarithmic axes after omitting the 100% contrast level, as it did not follow the same trend.

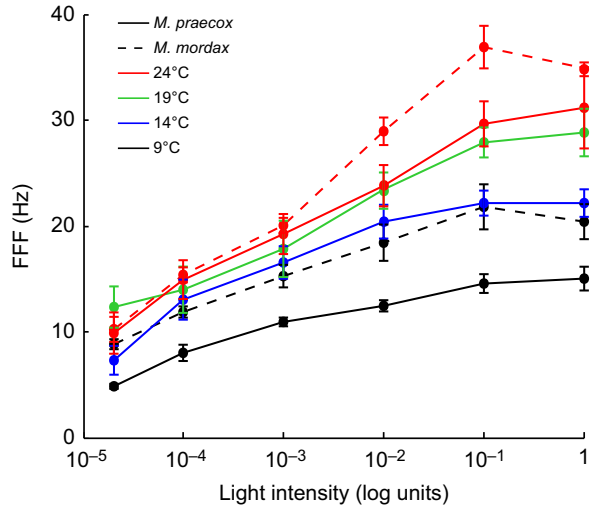


**Fig. 5. Relationship between FFF and light intensity.** FFF values were averaged across animals for each temperature tested (9, 14, 19 and 24°C) in *M. praecox* (*n*=5) and *M. mordax* (*n*=3). Full intensity, $4.5\times10^{-4}$ W cm$^{-2}$.

### Statistics

We compared the FFF and CS in the two species at different temperatures and light intensities using a linear mixed effects model from the *lme4* package (Bates et al., 2015) in RStudio (version 0.98.1056; R Core Team, 2016). All models were graphically checked for adherence to model assumptions.

## RESULTS

### FFF

The response power of eyecups (mean±s.d. diameter: *M. praecox* 1.79±0.06 mm and *M. mordax* 2.28±0.06 mm) to the flickering light stimulus decreased as stimulus frequency increased, with the maximum power occurring at frequencies between 2 and 9 Hz in *M. praecox* (Fig. 1A) and 2 and 8 Hz in *M. mordax* (Fig. 1B). This pattern of decreasing response power was consistent across all temperatures and light intensities. An increase in both temperature and light intensity resulted in an increase in the maximum response power, although in some recordings the power of the maximum response decreased at the brightest light intensity.

Temporal resolution in both *M. praecox* and *M. mordax* was significantly affected by temperature, with FFF increasing with an increase in temperature (Fig. 4). The magnitude of this increase depended on the light intensity, suggesting there is a significant interaction between the two factors (final model: species+intensity×temperature range, $P<0.001$, likelihood ratio=63.1, d.f.=15). The increase in FFF was greatest at the brightest light intensities, with FFF increasing approximately 15 Hz between 9 and 24°C in both

**Table 1. Average rate of increase in flicker fusion frequency with light intensity at the temperatures assessed in *Mordacia praecox* and *Mordacia mordax***

| | Increase in FFF (Hz/log unit) | | | |
|---|---|---|---|---|
| | 9°C | 14°C | 19°C | 24°C |
| *M. praecox* | 2.5 | 3.9 | 4.3 | 5.1 |
| *M. mordax* | 3.4 | | | 7.1 |

FFF, flicker fusion frequency.

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species at full intensity (Fig. 4B), compared with a smaller increase of approximately 7 Hz in *M. praecox* and 3.5 Hz in *M. mordax* between 9 and 24°C at a lower light intensity of $8.7\times10^{-8}$ W cm$^{-2}$ (Fig. 4A).

In both species, FFF increased as light intensity increased (Fig. 5, Table 1). Only at the highest intensity did the FFF show signs of plateauing in *M. praecox* and decrease in *M. mordax* (Fig. 5). Neither species showed any clear sign of change in slope across light intensity, which would be expected if there were a shift from rod to cone activity. There was a significant difference between the FFF in the two species recorded under the same conditions ($P$=0.019, likelihood ratio=5.5, d.f.=1). At both 9 and 24°C, the FFF of *M. mordax* was consistently higher across all light intensities than that of *M. praecox*. The average difference was 5.1 Hz at 9°C. The highest FFF achieved irrespective of light intensity (i.e. the cFFF) for *M. praecox* was 15.1 Hz and for *M. mordax* it was 21.8 Hz at 9°C. At 24°C the cFFF for *M. praecox* was 31.1 Hz and for *M. mordax* it was 36.9 Hz (Fig. 5).

### CS

The response power to a flickering 5 Hz white light stimulus decreased as the contrast level decreased (Fig. 3). The CS of *M. praecox* and *M. mordax* increased significantly with an increase in temperature (final model: temperature range, $P$<0.001, likelihood ratio=20.4, d.f.=1). Between 9 and 24°C, the average CS increased approximately 10-fold from 64 to 625 in *M. praecox* and 67 to 710 in *M. mordax*, at the brightest mean intensity (Fig. 6). The results demonstrate that both species are unusually sensitive to small intensity differences, particularly at higher temperatures.

CS was not significantly different between species ($P$=0.44, likelihood ratio=0.61, d.f.=1) or across light intensities ($P$=0.42, likelihood ratio=1.7, d.f.=2), suggesting that CS is comparatively constant across the light intensities we employed. However, there is variation in CS estimates between individuals which, given the small sample size, makes it difficult to predict the exact relationship between CS and light intensity.

## DISCUSSION

Our results show that the temporal resolution and CS of *Mordacia* spp. are highly temperature dependent. Increasing light intensity significantly increased FFF in an approximately logarithmic manner, except at the brightest intensities. In contrast, CS remained approximately constant across all light intensities. The temporal resolution of *M. mordax* was significantly greater than that of *M. praecox*, whereas CS was similar in the two species.

Increasing temperature from 9 to 24°C led to a significant increase in FFF in both species, paralleling the trend observed in goldfish (*Carassius auratus*), swordfish (*Xiphias gladius*), escolar (*Lepidocybium flavobrunneum*) and three species of elasmobranchs (Fritsches et al., 2005; Hanyu and Ali, 1963; Kalinoski et al., 2014; Landgren et al., 2014). Temporal CS improved 10-fold over the 9–24°C temperature range, suggesting that *Mordacia* spp. are exceptionally sensitive to small changes in contrast at higher temperatures. Our results are consistent with the effect of temperature on the biochemical processes of phototransduction (Tatler et al., 2000), as enzymatic reactions within the cGMP cascade (Baylor, 1996) and diffusion of phototransduction intermediates in the photoreceptor membrane (Lamb, 1984, 1996) are faster at higher temperatures (Tatler et al., 2000). Because lampreys are ectotherms (Saad et al., 1959), this strong temperature dependence has a fundamental impact on the visual function of *Mordacia* spp., ultimately limiting temporal resolution and CS at colder temperatures.

The cFFF values obtained from ERGs tend to be higher than those recorded using behavioural techniques (Dodt and Wirth, 1954; Hendricks, 1966; Lisney et al., 2011, 2012). For example, in the chicken *Gallus gallus domesticus*, ERGs produced a cFFF of 105 Hz (Lisney et al., 2012), while behavioural assessment provided a cFFF of 87 Hz (Lisney et al., 2011) measured across a similar light intensity range. The cFFF values obtained from ERGs should be considered as an upper limit of temporal resolution as ERGs measure neural activity (photoreceptor and bipolar cells) in the retina, while behavioural studies measure what an animal actually perceives as a flickering stimulus, which is dependent on more complex visual processing in the brain (Lisney et al., 2012; Schneider, 1968; Umino et al., 2012). To the best of our knowledge, direct comparison of ERG and behaviourally determined temporal CS is lacking. In mice, ERG and behaviourally assessed CS (at a temporal frequency of 3 Hz) followed similar trends, which suggests that behavioural temporal CS may be controlled within the retina (Umino et al., 2012). Future experiments will need to assess the temporal resolution and temporal CS of lampreys using behavioural studies.

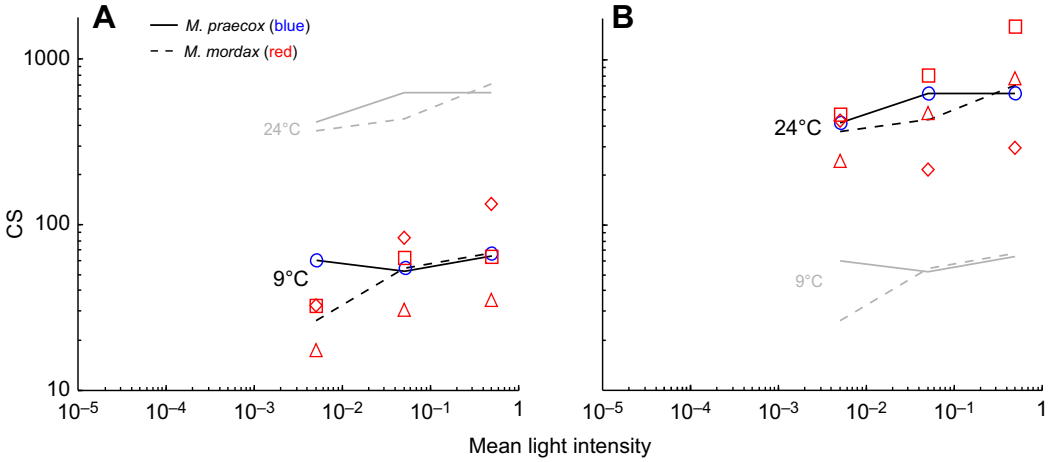


**Fig. 6. Relationship between temporal contrast sensitivity (CS) and mean luminance.** Average temporal CS values for each species (*M. praecox*, *n*=1; *M. mordax*, *n*=3) at two temperatures: (A) 9°C and (B) 24°C. Each symbol represents responses from a different animal. Grey lines are for comparison between the two temperatures. Highest mean intensity, $2.3\times10^{-4}$ W cm$^{-2}$.



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**Table 2. Critical FFF of fishes from different light environments assessed using different methods: behaviour, electroretinography and visual evoked potentials at the temperatures shown**

| | cFFF (Hz) | Light environment | Temperature (°C) | Method |
|---|---|---|---|---|
| Escolar (*Lepidocybium flavobrunneum*) | 9[1] | D | 23 | ERG |
| Longspine thornyhead (*Sebastolobus altivelis*) | 10[2] | D | 5–8 | VEP |
| Star-spotted dogfish (*Mustelus manazo*) | 10[3] | D | | ERG |
| Smooth dogfish (*Mustelus canis*) | 13[4] | D | 20 | ERG |
| European eel (*Anguilla anguilla*) | 14[5] | D | | Behaviour |
| Blacknose shark (*Carcharhinus acronotus*) | 18[6] | D | 24–25 | ERG |
| Horn shark (*Heterodontus francisci*) | 20[2] | D | 12–14 | VEP |
| Spiny dogfish (*Squalus acanthias*) | 20[4] | D | 12 | ERG |
| River lamprey (*Lampetra fluviatilis*) | 24[7] | D | 10 | ERG |
| Rainbow trout (*Oncorhynchus mykiss*) | 27[8] | D | 10 | Behaviour |
| Scalloped hammerhead (*Sphyrna lewini*) | 27[6] | D | 24–25 | ERG |
| Shovelnose guitarfish (*Rhinobatos productus*) | <30[2] | D | 15–17 | VEP |
| Little skate (*Raja erinacea*) | 30[9] | D | 20 | ERG |
| Winter skate (*Raja ocellata*) | 30[9] | D | 20 | ERG |
| Bonnethead shark (*Sphyrna tiburo*) | 31[6] | D & B | 24–25 | ERG |
| **Precocious lamprey (*Mordacia praecox*)** | 31* | D | 24 | ERG |
| **Short-headed lamprey (*Mordacia mordax*)** | 37* | D | 24 | ERG |
| Bigeye tuna (*Thunnus obesus*) | 37[10] | D | 27 | ERG |
| Lemon shark (*Negaprion brevirostris*) | 37[11] | D | 26–30 | ERG |
| Medaka (*Oryzias latipes*) | 37[12] | D | 25 | Behaviour |
| Bowfin (*Amia calva*) | 38[13] | D | | ERG |
| Tiger shark (*Galeocerdo cuvier*) | 38[14] | D | | ERG |
| Swordfish (*Xiphias gladius*) | 40[10] | D | 24 | ERG |
| Common snook (*Centropomus undecimalis*) | 40[15] | D | 24–25 | ERG |
| Weakfish (*Cynoscion regalis*) | 42[16] | D | 20–22 | ERG |
| Pinfish (*Lagodon rhomboides*) | 44[15] | B | 24–25 | ERG |
| Grey snapper (*Lutjanus griseus*) | 47[15] | B | 24–25 | ERG |
| Tautog (*Tautoga onitis*) | 48[17] | B | 20–22 | ERG |
| Sunfish (*Lepomis* sp.) | 50[18] | B | | Behaviour |
| Black sea bass (*Centropristis striata*) | 52[17] | B | 20–22 | ERG |
| Summer flounder (*Paralichthys dentatus*) | 52[19] | B | 20–22 | ERG |
| Red drum (*Sciaenops ocellatus*) | 54[16] | B | 20–22 | ERG |
| Sandbar shark (*Carcharhinus plumbeus*) | 54[14] | D & B | | ERG |
| Spot (*Leiostomus xanthurus*) | 55[16] | B | 20–22 | ERG |
| Bluefish (*Pomatomus saltatrix*) | 56[19] | B | 20–22 | ERG |
| Atlantic croaker (*Micropogonias undulatus*) | 59[16] | B | 20–22 | ERG |
| Thornback ray (*Platyrhinoidis triseriata*) | <60[2] | B | 15–17 | VEP |
| Atlantic spadefish (*Chaetodipterus faber*) | 60[17] | B | 20–22 | ERG |
| Sand bass (*Paralabrax nebulifer*) | 60[2] | B | 10–13 | VEP |
| Spotted seatrout (*Cynoscion nebulosus*) | 60[16] | B | 20-22 | ERG |
| Grunion (*Leuresthes tenuis*) | >60[2] | B | 21–23 | VEP |
| Cobia (*Rachycentron canadum*) | 65[19] | B | 20–22 | ERG |
| Brook trout (*Salvelinus fontinalis*) | 67[13] | B | | ERG |
| Goldfish (*Carassius auratus*) | 67[20] | B | 25 | ERG |
| Striped bass (*Morone saxatilis*) | 74[19] | B | 20–22 | ERG |
| Yellowfin tuna (*Thunnus albacares*) | 73[21] | B | 24–26 | ERG |
| Striped mullet (*Mugil cephalus*) | >100[2] | B | 24–26 | VEP |

cFFF, critical FFF; B, bright-light environment; D, dim-light environment; ERG, electroretinography; VEP, visual evoked potential. Study species are in bold (*data from the present study). [1]Landgren et al. (2014), [2]Bullock et al. (1991), [3]Kobayashi (1962), [4]Kalinoski et al. (2014), [5]Adrian and Matthews (1928), [6]McComb et al. (2010), [7]Dreyfert et al. (1979), [8]Carvalho et al. (2004), [9]Green and Siegel (1975), [10]Fritsches et al. (2005), [11]Gruber (1969), [12]Carvalho et al. (2002), [13]Ali and Kobayashi (1968), [14]Litherland (2009), [15]McComb et al. (2013), [16]Horodysky et al. (2008), [17]Horodysky et al. (2013), [18]Wolf and Zerrahn-Wolf (1936), [19]Horodysky et al. (2010), [20]Hanyu and Ali (1963), [21]Brill et al. (2005).

### Resolution and sensitivity reflect visual requirements

Fish that inhabit dim-light environments generally have lower cFFF values (<45 Hz) than those residing in bright-light environments (40–100 Hz; Table 2). Comparisons between the FFF and cFFF recorded in different studies should be treated with some caution as experimental conditions often vary among studies (e.g. methodology, temperature, adaptation state, stimulus and background light intensity, sine-wave or square-wave stimulation and how FFF is determined based on signal or threshold), which may impact temporal resolution. Comparisons between studies, however, tend to reflect an animal's ecology and life style (Horodysky et al., 2008).

Because of their burrowing habit and nocturnal life style (Potter et al., 1968), the two lamprey species we examined spend a significant portion of their life in a light-limited environment (except during spawning). The visual system of *Mordacia* spp. is adapted to these low light levels through optical (the presence of tapetum) and neural (temporal and spatial summation) mechanisms. In other words, *Mordacia* spp. sacrifice high temporal resolution (high FFF) to increase light sensitivity (Frank, 1999; Jenssen and Swenson, 1974; Warrant, 1999) and to improve contrast discrimination (Cronin et al., 2014; van Hateren, 1993). A visual system adapted to increase sensitivity is generally associated with low spatial resolution (i.e. high spatial summation), as demonstrated

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by the low anatomical spatial resolving power of 1.7 cycles deg$^{-1}$ in the downstream migrant of *M. mordax* (Collin et al., 2004). The possession of a retinal tapetum (Collin and Potter, 2000) also increases sensitivity by effectively doubling the length of the outer segment, which suggests that there is more visual pigment available for light absorption (Land and Nilsson, 2012; Rovamo and Raninen, 1988). In brief, all these characteristics imply that the visual systems of *Mordacia* spp. are adapted to improve photon capture in a dim-light environment.

The light levels at which *Mordacia* spp. are likely to be active, <10 lx at twilight (Johnsen, 2012), are about four orders of magnitude below the highest intensity we assessed. Therefore, at ecologically relevant light intensities (i.e. $10^{-4}$ in Fig. 5 or ∼6 lx), vision may be limited to a FFF of ∼15 Hz at 24°C, which declines further at colder temperatures (Fig. 5).

To locate prey, parasitic lampreys use a combination of senses; their remarkable olfactory ability allows them to detect sub-picomolar concentrations of certain compounds (Fine and Sorensen, 2008; Sorensen et al., 2005) and is used for long-distance orientation towards fish (Kleerekoper and Mogensen, 1963), while electroreception and vision are employed to localise prey at short distances (Farmer and Beamish, 1973; Lennon, 1954). The greater FFF values in fully metamorphosed *M. mordax* versus mature *M. praecox* could be due to the fact that the former are about to commence searching for prey, whereas the latter are about to spawn and do not feed. A higher FFF would enable the parasitic species *M. mordax* to track potential hosts, such as yellow-eyed mullet (*Aldrichetta forsteri*), barracouta (*Thyrsites atun*), brown trout (*Salmo trutta*) and black bream (*Acanthopagrus butcheri*) (Potter et al., 1968), which have swimming speeds of 3–5 km h$^{-1}$ (Peake, 2008; http://vro.depi.vic.gov.au/dpi/vro/vrosite.nsf/pages/marine_fish_tracking_black_bream, 26th June 2015), and to target a site for attachment (Cochran, 1986). The cFFF of *M. mordax* (20.4 Hz at 9°C, 49,000 lx) is similar to that of another parasitic lamprey, *L. fluviatilis* (24 Hz at 10°C, 170,000 lx; Dreyfert et al., 1979). There would be less selective pressure for the non-parasitic *M. praecox* to maintain high FFF, accounting for their lower temporal resolution (15.1 Hz at 9°C, 49,000 lx), and presumably better spatial resolution or sensitivity, or both.

The average temporal CS of both species appears to be approximately constant across all light intensities assessed. This can be explained by Weber's Law, where CS remains constant at high light intensities, as the signal to noise ratio is constant (van Hateren, 1993). The mean intensities we used, ∼250–25,000 lx, are likely to be greater than those to which *Mordacia* spp. are exposed during major periods of activity (luminance at twilight <10 lx; Johnsen, 2012). In dim-light conditions, the CS of *Mordacia* spp. may be lower than the values recorded, as it has been shown that peak sensitivity decreases as luminance is reduced (Bilotta et al., 1998; De Valois and De Valois, 1990) because photon noise becomes limiting (Warrant, 1999). Future studies should, therefore, record the CS of *Mordacia* spp. at an ecologically relevant light level.

In water, the visual contrast between adjacent stimuli tends to be lower than in air as a result of scattering and absorption of light by the water and the presence of suspended or dissolved substances (Hester, 1968). The situation is exacerbated as viewing distance and water turbidity increase (Lythgoe, 1988). Because the visibility of objects underwater is dependent on their contrast rather than their size (Cronin et al., 2014; Douglas and Hawryshyn, 1990), it may not be surprising that aquatic vertebrates have higher temporal contrast sensitivities than terrestrial vertebrates (except in humans; Table 3), which would be advantageous in scattering aquatic media. Some of the variation in CS between studies may be due to different experimental design (as discussed for FFF); however, the most important factor is the adaptation state of the eye (Douglas and Hawryshyn, 1990).

Our results show that *M. praecox* and *M. mordax* both have high average temporal CS (625 and 710 at 24°C, respectively), which equates to contrast thresholds of 0.16% and 0.14%, suggesting that *Mordacia* spp. can discriminate significantly lower contrasts than any other fish assessed thus far, which have contrast thresholds of 1–3% (Table 3). It appears that *Mordacia* spp. have optimised their CS for the dark and turbid aquatic environments they inhabit, which would be beneficial to detect predators at a distance, and prey and conspecifics under very low contrast conditions. The temporal CS recorded for *Mordacia* spp. may, however, be an overestimate as we illuminated the entire eyecup. In this situation, more neurons would be stimulated than under *in vivo* conditions and a larger summed response may be obtained.

### Characterising the photoreceptor type

Cones have faster light response kinetics than rods (Hestrin and Korenbrot, 1990; Thoreson, 2007), which allows them to have higher FFFs. Cones are, however, less sensitive to light, and their responses decline rapidly with decreasing light intensity (Crozier and Wolf, 1939, 1941; Hestrin and Korenbrot, 1990; Meneghini and Hamasaki, 1967). Examining the speed and sensitivity of the retina can therefore provide important information on the physiological characteristics of photoreceptor types within the retina.

**Table 3. Temporal contrast sensitivity of vertebrates studied using different methods: behaviour, electroretinography and visual evoked potentials**

| | CS | Class | Light environment | Method |
|---|---|---|---|---|
| Pigeon (*Columba livia*) | 10[1] | Actinopterygii | B | Behaviour |
| Ground squirrel (*Spermophilus beecheyi*) | 20[2] | Mammalia | B | Behaviour |
| Electric fish (*Gnathonemus petersii*) | 33[3] | Actinopterygii | D | VEP |
| Port Jackson shark (*Heterodontus portusjacksoni*) | 40[4] | Chondrichthyes | D | ERG |
| Brownbanded bamboo shark (*Chiloscyllium punctatum*) | 42[4] | Chondrichthyes | B | ERG |
| Epaulette shark (*Hemiscyllium ocellatum*) | 50[4] | Chondrichthyes | B | ERG |
| Smoothhound (*Mustelus mustelus*) | 50[4] | Chondrichthyes | D | ERG |
| Puffadder shyshark (*Haploblepharus edwardsii*) | 63[4] | Chondrichthyes | D | ERG |
| Goldfish (*Carassius auratus*) | 100[5] | Actinopterygii | B | Behaviour |
| Human (*Homo sapiens*) | 190[6] | Mammalia | B | Behaviour |
| **Precocious lamprey (*Mordacia praecox*)** | 625* | Cephalaspidomorphi | D | ERG |
| **Short-headed lamprey (*Mordacia mordax*)** | 710* | Cephalaspidomorphi | D | ERG |

CS, contrast sensitivity; B, bright-light environment; D, dim-light environment; ERG, electroretinography; VEP, visual evoked potential. Study species are in bold (*data from the present study). [1]Hodos et al. (2003), [2]Jacobs et al. (1980), [3]Pusch et al. (2013), [4]Ryan et al., 2017, [5]Bilotta et al. (1998), [6]Robson (1966).

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**Table 4. Increase in FFF with light intensity for different species and the composition of the retina**

| | Increase in FFF (Hz/log unit) | Retina composition | Temperature (°C) |
|---|---|---|---|
| Escolar (*Lepidocybium flavobrunneum*) | 2.5[1] | Pure rod | 23 |
| Tokay gecko (*Gekko gekko*) | 3.5[2] | Pure rod | 27–29 |
| Inagua least gecko (*Sphaerodactylus inaguae*) | 3.9[3] | Pure rod | 26–27.5 |
| **Precocious lamprey (*Mordacia praecox*)** | 5.1* | Rod-like | 24 |
| **Short-headed lamprey (*Mordacia mordax*)** | 7.1* | Rod-like | 24 |
| Iguana (*Iguana iguana*) | 20[2] | Pure cone | 27–29 |
| Horned lizard (*Phrynosoma cornutum*) | 20[4] | Pure cone | 27.5 |

Study species are in bold (*data from the present study). [1]Landgren et al. (2014), [2]Meneghini and Hamasaki (1967), [3]Crozier and Wolf (1939), [4]Crozier and Wolf (1941).

FFF increased gradually with increasing light intensity at a rate of 5.1 and 7.1 Hz/log unit in *M. praecox* and *M. mordax* at 24°C (Table 4). This rate of increase was slightly higher than that measured in the pure rod retina of the escolar *Lepidocybium flavobrunneum* (Landgren et al., 2014), and the nocturnal geckos *Gekko gekko* and *Sphaerodactylus inaguae* (Crozier and Wolf, 1939; Meneghini and Hamasaki, 1967), but was unlike the steeper increase noted in the pure cone retinas of the iguana *Iguana iguana* and the lizard *Phrynosoma cornutum* (Crozier and Wolf, 1941; Meneghini and Hamasaki, 1967). This suggests that the response kinetics of the photoreceptor within *Mordacia* spp. are slow (Thoreson, 2007) and may be rod like.

cFFF of *M. praecox* and *M. mordax* was 31.1 and 36.9 Hz at 24°C, respectively, which is comparable to that obtained from the pure rod retina of two species of skate, *Raja erinacae* and *Raja ocellata*, with an unusually high cFFF of 30 Hz at 20°C (Green and Siegel, 1975; Ripps and Dowling, 1990). Rod photoreceptors generally have cFFF values of less than 30 Hz (Horsten et al., 1962); therefore, the photoreceptors of *Mordacia* spp. may be at the higher end of rod functionality.

The absence of a change in slope in the FFF/intensity curve across approximately 6 log units indicates that all photoreceptors within the retinas of *M. praecox* and *M. mordax* have the same temporal response characteristics. The range of light intensities we assessed (0.02–49,000 lx) should be sufficient to reveal photoreceptors with different kinetic profiles. A previous study on the lamprey *L. fluviatilis* demonstrated that the point at which vision switched from rod dominated to cone dominated occurred at 9.8 Hz and 170 lx (Dreyfert et al., 1979). As the light intensity of 170 lx (the transition point) was within the range we tested, our results provide support for the hypothesis that *M. praecox* and *M. mordax* possess a single physiological photoreceptor type. This is consistent with the morphological characteristics of the retina of *M. mordax* (Collin and Potter, 2000). Collin and Potter (2000) suggested that the photoreceptor is rod like, because of the cylindrical shape of the outer segment and the presence of a typical rod inclusion, incisures. The photoreceptor, however, also possesses features that are indicative of cones, such as the presence of numerous infoldings of the plasma membrane along the length of the outer segment (Collin and Potter, 2000).

Previous research has suggested that lampreys do not possess 'true' rods because of the cone-like characteristics of their photoreceptors, with 'true' rods having evolved in gnathostomes after lampreys had diverged from their early agnathan ancestors (Collin, 2010; Collin et al., 2003; Lamb, 2013; Lamb et al., 2007). Recent electrophysiological studies conducted on the rod photoreceptors of the sea lamprey, *Petromyzon marinus* (Morshedian and Fain, 2015), and the European river lamprey, *Lampetra fluviatilis* (Asteriti et al., 2015), however, demonstrate their ability to detect single photons of light, confirming true rod functionality in lampreys (Baylor, 1987; Baylor et al., 1979).

The rod-like photoreceptor of *Mordacia* spp. contributes to vision over a large range of light intensities covering both scotopic and photopic light levels (0.02–49,000 lux), which corresponds to illumination levels from a quarter moon to daylight without direct sunlight (Johnsen, 2012). This unusual feature has also been demonstrated in the rod photoreceptor of another lamprey (*L. fluviatilis*), which continues to function at bright light levels (from 1 to 20,000 quanta $\mu m^{-2}$ $s^{-1}$) and shows no sign of saturation (Govardovskii and Lychakov, 1984). The photoreceptors of both *Mordacia* species, however, show signs of saturation at the brightest light intensity examined, as the FFF started to plateau in *M. praecox* and actually decreased in *M. mordax*, adding further support for a rod-like physiology (Fitzpatrick, 2004). A decrease in FFF suggests that the visual pigment is being bleached and the post-stimulus recovery is slow (Aguilar and Stiles, 1954; Hestrin and Korenbrot, 1990; Thoreson, 2007).

Suction electrode recordings (Morshedian and Fain, 2015) are needed to determine whether the photoreceptors of *Mordacia* spp. can also respond to single photons of light, and thus verify whether their photoreceptors are 'true' rods.

## Conclusion

The visual system of *Mordacia* spp. appears to have adapted to increase the capture of photons in their dim-light environments. This is achieved by having lower temporal resolution, so that more photons can be summed over time, which increases light sensitivity. This comes, however, at the expense of not being able to resolve fast visual events (i.e. low FFF). The lower temporal resolution potentially enables *Mordacia* spp. to have exceptionally high temporal CS, allowing discrimination between very small differences in contrast levels. This may be useful for both predator and prey detection in low-contrast aquatic environments. We have demonstrated that lower environmental temperatures significantly limit temporal resolution and CS in *Mordacia* spp., with the consequence that faster moving objects will be more difficult to detect or appear blurred, and contrast discrimination will be restricted. The ERG responses suggest that all photoreceptors of both species have the same temporal response characteristics, which include slow kinetics, high sensitivity and saturation at bright light intensities, suggesting a rod-like photoreceptor with the ability to operate over an unusually wide range of intensities, including photopic conditions.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conception: R.E.W., J.M.H., S.P.C., N.S.H. and I.C.P. Designed experiments: R.E.W. and J.M.H. Software development: J.M.H. and R.E.W. Performed experiments: R.E.W. Analysed data: R.E.W. and J.M.H. Provided resources: R.E.W., J.M.H., S.P.C. and I.C.P. Original draft preparation: R.E.W. Reviewed and edited manuscript: R.E.W., J.M.H., I.C.P., N.S.H. and S.P.C.

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