

The *d*-wave in fish and the state of light adaptation

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Abstract. Comparative electroretinographic studies of the *d*-wave evoked with long duration photo stimuli in dark- and light-adapted fish species (three marine and three freshwater) were performed. At the end of prolonged photo-stimulation in scotopic conditions a negative *d*-wave appears in electroretinograms of dogfish shark, eel and goldfish diminishing and eventually changing with intensity of photo-stimulation, while in rudd it only increases. Dark-adapted electroretinograms of two percids (perch and painted comber) exhibit a positive *d*-wave that approaches the *b*-wave amplitude under bright photopic conditions. Judging from the *d*-wave, only the rod pathway is active in dark-adapted dogfish shark, eel, and goldfish. Under the light adaptation, cone pathways are active in eel and goldfish, whereas the positive response to the end of light stimuli in dogfish shark could be explained by independent ON and OFF pathways from outer to inner retina *via* bipolar cells. In the case of two percids, dark adaptation has no influence on cone pathways. The *d*-wave of rudd behaves like cone-driven *d*-waves but in opposite sign. The data thus show that the *d*-wave form, amplitude and sign depend on interconnection of ON and OFF pathways as determined by the state of adaptation and/or type of photoreceptor.

Key words: *b*-wave — *d*-wave — Fish

Introduction

The electroretinogram (ERG) represents the extracellular recording of the complex electrical changes in the retina induced by light. The first component analysis of the ERG was done by Granit (1955) who divided the cat's ERG into three components: P-I, P-II and P-III, termed in sequence of their disappearance with the progress of ether anesthesia. In response to sufficiently long duration of photo stimuli, the ERG consists of 4 prominent waveforms: an initial negative deflection, the leading edge of the negative P-III component (*a*-wave), a relatively fast positive transient which reflects the summation of P-II and P-III (*b*-wave) followed by a slow corneal-positive summation of P-I and P-III (*c*-wave) and the reaction at the end of stimulation (*d*-wave). It is generally accepted that the *a*-wave represents a reaction of photoreceptor cells (Granit 1955; Brown 1968;

Andjus 1998). The *b*-wave is generated from potassium currents across the membranes of the ON bipolar cells and Müller cells (Dowling and Ripps 1970; Miller and Dowling 1970; Newman and Odette 1984; Stockton and Slaughter 1989; Shiells and Falk 1999). The third order neurons (amacrine and ganglion cells) can contribute as well (Dong and Hare 2000; Awatramani et al. 2001). Lowered stimulus intensity and adequate duration of stimuli could reveal the *dc*-component in its classical rectangular form (Brown 1968; Andjus 1998). The pigment epithelium is the origin of the *c*-wave, which corresponds to the P-I component of Granit (1955). The *d*-wave is seen only when the ON and OFF phases of the ERG response are separated in time, by using light stimuli of relatively long duration (>0.1 s). The *d*-wave of salamander is believed to be generated from cone-driven secondary retinal cells, such as the OFF-bipolar and/or horizontal cells (Stockton and Slaughter 1989; Naarendorp and Williams 1999). In the all-rod retina of skate parallel ON and OFF pathways *via* bipolar cells are functional as interplexiform afferent pathways, and OFF-bipolar cells, rather than horizontal cells, could be the source of a *d*-wave-like component of

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the ERG (Chappell and Rosenstein 1996). In giant danio (*Danio aequipinnatus*), the *d*-wave is selectively abolished when the specific α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)/kainate receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) is applied to the retina (Wong et al. 2000). It was suggested by way of AMPA/kainate receptors that OFF-bipolar cells receive signals from both type of photoreceptors, cones as well as rods.

An investigation on the frog isolated retina shows that the *d*-wave could be recorded from all retinal layers as well as the *b*-wave, and that its amplitude decreased as the electrode was advanced proximally until it disappeared at the inner limiting membrane (Yanagida et al. 1986). The peak latency of the *d*-wave was longer in the inner plexiform layer than in the distal portion of the inner nuclear layer so it is believed that the origin of this wave is in the bipolar cell layer, generated by OFF-bipolar cells. In late dark adaptation, the amplitude of the *b*-wave increases along with the increase in rod sensitivity and the *d*-wave changes sign to negative. The mechanism that underlies this *b*- and *d*-wave relation change is not fully understood. The transition may be due to an alteration of the dominance of rod-cone contribution to ERG sensitivity. During dark adaptation visual sensitivity is first dominated by cones and later by rods. It has been suggested that in zebrafish, during the transition from light to dark adaptation, *b*-wave represents the function of both rod and cone systems (Ren and Li 2004). In general, the positive *d*-wave represents mainly cone functions (Andjus 2001; Ren and Li 2004). However, different forms of *d*-waves were explained by cone and rod ERGs. In the case of the rod ERGs at light offset, *b*-wave and *dc*-component decay faster than the rod receptor potential causing the *d*-wave to be initially negative (Brown 1968).

The *d*-wave can be recorded only with prolonged light stimuli. In humans, with light stimuli of shorter durations the *d*-wave tends to combine with the *b*-wave. This phenomenon led early studies of patients with congenital stationary night blindness or with melanoma-associated retinopathy to conclude that the cone system was functioning normally and only the rod system was affected. The use of light stimuli of long duration revealed that the ON pathway in both rod and cone systems was affected and only the OFF pathway of the cone system exhibited normal function (Miyake et al. 1987; Alexander et al. 1992).

In order to show that the *d*-wave could be an indicator of the retinal photoreceptor content, we carried out an ERG study in three marine fish species: small-spotted dogfish shark (*Scyliorhinus canicula*), eel (*Anguilla anguilla*), and painted comber (*Serranus scriba*) and three fresh water species: rudd (*Scardinius erythrophthalmus*), perch (*Perca fluviatilis*) and goldfish (*Carassius gibelio*). The small spotted dogfish is a rhodopsin possessing marine species with a pure-

rod retina with a single layer of photoreceptors (Dowling and Ripps 1970, 1971; Bozzano et al. 2001), while other fish have duplex retinas with differing cone-rod ratio (Tamura and Niwa 1967; Muntz and Northmore 1970, 1973; Harosi and MacNichol 1974; Stell and Harosi 1976; Cameron 1982; Yang et al. 1983; Bowmaker 1995; Damjanović et al. 2005; Gačić et al. 2005; Golobokova and Govardovskii 2006).

Materials and Methods

Animals

Small-spotted dogfish sharks (150–250 g body mass) were caught by trawler nets in the south Adriatic, at a depth of about 100 m. At least one month prior to experiments they were maintained in a sea-water recirculation system for experimental aquaculture, located in a dark- and temperature-controlled room, at 15°C (Kotor Institute for Marine Biology, Montenegro). Care was taken not to expose the dogfish long to light, as this is known to be damaging to the elasmobranch photoreceptors (Hamasaki et al. 1967).

European eels of the yellow form were electrofished during summer months in coastal running waters along the Kotor Bay (Montenegro). Upon capture, the fish was kept for at least 20 days prior to experiments in a fresh-water aquarium located in the same dark- and temperature-controlled room (15°C) as the sea-water aquaculture system with dogfish.

Painted combers were caught by net in south Adriatic and kept for a month prior to experiment in a sea-water aquarium in the dark- and temperature-controlled room (15°C). Rudd, perch and goldfish were electrofished in the floodplain zone of the Danube River (1136 km). Fishes were kept in captivity for at least 15 days in order to acclimatize to experimental conditions (12 : 12 h light/dark regime in a temperature-controlled room at 15°C).

Isolated eyecup preparation

Isolated eyecups were prepared under dim red light from dogfish eyeballs (about 10 mm in diameter) excised after rapid decapitation of the fish. The preparations were surgically deprived of cornea, lens and most of the vitreous. The eyecup was filled with elasmobranch Ringer's solution (Rybak 1973) and placed on a cotton-wool bed soaked with the same solution, in a plastic temperature-controlled chamber inside a light-proof Faraday cage. After mounting, the preparations were dark-adapted for additional 30 min before actual ERG recording. In the case of photopic *b*-wave recording, the eyecup was continuously exposed to a 500 nm background illumination capable, at its onset, to evoke a *b*-wave response of saturating amplitude and to reduce sensitivity more than 3000-fold.

In situ eyecup preparation

Eel, goldfish, perch, rudd and painted comber were anesthetized (phenobarbital sodium) and curarized (tubocurarine) following procedures recommended by Hamasaki et al. (1967) adjusting the dosage so as to induce the arrest of respiratory movements. Artificial respiration was provided continuously by forcing aerated and temperature-controlled water through the gills. The immobilized fish were positioned laterally on a plastic platform inside a light-proof Faraday cage. The *in situ* eyecup was prepared in the same way as in the case of isolated preparations of the dogfish (removal of cornea, lens and most of the vitreous), and it was filled with teleosts Ringer.

Recordings and stimulation

ERG potentials were detected with non-polarizable chlorided silver (Ag-AgCl) electrodes (model EP2, World Precision Instruments, Inc.). The active electrode was introduced in the interior of the saline-filled eyecup while the reference electrode was in contact with the cotton-wool bed underneath the isolated preparations of the dogfish, or in the retro-orbital space behind the *in situ* eyecup in other experimental species. Electrodes were connected to the input stage of a directly coupled differential preamplifier, and responses were recorded by means of a Polaroid camera from a storage oscilloscope display (dogfish-shark, eel and painted comber) or transferring from a preamplifier to a computer by means of an AD-converter PCI-20428W-1 (8-bit; 125 Hz sampling rate). Photic stimuli were delivered by a single-beam optical system using an tungsten-halogen lamp (8 V, 50 W) as the light source, providing independent control of intensity (neutral density filters), duration (electromagnetic shutter, Uni-Blitz model T132), and spectral composition (interference filters) of the test flashes. Light intensities were calibrated and checked by placing the active surface of the custom-made radiometer probe in the position usually occupied by the eyecup preparation. When comparing intensity/amplitude relations in different preparations, relative intensity (I_R) scales were used, plotting normalized ERG amplitude voltage against attenuation in log units. Amplitude measurements are presented as mean \pm standard error.

All animal use was approved by the Ethical Council of Faculty of Biology, University of Belgrade.

Results

Fig. 1A shows dark- and light-adapted ERGs in dogfish shark evoked with prolonged light stimuli. At the end of a test flash, in dark-adapted preparations, a negative indentation appears on the rising phase of the *c*-wave. As Fig. 1Aa shows,

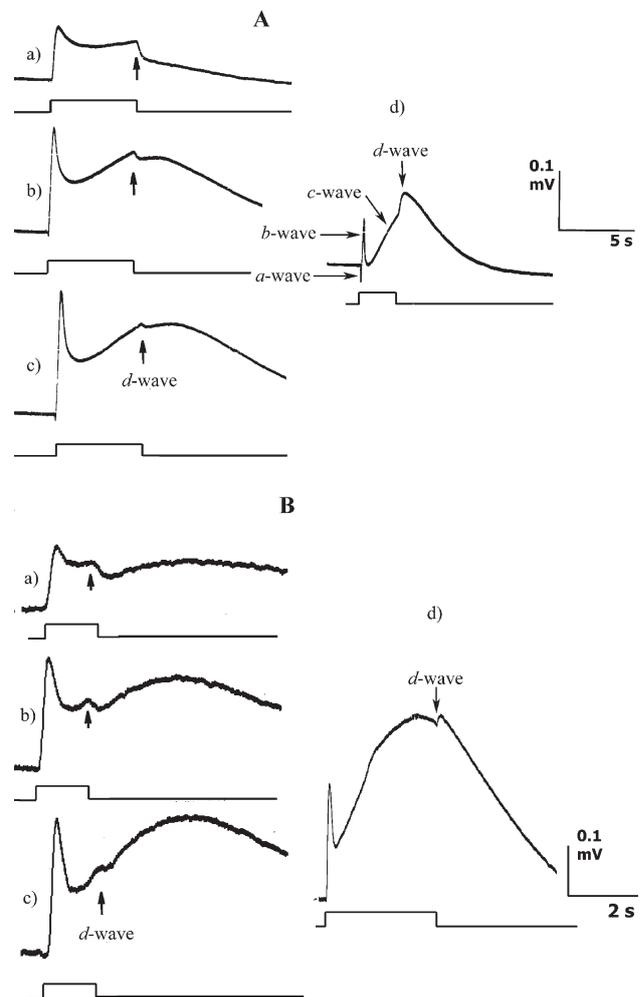


Figure 1. Scotopic and photopic electroretinograms in small-spotted dogfish shark and goldfish. **A.** At the end of a 5 s test flash of incremental intensities in dogfish dark adapted preparations a negative *d*-wave (vertical arrow) appears on the rising phase of the *c*-wave: a) 1.64, b) 8.9, c) 16.4 $\mu\text{W}/\text{cm}^2$. The negative *d*-wave is best visible when the *c*-wave is of reduced amplitude due to an attenuation of the test flash (a). In light adapted preparations of dogfish shark (d) the end of a test flash is signaled by a positive deflection, the *d*-wave. It appears on top of the *c*-wave, which outlasts the duration of the flash. Stimulus: white light – 2 s, 24 $\mu\text{W}/\text{cm}^2$. **B.** Dark-adapted goldfish (a,b,c). Negative *d*-wave (vertical arrow) appears at the end of test flashes of incremental intensities: a) 0.07, b) 0.18, c) 0.71 $\mu\text{W}/\text{cm}^2$. Duration of stimuli 1.3 s. d) After light adaptation, positive *d*-wave appears on the top of *c*-wave. Stimulus: white light – 3.9 s, 28.2 $\mu\text{W}/\text{cm}^2$.

this indentation, the negative *d*-wave, is best visible when the *c*-wave is of reduced amplitude due to an attenuation of the test flash. However, at the end of test flashes after light adaptation a positive *d*-wave appeared (Fig. 1Ad).

On the other hand, in the goldfish that possesses a high number of cones (Harosi and MacNichol 1974; Stell and

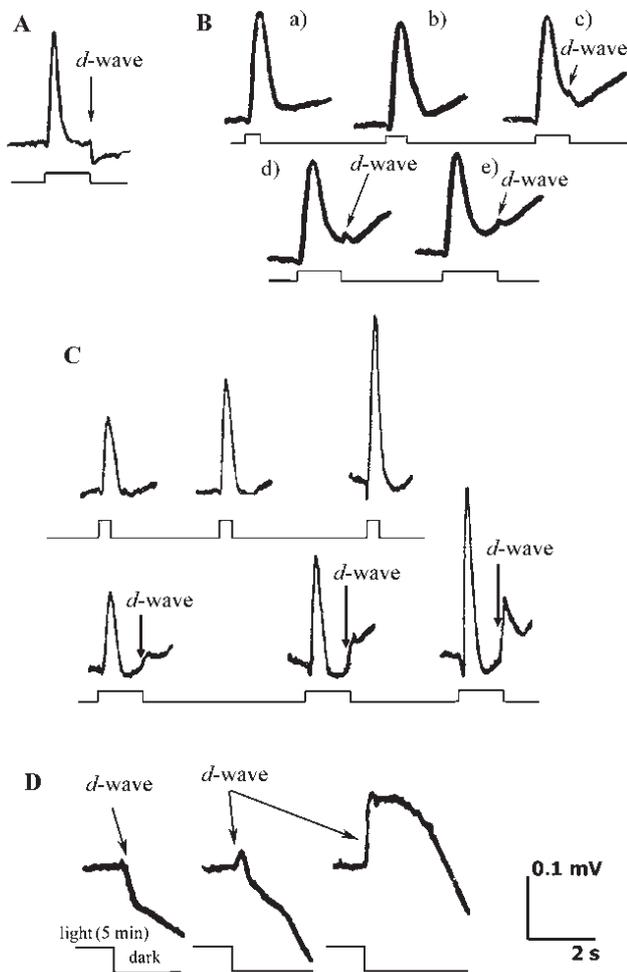


Figure 2. Electroretinographic responsiveness in eels in scotopic and photopic conditions. **A.** ERG recorded in a dark-adapted eye, with a negative deflection, *d*-wave (arrow) at the termination of light stimulus. **B.** ERGs in eel in photopic conditions; stimulus light – monochromatic (500 nm); duration of stimuli: a) 0.2, b) 0.4, c) 0.6, d) 0.8 s and e) 1 s; stimulus intensity – $8.9 \mu\text{W}/\text{cm}^2$; intensity of background illumination – $0.85 \mu\text{W}/\text{cm}^2$. **C.** ERG recorded in an eye light adapted to background illumination of $2.6 \mu\text{W}/\text{cm}^2$ with short (0.32 s upper panel) and prolonged stimuli (1.35 s lower panel); positive *d*-waves indicated by the vertical arrow. Stimulus intensity was 0.024, 0.24 and $24 \mu\text{W}/\text{cm}^2$ (from left to right for both stimulus durations). **D.** Dark-adapted preparations of eel were exposed to 5 min of adaptation light stimuli of incremental intensity (from left to right: 2.6×10^{-3} , 2.6×10^{-2} and $2.6 \mu\text{W}/\text{cm}^2$). Start of each trace presents the plateau of the corresponding *c*-wave. The negative and positive *d*-waves are indicated by arrows.

Harosi 1976; Bowmaker 1995) and a well developed color vision (Tamura and Niwa 1967; Yang et al. 1983; Golobokova and Govardovskii 2006) in scotopic conditions a similar negative *d*-wave appears after prolonged stimulation (Fig. 1Ba,b,c) changing its sign after light adaptation (Fig. 1Bd).

Note that duration of light stimuli in scotopic condition of goldfish preparation was almost 4-fold shorter than in dogfish. A similar reaction was also recorded in the dark-adapted preparation of eel. A negative *d*-wave appears at the termination of the light stimulus (Fig. 2A).

After adaptation to background illumination, similar to what was observed in the dogfish and goldfish, the small scotopic negative *d*-wave of the eel was replaced by a positive *d*-wave, its amplitude markedly increasing with flash duration (Fig. 2B). Amplitude of this positive *d*-wave is dependent on the intensity of light stimulation. After light adaptation to background intensity of $2.6 \mu\text{W}/\text{cm}^2$, three stimuli were applied with increasing intensity (Fig. 2C). It was apparent that *d*-wave of eel depends on the duration of stimuli. No reaction to the end of stimulation could be measured with stimulus duration of 0.32 s. Small positive *d*-waves, however, were observed even with the lowest intensity when stimulus duration was prolonged to 1.35 s (Fig. 2C). At higher intensities of stimuli positive *d*-waves increased. Fig. 2D demonstrates the dependence of the *d*-wave form and amplitude on the intensity of background illumination. Same dark-adapted preparations of the eel were exposed to steps of 5 min incremental background illumination: $I_{B1} = 24 \times 10^{-3}$, $I_{B2} = 24 \times 10^{-2}$ and $I_{B3} = 24 \mu\text{W}/\text{cm}^2$. After the end of ERGs at I_{B1} a negative *d*-wave appears on the top of the *c*-wave. On the other hand, cessation of I_{B2} was followed by a positive *d*-wave which reached more than $100 \mu\text{V}$ at the end of the brightest background, I_{B3} (Fig. 2D).

After prolonged dark adaptation (when 12 : 12 h light/dark regime was replaced with 24 h of constant darkness) ERGs of rudd and perch (Fig. 3A and C, respectively) were obtained with white light flashes (duration of photo stimuli 1.3 s) ranging in intensity from $28.2 \times 10^{-2} \mu\text{W}/\text{cm}^2$ ($-3 \log$ attenuation units) to $282 \mu\text{W}/\text{cm}^2$ (0 log units – maximum achievable intensity). At light offset the ERG of rudd shows a rapid negative *d*-wave rising in absolute amplitude at higher intensities of photo-stimulation (Fig. 3A). On the other hand ERGs of perch (Fig. 3C) show a prominent positive *d*-wave that is also directly dependent on stimulus intensity masking the *c*-wave.

Stimulus intensity-amplitude relation has been checked by fitting experimental data to the basic model:

$$V_0 = I^a / (I_0^a + I^a)$$

(Naka and Rushton 1966), where V_0 is normalized to the maximal amplitude (V_{max}) of the ERG corresponding signal (*b*-wave, *c*-wave, or *d*-wave), I_0 is the stimulus light intensity corresponding to $V_0 = 1/2$, and exponent a is the fitted constant (Fig. 3D,E). The average slopes (a values) of normalized log profiles in the rudd were 0.87 for the *b*-wave, 1.0 for the *d*-wave and 1.2 for the *c*-wave. The amplitudes of the *b*-wave (absolute maximum value of $160 \mu\text{V}$) and of

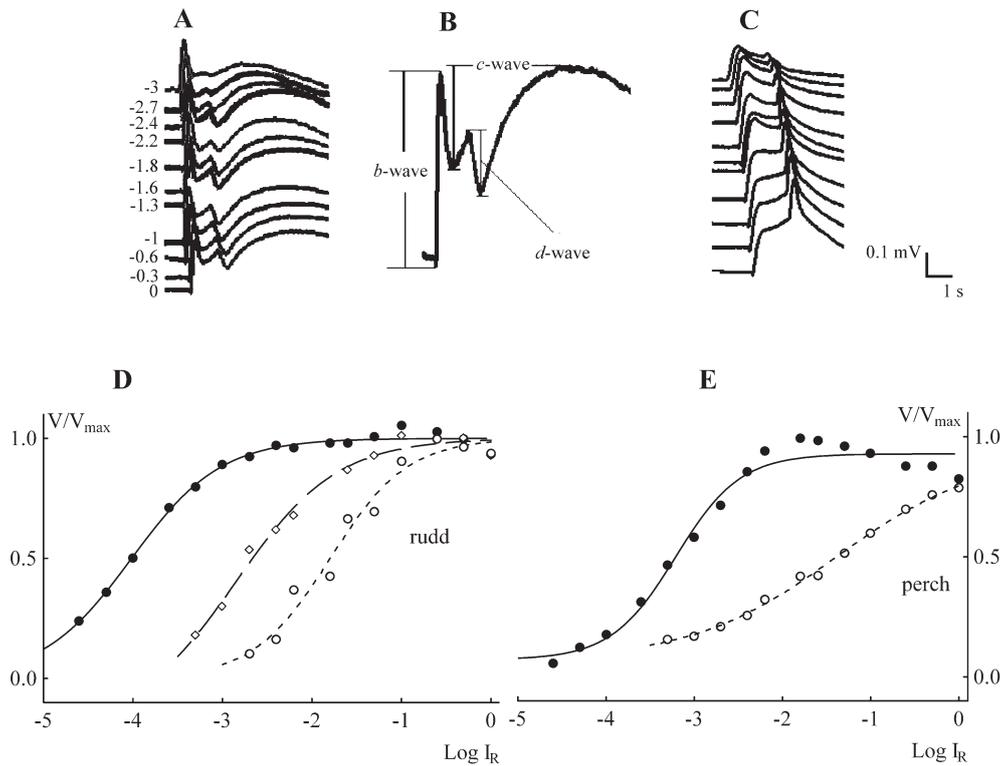


Figure 3. Relationship of normalized response amplitude (V/V_{\max}) and log intensity of stimulation in perch and rudd. **A.** A series of ERGs obtained with incremental stimulation from the eye of rudd (log attenuation units presented at each trace). Stimuli were white light (maximal intensity was $282 \mu\text{W}/\text{cm}^2$), 1.3 s. **B.** Scaling of measured ERG components. **C.** A series of ERGs obtained with incremental stimulation (same series as in A) from the eye of perch. Stimuli as in A. **D.** Amplitude/intensity relations in the rudd for *b*-wave (solid circle), *c*-wave (open diamond) and negative *d*-wave (open circle); $n = 3$. Curves were fitted according to the basic model of Naka and Rushton (1966). **E.** Amplitude/intensity relations in the perch for *b*-wave (solid circle) and positive *d*-wave (open circle); $n = 4$. Curves were fitted as in D.

the negative *d*-wave (absolute maximum value of $56 \mu\text{V}$) rose with increasing photo-stimulus intensity which was quite opposite to the negative *d*-wave in dogfish sharks and goldfish that decreased with higher intensity of stimulation (Fig. 1A and Ba,b,c). The saturation level for the *b*-wave was reached at a relatively low stimulus intensity, $28.2 \times 10^{-2} \mu\text{W}/\text{cm}^2$ (-3 log units, Fig. 3D). The saturation level for the *d*-wave and *c*-wave was reached with 10 times higher stimuli, as compared to the *b*-wave, i.e. with $2.82 \mu\text{W}/\text{cm}^2$ (-2 log units, Fig. 3D). Although the *c*-wave originates from the pigment epithelium, it depends upon the integrity of the photoreceptors, because light absorption in the photoreceptors triggers the chain of events leading to the decrease in extracellular concentration of potassium ions. Fig. 3E shows average amplitude-stimulus intensity relation of the *b*-wave and positive *d*-wave in dark-adapted perch. In this series, *c*-wave is masked by *d*-wave, and not directly measurable from the ERG. The slope of normalized log profiles for the *b*-wave was 1.2. The saturation level for the *b*-wave was reached at the stimulus intensity of $56.4 \times 10^{-5} \text{mW}/\text{cm}^2$ (amplitude

$232 \pm 10 \mu\text{V}$, $n = 4$). The slope for the *d*-wave was 0.52 but the saturation level was never reached, even when maximal achievable intensity of stimuli were applied (amplitude $163 \pm 2 \mu\text{V}$, $n = 4$).

With prolonged stimuli of up to 20 s, ERGs of the dark-adapted percids, *S. scriba* and *P. fluviatilis* contained a positive *d*-wave, exceeding the amplitude of scotopic *b*-wave (Fig. 4A). Fig. 4B shows a comparison of ERGs recorded from dark-adapted preparations in different teleosts at stimulus durations of 200 ms. Among 6 species explored, only in percid (*S. scriba* and *P. fluviatilis*) ERGs showed positive *d*-wave as a response to light cessation.

Discussion

Although in the early studies on elasmobranchs the presence of cones was not revealed, it was subsequently demonstrated that the lemon shark, *Negaprion brevirostris*, contains cones, as well as rods (Gilbert 1963). Additional investigations pro-

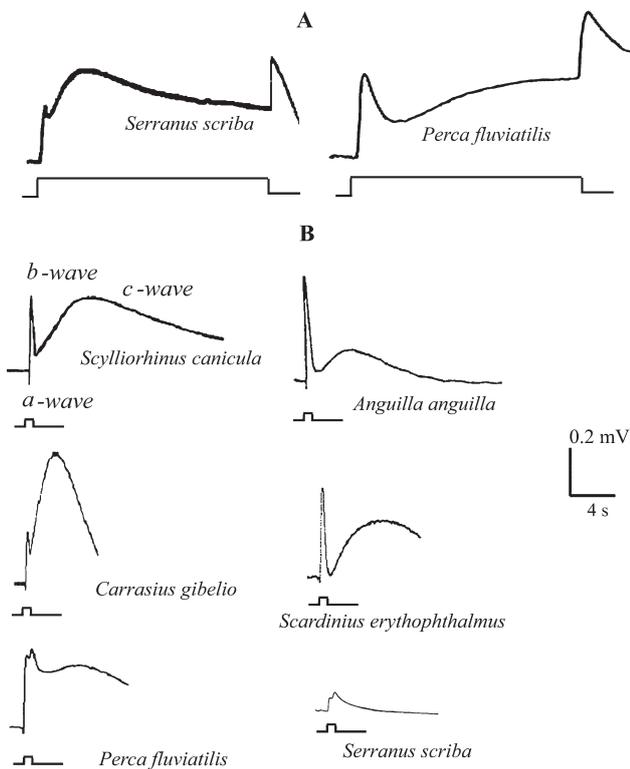


Figure 4. Comparative presentation of the electroretinographic responses in dark-adapted retinas of different fishes as indicated in the figure. **A.** A marked positive *d*-wave in *S. scriba* and *P. fluviatilis* can be seen after 20 s of illumination (white light, $24 \mu\text{W}/\text{cm}^2$). **B.** ERGs in different species evoked by short-term stimulation (0.2 s , 500 nm , $8.9 \mu\text{W}/\text{cm}^2$) in dark-adapted conditions.

vided evidence that cones are present in the retina of many rays and sharks (Gruber and Cohen 1978, 1985; Gruber et al. 1991). In the great white shark *Charcharodon carcharias* cone density was reasonably high (rod/cone ratio, 5 : 1) (Gruber and Cohen 1985). In shallow water guitarfish, *Rhinobatos lentiginosus*, which is active in shallow, sandy reef areas during the brightness of the midday sun when only cones are active, rod/cone ratio is also high – 6 : 1 (Gruber et al. 1991). On the other hand, dim-light species inhabiting sea depths 30–50 m, such as *Mustelus vulgaris*, can have rod/cone ratio as high as 100 : 1 (Stell and Witkovsky 1973). The specific function of elasmobranch cones has yet to be demonstrated, although some authors (Gruber et al. 1991) suspect that they subservise photopic vision and, perhaps, also color vision. In rays and sharks inhabiting depths 200–300 m, evidence of cone presence in the retina was not found (Dowling and Ripps 1970, 1971). *S. canicula* is known to inhabit similar depths (around 300 m) and in our previous studies (Andjus et al. 1998), the presence of cones in the retina of this shark was not proven. However, in the all-rod retina of the

skate Chappell and Rosenstein (1996) observed with prolonged stimuli (10 s) a *d*-wave-like component of the ERG. After the application of 2-amino-4-phosphonobutyric acid (APB), a glutamate agonist which blocks the ON pathway of the retina and abolishes the *b*-wave, enduring *d*-wave was blocked with the application of kynurenic acid. The selective block of ON and OFF components of the ERG demonstrates the existence of parallel ON and OFF pathways at the bipolar cell level of skate retina. Since dogfish shark inhabits similar depths with similar light conditions as skate, our findings of a positive *d*-wave recorded in photopic conditions could indicate the presence of independent ON- and OFF-bipolar cell pathways in dogfish shark retina as well. On the other hand, the positive *d*-wave in small spotted dogfish shark could present an exception as in some other “scotopic” animals, such as the cat (Brown 1968) or the rat (Naarendorp and Williams 1999).

Retina of the eel is of preponderantly rod type, with at least two types of cones (Gordon et al. 1978; Niwa 1979; Byzov et al. 1998; Damjanović et al. 2005). Rod/cone ratio in yellow eel from Adriatic Sea was 20 : 1 (eel cones degenerate during the process of sexual maturation, so that the rod/cone ratio in mature silver eels is very high and varies from 100 : 1 to 250 : 1; Braekevelt 1988). The goldfish possesses duplex retina with at least four types of cones (long-wave, middle-wave, short-wave and UV-cones; Bowmaker 1995) and two types of bipolar cells: bipolar cells that receive mixed input from rod and cone photoreceptors, and bipolar cells that receive input only from cones (Sherry and Yazulla 1993). In our study, absolute amplitude of negative *d*-wave increases with lowering stimulus intensity (Fig. 1Ba,b,c), that is consistent with a slower decay of the rod receptor potential as compared to the decay of Granit’s PII component (Brown 1968). Transition from dark to light adaptation is well documented from the fact that in eel and goldfish the *d*-wave changes its sign (Figs. 1B and 2D).

The most prominent *d*-wave was detected in two percids, painted comber and perch. Duplex retinas of painted comber and perch generated ERGs with positive *d*-waves, either in scotopic or in photopic conditions. Rod /cone ratio in perch increases with age from 4 : 1 in young 45 mm fish to 10 : 1 in fish 4–5 years old (Guma’a 1982). Perch and painted comber are highly active visual predators that forage more efficiently in high light intensity. In adult perch both cones and rods are mobile and function in the retinomotor response. When adapting to darkness, perch cones migrate from the light path, and the rods elongate. In the opposite situation, the cones migrate back to their original site, and rods are drawn back without effect on cone pathways (*d*-wave remains always positive; Milošević et al. 2006).

In comparison to a species of fish that inhabits a similar light environment (goldfish), the form and amplitude of rudd’s *d*-wave behaves differently. Namely, after prolonged

dark adaptation, absolute amplitude of negative *d*-wave increases with enlarged stimuli, which could be the consequence of synaptic modifications in cone pedicles resulting in reduced cone input to secondary retinal neurons and in redirecting cone response through rod pathways (Witkovsky et al. 1974; Yang and Wu 1989; Mangel et al. 1994).

In conclusion, the presented comparative studies of the *d*-wave in fish shows that form, amplitude and sign of this ERG component mainly depends on the state of adaptation and less on the photoreceptor content.

Acknowledgements. Supported by grant No. 143045 and No. 143054 of the Serbian Ministry of Science and Environmental Protection.

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