

## SHORT COMMUNICATION

# Differences in ocular media transmittance in classical frog and toad model species and its impact on visual sensitivity

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## ABSTRACT

The transmittance properties of the cornea, lens and humours of vertebrates determine how much light across the visible spectrum reaches the retina, influencing sensitivity to visual stimuli. Amphibians are the only vertebrate class in which the light transmittance of these ocular media has not been thoroughly characterised, preventing large-scale comparative studies and precise quantification of visual stimuli in physiological and behavioural experiments. We measured the ocular media transmittance in some commonly used species of amphibians (the bufonids *Bufo bufo* and *Rhinella ornata*, and the ranids *Lithobates catesbeianus* and *Rana temporaria*) and found low transmittance of short wavelength light, with ranids having less transmissive ocular media than bufonids. Our analyses also show that these transmittance properties have a considerable impact on spectral sensitivity, highlighting the need to incorporate this type of measurement into the design of stimuli for experiments on visual function.

**KEY WORDS:** Anura, Bufonidae, Light transmittance, Lens, Ranidae, Spectral sensitivity

## INTRODUCTION

The phenomenon of vision relies on photons hitting photoreceptors cells, and in vertebrates this implies that light must traverse the lens, cornea and humours before reaching the retina. These ocular media have different transmittance properties for different regions of the electromagnetic spectrum, thus influencing the availability of light of different wavelengths for vision. The transmittance of the ocular media is ultimately limited by the structure of molecules such as nucleic acids and aromatic amino acids that absorb almost all ultraviolet (UV) radiation up to 310 nm (Douglas and Marshall, 1999). Furthermore, absorption of short-wavelength light can be achieved by incorporating pigments into the ocular media, as in the corneas of many fishes (Gnyubkina and Kondrashev, 2001; Kondrashev, 2019) and lenses of various vertebrates, including humans (Douglas and Marshall, 1999).

In recent years, several comparative studies have examined the ranges and implications of ocular media transmittance (OMT) in vertebrates, including fishes (Douglas and McGuigan, 1989; Siebeck and Marshall, 2001, 2007), lizards (Pérez i de Lanuza and Font, 2014), snakes (Simões et al., 2016), birds (Lind et al.,

2013, 2014; Olsson et al., 2016) and mammals (Douglas and Jeffery, 2014). Somewhat surprisingly, no such comparative research exists for amphibians. The limited information available shows that the lenses of the leopard frog *Lithobates pipiens* (formerly *Rana pipiens*) and the edible frog *Rana esculenta* absorb almost all light below 400 nm (Kennedy and Milkman, 1956; Muntz, 1977) and that both the cornea and lens in *L. pipiens* have small amounts of pigment absorbing in the 300–350 nm range (Douglas and Marshall, 1999; Kennedy and Milkman, 1956). Furthermore, electroretinograms of whole eyes of *R. temporaria* have shown that the overall spectral sensitivity falls abruptly below 400 nm, even though sensitivities of about 10% of the maximum could still be recorded at 330 nm, and comparison to the results obtained with exposed eyecups these suggest that the ocular media are responsible for the reduction in sensitivity (Govardovskii and Zueva, 1974). None of these studies provides  $\lambda_{T50}$  values (wavelength at which the light transmittance is 50% of the maximum – the most commonly used parameter for characterisation of light transmittances), which has prevented comparisons between transmittances of frogs and other vertebrates.

The relevance of OMT for visual performance has an additional component in amphibians. This group is remarkable among vertebrates in having two, instead of one, spectral types of rod photoreceptors, which enables colour discrimination at very low light levels (Yovanovich et al., 2017). Their spectral sensitivity maxima are approximately 500 nm for the ‘typical’ green-sensitive rod and 430 nm for the blue-sensitive rod (Denton and Wyllie, 1955; Govardovskii et al., 2000). A decrease in sensitivity to light in the UV–violet part of the spectrum caused by the ocular media could be even more limiting for the unique colour discrimination abilities of frogs in very dim light compared with photopic conditions.

Determination of OMT in anurans would be useful not only to start filling that gap among vertebrate groups, but also to allow for more precise estimation of actual spectral sensitivities when designing stimuli for behavioural or physiological experiments, and when modelling visual abilities. We studied OMT and its relation to eye size and spectral sensitivities in four extensively studied anuran species representing distantly related lineages, the bullfrog *Lithobates catesbeianus* (Shaw 1802) (formerly *Rana catesbeiana*) and common European frog *Rana temporaria* Linnaeus 1758 (Ranidae), and the Cururuzinho toad *Rhinella ornata* (Spix 1824) and common European toad *Bufo bufo* (Linnaeus 1758) (Bufonidae). We focused on these model species as a first approach to this topic because the spectral sensitivities are already known for most of them, and, thus, the effect of OMT can be estimated and will be more readily applicable.

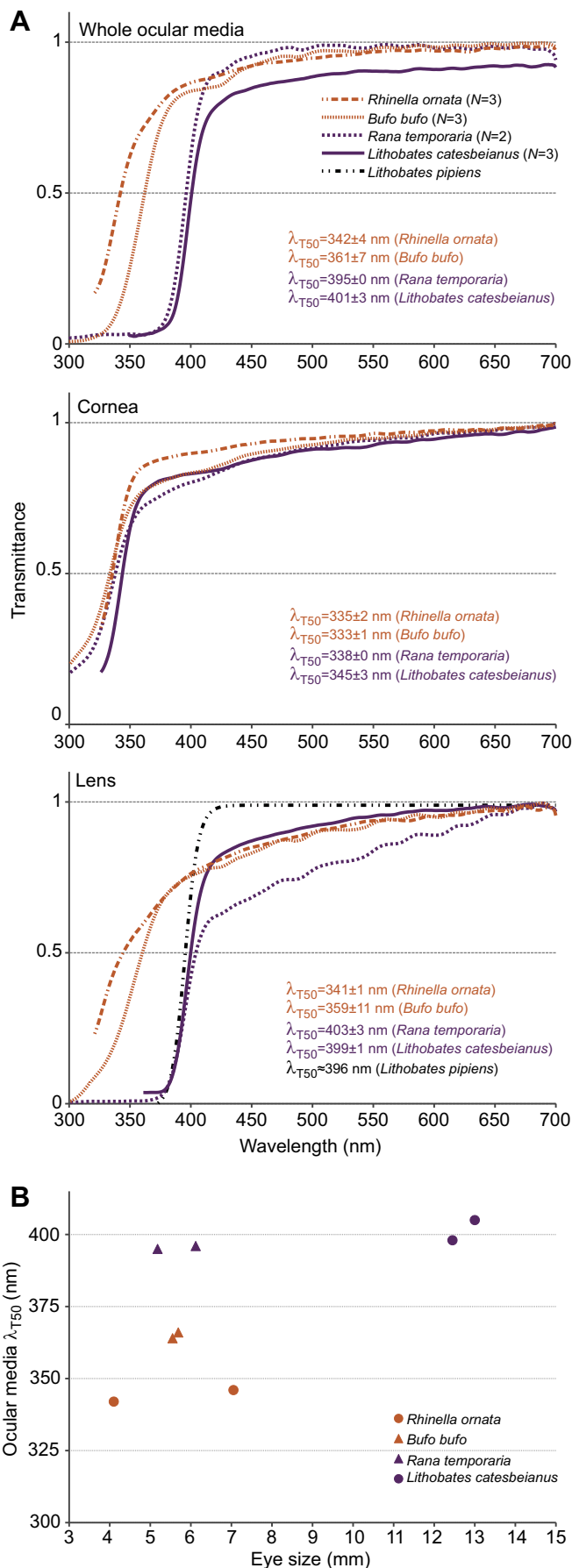
## MATERIALS AND METHODS

We used eyes from animals that were collected and euthanised for reasons unrelated to this study. The specimens originated from Lund University’s biological station in Skåne, Sweden (*B. bufo* and *R. temporaria*, collected under license no. 522-1574-2013 from

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**Fig. 1. Average transmittance curves and  $\lambda_{T50}$  for the different components of ocular media.** (A) Spectral transmittance curves of the whole ocular media (top) cornea alone (middle) and lens alone (bottom).

Values in parentheses indicate the numbers of eyes used.  $\lambda_{T50}$  are means  $\pm$  s.d. (see Table S1 for individual datasets). The curve and  $\lambda_{T50}$  for *L. pipiens* were estimated from Kennedy and Milkman (1956) (see Materials and Methods for details). (B) Relationship between ocular media  $\lambda_{T50}$  and eye size. Each point represents a single eye.

Länsstyrelsen Skåne issued to A.K.) and the surroundings of São Paulo in Brazil (*R. ornata* and *L. catesbeianus*, collected under license no. 13173 from SISBIO issued to T.G.). We enucleated the eye, freed the cornea from the eyecup cutting along the ora serrata, and lifted it, cutting through the vitreous to pull the lens, releasing the whole ocular media in one piece. After measuring this preparation (see below), the sample was dissected by cutting through the aqueous humour and removing the iris to obtain isolated corneas and lenses. The humours have negligible effects on transmittance (Douglas and Marshall, 1999) and were not measured separately.

We measured the light transmittance using the approach of Lind and co-workers (Lind et al., 2013), as follows. We placed the samples in a custom-made matte black plastic cylinder (12 mm diameter  $\times$  10 mm height) with a circular (5 mm diameter) fused silica window in the bottom and filled with phosphate-buffered saline (PBS). For some small samples, a black plastic disc with a pinhole of 2 mm diameter was added on top of the silica window to ensure that all incoming light passed through the sample. We used an HPX-2000 Xenon lamp (Ocean Optics, Dunedin, FL) to illuminate the samples via a 50  $\mu$ m light guide (Ocean Optics) through the fused silica window and collected transmitted light using a 1000  $\mu$ m guide connected to a Maya2000 spectroradiometer controlled by SpectraSuite v4.1 software (Ocean Optics). The guides were aligned with the container in a microbench system (LINOS, Munich). The reference measurement was taken from the container filled with PBS. We took 3–5 measurements from each sample, averaged them, smoothed the curve using an 11-point running average, and normalised to the highest value within the range 300–700 nm. From these data, we determined  $\lambda_{T50}$  as the wavelength at which the light transmittance was 50% of the maximum. The curves were cut for clarity in those cases in which the measurements at very short wavelengths were too noisy due to the reduced sensitivity of the spectrometer in that region of the spectrum.

In the case of the *L. pipiens* lens, we generated the transmittance curve from the optical density (OD) data published by Kennedy and Milkman (1956). The numerical values of OD shown in Fig. 1 of their article were estimated by eye and converted to transmittance (*T*) values using Eqn 1 (Land and Nilsson, 2012) (Table 1):

$$T = \frac{1}{10^{OD}} \quad (1)$$

The transmittance dataset was best fitted ( $R^2=0.995$ ) to the symmetrical sigmoidal function described by:

$$y = 0.9891606 + \frac{-0.01368352 - 0.9891606}{1 + (x/395.2257)^{71.82512}} \quad (2)$$

Eqn 2 was used to calculate the transmittance values (*y*) for the range of wavelengths 300–700 nm in 1 nm steps (*x*) that are plotted in Fig. 1A.  $\lambda_{T50}$  was obtained in the same way as described above for the other species. We used the transmittance spectra from *B. bufo* and *R. temporaria* to calculate the influence of the transmittance

**Table 1. Lens transmittance of *Lithobates pipiens* calculated from optical density data extracted from Kennedy and Milkman (1956)**

Wavelength (nm)	OD	Transmittance
375	2.10	0.008
380	1.30	0.05
390	0.60	0.251
400	0.15	0.708
420	0.05	0.891
440	0.00	1
460	0.00	1
480	0.00	1
500	0.00	1
520	0.00	1
540	0.00	1
560	0.00	1

properties on spectral sensitivity by determining  $CS_i$ , the corrected spectral sensitivity to wavelength  $i$ , as:

$$CS_i = S_i \times OMT_i, \quad (3)$$

where  $S_i$  is the spectral sensitivity at wavelength  $i$  (accounting for the self-screening effect of the photoreceptors' outer segments) as calculated in Yovanovich et al. (2017), and  $OMT_i$  is the ocular media transmittance at wavelength  $i$ .

As a measure of eye size, we used the axial length (distance from corneal vertex to posterior sclera), either measured with a calliper in the freshly enucleated eye prior to dissection or in the contralateral eye to the one used for transmittance measurements, freshly frozen and cryosectioned, following the method of Lind and Kelber (2009). The effect of freezing on axial length is negligible in this context (Hart, 2002).

## RESULTS AND DISCUSSION

### Variation in ocular media transmittance and eye size among frog species

Both the corneas and lenses looked transparent upon direct observation in all specimens, suggesting that none of them would have cut-off wavelengths far into the visible spectrum. The measurements confirmed this prediction and showed that the lens sets the limits for the light transmittance of the ocular media as a whole (Fig. 1A), as is the case in most other vertebrates. None of the species we studied had lenses with high UV transmittance, but there are two clusters that match their phylogenetic relationship: the bufonids have lenses with  $\lambda_{T50}$  of 341–359 nm, while the ranids have  $\lambda_{T50}$  of 399–403 nm (Fig. 1A), in agreement with previous findings for *L. pipiens* (Kennedy and Milkman, 1956), another model species of ranid. In *R. temporaria*, the spectra and  $\lambda_{T50}$  that we obtained seem to match the transmittance properties inferred by Govardovskii and Zueva (1974), although a direct comparison is not possible due to the difference in the methods.

We also measured the axial lengths of the eyes of eight of the eleven animals used in this study ( $N=2$  for each species). When plotted against the  $\lambda_{T50}$  for each individual, there seems to be a weak trend ( $r=0.38$ ) for larger eyes to have higher  $\lambda_{T50}$  (Fig. 1B), but this is driven by *L. catesbeianus* having larger eyes than the other three species. In the absence of pigments in the lens and in the cornea, as a general rule  $\lambda_{T50}$  is expected to have a positive correlation with path length, because the optical path filled with UV-absorbing proteins is longer in large eyes bearing thick lenses than in small eyes. This has been demonstrated to varying degrees in some fishes (Siebeck and Marshall, 2001, 2007) and birds (Lind et al., 2014). In our case, we rather suggest that the low UV transmittance of the lenses of the

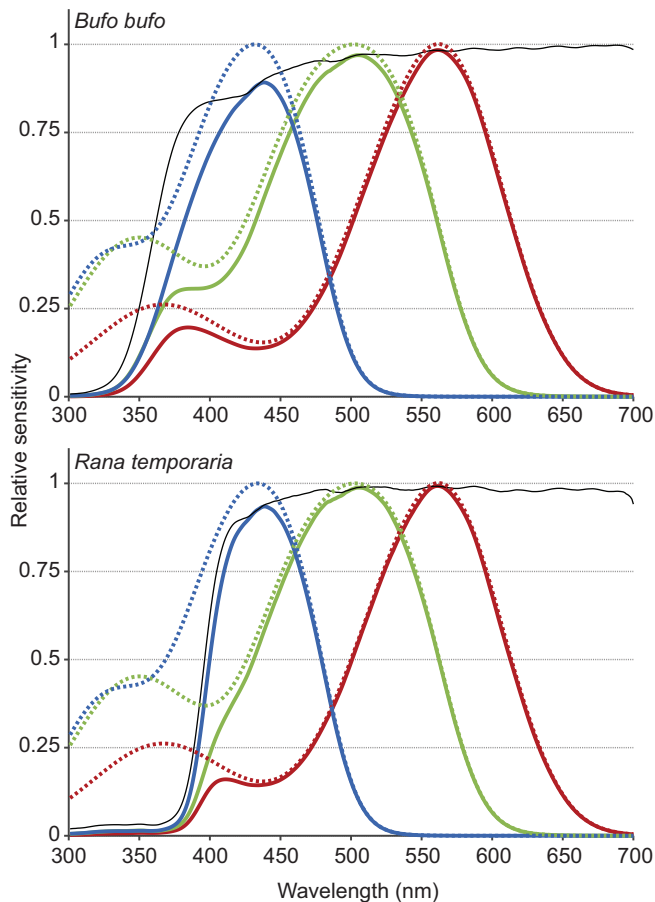
ranid frogs is probably due to the presence of a lens pigment, such as that found in *Lithobates pipiens* (Kennedy and Milkman, 1956).

Differences in transmittance are associated with daily activity patterns in mammals and snakes, with diurnal species having higher  $\lambda_{T50}$  values compared with their close nocturnal relatives (Douglas and Jeffery, 2014; Simões et al., 2016). Transmittance is also related to photoreceptor spectral sensitivity; species that possess UV-sensitive photoreceptors tend to have lower  $\lambda_{T50}$  (Douglas and Jeffery, 2014; Lind et al., 2014). Such functional explanations are not applicable for the segregation of OMT in our sample, as all species are crepuscular to nocturnal (Anderson and Wiens, 2017) and have virtually the same spectral sensitivities (see Yovanovich et al., 2017 for a summary). Thus, phylogeny seems to be the factor driving the grouping of OMT in this case. Further studies with broader phylogenetic and ecological sampling are needed to corroborate this pattern and assess whether it is influenced by ecological differences among closely related species. Similarly, the range of  $\lambda_{T50}=341\text{--}403$  nm obtained by us is narrower than those known for fishes (324–437 nm; Siebeck and Marshall, 2001), birds (313–390 nm; Lind et al., 2014), snakes (306–428 nm; Simões et al., 2016) and mammals (313–478 nm; Douglas and Jeffery, 2014), so a broader sample would also help determine the boundaries of the OMT range among anurans.

### Impact of ocular media transmittance on visual sensitivity

We quantified the effect of OMT on frog spectral sensitivity by using the transmittance spectra measured here to correct the photoreceptor spectral sensitivity curves available in the literature for *B. bufo* and *R. temporaria* (Yovanovich et al., 2017). This calculation allows quantification of the amount of the incoming light of a given wavelength that is actually available to the visual system. We used the transmittance of the whole ocular media, as this type of preparation is closest to the reality of an intact eye in a living animal. A comparison of the relative sensitivities of photoreceptors known in the species used for this study before and after correcting for the effect of OMT is given in Fig. 2. While the peaks are hardly affected by the UV-filtering effect of the ocular media, the overall sensitivity in the UV–blue region is more strongly influenced in *R. temporaria* than in *B. bufo*. These differences will be reflected in the quantum catches elicited by objects of a given colour between the two species, which have the potential to be ecologically relevant: behavioural experiments have shown that males of *B. bufo* prefer blue female models, while male *R. temporaria* prefer red ones (Kondrashev et al., 1976). Our results offer a possible interpretation of this observation: the higher sensitivity of the blue photoreceptors of *B. bufo* might allow them to take advantage of the conspicuousness of blue-hued objects, while *R. temporaria* might rely on the higher sensitivity of their red photoreceptors to spot potential partners. Another example of the potential ecological relevance of the transmittance of the ocular media among anurans is the vivid blue colouration that males of *Rana arvalis* acquire during the breeding season. This ephemeral colour pattern has a strong UV component (Ries et al., 2008), and it would be interesting to know how the ocular media of this particular species affect the strength of the UV–blue signal for conspecifics.

All photoreceptors have some sensitivity to UV light, which is noticeably decreased in *B. bufo* and virtually disappears in *R. temporaria*, when OMT is incorporated into the sensitivity spectra. Even though these frogs lack photoreceptors with sensitivity maxima in the UV – and thus would not perceive UV as a separate colour even if they had UV-transparent ocular media – the absorption of short-wavelength photons by the ocular media has a considerable



**Fig. 2. Effect of ocular media transmittance on spectral sensitivity in *Bufo bufo* and *Rana temporaria*.** Blue: blue-sensitive rods; green: green-sensitive rods; red: red-sensitive cones. The individual photoreceptor spectral sensitivities (dashed lines) are from Yovanovich et al. (2017). The corrected spectra (solid lines) are the result of multiplying by the ocular media transmittance curve (black line) for each species (see Table S2 for full datasets).

impact on the overall quantum catches of all photoreceptors. This is particularly relevant for crepuscular animals, like these and many other frogs, because the spectral composition of light at sunset has a relatively high proportion of shorter wavelengths compared with light composition at other times of the day (Johnsen et al., 2006). Furthermore, in very dim light conditions, in which the visual system functions close to its absolute sensitivity limit, the removal of even a small proportion of photons by the ocular media has the potential to affect performance. In a recent study using phototactic behaviour, *R. temporaria* showed the remarkable ability to discriminate colours at extremely low light levels using its two different spectral types of highly sensitive rod photoreceptors (Yovanovich et al., 2017). It would thus be interesting to see how the threshold for this ability changes in cases where the visual stimuli have a considerable amount of UV in their spectral composition. This example illustrates the crucial role that the ocular media transmittance can have in setting the limits for visual sensitivity and the need to take it into account when using spectral sensitivity curves to characterise visual stimuli. Additionally, the variation we found between bufonids and ranids showcases the relevance of using transmittance data from species as closely related as possible to the experimental model. In particular, work using other popular amphibian models such as salamanders would benefit from OMT measurements obtained specifically from urodeles. Conversely, the similarity within families suggests that our

data can be reliably applied as an approximation for other anuran species commonly used in visual research, such as the bufonids *Anaxyrus americanus* and *Rhinella marina* and the ranid *Pelophylax ridibundus*, among others.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: C.A.M.Y., T.G., A.K.; Formal analysis: C.A.M.Y., A.K.; Investigation: C.A.M.Y.; Resources: C.A.M.Y.; Writing - original draft: C.A.M.Y.; Writing - review & editing: C.A.M.Y., T.G., A.K.; Visualization: C.A.M.Y.; Supervision: C.A.M.Y., T.G.; Project administration: C.A.M.Y.; Funding acquisition: C.A.M.Y., T.G., A.K.

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#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.204271.supplemental>

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