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Effects of mydriatics on rod/cone- and melanopsin- driven pupil responses

--Manuscript Draft--

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Abstract:	Significance: Pupillometry protocols evaluating rod/cone- and melanopsin-driven responses often utilize mydriatics to ensure maximal stimulus exposure; however, retinal effects of mydriatics are not fully understood. We demonstrate that dilation with either atropine or phenylephrine results in similar enhancements of rod/cone- and melanopsin- driven pupil responses. Purpose: To compare effects of atropine, a muscarinic antagonist, and phenylephrine, an adrenergic agonist, on consensual pupil responses, and to assess repeatability of pupil metrics without mydriasis. Methods: Right eye pupil responses of 20 adults, aged 21-42, were recorded before and 45 minutes after instillation of 0.5% atropine or 2.5% phenylephrine in the left eye. Stimuli were presented to the left eye and included six alternating 1 second (s) 651 nm “red” and 456 nm “blue” flashes. Metrics included baseline pupil diameter, maximum constriction, 6 s and 30 s post illumination pupil responses, and early (0-10 s) and late (10-30 s) areas under the curve. Results: Dilation of the stimulated eye with either mydriatic significantly increased the 6 second post illumination pupil response and early and late areas under the curve for blue stimuli, and early area under the curve for red stimuli ($P < .05$ for all). Melanopsin-driven post illumination pupil responses, achieved with either phenylephrine or atropine, did not significantly differ from each other ($P > .05$ for all). Without mydriasis, intersession intraclass correlation coefficients for pupil metrics were 0.63 and 0.50 (6 s and 30 second post illumination pupil responses, respectively), and 0.78 and 0.44 (early and late areas under the curve, respectively) for blue stimuli, with no significant difference between sessions ($P > .05$ for all). Conclusion: Dilation with phenylephrine or atropine resulted in similar enhancements of the rod/cone- and melanopsin-driven pupil responses, despite differing mechanisms. Early pupil metrics without mydriasis demonstrated moderate to good intersession repeatability.

Synopsis image

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Second Revision

Editors' comments:

The reviewers make some good points and I advise the authors address these in a revised manuscript.

Pupil measurements are reported to the tenth of a micron. Please round to the nearest .1 mm in the tables and text.

Completed

In accordance with the journal instructions for authors, please eliminate the PIPR acronym and spell out this phrase.

Completed

I am accepting this provisional upon receiving your satisfactory response to these issues.

Reviewers' comments:

Thank you for your effort to revise the manuscript. I believe the quality of the paper improved a lot.

A few issues to address:

- Line 10-11: The description is not correct. ipRGC does not fire only after short wavelength stimulus. It's more sensitive to short-wavelength, but it still response to any light stimulus depending upon the luminance level.

Thank you for your comments. We have corrected this oversight.

'Single cell recordings demonstrate sustained firing of intrinsically photosensitive retinal ganglion cells after light offset when melanopsin is activated.[15] This contributes to the observed, *in vivo*, melanopsin-driven post illumination pupil response which is characterized by a sustained pupil constriction following light offset. Melanopsin is most sensitive to short wavelength stimuli.'

- Line 179: "MATLAB filtering" is not clear enough. Please describe what kind of filtering method was applied.

This sentence has been re-phrased to draw attention to the detailed filtering details within the methods. (Line 116-119)

'For figure 2, pupil diameter data were visually inspected subsequent to filtering by a custom written MATLAB program described in the methods. Any remaining points that were identified as artefacts (i.e. due to blinks) were manually removed prior to averaging.' (Line 180-183)

- Line 218-226: I have a little problem with this approach. As many researchers do, it's possible to modulate the PLR based on the main contributor (cone, rod, or melanopsin).

But the problem is that ipRGC is a main conduit of most PLR and if certain factor affect ipRGC, in this case, dopamine, it will affect ALL PLRs, not not only melanopsin-mediated component. Some of the description here and introduction seem to confuse the role of ipRGC and melanopsin; they are close but not identical. ipRGC still control most of PLRs whether melanopsin is triggered or not. Maybe the authors make the argument a bit more clear by mentioning this

Thank you for pointing out this distinction. We have now clarified the role of ipRGCs in both rod/cone and melanopsin driven pupil pathways in several locations throughout the manuscript.

In the introduction at line 9, we have added the statement, “The intrinsically photosensitive retinal ganglion cells are the main conduit of the light mediated afferent pupil pathway for both rod/cone- and melanopsin- driven pupil responses.”

We have replaced “ipRGCs” with “melanopsin” in line 15, “Melanopsin is most sensitive to short wavelength stimuli.”

We have added at Line 227 that all light information is carried to higher pupil centers via iprgcs, “In this study, we examined the effects of different mydriatic drugs on the melanopsin-driven post illumination pupil response, as well as the rod/cone-driven pupil response. For both rod/cone- and melanopsin-driven pupil responses, light information is primarily carried from the retina to the olivary pretectal nucleus via the intrinsically photosensitive retinal ganglion cells.”

At line 265, we added “rod/cone-driven pupil response…” to make the point that the rod/cone pathway also goes through ipRGCs, “Future research evaluating the effects of various concentrations of atropine on the rod/cone-driven pupil response and melanopsin-driven post illumination pupil response would be valuable to determine the nature of atropine’s interactions with intrinsically photosensitive retinal ganglion cells.”

In the abstract, we added “rod/cone-“ in addition to “melanopsin-“, in referring to which pupil metrics were assessed in this study, “Pupillometry protocols evaluating rod/cone- and melanopsin-driven responses often utilize mydriatics to ensure maximal stimulus exposure…”

Finally, the title was changed to reflect the reviewer’s comment, and now reads, “Effects of mydriatics on the rod/cone- and melanopsin-driven pupil responses”

Effects of mydriatics on rod/cone- and melanopsin- driven pupil responses

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Short title**:** Effects of mydriatics on consensual pupil responses

Word count: 4105

Tables: 3

Figures: 4

ABSTRACT

Significance: Pupillometry protocols evaluating rod/cone- and melanopsin-driven responses often utilize mydriatics to ensure maximal stimulus exposure; however, retinal effects of mydriatics are not fully understood. We demonstrate that dilation with either atropine or phenylephrine results in similar enhancements of rod/cone- and melanopsin- driven pupil responses.

Purpose: To compare effects of atropine, a muscarinic antagonist, and phenylephrine, an adrenergic agonist, on consensual pupil responses, and to assess repeatability of pupil metrics without mydriasis.

Methods: Right eye pupil responses of 20 adults, aged 21-42, were recorded before and 45 minutes after instillation of 0.5% atropine or 2.5% phenylephrine in the left eye. Stimuli were presented to the left eye and included six alternating 1 second (s) 651 nm “red” and 456 nm “blue” flashes. Metrics included baseline pupil diameter, maximum constriction, 6 s and 30 s post illumination pupil responses, and early (0-10 s) and late (10-30 s) areas under the curve.

Results: Dilation of the stimulated eye with either mydriatic significantly increased the 6 second post illumination pupil response and early and late areas under the curve for blue stimuli, and early area under the curve for red stimuli ($P < .05$ for all). Melanopsin-driven post illumination pupil responses, achieved with either phenylephrine or atropine, did not significantly differ from each other ($P > .05$ for all). Without mydriasis, intersession intraclass correlation coefficients for pupil metrics were 0.63 and 0.50 (6 s and 30 second post illumination pupil responses, respectively), and 0.78 and 0.44 (early and late areas under the curve, respectively) for blue stimuli, with no significant difference between sessions ($P > .05$ for all).

Conclusion: Dilation with phenylephrine or atropine resulted in similar enhancements of the rod/cone- and melanopsin-driven pupil responses, despite differing mechanisms. Early pupil metrics without mydriasis demonstrated moderate to good intersession repeatability.

Melanopsin containing retinal ganglion cells are a unique, intrinsically photosensitive, subset of ganglion cells located in the inner and outer regions of the inner plexiform layer.[1] They serve as irradiance detectors and have a maximum sensitivity to short-wavelength light (approximately 482nm).[2,3] In addition to intrinsic melanopsin stimulation, photic information is integrated from extrinsic rod and cone pathways via synaptic connections with bipolar and dopaminergic amacrine cells.[4–9] Intrinsically photosensitive retinal ganglion cells are known to project to multiple brain regions including the hypothalamic suprachiasmatic nucleus to facilitate circadian photo-entrainment, the pretectal olivary nucleus to regulate pupil size, and the lateral geniculate nucleus of the thalamus for image forming visual functions.[7,10–14] The intrinsically photosensitive retinal ganglion cells are the main conduit of the light mediated afferent pupil pathway for both rod/cone- and melanopsin- driven pupil responses.

Single cell recordings demonstrate sustained firing of intrinsically photosensitive retinal ganglion cells after light offset when melanopsin is activated.[15] This contributes to the observed, *in vivo*, melanopsin-driven post illumination pupil response which is characterized by a sustained pupil constriction following light offset. Melanopsin is most sensitive to short wavelength stimuli.[15] Post illumination pupil responses can be quantified through chromatic pupillography, which, as a biomarker for melanopsin function, is increasingly employed in clinical and research areas of ophthalmology, psychology and chronobiology.[16] Melanopsin-driven post illumination pupil responses have not been found to vary with age or refractive error.[17,18] However, altered melanopsin function has been demonstrated in ocular pathologies including glaucoma,[19–21] age-related macular degeneration,[22,23] diabetes,[24,25] and retinitis pigmentosa.[26]

The broad application, and the wide variability of pupillography protocols, motivated a recent review outlining minimum standards in pupillography.[27] Pupil status, i.e. whether the pupil has undergone pharmacological mydriasis during pupillography, is an important variable discussed within this aforementioned review. A natural pupil will fluctuate in size during stimuli

presentations, subsequently altering retinal irradiance.[16] This is particularly problematic when Newtonian, full field, stimuli are presented. Retinal irradiance can be controlled by presenting Maxwellian stimuli, by using artificial pupils or by dilating the stimulated eye with mydriatics whilst recording the consensual pupil response.[27] Maxwellian apparatus is typically custom-built therefore, dilation is often favored. Dilation is achieved using, either alone or in combination, muscarinic antagonists, such as tropicamide, cyclopentolate, or atropine, or alpha-adrenergic agonists, such as phenylephrine. The extent to which these mydriatic drugs differentially influence retinal physiology is not fully understood.[28,29]

Atropine eye drops are increasingly prescribed to reduce myopia progression in children.[30–32] However, the exact mechanism by which atropine protects against myopia is unknown. It has been hypothesized that atropine may function to control myopia through a dopaminergic pathway via a retinal neurochemical cascade.[29,33] Interestingly, retinal dopamine has been implicated in the protection against myopia.[28,34] In addition, retinal dopamine concentration has been found to increase with light exposure[35] and by intravitreal injections of atropine to the chick eye.[28] Retinal dopamine is diurnally released from dopaminergic amacrine cells, in part, via 'light' signals from intrinsically photosensitive retinal ganglion cells,[36–38] and has been linked to the regulation of melanopsin mRNA.[39] Therefore, intrinsically photosensitive retinal ganglion cells may be implicated in the mechanism by which atropine constrains eye growth. If so, instillation of muscarinic antagonists prior to pupillometry may present a confounding factor when evaluating melanopsin function.

The present study utilized Newtonian stimuli to examine the effects of two different mydriatic agents (atropine 0.5%, a muscarinic antagonist, and phenylephrine 2.5%, an adrenergic agonist) on rod/cone- and melanopsin- driven post illumination pupil responses. While enhancement of consensual pupil responses is anticipated with both mydriatic agents due to higher retinal irradiance, differential, drug-specific effects may also be postulated resulting from the differing drug mechanisms. Phenylephrine has no documented myopia control effects

or interactions with dopaminergic or melanopsin pathways, and will act as a control in this experiment. Understanding the effects of these mydriatics is important in protocol development. Differences in mydriatic effects may elucidate interactions between muscarinic, adrenergic, and melanopsin pathways. The intersession repeatability of rod/cone- and melanopsin-driven pupil metrics without dilation was also investigated in the present study, providing valuable information for chromatic pupillometry studies where mydriasis is contraindicated or unavailable.

Methods

Twenty healthy adults, aged 21-42 years, were recruited from the University of Houston's College of Optometry faculty, staff and student population. The study was approved by the institutional review board at the University of Houston and followed the tenets of the Declaration of Helsinki. Interested individuals were fully informed on the procedures and written consent was obtained.

Initial lab visits were scheduled between 9:00 am and 4.30 pm. Repeat sessions were scheduled at the same time of day for each subject to minimize effects of circadian variation on the post illumination pupil response.[40] Visual acuity was measured with habitual correction, and an anterior eye exam using slit lamp biomicroscopy was performed to confirm open anterior chamber angles and suitability for dilation. Best corrected visual acuity for all subjects was 20/25 or better. No subjects had ocular pathology, nor had they been dilated in the five days prior to the experiment. No subjects were taking prescription or recreational drugs known to affect pupil size or sleep, and no subjects reported being pregnant or breastfeeding.

Experimental protocol

Each subject underwent two experimental sessions. At the first visit, spherical equivalent refraction was calculated for each eye following non-cycloplegic autorefraction (WAM-5000, Grand Seiko, Japan), and axial length and pupil diameter were determined (LenStar, Haag-Streit, Germany). Following these measures, non-mydriatic pupillometry was performed. For pupillometry, stimuli were presented to the left eye, and the consensual pupil response was

measured in the right eye. The left eye was then dilated with either 2.5% phenylephrine (Paragon BioTeck, USA) or 0.5% atropine (Greenpark Compounding Pharmacy, Houston, TX, USA). An atropine concentration of 0.5% was chosen to minimize recovery time between visits whilst still eliciting a significant effect on the pupil. The pharmacological agent used at the first session was randomized. Two drops of the selected mydriatic were delivered five minutes apart to the left eye. After a 45-minute dilation period, diameter of the dilated left pupil was measured, and pupillometry was repeated. To allow drug wash-out, visit two was scheduled at least five days later if phenylephrine 2.5% had been instilled first, and at least ten days later if atropine 0.5% had been instilled first.

Pupillometry procedure

The pupillometry protocol has been described in detail elsewhere.[41] Subjects were fitted with a frame mounted 60 Hz infrared illumination eye tracker (ViewPoint EyeTracker, Arrington Research, USA) to record pupil diameter of the right eye. The system provides better than 0.03 mm resolution for pupil diameter. The infrared light emitting diode light source has a lambda max of 943 nm with a half-max width of 46 nm (Spectrometer, Ocean Optics, USA). At the start of each session, the camera was positioned and focused on the iris, and pupil diameter was calibrated by capturing an image of a 5 mm printed black circle positioned close to the subject's corneal plane. Following calibration, the room lights were switched off, and subjects dark adapted behind a black-out curtain for five minutes (<0.1 lux). The five minute dark adaptation period allowed adaptation of cones; rods and ipRGCs were not expected to be fully adapted. Subjects were then instructed to place their head on a chinrest with a light emitting diode-driven Ganzfeld system (Color Burst, Espion, Diagnosys LLC, USA) centered 10 mm in front of the left eye. Subjects viewed a red fixation point at approximately 3 m with the right eye; the single red fixation point was used to minimize accommodation cues and preclude a light-driven pupil response. Baseline pupil diameter was recorded for 10 seconds, then six alternating 1 second long wavelength "red" and short wavelength "blue" Newtonian stimuli were presented to the left

eye, with a 60 second interstimulus interval (Figure 1). Red stimuli, always presented first, were 651 nm with a half-max width of 25 nm (Spectroradiometer CS1W, Konica Minolta, USA) and set to 33.3 cd/m^2, and with a measured corneal irradiance of 5.58 x 10^{13} photons $cm^{-2}s^{-1}$ (Power Meter, Newport, USA). The pupillary light reflex to red stimuli is known to be primarily driven by medium and long wavelength cones. Blue stimuli were 456 nm (half-max width of 20 nm) and set to 16.67 cd/m^2, with a measured corneal irradiance of 5.85 x 10^{13} photons $cm^{-2}s^{-1}$. These intensities of red and blue stimuli were chosen as they have similar photon flux and elicit similar pupil constriction. The pupillary light reflex to blue stimuli is driven by rods, short, medium, and long wavelength cones, and the intrinsically photosensitive retinal ganglion cells.[42] The blue stimulus used in the present study is above the melanopsin threshold.[10,43] Previous findings show that these intensities of red and blue stimuli elicit approximately equal pupil constriction when the stimulated eye is dilated with both 2.5% phenylephrine and 1% tropicamide.[44]

Data analysis

Raw pupil data were analysed off-line using a custom program (MATLAB, The MathWorks, Inc., USA). Blinks were identified as intervals of pupil aspect ratio outside 6 standard deviations of the mean pupil aspect ratio during stable fixation and were removed from the data file along with samples that were deemed poor quality by the instrument. Individual data were then exported to an Excel file (Microsoft Office 2013). Data for the three red stimuli were averaged together, and data for the three blue stimuli were averaged together. Pupil metrics used to evaluate the pupil response included baseline pupil diameter, relative maximum constriction, relative 6 s post illumination pupil response, relative 30 s post illumination pupil response, and early and late area under the curve, defined in Table 1. The baseline pupil diameter was calculated by averaging pupil diameter during the 10 s recording period prior to the first red stimulus. Relative responses (maximum constriction, 6 s post illumination pupil response, and 30 s post illumination pupil response) were calculated based as the percentage change from baseline. The 6 s and 30 s post illumination pupil response were calculated as the

pupil size averaged over 6–7 s and 30–31 s, respectively, after each stimulus offset. Early and late areas under the curve were computed for the intervals post stimulus offset 0 to 10 s and 10 to 30 s, as the trapezoidal approximation of the integral of 100% minus the interpolated percent pupil diameter (i.e., the difference between the pupil and baseline) for the respective intervals.

Statistical analysis

Statistical analysis was performed in SPSS (SPSS, IBM Corp., USA). Data are presented as mean ± standard deviation. Data were analyzed for normality using the Shapiro-Wilk test. Parametric data were analyzed using a paired sample t-test. Non-parametric data were analyzed using a related-sample Wilcoxon signed rank test. In all instances, $P < 0.05$ was considered statistically significant. A two-way mixed effects single measurement intraclass correlation coefficient for absolute agreement was calculated to determine the repeatability of non-mydriatic pupil metrics, and interpreted based on recently published guidance.[45] The intrasession and intersession pupil metrics for red and blue stimuli were calculated and compared across four conditions: non-mydriatic pupillometry during the phenylephrine session (from this point on referred to as non-mydriatic session 1), non-mydriatic pupillometry during the atropine session (non-mydriatic session 2), 45 minutes post-phenylephrine instillation, and 45 minutes post-atropine instillation. Pupil metrics were compared using a paired sample t-test or appropriate non-parametric test where indicated.

Results

One subject's data were excluded from analysis due to extreme fluctuations in the demarcation of the pupil boundary during all sessions. The remaining subjects (n = 19) had a mean age of 28.1 ± 5.1 years and included 6 males and 13 females. Mean spherical equivalent refraction of right eyes was -1.91 ± 2.08 D (range -5.75 to +1.87 D) and of left eyes was -2.11 ± 2.22 D (range -6.31 to +1.62 D), with no significant difference between eyes ($P = .09$). Mean axial length of right eyes was 24.33 ± 1.21 mm (range 22.49 to 27.57 mm) and of left eyes was

24.31 ± 1.22 mm (range 22.35 to 27.65 mm), with no significant difference between eyes (*P* = .64).

Pupil diameter in photopic room illumination (approximately 400 lux) prior to non-mydriatic pupillometry was 4.8 ± 0.8 mm for the left eye and 4.7 ± 0.8 mm for the right eye, with no significant differences between eyes (*P* = .46). The pupil diameter of the left eye 45 minutes after dilation with phenylephrine increased to 6.3 ± 1.1 mm (*P* < .0001), and after dilation with atropine increased to 8.2 ± 0.5 mm (*P* < .0001). Pupil diameter after atropine was significantly larger than after phenylephrine (*P* < .0001). Pupil area under photopic conditions of the left eye during non-mydriatic sessions was 18.0 mm^2, after phenylephrine was 30.7 mm^2, and after atropine was 53.2 mm^2.

For non-mydriatic conditions, following 5 minutes of dark adaptation, the right eye pupil diameter increased to 6.2 ± 1.1 mm (for non-mydriatic session 1, *P* < .0001), and to 6.1 ± 0.6 mm (for non-mydriatic session 2, *P* < .0001) with no significant differences between right eye pupil diameters during non-mydriatic sessions prior to stimulus onset (*P* = .63). When the left eye was dilated with phenylephrine, dark adapted right eye pupil diameter was 6.1 ± 0.9 mm, and when the left eye was dilated with atropine, dark adapted right eye pupil diameter was 6.2 ± 0.7 mm. These pupil diameters were not significantly different from each other (*P* = .9) or from their respective non-mydriatic measures (*P* = .34 and .3 respectively). Dark adapted pupil diameter of the left eye prior to pupillometry was not measured as the light stimulus equipment was placed in front of the left eye precluding imaging.

For all sessions, pupils re-dilated rapidly following red stimulus offset, and re-dilated at a slower rate following blue stimulus offset, i.e. pupils demonstrated an enhanced post illumination pupil response following blue stimuli, which is the signature for a melanopsin-driven pupil response. These dynamics resulted in a larger percentage for 6 s and 30 s post illumination pupil response, and a larger value for early and late area under the curve for blue stimuli versus red stimuli for all conditions. Dynamic pupil responses for all pupillometry

sessions are presented in Figure 2. For figure 2, pupil diameter data were visually inspected subsequent to filtering by a custom written MATLAB program described in the methods. Any remaining points that were identified as artefacts (i.e. due to blinks) were manually removed prior to averaging. Relative response diameters for the three red stimuli were averaged together, and diameters for the three blue stimuli were averaged together. Associated pupil metrics are shown Table 2.

Non-mydriatic session 1 versus 2

Non-mydriatic sessions were conducted on separate days to assess repeatability. There was no significant difference in the time of day of the visits ($P = .89$). Pupil metrics did not differ significantly across non-mydriatic sessions ($P > .05$ for all metrics). Intersession intraclass correlation coefficient [95% confidence interval] demonstrate moderate to good repeatability for maximum constriction, 6 s post illumination pupil response, and early area under the curve for both red and blue stimuli (Table 3). The 30 s post illumination pupil response for red stimuli and the late area under the curve for blue stimuli revealed an intraclass correlation coefficient value below 0.5, indicating poor repeatability. The 95% confidence intervals also suggest poor repeatability of the 30 s post illumination pupil response for blue stimuli and late area under the curve for red stimuli. Bland-Altman analysis examining the agreement of repeated non-mydriatic measures demonstrates close to zero bias between sessions (i.e. the mean difference between sessions is close to zero) and good agreement for maximum constriction, 6 s post illumination pupil response, and early area under the curve for red and blue stimuli (Figure 3).

Non-mydriatic versus phenylephrine session

Following phenylephrine induced mydriasis of the stimulated eye, maximum constriction of the consensual pupil increased significantly for red ($P < .0001$) but not for blue ($P = .1$) stimuli. The consensual post illumination pupil response was enhanced, as seen by a significantly higher 6 s post illumination pupil response ($P = .045$ for red stimuli; $P = .01$ for blue stimuli), and early ($P = .01$ for red stimuli; $P = .001$ for blue stimuli) and late area under the

curve values (*P* = .03 for red stimuli; *P* = .01 for blue stimuli). Differences in the 30 s post illumination pupil response did not reach significance (*P* = .06 for red stimuli; *P* = .47 for blue stimuli) (Table 2, Figure 4).

Non-mydriatic versus atropine session

Following atropine induced mydriasis of the stimulated eye, maximum constriction of the consensual pupil increased for red (*P* = .05) and blue (*P* = .18) stimuli, but neither increase was statistically significant. The consensual post illumination pupil response was enhanced for blue stimuli as seen by the significantly higher 6 s and 30 s post illumination pupil response (*P* = .03 and .01, respectively), and early and late area under the curve values (*P* = .01 and .02, respectively). The early area under the curve also significantly increased for red stimuli (*P* = .047) (Table 2, Figure 4).

Phenylephrine versus atropine session

Maximum constriction was not significantly different after phenylephrine compared to after atropine for red or blue stimuli (*P* = .36 and .69 respectively). For blue stimuli, there were no significant differences between phenylephrine and atropine post illumination pupil response metrics. For red stimuli, the 6 s and 30 s post illumination pupil response were significantly higher (*P* =.04 and .02 respectively) after phenylephrine compared to atropine.

Discussion

In this study, we examined the effects of different mydriatic drugs on the melanopsin-driven post illumination pupil response, as well as the rod/cone-driven pupil response. For both rod/cone- and melanopsin- driven pupil responses, light information is primarily carried from the retina to the olivary pretectal nucleus via intrinsically photosensitive retinal ganglion cells. As expected, dilation of the stimulated eye with either mydriatic (phenylephrine, an adrenergic agonist, or atropine, a muscarinic antagonist) enhanced several consensual post illumination pupil metrics. Interestingly, there were no significant differences between the effects of atropine 0.5% and phenylephrine 2.5% on the melanopsin-driven pupil response, despite greater dilation

and higher levels of retinal irradiance achieved with atropine. Furthermore, we demonstrated that rod/cone- and melanopsin- driven pupil metrics, assessed without mydriatics, show moderate to good intersession repeatability and agreement when Newtonian stimuli are presented.

In light of the emerging role of atropine in myopia management,[46] we hypothesized that through evaluating the influence of different mydriatics on the melanopsin-driven post illumination pupil response, insight may be gained into the mechanism by which atropine acts on axial growth regulation. Dilation with either phenylephrine or atropine increased post illumination pupil metrics to red and blue stimuli (Table 2). The increase in maximum constriction was statistically significant with phenylephrine dilation and for red stimuli only. This is unlikely to be a clinically significant result. Statistically significant increases were also noted for early metrics (< 10 seconds following stimulus offset) of the post illumination pupil response to blue stimuli. Blue stimuli activate both rod/cone- and melanopsin-driven pupil pathways. For a 1 second blue stimulus, the pupil response up to 1.7 seconds post stimulus is attributed to major inputs from rods and intrinsically photosensitive retinal ganglion cells, with minimal cone contribution.[47] Intrinsically photosensitive retinal ganglion cells are distributed throughout the retina with wide dendritic coverage.[48] Previous research has shown the post illumination pupil response increases with stimulus intensity[15,44,49] and pupil size.[50] Therefore, it is to be expected that with a larger pupil size of the stimulated eye, a greater number of intrinsically photosensitive retinal ganglion cells will be directly activated by the blue stimulus, and the melanopsin-driven post illumination pupil metrics enhanced, as observed here.

Remarkably, dilation of the stimulated eye with either mydriatic resulted in comparable enhancement effects despite a 32% larger photopic pupil diameter following dilation with atropine compared to phenylephrine. It is possible that saturation of the melanopsin-driven photoresponse occurred and resulted in a maximal post illumination pupil response with phenylephrine dilation (6.3 mm). However, our previous study,[18] as well as others,

demonstrates that a stronger post illumination pupil response can be elicited with higher stimulus intensity, so it is unlikely that the response was saturated at the stimulus intensity used here. Alternatively, atropine instillation may have inhibited the expected boost in post illumination pupil response with increased pupil size. Future research evaluating the effects of various concentrations of atropine on the rod/cone-driven pupil response and melanopsin-driven post illumination pupil response would be valuable to determine the nature of atropine's interactions with intrinsically photosensitive retinal ganglion cells. Future studies should include low dose atropine (0.01%), which is suggested to control myopia via a neurochemical cascade that begins with muscarinic receptors in the retina, with the aim of elucidating potential retinal sites of atropine's action.[29] To control pupil size, custom built Maxwellian presented stimuli, or artificial pupils, should be employed to standardize retinal irradiance within and between subjects.[16,27]

Phenylephrine and atropine produce mydriasis through different mechanisms, with phenylephrine stimulating the dilator muscle and atropine blocking the sphincter muscle, as well as the ciliary muscle, leading to mydriasis in conjunction with cycloplegia. As a consequence, accommodative tone in the stimulated eye will have differed between the two mydriatic protocols in the present study. However, it is unlikely that accommodation in the fixating, consensual eye was affected. The experimental set up was designed to minimize stimulating accommodation in the fixating eye.

It has been suggested that mydriatics are not necessary in pupillometry protocols if the intensity of the light stimulus is sufficiently bright; Bruijel, et al. (2016) intensified blue stimuli to 15.11 log photon flux without mydriasis and revealed reasonable agreement to an earlier protocol which incorporated mydriasis.[51] Authors also reported that pupillometry without mydriasis had a very high test-retest reliability for post illumination pupil metrics across consecutive days and across seasons; albeit, reliability was lower across seasons compared to across consecutive days.[51] The blue stimulus in the present study was 13.77 log photon flux,

presented over a wide visual field of approximately 140 degrees, and was shown to be sufficiently bright to elicit a melanopsin-driven pupil response. The results of the present study provide further evidence that recording the post illumination pupil response without mydriasis of the stimulated eye is reliable and repeatable and should be acceptable if pharmacological pupil dilation is contraindicated or unavailable. Protocols without mydriasis have fewer ethical and risk assessment considerations, and benefits include conserving research time and minimizing both ocular and systemic risks of mydriatic drug instillation. In addition, standardizing retinal irradiance by presenting Maxwellian stimuli or by using artificial pupils will likely boost repeatability metrics of non-mydriatic protocols.

While we show that non-mydriatic pupil metrics did not differ significantly across sessions, some variability was present (Table 3). The intraclass correlation coefficient suggests moderate to good repeatability for early metrics of the post illumination pupil response (6 s post illumination pupil response and early area under the curve) and poor repeatability for late metrics of the response (30 s post illumination pupil response and late area under the curve). We speculate that early metrics are predominantly driven by melanopsin activation, whereas later metrics are influenced by autonomic tone once intrinsically photosensitive retinal ganglion cells decrease firing, and therefore subject to greater variability. Another factor contributing to variability is habitual light exposure. Abbott, et al. (2018) demonstrated that an enhanced post illumination pupil response was evident with greater habitual light exposure in adult participants.[18] Similarly, Ostrin (2018) showed that the 6 s post illumination pupil response and early area under the curve to high intensity blue stimuli were associated with light exposure in the 24 hours prior to pupillometry in children.[44] Prior light exposure may explain the variability across sessions and between subjects. Light exposure data were not collected in this experiment and should be considered in future research. Furthermore, pupil size can be affected by several other variables, including age, attention, accommodative tone, fatigue, and autonomic input, including alterations in systemic adrenaline circulation.[52,53] Whilst intrinsic

inputs to the pupil cannot be entirely eliminated, efforts were made to minimize these factors. All experiments were conducted in a controlled dark environment, with fixation directed at a minimally accommodative target, mydriatic selection was randomized, and pupillometry was performed at the same time of day.

In conclusion, dilation with either phenylephrine 2.5% or atropine 0.5% resulted in similar short-term enhancement effects on rod/cone- and melanopsin- driven pupil responses, despite differing mechanisms of mydriatic action and differential effects on pupil size of the stimulated eye. Furthermore, we have demonstrated that non-mydriatic post illumination pupil metrics within 10 seconds of stimulus offset show moderate to good repeatability and agreement between across different days.

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Figure legends

Figure 1: Pupillometry protocol. Subjects dark adapted for 5 minutes. Baseline (BL) pupil diameter was recorded for 10 seconds (s), then six alternating red or blue 1 second stimuli were presented to the left eye, with a 60 second interstimulus interval (ISI) between each stimulus presentation.

Figure 2: Mean relative pupil diameter of right eyes (n=19) before (-5 s to 0 s), during (0 s to1 s) and after (1 s to 32 s) 1 second red and blue stimuli presented to the left eye for four conditions: A) non-mydriatic and 45 minutes post-phenylephrine, and B) non-mydriatic and 45 minutes post-atropine. Shaded areas represent 95% confidence intervals.

Figure 3: Bland-Altman plots for non-mydriatic sessions 1 and 2 for maximum constriction for red (A) and blue (B) stimuli; 6 s post illumination pupil response for red (C) and blue (D) stimuli; and early area under the curve for red (E) and blue (F) stimuli. Dashed lines represent the mean difference between sessions. Dotted lines represent 95% limits of agreement.

Figure 4: Maximum (Max.) constriction for red (A) and blue (B) stimuli; 6 s post illumination pupil response for red (C) and blue (D) stimuli; and early area under the curve for red (E) and blue (F) stimuli, during each pupillometry condition [non-mydriatic session 1 (NM 1), 45 minutes post-phenylephrine (Phenyl), non-mydriatic session 2 (NM 2), and 45 minutes post-atropine (Atropine)]. *indicates significance at $P \leq .05$ for non-mydriatic compared to mydriatic conditions.

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Table 1: Pupil metrics used to quantify the post illumination pupil response (PIPR)

Pupil metric	Unit	Description
Baseline pupil diameter	mm	Mean dark-adapted pupil diameter 10 s prior to first stimulus
Maximum constriction	% change from baseline pupil diameter	Maximum pupil constriction
6 s PIPR	% change from baseline pupil diameter	Mean pupil diameter 6–7 s after stimulus offset
30 s PIPR	% change from baseline pupil diameter	Mean pupil diameter 30–31 s after stimulus offset
Early AUC	No unit	Integral of 100% minus the interpolated % pupil diameter, 0–10 s after stimulus offset
Late AUC	No unit	Integral of 100% minus the interpolated % pupil diameter, 10–30 s after stimulus offset

Post illumination pupil response (PIPR), area under the curve (AUC)

Table 2: Pupil metrics for 1 second red and blue stimulations during four experimental sessions. Metrics include maximum constriction (% change from baseline), 6 s and 30 s post illumination pupil response (PIPR, % change from baseline), and early and late area under the curve (AUC, unitless).

Pupil Metric	Phenylephrine 2.5%				Atropine 0.5%			
	Non-mydriatic session 1		45 minutes post-phenylephrine		Non-mydriatic session 2		45 minutes post-atropine	
	Red	Blue	Red	Blue	Red	Blue	Red	Blue
Maximum constriction	43.8 ± 5.4	49.4 ± 6.4	46.0 ± 5.4*	50.6 ± 5.4	43.2 ± 6.5	48.9 ± 6.3	45.3 ± 6.9	50.2 ± 7.4
6 s PIPR	10.6 ± 4.3	26.9 ± 8.9	12.7 ± 4.5*	31.0 ± 9.2*	10.1 ± 3.8	28.3 ± 6.8	11.4 ± 3.5	32.3 ± 8.4*
30 s PIPR	4.7 ± 5.4	6.6 ± 4.4	6.8 ± 5.4	7.30 ± 4.0	4.3 ± 3.5	5.2 ± 2.7	4.6 ± 3.0	7.3 ± 3.3*
Early AUC	1.7 ± 0.4	3.1 ± 0.8	1.9 ± 0.4*	3.5 ± 0.8*	1.6 ± 0.4	3.2 ± 0.6	1.8 ± 0.4*	3.6 ± 0.7*
Late AUC	1.0 ± 0.9	2.1 ± 0.9	1.4 ± 0.9*	2.6 ± 1.0*	1.0 ± 0.7	1.9 ± 0.9	1.2 ± 0.6	2.6 ± 0.9*

Post illumination pupil response (PIPR), area under the curve (AUC), *$P < .05$ for non-mydriatic versus mydriatic conditions

Table 3: Intraclass correlation coefficient [95% confidence interval] for pupil metrics compared across non-mydriatic sessions 1 and 2 on different days. Metrics include maximum constriction, the 6 s and 30 s post illumination pupil response (PIPR) and early and late areas under the curve (AUC).

	Intraclass Correlation Coefficient	
Pupil Metric	Red	Blue
Maximum constriction	0.83 [0.61 to 0.93]	0.77 [0.50 to 0.91]
6 s PIPR	0.59 [0.20 to 0.82]	0.63 [0.26 to 0.84]
30 s PIPR	0.30 [-0.19 to 0.66]	0.50 [0.09 to 0.77]
Early AUC	0.62 [0.24 to 0.84]	0.78 [0.52 to 0.91]
Late AUC	0.53 [0.10 to 0.79]	0.44 [-0.02 to 0.74]

Post illumination pupil response (PIPR), area under the curve (AUC)

Figure 1


5 min
Dark
10 s
BL
1 s
Red
60 s
ISI
1 s
Blue
60 s
ISI
1 s
Red
60 s
ISI
1 s
Blue
60 s
ISI
1 s
Red
60 s
ISI
1 s
Blue
60 s
ISI

Figure 2

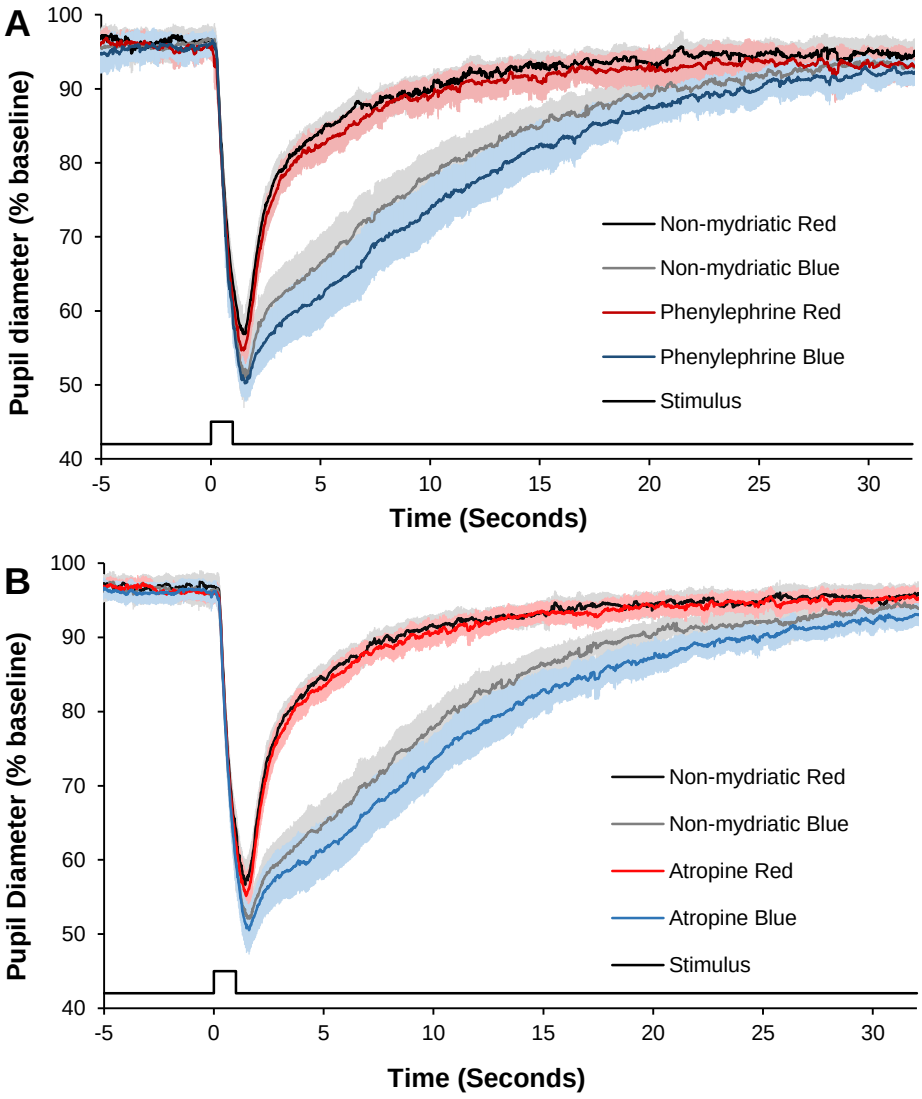

A
Pupil diameter (% baseline)
100
90
80
70
60
50
40
-5
0
5
10
15
20
25
30
Time (Seconds)
Non-mydriatic Red
Non-mydriatic Blue
Phenylephrine Red
Phenylephrine Blue
Stimulus
B
Pupil Diameter (% baseline)
100
90
80
70
60
50
40
-5
0
5
10
15
20
25
30
Time (Seconds)
Non-mydriatic Red
Non-mydriatic Blue
Atropine Red
Atropine Blue
Stimulus

Figure 3

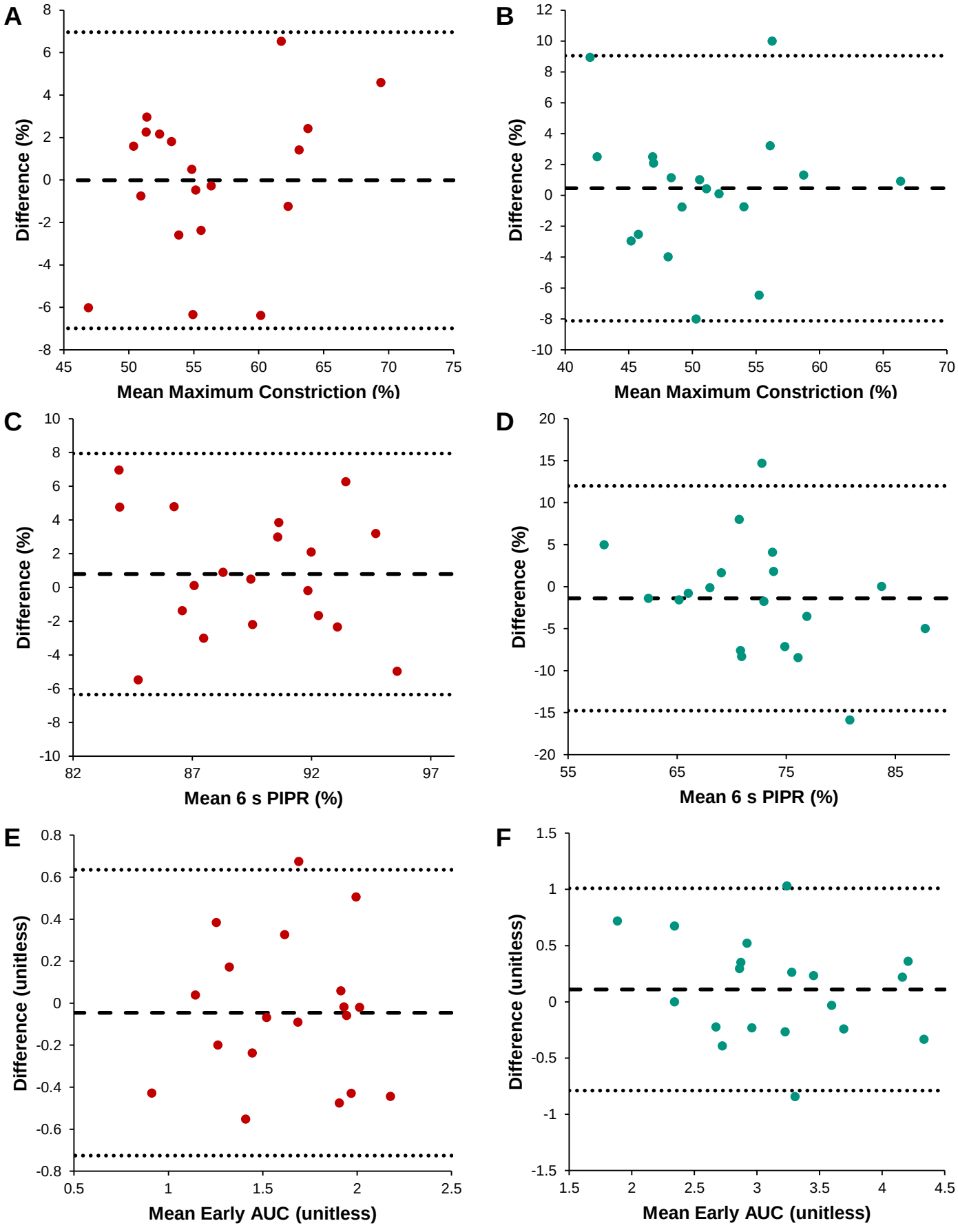
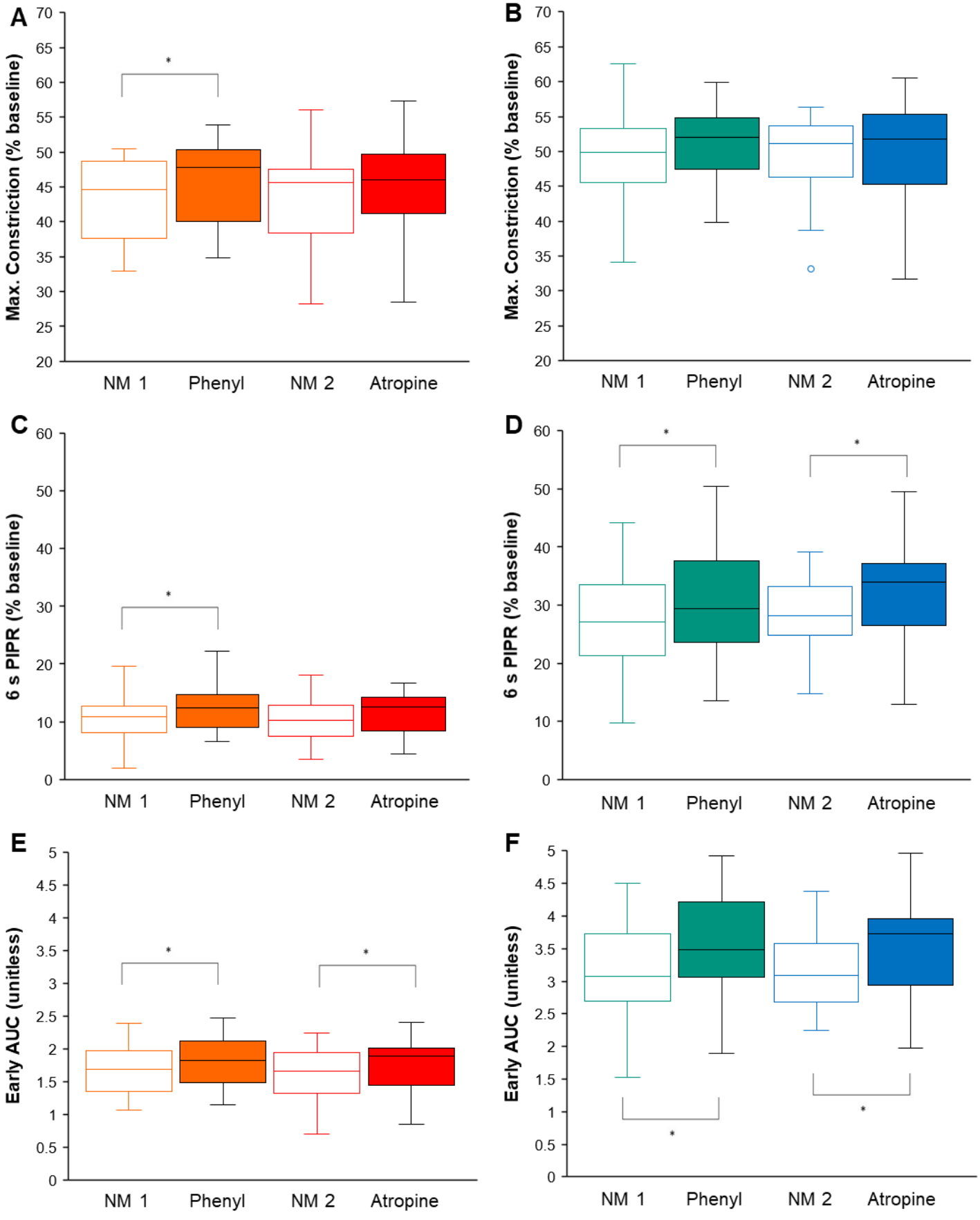

A
Difference (%)
Mean Maximum Constriction (%)
B
Difference (%)
Mean Maximum Constriction (%)
C
Difference (%)
Mean 6 s PIPR (%)
D
Difference (%)
Mean 6 s PIPR (%)
E
Difference (unitless)
Mean Early AUC (unitless)
F
Difference (unitless)
Mean Early AUC (unitless)

Figure 4


A
Max. Constriction (% baseline)
70
65
60
55
50
45
40
35
30
25
20
*
NM 1
Phenyl
NM 2
Atropine
B
Max. Constriction (% baseline)
70
65
60
55
50
45
40
35
30
25
20
NM 1
Phenyl
NM 2
Atropine
C
6 s PIPR (% baseline)
60
50
40
30
20
10
0
*
NM 1
Phenyl
NM 2
Atropine
D
6 s PIPR (% baseline)
60
50
40
30
20
10
0
*
*
NM 1
Phenyl
NM 2
Atropine
E
Early AUC (unitless)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
NM 1
Phenyl
NM 2
Atropine
F
Early AUC (unitless)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
NM 1
Phenyl
NM 2
Atropine