



## Post-illumination pupil response after blue light: Reliability of optimized melanopsin-based phototransduction assessment



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### ABSTRACT

Melanopsin-containing retinal ganglion cells have recently been shown highly relevant to the non-image forming effects of light, through their direct projections on brain circuits that regulate alertness, mood and circadian rhythms. A quantitative assessment of functionality of the melanopsin-signaling pathway could be highly relevant in order to mechanistically understand individual differences in the effects of light on these regulatory systems. We here propose and validate a reliable quantification of the melanopsin-dependent Post-Illumination Pupil Response (PIPR) after blue light, and evaluated its sensitivity to dark adaptation, time of day, body posture, and light exposure history. Pupil diameter of the left eye was continuously measured during a series of light exposures to the right eye, of which the pupil was dilated using tropicamide 0.5%. The light exposure paradigm consisted of the following five consecutive blocks of five minutes: baseline dark; monochromatic red light (peak wavelength: 630 nm, luminance: 375 cd/m<sup>2</sup>) to maximize the effect of subsequent blue light; dark; monochromatic blue light (peak wavelength: 470 nm, luminance: 375 cd/m<sup>2</sup>); and post-blue dark. PIPR was quantified as the difference between baseline dark pupil diameter and post-blue dark pupil diameter (PIPR-mm). In addition, a relative PIPR was calculated by dividing PIPR by baseline pupil diameter (PIPR-%). In total 54 PIPR assessments were obtained in 25 healthy young adults (10 males, mean age ± SD: 26.9 ± 4.0 yr). From repeated measurements on two consecutive days in 15 of the 25 participants (6 males, mean age ± SD: 27.8 ± 4.3 yrs) test-retest reliability of both PIPR outcome parameters was calculated. In the presence of considerable between-subject differences, both outcome parameters had very high test-retest reliability: Cronbach's  $\alpha > 0.90$  and Intraclass Correlation Coefficient  $> 0.85$ . In 12 of the 25 participants (6 males, mean age ± SD: 26.5 ± 3.6 yr) we examined the potential confounding effects of dark adaptation, time of the day (morning vs. afternoon), body posture (upright vs. supine position), and 24-h environmental light history on the PIPR assessment. Mixed effect regression models were used to analyze these possible confounders. A supine position caused larger PIPR-mm ( $\beta = 0.29$  mm, SE = 0.10,  $p = 0.01$ ) and PIPR-% ( $\beta = 4.34\%$ , SE = 1.69,  $p = 0.02$ ), which was due to an increase in baseline dark pupil diameter; this finding is of relevance for studies requiring a supine posture, as in functional Magnetic Resonance Imaging, constant routine protocols, and bed-ridden patients. There were no effects of dark adaptation, time of day, and light history. In conclusion, the presented method provides a reliable and robust assessment of the PIPR to allow for studies on individual differences in melanopsin-based phototransduction and effects of interventions.

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**Abbreviations:** PIPR, Post-Illumination Pupil Response; ipRGC, intrinsically photosensitive Retinal Ganglion Cell; ICC, Intraclass Correlation Coefficient.

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## 1. Introduction

Light reaching the retina of the eyes does not only provide the brain with images of the environment, but also generates several non-image forming effects. These include constriction of the pupil diameter, changes in the arousal level of the brain, and entrainment of the biological clock of the brain to the environmental 24-h light–dark cycle. The observation that this circadian photo-entrainment was preserved in some blind individuals (Czeisler et al., 1995) as well as in mice lacking rods and cones (Freedman et al., 1999; Lucas et al., 1999) has led to the discovery of an entirely new photoreceptor system. Indeed, we now know that a small subset of retinal ganglion cells express an opsin/vitamin A-based photopigment, called melanopsin. This photopigment is maximally sensitive to short wavelengths (peak sensitivity ~ 480 nm) and renders these cells intrinsically photosensitive (Berson et al., 2002; Brainard et al., 2001). Intrinsically photosensitive retinal ganglion cells (ipRGCs) were demonstrated to be strongly involved in the mentioned non-image forming effects of light on pupil diameter (Lucas et al., 2001), enhancement of mood and alertness (Lockley et al., 2006), and modulation of circadian rhythms (Thapan et al., 2001). Given these strong and important effects, a quantitative assessment of functionality of the melanopsin-signaling pathway could be highly relevant in order to mechanistically understand individual differences in the effects of light on the regulation of mood, alertness and circadian rhythms.

The pupillary light reflex may provide the most feasible non-invasive method to assess functionality of the melanopsin-signaling pathway. The reflex is mediated through direct connections between ipRGCs and the Olivary Pretectal Nucleus (Hattar et al., 2006), the nucleus that controls pupil size (Trejo and Cicerone, 1984). However, this reflex is not exclusively driven by the melanopsin-signaling pathway, but also highly dependent on the input from rods and cones (Lall et al., 2010). Still, there is one characteristic of the pupillary light reflex that is specific to the melanopsin-signaling pathway. In contrast to rods and cones, ipRGCs show a delayed repolarization after light offset, resulting in a sustained pupil constriction. This phenomenon has been dubbed ‘post-illumination pupil response’ (PIPR) (Dacey et al., 2005). The PIPR that can be recorded following exposure to bright blue light is almost entirely attributable to ipRGC activity (Gamlin et al., 2007; Markwell et al., 2010) and can therefore be used to estimate functioning of the melanopsin signaling pathway (Park et al., 2011). Several studies suggest that the processing of light by the ipRGC may be altered in disorders including diabetes type II (Feigl et al., 2012), neuroretinal visual loss (Kardon et al., 2009), glaucoma (Kankipati et al., 2011), and seasonal mood disorder (Roeklein et al., 2013). In order to be of value in case–control, intervention, and mechanistic studies, it is of great importance to assess the PIPR according to a maximally reliable standardized protocol. Test–retest reliability of PIPR assessment has previously been evaluated for two other specific protocols and was rated as moderate to high (Herbst et al., 2011; Lei et al., 2015). The light stimuli in these two paradigms were of short duration (i.e., 400 ms and 20 s). In view of the characteristic low sensitivity and slow kinetics of ipRGCs, however, longer stimulus duration allows for more specific assessment of the melanopsin-signaling pathway (Berson et al., 2002; Do et al., 2009). Accordingly, previous animal work showed that phase shifts in circadian rhythms were larger with a 300-s light stimulus compared to light stimuli with a shorter duration (Nelson and Takahashi, 1991). In addition, these circadian phase shift effects did not grow any further with extending the light stimulus beyond 300 s. Others explained this saturation effect by showing that light adaptation of ipRGCs was completed after 300 s of light exposure (Wong et al., 2005). We therefore here propose a both feasible and

reliable PIPR assessment protocol using prolonged light exposure with a duration of 300 s.

We first assessed the within-subject between-day test–retest reliability. Because the pupil response to light is dependent on many inputs, in part originating from the autonomic nervous system (Heller et al., 1990), we moreover addressed sensitivity of our PIPR assessment protocol to four possible confounders: 1) Dark adaptation, to check whether the eyes were dark adapted and pupil diameter was stabilized prior to the light exposure (PIPR is quantified relative to pre-exposure pupil diameter); 2) Time of day (i.e., morning vs. afternoon), to test whether the protocol provides similar estimates across office hours; 3) Body posture, to check whether the test could be applied in both upright and supine position (i.e., application in bed-ridden participants and in magnetic resonance imaging environments (Chellappa et al., 2014)); 4) Environmental light history, to confirm that the outcome measures were unaffected by previous light exposure, which is of relevance with respect to planning of experimental and clinical evaluations. Previous studies (Gooley et al., 2012; Mure et al., 2009, 2007; Wong et al., 2005) showed an effect of short-term light history on pupil response. We here add to these findings by assessing also long-term effects of prior light exposure (i.e., from 24-h prior to the test).

## 2. Methods

To evaluate and validate our PIPR protocol, two experiments were performed, using similar light exposure and pupillometry procedures. The aim of the first experiment was to estimate the within-subject, between-day test–retest reliability. The second experiment aimed to evaluate possible effects of dark adaptation, time of day, body posture, and environmental light history on the outcomes of the PIPR protocol.

### 2.1. Participants

In total 25 healthy young adults were recruited by advertisement and word of mouth. These 25 participants were distributed over the two experiments as follows: 2 of the 25 participated in both experiments, 13 of the 25 in experiment 1 only, and 10 of the 25 solely in experiment 2. All participants were in good health, free of medication, non-smoking, and had neither sleep complaints nor a history of ocular pathology, as indicated by the Duke clinical Structured Interview for Sleep Disorders (Edinger et al., 2004). All participants worked regular office hours and did not travel across time-zones for at least a month prior to participation. Results from the Munich Chronotype Questionnaire (MCTQ) showed that none of the participants was an extreme chronotype (mean mid-sleep on free days  $\pm$  SD: 5:05 AM  $\pm$  1:05) and all in the center part of the normative distribution for the age range of our participants (5:00 AM  $\pm$  1:23) (Zavada et al., 2005). According to Nagel anomaloscope tests none of the participants suffered from color vision deficiency. Participants received oral and written information on the study, signed informed consent before study participation, and did not receive any incentive. The study was approved by the Medical Ethical Committee of the VU University Medical Center Amsterdam (protocol NL43319.029.13) and adhered to the tenets of the Declaration of Helsinki.

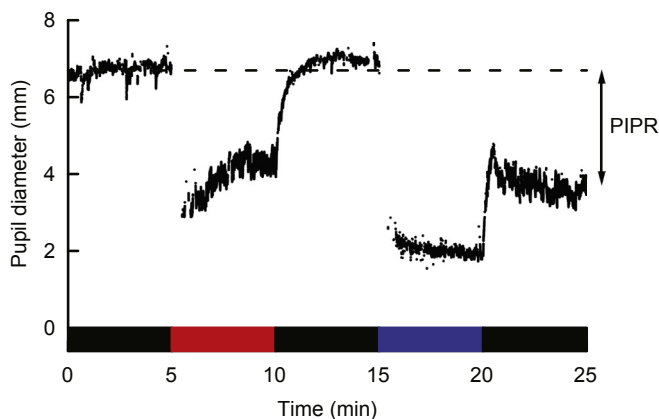
### 2.2. Light exposure protocol

Our light exposure protocol was designed to obtain a prolonged steady-state PIPR after blue light, with a high signal-to-noise ratio (Mure et al., 2009). In order to obtain maximal stimulation of the melanopsin-based phototransduction system, the pupil of the right eye was dilated using 0.5% tropicamide (Nissen et al., 2011). In

accordance with previous work (Mure et al., 2009, 2007), the right eye was first pre-exposed to five minutes of bright monochromatic red light (LED Cree C503B-RAS, Durham, NC, USA; peak wavelength (full width half maximum): 630 (20) nm; luminance: 375 cd/m<sup>2</sup>) in order to maximize the PIPR after blue light (Supplementary Material, Fig. S1). The right eye was subsequently exposed to 5 min of bright monochromatic blue light (LED Cree C503B-BAN, Durham, NC, USA; peak wavelength (full width half maximum): 470 (20) nm; luminance: 375 cd/m<sup>2</sup>). Wavelength and luminance of the light stimuli were calibrated using a spectrometer (AvaSpec-3648-USB2, Avantes, Apeldoorn, The Netherlands). Both monochromatic lights were transmitted through a diffuser and presented in free view. There were 5-min blocks of darkness before (here labeled 'baseline'), between, and after ('post blue') the light blocks (Fig. 1).

### 2.3. Pupillometry

Participants had little to no experience with ophthalmological tests and were naïve to the experimental paradigm (i.e., there was no rehearsal trial). They were placed in front of a custom-made infrared pupillometry set-up built around a printed circuit board charge coupled device camera (Sony d2463r, Sony Electronics Inc., San Diego, CA, USA). Participants were asked to focus on a fixation target, integrated in the set-up in front of their left eye. This fixation target was projected at infinity to prevent accommodation. The eyes were separated by a septum. The pupil diameter of the left eye was measured throughout the entire protocol using infrared radiation. The pupil was illuminated from the lateral side with an 880 nm infrared radiation emitting diode, in such a way that the pupil appeared as a black disk in a bright iris on the camera. Visible radiation was blocked by a Wratten 87 gelatin filter (Kodak, Rochester, NY, USA). The images were digitized at 25 Hz with a USB frame grabber (Grabby, Terratec, Alsdorf, Germany) and analyzed real time with custom software written in C++ using the OpenCV image analysis library (Itseez, Nizhny Novgorod, Russia). Missing data points (e.g. due to eye blinks) were interpolated using nearest neighbors interpolation. Pupil diameter was assessed continuously from the baseline block until the post-blue block. During baseline darkness the pupil diameter remained stable over the entire block.



**Fig. 1.** Example of the change of pupil diameter in the left eye throughout the light exposure protocol. The bar in the bottom indicates when the right eye was exposed to light; black = darkness, red = monochromatic red light, blue = monochromatic blue light. The dashed line represents mean pupil diameter during the first dark block (i.e., baseline pupil diameter). Post-Illumination Pupil Response (PIPR) indicates the difference between baseline pupil diameter and the mean pupil diameter during the last dark block (i.e., post-blue pupil diameter) and can be expressed either as the absolute difference in mm (PIPR-mm, here 2.99 mm) or the percentage change from baseline (PIPR-%, here 44.7%).

During post-blue darkness we observed in several assessments that during the first minute after light offset the pupil first dilated to an intermediate level before it constricted again to a level at which the constriction was sustained (Fig. 1). Similar pupil behavior has been shown previously after cessation of bright light stimuli with durations of 2 min (Newsome, 1971) and 3 min (Alpern and Campbell, 1963). These complicated dynamics are a result of the interaction between the image forming and non-image forming photoreceptors after light offset (Dacey et al., 2005; Gamlin et al., 2007). Another between-trial variation was observed during the final minute of the post-blue block: in most trials the pupil constriction was maintained over the entire minute, but in some trials the pupil already started redilating towards baseline size within this minute. In view of these observed inter-assessment differences in pupil dynamics, the first and last minute of the post-blue block were excluded from the analysis: we used the averaged pupil diameter over minutes 2 to 4 of the post-blue block. In order to optimize the comparison between the baseline and post-blue dark block we decided to use equal metrics for both blocks. Accordingly, we calculated the averaged pupil diameter over minutes 2 to 4 of the baseline block. From the baseline and post-blue pupil diameter we calculated two PIPR outcome parameters (Kankipati et al., 2011; Roeklein et al., 2013).

1. PIPR-mm = baseline pupil diameter – post-blue pupil diameter
2. PIPR-% = 100 \* PIPR-mm/baseline pupil diameter

### 2.4. Experiment 1

#### 2.4.1. Procedures

Fifteen participants (6 males, 9 females, mean age  $\pm$  SD: 27.8  $\pm$  4.3 yr) underwent the PIPR assessment in upright position twice on consecutive days. Both tests within one participant were at the same time of day. Between participants this time point ranged from 09:00 AM to 16:30 PM. Measurements were performed using the same set-up at two different locations: ten participants (5 males, 5 females, mean age  $\pm$  SD: 26.1  $\pm$  3.3 yr) were assessed at the Netherlands Institute for Neuroscience in Amsterdam and five (1 male, 4 females, mean age  $\pm$  SD: 31.2  $\pm$  4.1 yr) at PsyQ Expertise Center Adult ADHD in The Hague.

#### 2.4.2. Statistical analysis

Mixed effect regression models were used to assess whether between-subject differences in PIPR-mm and PIPR-% were confounded by the time point of measurement and the assessment location. Mixed effect models are optimally suited to account for nested data structures. The data were structured in a 2-level hierarchy: PIPR outcome parameters were measured in two assessments that were nested in fifteen participants. Time point and location were included in the model as regressors. The significance of their estimated effects was evaluated using the Wald test and Likelihood-ratio tests were performed to compare models (Twisk, 2013).

Cronbach's  $\alpha$  was calculated to examine the within-subject reliability of the PIPR assessment (Cronbach, 1951). Values of Cronbach's  $\alpha > 0.90$  are considered as satisfactory for clinical application (Bland and Altman, 1997). To examine test–retest reliability, we computed the two-way random effects single measures intraclass correlation coefficient (ICC) for absolute agreement (Shrout and Fleiss, 1979). Bland–Altman plots were made to visually inspect test–retest reliability (Bland and Altman, 1986). Data processing was conducted using MATLAB (Version R2013A, The MathWork Inc, Natick, MA). Statistical analyses were conducted using the software packages 'lme4' (Bates et al., 2013), 'cocron'

(Diedenhofen, 2013), and 'ICC' (Wolak et al., 2012) for R (Version 3.1.1, R Foundation for Statistical Computing, Vienna, Austria).

## 2.5. Experiment 2

### 2.5.1. Procedures

Twelve participants (6 males, 6 females, mean age  $\pm$  SD:  $26.5 \pm 3.6$  yr) underwent the PIPR assessment twice with 3 days between assessments. An RGB multiband light sensor (Dimesimeter, Rensselaer Polytechnic Institute, Troy, NY, USA) (Bierman et al., 2005), integrated in a brooch, was worn to assess environmental light spectrum and intensity exposure history over 24 h prior to the start of each assessment. The ability of the light sensor to measure light in multiple bands enabled us to take the composition of the light into account. The separate output values from the red, green, and blue band were weighted, based on the sensitivity of the ipRGC network for each bandwidth, and integrated into a single light exposure parameter, which was quantified as: irradiance, spectrally weighted for effects on circadian rhythm (weighted  $W/m^2$ ) (Rea et al., 2005).

To investigate effects of dark adaptation duration and stabilization of baseline pupil diameter, the start of the light exposure protocol was delayed by either 0, 5, or 10 min in darkness ( $0 \text{ cd/m}^2$ ), randomly assigned to the different participants, resulting in dark adaptation durations of 5, 10 or 15 min. If the eyes are sufficiently adapted to the dark environment within 5 min, baseline pupil diameter would not change with further extension of the dark exposure duration. Otherwise, ongoing dark adaptation would result in a larger baseline pupil diameter with increasing delay.

To investigate potential time-of-day effects of the PIPR outcomes during office hours, one trial started in the morning (09:00 AM) and the other in the afternoon (01:00 PM). To assess the effect of body posture on the PIPR outcomes, one assessment was performed in upright (i.e. sitting) and the other in supine position. In supine position the pupillometry set-up was placed above the participant's head in such a way that the angle and distance relative to the eyes were similar to the upright position. The orders of posture and time of day were counterbalanced across participants. At the start of each trial, participants were placed in the upright or supine position. Room lights were dimmed for 30 min ( $0.5 \text{ cd/m}^2$ ) and subsequently the light exposure protocol was commenced.

### 2.5.2. Statistical analysis

Mixed effect regression models were used to analyze the repeated assessments of PIPR-mm and PIPR-%, and the effects of dark adaptation, time of day, posture and light history. To dissect pre- and post-exposure effects, the same models were performed on baseline and post-blue pupil diameter. The assessed data represented a 2-level hierarchy: variables were measured in two assessments that were nested in twelve participants. Dark adaptation duration, time of day, posture, and 24-h environmental light history were added to the model as regressors. All analyses were performed using the R-package 'lme4'.

## 3. Results

### 3.1. Experiment 1

Interindividual differences in PIPR outcome measures were confounded neither by time point of measurement (PIPR-mm:  $\beta = 0.09$ ,  $SE = 0.10$ ,  $P = 0.37$ ; PIPR-%:  $\beta = 0.9$ ,  $SE = 1.0$ ,  $P = 0.38$ ) nor by assessment location (PIPR-mm:  $\beta = 0.49$ ,  $SE = 0.52$ ,  $P = 0.36$ ; PIPR-%:  $\beta = 7.8$ ,  $SE = 5.0$ ,  $P = 0.15$ ).

Cronbach's  $\alpha$  was larger than 0.90 for both PIPR-mm and PIPR-% (Table 1). The ICC point estimations for PIPR-mm and PIPR-% were

both  $> 0.85$ , which indicates almost perfect test–retest reliability. Bland–Altman plots for both parameters indicate almost zero bias between the two assessments (Fig. 2).

### 3.2. Experiment 2

#### 3.2.1. Dark adaptation duration

PIPR-mm was smaller ( $P = 0.04$ ), and PIPR-% had a tendency to be smaller ( $P = 0.09$ ), with increasing dark adaptation duration prior to the first light exposure (Table 2). This was caused by a decrease in baseline pupil diameter with increasing dark adaptation duration prior to the start of the light exposure protocol ( $P = 0.02$ ). Post-blue pupil diameter was not affected by dark adaptation duration ( $P = 0.99$ ).

#### 3.2.2. Time of day

No differences in PIPR-mm ( $P = 0.11$ ) and PIPR-% ( $P = 0.22$ ) were found between the morning and afternoon assessments (Fig. 3).

#### 3.2.3. Body posture

PIPR-mm ( $P = 0.01$ ) and PIPR-% ( $P = 0.02$ ) were larger during assessments in a supine posture relative to an upright posture. The difference was explained by a larger baseline pupil diameter during the supine posture ( $P = 0.007$ ), whereas the post-blue pupil diameter did not change with posture ( $P = 0.81$ ).

#### 3.2.4. Environmental light history

The 24-h mean environmental spectrally weighted irradiance per subject was on average  $0.16$  ( $SD = 0.03$ ) weighted  $W/m^2$ . Previous environmental light history did not affect PIPR-mm ( $P = 0.72$ ) and PIPR-% ( $P = 0.75$ ).

## 4. Discussion

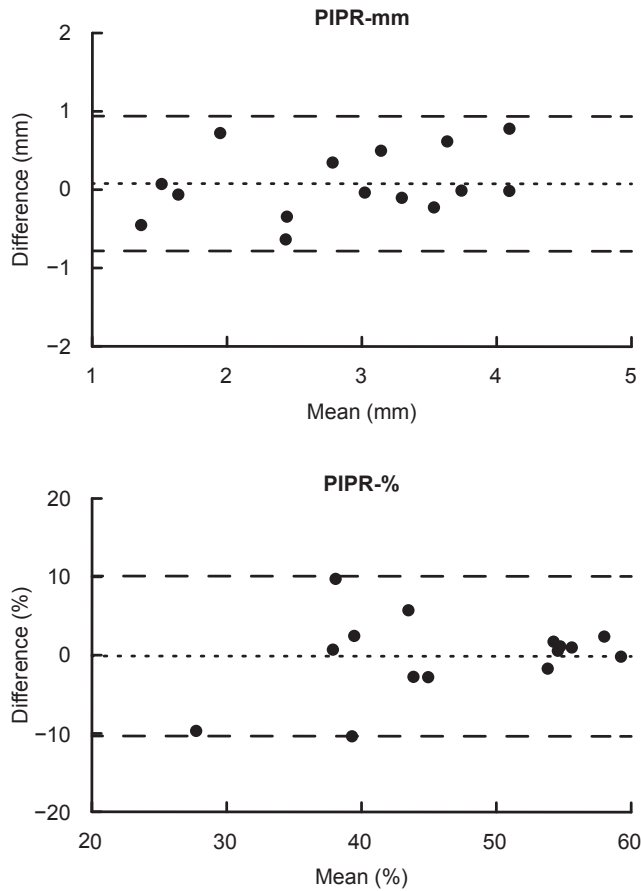
The aim of the present study was to present and validate a PIPR protocol with high robustness and reliability to assess interindividual differences in the melanopsin-signaling pathway. The two proposed outcome parameters both showed a Cronbach's  $\alpha > 0.90$  and an ICC  $> 0.85$ , indicating a very high reliability. PIPR outcome parameter estimates were larger in the supine position, due to its dilating effect on the pupil diameter during baseline darkness. Outcome measures were not affected by dark adaptation, time of day, and environmental light history.

Single measure reliability of PIPR assessment using monochromatic short-wavelength light ( $\sim 470 \text{ nm}$ ) was evaluated in two previous studies. One study reported an ICC of 0.80 when using 20-s light stimulation ( $300 \text{ cd/m}^2$ ) twice with 3 min between sessions (Herbst et al., 2011). In the other study PIPR was induced by exposing specific retinal areas to a 400-ms light stimulus ( $400 \text{ cd/m}^2$ ) (Lei et al., 2015). All areas were stimulated three times each with several minutes between sessions. The ICC was 0.84 for full-field and 0.87 for central-field stimulation. These PIPR protocols with relative short durations may be more user-friendly. Others moreover showed that comparable stimulus durations were sufficient to detect group differences (Feigl et al., 2012; Kankipati et al., 2011; Roeklein et al., 2013). Light stimuli of longer duration, as we evaluated here, however allow for a more specific targeting of the melanopsin-signaling pathway; ipRGCs are less photosensitive and have a slower photoresponse than rods and cones (Berson et al., 2002; Do et al., 2009). In addition, the brief stimuli applied in previous work evoked a transient PIPR (i.e., the post-exposure pupil diameter returned to baseline already during the assessment), while the prolonged stimulus in our protocol induced a PIPR that remained stable during the entire 5-min post-blue assessment interval. This high PIPR robustness in our protocol may explain the

**Table 1**  
PIPR outcome parameters from session 1 and 2 and test–retest reliability outcomes.

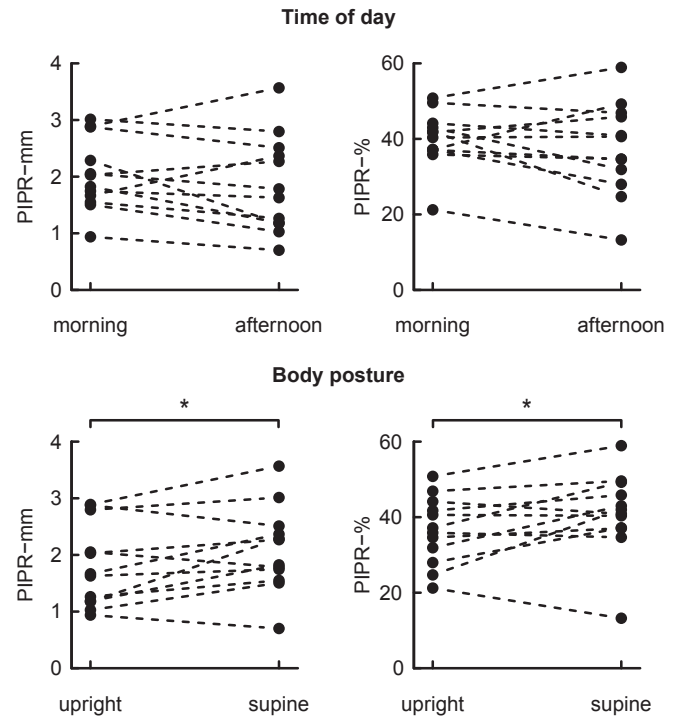
Parameter	Session 1			Session 2			Cronbach's $\alpha$ (95% CI)	ICC (95% CI)	Bland–Altman bias (95% limits of agreement)
	Mean $\pm$ SD	Min	Max	Mean $\pm$ SD	Min	Max			
PIPR-mm	2.88 $\pm$ 1.01	1.14	4.49	2.81 $\pm$ 0.88	1.48	4.10	0.95 (0.84–0.98)	0.90 (0.74–0.96)	0.08 (–0.78 to 0.94)
PIPR-%	46.9 $\pm$ 10.4	22.9	59.2	47.0 $\pm$ 9.0	32.6	59.3	0.93 (0.78–0.98)	0.87 (0.67–0.95)	–0.11 (–10.3 to 10.1)

PIPR, Post-Illumination Pupil Response; ICC, Intraclass Correlation Coefficient.



**Fig. 2.** Bland–Altman plots for PIPR-mm and PIPR-%. Differences between the two measurements on two consecutive days (i.e., day 1 minus day 2) are plotted against the mean of the two measurements. The bias (dotted line) is the mean difference between all measurements on the first day and all measurements on the second day. The 95% limits of agreement (dashed lines) define the range between which 95% of the differences between measurements by the two devices will lie. The smaller the limits of agreement, the better the agreement between two measurements.

high ICC values of 0.90 for PIPR-mm and 0.87 for PIPR-% with a relatively large interval of 24 h between sessions. Although our protocol takes longer to complete than previously proposed



**Fig. 3.** Effects of the factors time of day and body posture on the outcome parameters PIPR-mm and PIPR-%. In the plots for both time of day (top row) as well as body posture (bottom row) each dashed line connects the two repeated measures within a participant to visualize the difference between the two conditions. Horizontal lines with an asterisk (\*) above a plot indicate a significant effect of the factor on the outcome parameter ( $P < 0.05$ ).

protocols, and may thus be slightly more difficult to accomplish in clinical settings, this duration seems necessary to obtain a robust assessment that is not sensitive to the onset and offset variability discussed in the introduction section (Alpern and Campbell, 1963; Dacey et al., 2005; Gamlin et al., 2007; Newsome, 1971). We would even suggest that it could be interesting for future studies, to extend the post-blue assessment interval and quantify the time course of normalization of the pupil diameter, as a further probe to individual differences in ipRGCs kinetics (Gooley et al., 2012). An example of such normalization curve is provided in the

**Table 2**  
Estimates of the effects of dark adaptation duration, time of day, body posture, and light history on PIPR outcome parameters and baseline and post-blue pupil diameter.

	PIPR-mm	PIPR-%	Baseline pupil diameter (mm)	Post-blue pupil diameter (mm)
Intercept	1.79 $\pm$ 0.32***	38.97 $\pm$ 5.11***	4.62 $\pm$ 0.32***	2.93 $\pm$ 0.33***
Dark adaptation duration (/hour)	–1.96 $\pm$ 0.87*	–27.91 $\pm$ 14.98	–1.98 $\pm$ 0.72*	0.02 $\pm$ 1.00
Time of day (morning vs. afternoon)	0.17 $\pm$ 0.10	2.22 $\pm$ 1.74	0.17 $\pm$ 0.08	–0.01 $\pm$ 0.12
Body posture (supine vs. upright)	0.29 $\pm$ 0.10*	4.34 $\pm$ 1.69*	0.26 $\pm$ 0.08**	–0.03 $\pm$ 0.11
Light history (weighted W/m <sup>2</sup> )	0.51 $\pm$ 1.40	–7.89 $\pm$ 23.92	1.54 $\pm$ 1.17	0.43 $\pm$ 1.59

Mean values  $\pm$  SE are displayed. PIPR, Post-Illumination Pupil Response.  
\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

supplementary material (Fig. S2).

Both PIPR-mm and PIPR-% met the test–retest criteria for clinical use (Bland and Altman, 1997), and the potential of our PIPR protocol as a diagnostic tool was furthermore expressed by the consistency of these outcome parameters assessed at different regular office hours. Caution is however needed when performing the test outside these hours, since previous studies showed that PIPR was altered during the evening and night as a result of a modulation of melanopsin-based phototransduction (Figueiro et al., 2005; Munch et al., 2012; Zele et al., 2011). We found that both PIPR outcome measures were dependent on body posture, secondary to the effect of posture on the baseline pupil diameter during darkness. This should be taken into account when performing future research on the effects of light when subjects have to maintain a supine position such as in magnetic resonance imaging studies (Chellappa et al., 2014). Previous work found a smaller pupil diameter in a supine position than in a sitting position, in agreement with cardiovascular studies showing less sympathetic activation in a supine position (Lee et al., 2007). We found the opposite, and consider that this indication of increased sympathetic activity could be caused by ‘fighting against sleep’ (van der Meijden et al., 2015). Such seemingly paradoxical changes have also been reported in EEG beta-power, which indexes central nervous system activation (Ramautar et al., 2013). Because of the dependency of PIPR measures on baseline pupil diameter it is recommended to reporting them as well in any PIPR study.

We found that PIPR was not affected by 24-h environmental light history. This indicates that correction for previous light exposure is not necessary, which simplifies the implementation of PIPR assessment. To our knowledge we are the first to assess the effects of 24-hr light history on PIPR. Previous work on short-term light history did show ipRGC modulation by previous light exposure: 5 min of prior long-wavelength light increased the pupil response to blue light, while prior short-wavelength light blunted this response (Mure et al., 2009, 2007). The lack of effect of prior light exposure on our outcome measures should not be interpreted as absence of effects of light history; rather, it indicates that our approach is robust to differences in light history.

We found a decline in baseline pupil diameter with increasing dark adaptation duration prior to the start of the light exposure protocol indicating that the eyes were adapted to the dark using 5 min of darkness. If dark adaptation was incomplete the pupil would be growing instead of declining with an extension of the baseline dark period. The enhancement of pupil constriction over time spent in the dark may be caused by increasing sleepiness (Lowenstein and Loewenfeld, 1964), but this effect is not expected to be large enough to be able to mask a possible effect of uncompleted dark adaptation. We therefore assume that 5 min of dark pre-exposure is both required and adequate for PIPR assessment.

The high reliability of our PIPR assessment protocol renders it a sensitive tool for research on group differences in human ipRGC functioning and ipRGC modulation in response to intervention, allowing for acceptable sample sizes. In view of ipRGC projections to the biological clock and brain areas involved in sleep/wake regulation (Hattar et al., 2006), it would be interesting to assess the association between PIPR and interindividual differences in circadian phase. Interesting patient populations for future PIPR research include not only patients with ophthalmological diseases but also patients with disorders associated with sleep and alertness complaints (e.g., insomnia, narcolepsy, mood disorders and attention deficit/hyperactivity disorder (Kooij and Bijnenga, 2014)).

A possible limitation of our study could be that we used a period

of only 5 min between red and blue light exposure, which may not have been sufficient to completely exclude rod and cone interference on the priming effect of red light (Mure et al., 2009). We however felt that a dark period of 5 min ruled out most rod and cone interference while preserving feasibility of the test. Accordingly, in future studies it may be interesting to investigate whether the PIPR after blue light assessment can be further enhanced by increasing the intermediate dark period or, alternatively, by using other priming light exposures. Accordingly, others showed that using low light levels the sustained pupil constriction after light offset was enhanced by using intermittent green light instead of continuous stimulation, which was explained by increased cone activation (Gooley et al., 2012). We however do not expect that implementing intermittent light stimuli in our paradigm would further increase the PIPR outcome parameters: the intensity and duration of our light stimulus are expected to already saturate ipRGCs (Wong et al., 2005). Whereas in our protocol acute effects of cones are likely minimal, because we excluded the first minute after light offset and moreover the red light pre-exposure, it could be most interesting to add complementary paradigms using intermittent light to dissect ipRGC activity from rods and cones to the pupillary light reflex. In future studies it is therefore interesting to include multiple light exposure protocols in order to make a multivariate finger print of rod, cone, and ipRGC involvement in the PIPR.

Experiment 1 was performed at two different environments by different experimenters. We observed no systematic differences when comparing data from both settings, which supports the applicability of our PIPR paradigm at multiple sites. Experiment 2 was limited by the lack of objective measurements of circadian rhythms (e.g., melatonin levels (Cajochen et al., 2003)). Individual differences in sleep/wake rhythm may lead to different sleepiness levels, which may be a potential bias in the assessment of the time-of-day effects on pupil diameter (Lowenstein and Loewenfeld, 1964). In view of the absence of extreme chronotypes in our population, however, we expect circadian sleep/wake timing to be similar in all participants. Another limitation of experiment 2 was that during the 30-min habituation period prior to the start of the light exposure protocol, room lights were set at a very dimmed level instead of switching them completely off. This small amount of light was maintained in order to prevent participants from falling asleep, especially in supine condition (Romeijn et al., 2012). Complete darkness may have been preferred in order to maximize repolarization of rods and cones in order to increase their response to the light exposure protocol. Although the dim light exposure was standardized, it may have biased the pupillary light reflex as a result of variance in membrane potentials of rods and cones (Lall et al., 2010). The contribution of rods and cones to the pupillary light reflex is particularly substantial during stimulation and immediately after stimulus offset (Dacey et al., 2005; Gamlin et al., 2007). We however included pupil diameter measures from 1 min after light offset and therefore do not expect that our PIPR measures were affected by using dim light during the habituation period instead of darkness. A limitation of both experiments in this study was that we only included young healthy adults. Future studies can use the proposed assessment protocol to evaluate the reliability of the proposed PIPR assessment protocol in clinical, and in pediatric or older populations. In conclusion, the present protocol can reliably quantify the PIPR outcomes to evaluate ipRGC functioning in case–control and intervention studies.

#### Conflicts of interest

No conflicting relationship exists for any author.

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## Appendix A. Supplementary material

Supplementary material related to this article can be found at <http://dx.doi.org/10.1016/j.exer.2015.07.010>.

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