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ORIGINAL RESEARCH ARTICLE

Consecutive unilateral recording of the two eyes affects dark-adapted ERG responses, when compared to simultaneous bilateral recording

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Abstract

Purpose Our aim was to compare the electroretinographic (ERG) responses of two eyes obtained by consecutive unilateral recordings to those obtained by a simultaneous bilateral recording in sheep.

Methods Eight sheep underwent two full-field ERG recordings, using two recording strategies of the standard ISCEV protocol: consecutive unilateral recordings of one eye after the other, and simultaneous bilateral recording of both eyes. The order of recording strategy within an animal (unilateral/bilateral), eye recording sequence in the unilateral session (OD/OS), and amplifier channel assignment for each eye were all randomized. To test whether duration of dark adaptation and/or anesthesia affect the results, the ISCEV protocol was recorded bilaterally in six additional eyes following 38 min of patched dark adaptation, as was done for the second eye recorded in the consecutive unilateral recordings.

Results The second recorded eye in the unilateral session had significantly higher scotopic b-wave amplitudes compared to the first recorded eye and to the bilaterally recorded eyes. A-wave amplitudes of the dark-adapted mixed rod–cone responses to a high-intensity flash were also significantly higher in the second eye compared to the first eye recorded unilaterally and to the bilaterally recorded eyes. Light-adapted responses were unaffected by the recording strategy. When the ISCEV protocol was recorded after 38 min of dark adaptation, the scotopic responses were higher than in the first eyes, and similar to those of the second eyes recorded unilaterally, suggesting that indeed the longer duration of anesthesia and dark adaptation are responsible for the increased scotopic responses of the second eye.

Conclusions Consecutive unilateral ERG recordings of two eyes result in higher amplitudes of the dark-adapted responses of the eye recorded second, compared to the eye recorded first and to bilaterally recorded eyes. The differences in scotopic responses can be attributed to different duration of dark adaptation and/or anesthesia of the two consecutively recorded eyes. Photopic responses are not affected. Therefore, simultaneous bilateral ERG responses

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should be recorded when possible, especially for evaluation of scotopic responses.

Keywords Electroretinography (ERG) · Unilateral · Bilateral · Dark-adapted responses · ISCEV · Sheep

Introduction

Electroretinography (ERG) is an important, noninvasive, diagnostic technique used to evaluate retinal function in both clinical and research settings [1]. In clinical practice, ERG is used to diagnose inherited and acquired retinal pathologies, to differentiate between retinal and neurological disease and to evaluate retinal function when the retina cannot be visualized [2]. In research settings, ERG is often used to study retinal physiology [3] and pathophysiology [4], to assess safety and toxicity of drugs [5–7], and to evaluate the efficacy of new therapies for retinal degenerations. In recent years, intensive research has been conducted to develop gene- and cell-based treatments for inherited retinopathies and ERG is a key modality in the evaluation of treatment outcome [8–12].

One of the inherent benefits of the eye as a candidate for therapeutic studies is that, being a paired organ, one eye can serve as control for the fellow, experimental eye of the same subject. Indeed, the comparison of ERG responses of control and experimental eyes is very common in ophthalmic research. This could be achieved either by recording one eye after the other using a unilateral ERG unit that stimulates and records each eye separately [13–16], or by using a dual unit that stimulates and records both eyes simultaneously [7, 17–20].

In recent years, our group has been working on the characterization of day blindness in sheep [21–23] and consequently, we successfully performed gene augmentation therapy in this naturally occurring large animal model of *CNGA3* achromatopsia [10, 24, 25]. In our earlier work we recorded the two eyes consecutively using a unilateral ERG unit [10, 21, 24], but in our recent work we have used a dual unit for simultaneous bilateral recording of both eyes [25]. In our efforts to standardize the data we could not find any information regarding potential differences between consecutive unilateral and simultaneous bilateral ERG recordings in any species. In the present study, we wished to examine whether there may be an effect of the recording strategy, by comparing ovine ERG responses obtained through consecutive unilateral recordings of the two eyes to those obtained through a simultaneous bilateral recording. Specifically, our aim was to determine whether there are differences in the results of the second eye, depending on whether it is recorded consecutively (after the first eye, in sequential unilateral recordings) or simultaneously with the first eye, since variables such as duration of anesthesia and dark adaptation will be different in the two recording strategies.

Materials and methods

Subjects and experimental design

Eight healthy Afec Assaf male lambs aged 153 ± 8 days (mean ± SD) were used in this study. Animals were housed in an outdoor facility at the experimental flock of the Volcani Center at Bet Dagan, Israel. Experimental protocols were approved by the Volcani Center Animal Care and Use Committee and conformed with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Each sheep was recorded twice, in a unilateral session (in which the eyes were recorded unilaterally and consecutively) and a bilateral session (in which both eyes were recorded simultaneously) 1 week apart. The order of recording sessions, the eye recording sequence in the unilateral session (OD/OS), and the amplifier channel assignment for each eye (A/B) were all randomized (Table 1). All recordings were conducted by a single researcher (MR), between 8 am and 1 pm to reduce potential circadian effects. Prior to anesthesia animals were kept outdoors, in an open shed, under similar daylight conditions (cloudless, blue sky) in order to minimize variation. Illuminance was measured in the outdoor shed, in the recording room before recording and during dark adaptation using a Zico Zi-7810 light meter (Zico Tech Ltd, Israel). The average illumination intensity (mean ± SE) was 349.25 ± 44.07 lx, 198.63 ± 18.62 lx, and 0.63 ± 0.18 lx respectively.

Table 1 Randomization of study animals

Animal number	First session	Unilateral recording		Second session	Unilateral recording	
		First eye	Second eye		First eye	Second eye
9765	Unilateral	OD	OS	Bilateral		
9893	Bilateral			Unilateral	OS	OD
9846	Unilateral	OS	OD	Bilateral		
9815	Bilateral			Unilateral	OD	OS
9853	Unilateral	OS	OD	Bilateral		
9882	Bilateral			Unilateral	OD	OS
9927	Unilateral	OD	OS	Bilateral		
9955	Bilateral			Unilateral	OS	OD

Anesthesia

Animals were premedicated with an intramuscular injection of pethidine (3 mg/kg; Dolestine, Teva Pharmaceutical Industries, Kfar Saba, Israel) and acepromazine (0.1 mg/kg; 10 mg/ml compounded preparation, Vetmarket Pharmacy, Shoham, Israel). Anesthesia was induced with an intravenous injection of propofol (4 mg/kg; Propofol-lipuro, B. Braun Medical Supplies, Manila, Philippines) and diazepam (0.15 mg/kg; Assival, Teva Pharmaceutical Industries, Kfar Saba, Israel) and maintained with 3% isoflurane (Forane, Abbott Laboratories, Maidenhead, England). During anesthesia, animals were ventilated, hydrated with intravenous 0.9% saline infusion, and monitored by a board-certified specialist in small ruminant medicine. Heart rate, oxygenation, and depth of anesthesia were continuously monitored (Vitalogik 6000 Compact itor, Mennen Medical, Rehovot, Israel) and remained stable throughout the course of the procedure. Duration of anesthesia for the bilateral recording was approximately 40 min and for the two consecutive unilateral recordings approximately 60 min.

ERG

Pupils were dilated with topical 0.5% tropicamide (Mydramide, Fischer Pharmaceutical Labs, Israel) and 10% phenylephrine hydrochloride (Efrin-10, Fischer Pharmaceutical Labs, Israel) solutions at least 20 min before recording, once again before induction of anesthesia, and before the second recording in the unilateral session. Animals were positioned in sternal recumbency, and eyelids of the recorded eye retracted with Barraquer eyelid retractors. Since the globe was centrally positioned throughout anesthesia, a stay suture for globe centralization was unnecessary. For the consecutive unilateral recordings, the second, unrecorded, eye was covered with a black patch while the first eye was being recorded, a practice commonly used when performing sequential unilateral recordings in various species [15, 26–29]. After the first eye had been recorded, the second eye was uncovered and recorded.

To improve conduction, a drop of 1.4% hydroxymethylcellulose (Celluspan, Fischer Pharmaceutical Labs, Tel-Aviv, Israel) was applied to the recorded eye. ERG responses were recorded using a jet contact lens electrode (ERG-Jet, Fabrinal SA, La Chaux-de-Fonds, Switzerland), with reference and ground subcutaneous needle electrodes (CareFusion, WI, USA) placed at the ipsilateral lateral canthus and the forehead, respectively. Impedance was kept under 5 KΩ. Recordings were conducted using a Handheld Multi-species Electroretinography (HMsERG) system (OcuScience, Henderson, NV, USA) with a bandpass of 0.3–300 Hz. Background adaptation light and stimuli were delivered using dual handheld mini Ganzfeld units for the simultaneous bilateral recordings or using only the "master unit" for the consecutive unilateral recordings.

After 20 min of dark adaptation, scotopic and photopic responses were recorded using the ISCEV protocol [30]. Responses to 10 flashes, presented at 0.5 Hz and a stimulus strength of 0.01 cd * s/m^2, were averaged to generate the single-flash scotopic

response. Next, mixed rod–cone function was recorded by averaging responses to four flashes of standard and high intensity (3 and 10 cd * s/m^2) presented at 0.1 and 0.05 Hz, respectively. Then, following 10 min of light adaptation (30 cd/m^2), photopic responses were recorded at two stimulus strengths (3 and 10 cd * s/m^2). At each strength, 32 flashes, presented at 1 Hz, were averaged to generate the single-flash photopic response. This was followed by a cone flicker test at 30 Hz frequency, with 128 responses recorded and averaged. In the unilateral session the second eye was then uncovered, electrodes were placed under a dim red light headband (OcuScience, Henderson, NV, USA), and the recording of the ISCEV protocol was repeated as described, without additional dark adaptation.

Consequently, in the sequential recording, the second eye was dark-adapted for about 38 min (20 min of initial dark adaptation, 13 min for the full ISCEV protocol, and 5 min to change electrodes) rather than the 20 min used to dark adapt the first eye recorded unilaterally and the two eyes recorded simultaneously. To determine whether our results in this eye were affected by the longer dark adaptation, quality of dark adaptation (due to patching of the unrecorded eyes), and/or anesthesia duration, we conducted a second experiment. Six eyes of three other sheep were patched and dark-adapted for 38 min. After 33 min of dark adaptation, both eyes were uncovered, and electrodes were placed using a dim red light headband. A full ISCEV protocol was recorded after 38 min of dark adaptation in both eyes simultaneously.

Data and statistical analysis

Power analysis was performed using WinPEPI software 11.36 [31] to justify the number of animals needed, based on the difference in means and in standard deviation of the means from our previous work in sheep [10, 21, 24, 25]. Amplitudes and implicit times of the a- and b-waves of all single-flash responses, and flicker amplitudes were measured. The a-wave amplitude was measured from baseline to the first trough, and the b-wave amplitude from the a-wave trough to the next positive peak. The a-wave implicit time was measured from the flash onset to the a-wave trough and the b-wave implicit time was measured from the flash onset to the b-wave peak. ERG data from the bilateral sessions are presented for the total number of eyes, i.e., it was not averaged per animal.

Statistical analysis was performed using two methods. JMP® Pro 13.0.0 (SAS institute Inc., 2016. Cary, NC, USA) was used to compare ERG parameters by repeated-measures ANOVA and to confirm normal distribution of the data by the Shapiro–Wilk test. Stata14 (StataCorp. 2015. Stata Statistical Software: Release 14. StataCorp LP, College Station, TX, USA) was used for further statistical analysis; linear mixed effects regression models were used to account for the variability between sheep. The random effect was the sheep, and the fixed effects were recording session (unilateral/bilateral), eye order within the consecutive unilateral recording (recorded first/second), and amplifier channel (A/B). Values were considered significant for $p < 0.05$. When multiple pairwise comparisons were made, the Bonferroni and Holmes corrections were applied to the significance level and values were considered significant for $p < 0.017$.

Results

Box-and-whisker plots for the distribution of a- and b-wave amplitudes of responses recorded unilaterally and bilaterally are presented in Fig. 1, and averaged traces of the dark- and light-adapted responses are presented in Fig. 2. When all data (for both unilateral and bilateral recordings) were combined, there were no significant differences between average amplitudes and implicit times of responses of the left and right eyes (Online Resource 1) or the amplifier channels (Online Resource 2). Furthermore, there were no significant differences between the two eyes recorded during the bilateral session alone (Online Resource 3). Overall, the waveforms of the responses were uniform in the two recording sessions (Fig. 2).

Light-adapted responses were not significantly different in the two recording sessions and were not affected by the order of recording during the unilateral session (Figs. 1, 2). Nor were there significant differences in dark-adapted response amplitudes between the first eye recorded in the unilateral session, and the responses recorded in the bilateral session. However, the second eye recorded in the unilateral session showed significantly higher scotopic b-wave amplitudes compared both to the first recorded eye and to the bilaterally recorded eyes (corrected to the Bonferroni

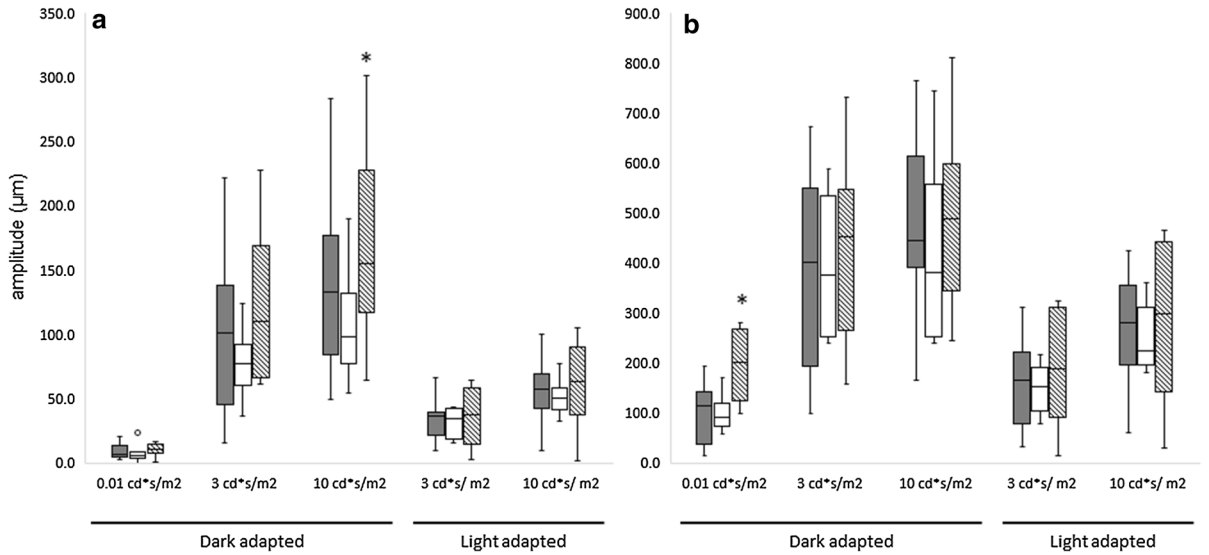

Fig. 1 A-wave (**a**) and b-wave (**b**) amplitudes of unilaterally and bilaterally recorded eyes. Amplitudes of dark- and light-adapted responses are presented as box-and-whisker plots showing 5 and 95% quantiles (whiskers), 25 and 75% quartiles (box), and the median (marked by a horizontal line). Outliers are marked with a circle. Gray boxes represent bilaterally recorded eyes (n = 16), white boxes represent the first eyes recorded unilaterally (n = 8), and striped boxes represent the second eyes recorded unilaterally (n = 8). Significant differences corrected to the Bonferroni and Holmes criteria are marked with *($p < 0.017$)

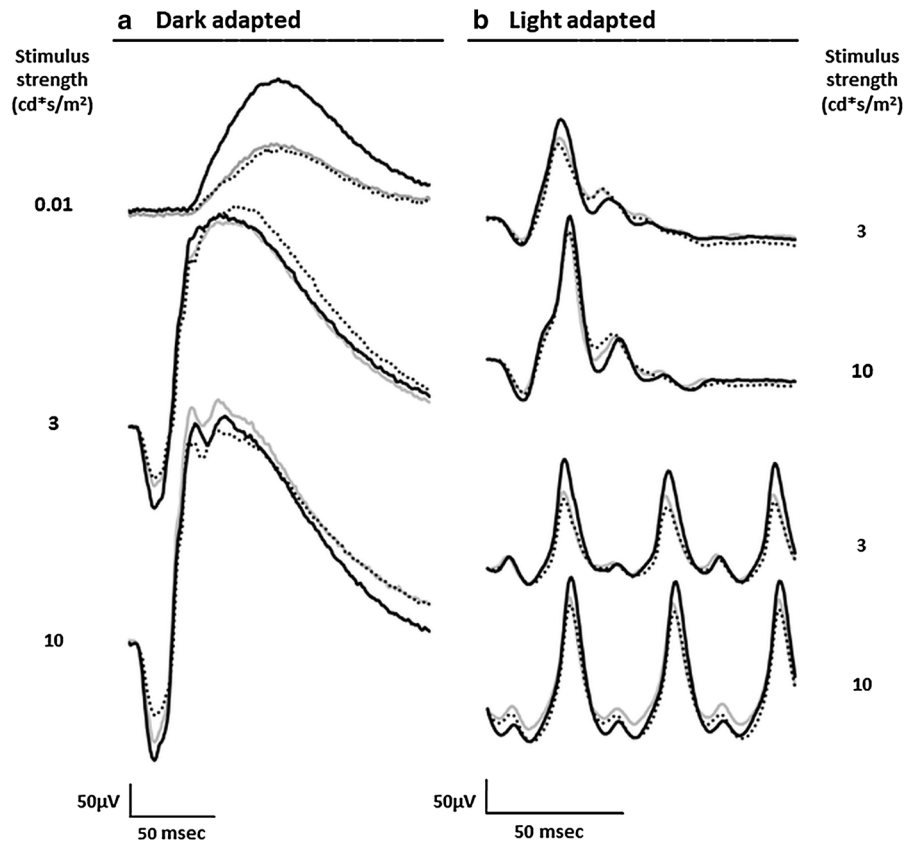

Fig. 2 Average traces of dark- and light-adapted responses recorded from 8 sheep. Dark-adapted responses at three intensities (a), and light-adapted, single-flash (at two strengths) and 30-Hz flicker responses (b) are presented. Dotted and black traces are the averaged responses of the first and second eyes, respectively, recorded unilaterally and consecutively. Gray traces are the averaged responses of the bilaterally recorded eyes

and Holmes criteria, $p < 0.017$) (Figs. 1b, 2a, $p = 0.001$). A-wave amplitudes of the dark-adapted high-intensity mixed rod–cone response were also significantly higher in the second recorded eye compared to the first eye recorded unilaterally and to the bilaterally recorded eyes (Figs. 1a, 2a, $p = 0.009$). A-wave amplitudes of the dark-adapted standard-intensity mixed rod–cone response were higher in the second recorded eye compared to the first eye recorded unilaterally and to the bilaterally recorded eyes although the difference did not reach statistical significance when corrected to the Bonferroni and Holmes criteria (Fig. 1a, $p = 0.030$). Implicit times of the dark-adapted responses were not significantly affected by the recording session, nor by the order of recording in the unilateral session, when corrected to the Bonferroni and Holmes criteria (Online Resource 4).

The second eye recorded in the unilateral session underwent longer, patched dark adaptation, and the duration of anesthesia was longer. To test if these variables had an effect on the dark-adapted responses of this eye, the ISCEV protocol was recorded in six more eyes (of 3 additional sheep) after 38 min of patched dark adaptation (similar to the conditions that preceded the recording of the second recorded eye in the unilateral session). Box-and-whisker plots for the distribution of a- and b-wave amplitudes of dark- and light-adapted responses of these six eyes are presented in Fig. 3. Following 38 min of bilateral dark adaptation, the mean scotopic b-wave amplitude was similar to that of the second unilaterally-recorded eyes and significantly higher than that of the first unilaterally-recorded eyes ($p = 0.001$). A-wave amplitudes of the dark-adapted mixed rod–cone responses in both strengths were also significantly higher in the eyes that underwent 38 min of bilateral dark adaptation compared to those of the first unilaterally-recorded eyes ($p = 0.010$ and $p = 0.014$ for the standard- and high-strength stimuli, respectively). The photopic response amplitudes were not significantly affected by the longer bilateral dark adaptation, compared to the photopic responses of both the first and second eyes recorded in the unilateral session ($p > 0.017$, corrected for the Bonferroni and Holmes criteria). Implicit times of both scotopic and photopic responses of these six eyes were not significantly affected (Online resource 5), except for the standard-intensity mixed rod–cone b-wave implicit time that was shorter in the eyes that underwent 38 min on dark adaptation compared to the first unilaterally recorded eyes ($p = 0.009$).

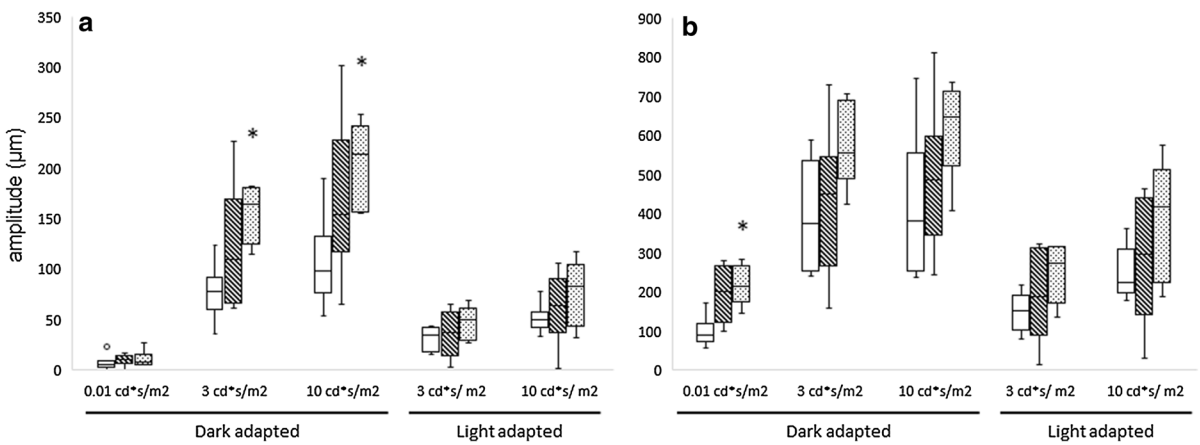

Fig. 3 A-wave (**a**) and b-wave (**b**) amplitudes of eyes recorded unilaterally and consecutively, and of bilaterally recorded eyes after 38 min of dark adaptation. Amplitudes of dark- and light-adapted responses are presented as box-and-whisker plots showing 5 and 95% quantiles (whiskers), 25 and 75% quartiles (box), and the median (marked by a horizontal line). Outliers are marked with a circle. White boxes represent the first eyes recorded unilaterally ($n = 8$), striped boxes represent the second eyes recorded unilaterally ($n = 8$), and dotted boxes represent the eyes recorded bilaterally after 38 min of dark adaptation ($n = 6$). Significant differences corrected to the Bonferroni and Holmes criteria are marked with *($p < 0.017$)

Discussion

The purpose of the present study was to compare ERG results obtained by consecutive unilateral recordings of two eyes of the same subject, to those obtained by simultaneous bilateral recording of the same eyes. The results show an effect of the unilateral session on the dark-adapted responses of the second eye recorded. When compared to the first unilaterally recorded eye and to the bilaterally recorded eyes, the second recorded eye in the unilateral session has a significantly higher scotopic b-wave amplitude and higher dark-adapted mixed rod–cone a-wave amplitude. Overall, the response waveforms are similar in the two eyes recorded unilaterally and in the bilaterally recorded eyes (Fig. 2). They are also compatible with traces of normal sheep previously published by our group [21], confirming a lack of effect on the response form or kinetics and demonstrating an effect on the amplitudes alone.

Many variables may affect ERG recordings. These include hardware variables such as electrode type and positioning [32, 33], and the ERG system and flash characteristics [34] being used. Physiological factors such as pupil size [29], age, temperature, oxygenation [35, 36], animal species and breed, and the anesthetic protocol [13, 37–39] also have significant effects on ERG. All these variables should be considered when interpreting the recordings.

In the present study, the recording of the second eye in the unilateral session required longer anesthetic duration compared to the recording of the first eye and to the bilateral recording. The effect of anesthesia on ERG was evaluated in dogs by Freeman et al. [39]. In their study, anesthetized dogs had lower ERG amplitudes and longer implicit times compared to alert dogs. Therefore, if the duration of anesthesia had any influence on our animals, we would expect it to be opposite to the effect witnessed in the second recorded eye. Namely, we would have demonstrated attenuated, rather than increased, responses in this eye due to prolonged anesthesia. On the other hand, the longer duration of anesthesia might result in washout of the effects of the premedication and induction drugs we used, which could lead to higher amplitudes as seen in our study. Indeed, a pharmacokinetic study of propofol as an induction agent in small ruminants reveals a mean elimination half-life of 15.5 min [40], meaning that during the recording of the second eye in our unilateral session the level of serum propofol would likely be lower. While we would expect the effect of the drugs' washout to be similar for both scotopic and photopic recordings, we cannot rule out a possible effect it might have had on our scotopic results.

The second eye in the unilateral session also underwent an extra 18 min of dark adaptation compared both to the first eye and to the bilaterally-recorded eyes . Duration of dark adaptation was shown to significantly affect scotopic b-wave amplitudes in humans, [41, 42]. Specifically, shorter dark adaptation periods have been shown to decrease the scotopic b-wave amplitudes [42]. Therefore, it is tempting to think that the significant increase in scotopic b-wave amplitude of the second eye recorded in the unilateral session demonstrates increased dark adaptation in this eye compared to the fellow (first) eye and to the bilaterally recorded eyes. The quality of dark adaptation in the second eye might have also had an influence on the results since the second eye recorded in the unilateral session was patched during the recording of the first eye, and therefore, it qualitatively differed from the dark adaptation in the bilateral recordings.

To further assess the potential influence of the longer dark adaptation and anesthesia on the scotopic responses of the second eye recorded in the unilateral session, six other eyes were recorded after 38 min of continuous dark adaptation with patching, thus simulating the dark adaptation conditions of this second eye. Indeed, a significant increase in scotopic amplitudes was seen in those six eyes, similar to the responses of the second eye in recorded in the unilateral session (Fig. 3). Therefore, it is possible that the longer duration of dark adaptation and anesthesia, and the quality of dark adaptation (namely patching of the unrecorded eyes), are indeed involved in the effect on scotopic responses seen in the second unilaterally recorded eyes.

We believe that our results are relevant, as sequential recordings are commonly practiced in ERG laboratories. Sequential unilateral recordings, in which the eye that will be recorded second was patched, just as it was patched in our study, have been reported in rats [43], rabbits [15], pigs [26], and monkeys [27–29]. Our results demonstrate that such practice is valid for photopic recordings, but may affect the dark-adapted responses of the second eye. It is conceivable that longer dark adaptation of both eyes prior to the sequential unilateral recordings might

minimize the differences; however, such a protocol would require substantially longer anesthesia that is not always feasible.

It should be noted that we observed a trend of increased light-adapted b-wave amplitudes in the second recorded eye in the unilateral session and in the eyes that underwent 38 min of dark adaptation (Figs. 1, 2, 3). These results did not reach statistical significance, though it is conceivable that a larger sample size would have strengthened this finding. However, Lachapelle analyzed the photopic ERG recorded before and after dark adaptation and demonstrated reduced photopic b-wave amplitudes and prolonged implicit times in eyes that were readapted to light after dark adaptation, compared to those that did not undergo prior dark adaptation [44]. This suggests that even with a larger sample we would not have seen a significant increase in the photopic b-wave amplitude of the second eye.

In conclusion, in the present study sequential unilateral ERG recordings of the two ovine eyes significantly affected the dark-adapted amplitudes of the second eye, when compared to a simultaneous bilateral recording. The effect can be attributed to the longer duration of dark adaptation, quality of dark adaptation, and possibly the duration of anesthesia. Therefore, it is advised to favor simultaneous bilateral ERG recordings, specifically in the evaluation of dark-adapted responses. If bilateral recordings are not feasible, the potential implications of consecutive recordings in two eyes have to be considered when results are interpreted.

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Compliance with ethical standards

Conflict of interest RO received speaker honoraria from OcuScience.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Statement of human rights This article does not contain any studies with human participants performed by any of the authors.

Statement on the welfare of animals All procedures performed in studies involving animals were in accordance with the ethical standards of the Volcani Center Animal Care and Use Committee and conformed with the ARVO Statement for the use of animals.

Informed consent Informed consent was not applicable.

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