Pupillometry reveals autonomic adjustments during diving reflex in face immersion apnea

Rizzuto V.[1,9*†], Montanari R.[2*†], Mesin L.[3], Bortolozzo-Gleich M. H.[4], Laurino M.[5], Bonneh Y.[6], Yellin D.[7], Laganovska G.[8,9], Vanags J.[8,9], Gemignani A[10,11].

*Authors who contributed equally to this work
† Corresponding author.

[1] School of Advanced Science, University of Camerino, Camerino, Italy
[2] Institute of Pharmacology, Heidelberg University Hospital, 69120 Heidelberg, Germany
[3] Department of Electronics and Telecommunications, Mathematical Biology and Physiology, Politecnico di Torino, Turin, Italy
[4] Instituto de Neurociencias UMH-CSIC (Alicante), Avenida Santiago Ramón y Cajal s.n., 03550, Spain
[5] Institute of Clinical Physiology, National Research Council, Pisa, Italy
[6] School of Optometry and Vision Science, Faculty of Life Science, Bar Ilan University, Ramat Gan, Israel
[7] Department of Brain Sciences, Weizmann Institute of Science, Rehovot, Israel
[8] Clinic of Ophthalmology, P. Stradins Clinical University Hospital, Riga, Latvia
[9] Department of Ophthalmology, Riga Stradins University, Riga, Latvia
[10] Department of Surgical, Medical and Molecular Pathology and Critical Care Medicine, Pisa, Italy
[11] Department of Neuroscience, University of Pisa Hospital, Pisa, Italy

Abstract

The human diving reflex (DR), an innate defensive reflex triggered during periods of apnea, concurrently activates both the sympathetic and parasympathetic branches of the autonomic nervous system to regulate physiology against challenging demands. Despite pupil dilation and constriction being antagonistically controlled by the two autonomic branches, the effect of the diving reflex on pupil diameter fluctuations is still unknown. Thus, we compared participants' pupil diameter fluctuations while breathing or performing apnea either with (Wet) or without (Dry) face immersion in cold water. We found that pupil diameter fluctuations in both apneic conditions are associated with lower power in the low-frequency band (< 0.25 Hz) while in the Wet condition, a reallocation of power towards higher frequencies (> 0.25 Hz) occurs, together with an increased entropy, contrary to the remaining conditions. This indicates a shift in autonomic balance and an increased complexity of pupil fluctuations during the diving reflex. Our findings present pupil dynamics as a valuable entry point to the exploration of autonomic adjustments for this reflex.

Introduction

Pupillary physiology extends beyond a simple light-evoked reflex, reflecting complex neurological pathways related to changes in ambient illumination, autonomic activity, and cognitive functions [1, 2, 3, 4, 5, 6, 7, 8, 9]. Moreover, pupil diameter can be monitored non-invasively, serving as a proxy for studying oscillatory responses of the autonomic nervous system [3, 38].

In the absence of external stimuli, mood changes, or fixation adjustments, the pupil exhibits spasmodic, cyclical, and bilaterally synchronous fluctuations known as hippus (or pupil unrest) [10]. The frequency of hippus ranges from 0.047 to 2 Hz [11], with most studies finding a peak frequency around 0.3 Hz [10], and the magnitude of pupil diameter variations ranging from not detectable (usually with naked-eye observation) to over 0.5 mm [12].

The roles of the parasympathetic nervous system (PNS) and sympathetic nervous system (SNS) in regulating iris function and overall pupil diameter are well-documented [9, 13, 14, 41]. The PNS controls the constriction pathway through the iris sphincter muscle, while the SNS governs the dilation pathway via the radial-oriented iris dilator muscle, associated with excitement and the fight-or-flight response [9, 13, 14]. Despite these distinct pathways, the central nervous system can control both in unison [15]. The locus coeruleus activates the sympathetic dilation pathway and inhibits the parasympathetic constriction pathway at the Edinger-Westphal nucleus [14, 15].

Pupillary measurements can thus serve as neurological markers for autonomic, mesencephalic, and even neocortical activity related to attention [42]. As pupil diameter is influenced by the dynamic interplay of SNS and PNS inputs, hippus may arise from their continuous neurological interaction [10, 16, 18].

Despite these insights, the exact mechanisms behind hippus remain elusive, with variable frequency reports and lacking precise definition [16, 10, 19, 20, 21, 22, 23].Indeed, the dynamics of pupil diameter show nonlinear properties which result in complex behavior [23, 24, 25, 27, 28, 39] . Moreover, pupillary fluctuations have been found to correlate with respiratory activity, cardiac activity, breathing patterns , and resting-state cortical activity [18, 21, 23, 28, 40, 74], suggesting a shared neural control of these processes.

The diving reflex (DR) is an autonomic response in humans, evolutionarily originated in vertebrates and conserved in mammals [29, 30, 31]. DR triggers a coordinated set of physiological changes, including bradycardia and peripheral vasoconstriction, engaging both the sympathetic and parasympathetic nervous systems [30, 32]. Its primary function includes the preservation of vital oxygen reserves, prioritizing perfusion to critical organs such as the heart and brain [33]. The DR is significantly influenced by the activation of facial cold receptors [30]. Indeed, cold water immersion amplifies the DR response by inducing profound bradycardia and vasoconstriction, thus conserving oxygen during underwater activities [34, 35].
Given the simultaneous recruitment of SNS and PNS by the DR and their roles in regulating pupil diameter, we investigated whether pupil diameter fluctuations are modulated during DR. To address this question, five free divers participated in an experiment where they performed either controlled breathing or apnea (breath-holding), with and without face immersion in cold water. Pupillary measurements were taken to assess changes in pupil dynamics across these conditions.
The pupillary signals were analyzed in two frequency bands, with specific contributions to the autonomic balance associated with pupillary control assigned to each band: low-frequency (LF, 0.04-0.25 Hz) primarily reflecting SNS contribution, and high-frequency (HF, 0.25-2 Hz) reflecting PNS contribution.

Our results indicate a stronger contribution of the PNS, and a weaker contribution of SNS, to pupil diameter fluctuations, accompanied by increased complexity during DR in face-immersion apnea.

Methods

Participants. The study enrolled 5 healthy male volunteers aged between 30 and 63 years, all of whom were emmetropic and had no comorbidities or medication intake.

Inclusion criteria were: nonsmokers, light caffeine and alcohol consumers, drug-free for at least 2 months with no history of cardiovascular, pulmonary, or neurological/psychiatric diseases.

Participants reported no history of ocular surgeries, ocular traumas, ocular infections, or previous uveitis and provided informed consent for their participation in the experiment. They were categorized based on their experience level in freediving into two distinct groups: “Advanced” and ”Beginner.” The criteria for each group were defined by the duration of freediving experience, maximum static apnea duration, and maximum depth reached in water. Advanced participants had more than three years of freediving experience, could sustain static apnea for over three minutes, and reached freediving depths exceeding 25 meters. In contrast, Beginner participants had less than three years of experience, with static apnea durations below three minutes and depths reached below 25 meters.

All experimental procedures were performed according to the ethical guidelines of the Local Ethical Committees of Pisa University with resolution n. 69/2024 of 17/12/2024 and all athletes gave their informed consent.

Fig 1. Experimental setup. a) Overview of the setup showing, from left to right, a custom-designed operating table with a headrest for head stabilization, a pupillometer with an infrared camera (IRC), an oximeter for measuring oxygen saturation and heart rate, and a transparent glass basin filled with water maintained at a constant temperature of 15°C, monitored via an internal thermometer. Anesthetic eye drops were used for the Wet condition. b) Illustration of the positioning of subjects, who were instructed to lie face down on the operating table

with their heads centered and stabilized using the headrest. The eye tracker was positioned beneath the subjects' faces, and a calibration system was employed. Inset shows an IRC capture of the eye in Wet condition through the water-filled transparent basin.

Design, materials, and measurements. Subjects were instructed to perform controlled breathing, consisting of 4 seconds of inspiration followed by 6 seconds of expiration (Breathing) (6 respiratory cycles per minute), a dry breath-hold (Dry), and a breath-hold with the face submerged in a glass basin of water at 13 degrees Celsius (Wet). In the Breathing condition, an audio track of deep ventilatory noises was given via an audio track to indicate the target respiration rate (inspiration 4 seconds, expiration 6 seconds) at the beginning of the trial for the training, without any change of visual presentation for 3 minutes. Pupillometry was performed using the BioEye eye-tracking solution (https://braineye.com/how-it-works/ and Supplementary), in which acquisition software application was installed on a Xiaomi 9 smartphone, connected to a BioEye's near-infrared (NIR) camera specifically built for optimizing pupil acquisition by preserving wavelengths that enhance the pupil-to-iris contrast in the obtained image. The device emitted a constant infrared light with a wavelength of 850 nm.

We chose the BioEye device for its portability and adaptability, allowing us to customize it for underwater pupillometry at various depths. Initial tests were conducted in a pool to refine the setup, enabling reliable data collection underwater. This portable solution facilitated the transition from preliminary trials to the main experiment, where participants immersed their faces in the water, aligning with the conditions needed to study the diving reflex.

Simultaneously, an integrated NIR camera (optimized for best sensitivity at a wavelength centered at 850 nm) recorded changes in pupil diameter at around 30 frames per second (fps). Heart rate (BPM) and peripheral oxygen saturation data were collected using Gima oxy-2 and Gima oxy-3 pulse oximeters by sampling at 0.1 Hz. An experimental setting was created to minimize movement artifacts caused by contractions and body movements. Each subject was asked to lie horizontally on a customised operating table with a device for head stabilization and to focus on a fixation point located 40 cm in front of them into the eye-tracker structure. Head immobilization was carefully monitored during the course of each experiment. Two to three performances were recorded for each subject for each of the three different experimental conditions (Dry, Wet, Breathing). In the Wet condition, participants submerged their faces in a transparent basin filled with water, keeping their eyes open without using a diving mask. They were instructed to focus on the outline of the eye-tracker. To avoid excessive blinking underwater, drops of topical anesthetic (Benoxinato Cloridrato Intes 4 mg/ml eye drops) were instilled into the ocular surface. Considering that water temperatures affect bradycardia [36], the water in the basin was maintained at a constant temperature of 13 °C, measured with a mercury thermometer, and the water was changed and mixed to reach the target temperature for each trial. The experiment was conducted in a room with controlled lighting (lumen = 70 ± 5).

Apnea metrics. Values related to apnea performance metrics in Tab 1, such as apnea time in different conditions, peripheral oxygen saturation at the end of apnea, and average pupil diameter, were obtained by considering all subject performances and then calculating the mean unless otherwise specified.

Pupillometry inclusion criteria. Pupillometry traces were analyzed quantitatively to exclude traces exhibiting acquisition errors and artifacts. Specifically, a subject's performance was excluded if the median pupil score (obtained via Bioeye software) was less than 8, as per the criterion specified by Bioeye (defined by the sharpness of the iris-to-pupil contrast in the obtained image, for which the threshold value >= 8 indicates an excellent separation level between the darker pupil and its iris peripheral surround). Additionally, for each performance, one of the two eyes was selected for analysis based on the acquisition of the cleanest data, determined by comparing the lower standard deviation of iris measurements between the two eyes.

Pupil-size pre-processing and spectral analysis. The signals related to pupil diameter in the three conditions were processed to estimate their power content in different frequency bands. All analyses were performed using Matlab software (version 2023a). The spectral bands considered were as follows: 0.04-0.25 Hz and 0.25-2 Hz, denoted as low and high frequency, respectively. This subdivision was chosen after a review of the literature, based either on provided compelling evidence [10] or on overlapping ranges with the most often used subdivisions [39] (see Discussion). Given the non-uniform sampling of the signals collected by the eye tracking used, we proceeded as a first step to resample each signal at a constant frequency of 30 Hz, using piecewise cubic interpolation (*pchip* option in Matlab). Blinks were removed from the pupil diameter signal, and the resulting trace was interpolated (again using a piecewise cubic interpolation) to construct a continuous signal (joining the otherwise separated segments of epochs without blinks). Subsequently, data were band-pass filtered between 0.04 and 2 Hz (Chebyshev type II filter, with 0.5 dB of maximum ripple and 25 dB of minimum attenuation below 0.02 Hz and above 2.5 Hz). The power spectral density (PSD) was estimated by Welch's method, performed on temporal windows of 10 seconds (Tukey window with cosine fraction of 0.1) with 50 percent overlap. Then, the power associated with the signal for each band was obtained by integration.

Analysis of complexity. Approximate Entropy (ApEn) is a popular index of complexity introduced to obtain stable information from short and noisy signals [43]. However, careful choice of the parameters is recommended, as ApEn depends on many factors, i.e., the sampling frequency, the embedding dimension, the tolerance (under which a recurrence is identified), the epoch duration, and the presence of low-frequency trends [26]. For this reason, ApEn was here computed with a small embedding dimension (i.e., 2), after removing low-frequency trends and resampling the signals at about 3 times their bandwidths. Specifically, low and high-frequency components were obtained by filtering the already cleaned and band-pass filtered pupil diameter time series (low-frequency component obtained by using a Chebyshev low-pass type II filter, with 0.5 dB of maximum ripple and 20 dB of minimum attenuation, with a transition band between 0.24 and 0.27 Hz; high-frequency component was defined as the difference between the signal and the low-frequency component). Then, the two components were resampled at 0.8 and 8 Hz, for the low and high frequencies, respectively. The tolerance was fixed at 20% of the standard deviation of the processed signal. Moreover, the modified ApEn proposed in [26] was

computed on the same signals: it chooses the tolerance in order to select a number of recurrences equal to a percentage of the total samples (10% was chosen in this work). This way, compared to the classical ApEn, the effect of self-recurrences is much reduced and stable indications in a larger range of parameters have been documented [26].

Statistical analysis. To compare percentages of power spectral density and approximate entropy values in the 3 experimental conditions, one-way analysis of variance (ANOVA) was used after testing for normality (Shapiro-Wilk test). As we performed multiple observations on the same subjects, we used nested statistical testing to account for the reduced degree of freedom. In case of statistical significance, this was followed by a multiple comparison test (Tukey-Kramer test), providing adjusted p-values for pairwise comparisons. The alpha level was set at 0.05.

Results

In Table 1, participants are characterized according to their expertise level and apnea performance. The study involved 5 male subjects: 3 advanced and 2 beginners, with an average age of 39.2 ± 13.91 years (mean ± standard deviation). They achieved an average Dry apnea duration of 139 ± 15.13 seconds and an average Wet apnea duration of 150.8 ± 29.86 seconds.
Despite the low levels of SaO_2 they reached at the end of apnea (87,53 % ±10,9 % in Dry, 93,03 % ± 6,73 % in Wet) no subject had any medical and/or neurological complication as a consequence of the apnea.

Table 1: Summary of Apnea Results

Subject	Sex	Age	Exp. Level	Duration of Dry Apnea (s)	Duration of Wet Apnea (s)	Avg. PD during Breathing (mm)	Avg. PD during Dry Apnea (mm)	Avg. PD during Wet Apnea (mm)	Min SpO_2 in Dry Apnea (%)	Min SpO2 in Wet Apnea (%)
1	M	63	Advanced	140 ± 17	175 ± 49	3.65 ± 0.29	3.23 ± 0.43	3.59 ± 0.35	69.33 ± 7.09	81.5 ± 6.92%
2	M	40	Advanced	163 ± 20	180	4.13 ± 0.62	3.66 ± 0.51	3.66 ± 0.24	89.33 ± 3.21	95 ± 5.20%
3	M	33	Advanced	140 ± 20	160 ± 20	4.45 ± 0.67	4.48 ± 0.44	3.58 ± 0.32	87	93.67 ± 1.53%
4	M	30	Beginner	126 ± 15	126 ± 46	5.09 ± 0.69	5.52 ± 0.59	3.88 ± 0.15	96 ± 3.0	96 ± 2.83%
5	M	30	Beginner	126 ± 11	113 ± 11	4.52 ± 0.31	4.48 ± 0.31	3.98 ± 0.09	96 ± 1.73	99

Table Legend for Abbreviations

Duration of Apnea: Represents the mean values of apnea performance for the subject under wet and dry conditions in seconds ± standard deviation.

PD (Pupil Diameter): Indicates the pupil diameter value expressed in millimeters ± standard deviation.

Min SpO_2 (Minimum Peripheral Oxygen Saturation): Denotes the minimum value of peripheral oxygen saturation at the end of apnea, expressed in percentage ± standard deviation.

During apnea performances, in both Dry and Wet conditions (Fig 2a-b), participants' heart rate showed a decrease-increase pattern, that is reaching a minimum from resting values to then increase around original levels before performance end [mean ± S.E.M. (BPM): start: 66.9 ± 1.5, middle: 62.3 ± 1.8, end: 68.2 ± 2.3], consistent with other descriptions [33, 46, 47]. As expected, this was accompanied by a reduction in SpO_2 during the course of the performance, with pupil diameter showing the typical hippus [10].

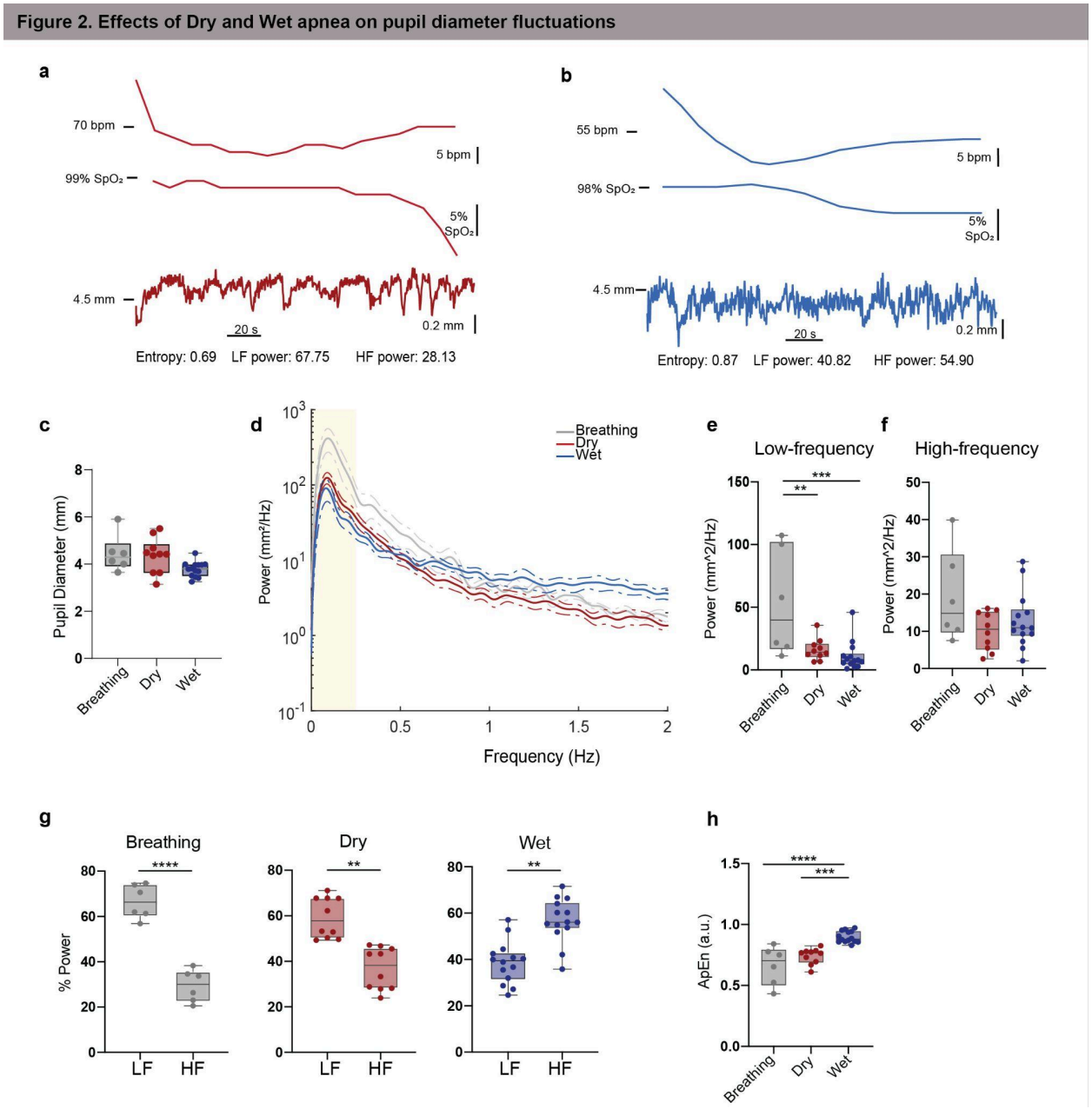

Fig 2. Effects of Dry and Wet apnea on pupil diameter fluctuations a) Example parallel recording of heart rate (upper panel) and oxygen saturation (middle panel) associated with pupil diameter fluctuations and its characterisation (bottom panel) during apnea in Dry condition. b) Same as *a* for apnea in the Wet condition. c) Average pupil diameter in the three experimental conditions . d) Power spectral density (PSD) semi-log plot of pupil diameter fluctuations in the three experimental conditions ± S.E.M (broken lines). Shaded area indicates the selected sympathetic frequency band. e) Statistical comparison of Power contained in the low- frequency band in the three conditions. Each data point represents a single performance. f) Same as *e* for the high-frequency band. g) Comparison of %Power between low- and high-frequencies for each experimental condition. h) Comparison of Approximate Entropy in the three experimental conditions. LF= low-frequency; HF= high-frequency.

Average pupil diameter across single performances (Fig. 2c) tended to be significantly smaller in Wet than in Dry and Breathing conditions (absolute medians' difference (mm): Breathing - Dry=

0.08; Breathing - Wet= 0.54; Dry - Wet= 0.62) [One-way ANOVA: $F(2,27)= 4.03$, $p= 0.0293$], however post-hoc analysis only indicated tendencies for pairwise differences [p(Breathing vs Dry)= 0.9576, p(Breathing vs Wet)= 0.0720, p (Dry vs Wet)= 0.0634], likely due to the relatively low sample size.

To examine whether apneic conditions would alter the hippus compared to breathing, we calculated the Power Spectral Density (PSD) of pupil diameter fluctuations in the three conditions (Fig 2d). In the low-frequency band (Fig 2e), we found that both Dry and Wet apnea contained significantly lower power than Breathing [Nested one-way ANOVA: $F(2,27)= 8.95$, $p= 0.001$; Tukey's multiple comparison: p (Breathing vs Dry)= 0.0054, p (Breathing vs Wet)= 0.0009, p (Dry vs Wet)= 0.8272], indicating a diminished contribution of SNS when breath-holding. However, power in the high-frequency band was not different (Fig 2f) [Nested one-way ANOVA: $F(2,27)= 2.25$, $p= 0.147$].
Despite this statistical similarity, we noticed that the power in Wet was more sustained across the high-frequency band compared to the remaining conditions. Thus, we hypothesized that a redistribution of power between low- and high-frequencies could have occurred in Wet apnea. To test this hypothesis, we extracted the fractions of power for low- and high-frequencies over the total power within the 2Hz range, and compared such fractions as percentage of power (%Power) for each condition (Fig 2f). As hypothesized (Fig 2g), we found that while a significantly higher %Power was contained in the low-frequency band for Breathing [Nested t-test: $F(1,10)= 77.46$, $p< 0.0001$] and Dry [Nested t-test: $F(1,8)= 11.39$, $p = 0.0097$], Wet exhibited the opposite pattern instead, with the high-frequency band containing significantly %Power compared to the low-frequency band [Nested t-test: $F(1,8)= 15.09$, $p = 0.0046$].
Together these data indicate that apneic conditions reduce the power of low-frequency pupil fluctuations associated with the SNS, and that in cold water face-immersion apnea a reallocation of power towards higher frequencies occurs, suggesting an increased contribution of the PNS.
Since apnea and face contact with cold water are known to prime the DR, and that tendencies in BPM and SpO_2 recapitulate the expected DR phenotype, our results suggest that activating the DR affects pupillary dynamics. This consists in an increased contribution to pupil control of the PNS component in response to face-immersion apnea, along with a reduction in the SNS one. Importantly, this effect can be largely attributed to cold water contact with the skin of the face given that pupil dynamics do not significantly differ between Dry and Breathing conditions in terms of allocation of power in the two frequency bands.

Next, we hypothesized that the DR priming in the Wet condition would have reduced the complexity (i.e., lower entropy) of hippus, due to the stereotypical nature of reflexes that would take over the control of pupil diameter fluctuations. However, the Approximate Entropy associated with hippus in Wet condition was significantly higher than in Breathing and Dry conditions [Nested one-way ANOVA: $F(2, 27) = 19.89$, $p <0.0001$; Tuckey's multiple comparison: p (Breathing vs Dry)= 0.196, p(Breathing vs Wet) <0.0001, p (Dry vs Wet)= 0.0003] (Fig. 2h), indicating an increase in complexity with DR instead.

Overall, these findings indicate that the human DR significantly impacts the dynamics of pupillary fluctuations. The decreased low-frequency power and higher entropy, along with the

shift towards high-frequency power in Wet apnea, suggest an autonomic shift and heightened complexity of pupillary behaviour during the DR.

Discussion

The DR involves a series of physiological responses to immersion that override the basic homeostatic reflexes and is found in all air-breathing vertebrates [29, 30, 31].
The most noticeable effects are on the cardiovascular system, which displays peripheral vasoconstriction, slowed heart rate, redirection of blood to the vital organs to conserve oxygen, release of red blood cells stored in the spleen, sometimes leading to heart rhythm irregularities in humans [30]. When compared to cold facial stimulation and breath-holding alone, these responses produce a greater effect than the sum of individual responses [34, 35]. This motivated us to design the study and analyze the performance of subjects between Dry and Wet conditions.

Our measures of BPM variations during apnea, the peripheral oxygen saturation at the performances' end, which tended to show higher values for Wet than for Dry , along with a more extended duration of apnea in the Wet compared to Dry, strongly suggest DR activation. The observed trends, albeit non-significant, support the notion that cold facial immersion can enhance the DR [34, 44]. This is congruent with Wet performances, which exhibited a significantly stronger pupil behavior modulation (i.e., autonomic shift) compared to Dry ones or Breathing (Fig 2d-g).

Pupillary fluctuations and choice of frequency bands. Hippus, a spontaneous oscillation of the pupil, is believed to be driven by the continuous antagonism between the PNS and the SNS [2, 10]. Given that both the PNS and SNS are recruited by the DR, we hypothesized that pupillometry could reveal its impact on pupillary dynamics. Our results demonstrated significant changes in pupillary behavior during apnea, more extensive with face immersion in cold water. In this condition, we observed an overall reallocation of power in high-frequency at the expense of low-frequency components of pupil diameter fluctuations (Fig 2g), accompanied by a more erratic hippus, as indicated by increased Approximate Entropy (Fig 2h).

Much effort has been made in attempting to classify specific frequency bands of pupillary fluctuations in relation to other physiological processes such as respiration, cardiac dynamics, and emotional autonomic components [10, 18, 21, 23, 28, 39, 49]. In our study, we classified the bands by assigning the sympathetic contributions to the 0.04-0.25 Hz band and the parasympathetic contributions to the 0.25-2 Hz band. This was based on a prior review of the literature that we carried on, which included studies on the division of signal bands, like heart rate and skin conductance, in relation to pupillary fluctuation [10, 18, 20, 22, 23, 39, 50, 51, 52, 53, 54]. To overcome the wide variety of frequency band assignments, we attributed higher value to the interventionist approach. Specifically, Turnbull et al.'s work found a reduction of energy in the previously reported hippus range of 0.24-1 Hz after the application of tropicamide, a parasympathetic blocking agent [10]. This provided us with a lower frequency limit for the parasympathetic system. Since other works found relevant parasympathetic effects beyond 1Hz [21, 10], we selected high frequency (HF) 0.25-2 Hz as the parasympathetic contribution to pupillary fluctuation. As a consequence, lower frequencies (< 0.25 Hz) were attributed to the sympathetic one.

Although there is much debate regarding the biological attribution of pupillary fluctuations at low frequencies [10, 18, 21, 23, 28], at such frequencies, pupillary, respiratory, and cardiac dynamics may be co-modulated. As initially hypothesized by Borgdorff and later supported by subsequent studies [18, 21 23, 28, 64], the autonomic modulation of pupillary fluctuation is reflected in the oscillations classically attributed to the autonomic control of cardiorespiratory activity. Also, by recording other autonomic parameters with ECG and skin conductance, Schumann et al. [18] demonstrated a strong correlation between vagal heart rate variability and the pupillary unrest index, which estimates the deviation of pupillary diameter at low frequencies (< 0.15 Hz). Moreover, in the same study, subjects breathed deeply at 6 respiratory cycles per minute (0.1 Hz), the same respiratory frequency used in our experiment. This could be the reason underlying higher power around 0.1 Hz in our Breathing condition (Fig. 2d). Moreover, we observed that, in the low-frequency range, the Breathing condition contained higher power than apneic conditions. This suggests that the modulation of pupillary dynamics in response to respiratory and cardiovagal influences is altered during apnea, particularly under Wet conditions where the DR is more pronounced.

In Onorati et al. [21], besides finding a significant correlation between respiratory dynamics, pupillary diameter, and heart rate variability within the low-frequency band, authors explored pupillary dynamics in high and very high frequencies (0.45-5 Hz) to assess affective states and autonomic correlates. They found significant correlations between high-frequency pupillary fluctuations and emotional states, highlighting the influence of central autonomic control on pupillary dynamics. In our study, such influences may have affected pupil dynamics predominantly in the Wet condition, which was the only instance characterized by a proportional elevation of high-frequency power at the expense of the low-frequency power (Fig 2g). A characterisation of emotional states in future studies may enable disentangling their effect on pupil dynamics in our task.

Central neural substrates underlying autonomic shift in pupil control. Noble and Hochman [66] proposed a network model explaining how the control of respiration provides a powerful voluntary modulation of central autonomic networks [67, 68, 69]. In their model, they suggest that pulmonary afferents project to the nucleus tractus solitarius (NTS), in turn projecting to the locus coeruleus (LC), which regulates pupillary fluctuations [15]. By involving a complex neural network that integrates respiratory and cardiovascular systems, the NTS is central to the DR [32, 33]. Key inputs from facial cold receptors, carotid chemoreceptors, baroreceptors, and atrial stretch receptors project to the NTS, which then communicates with respiratory, cardiac parasympathetic, and vasomotor centers, producing responses such as bradycardia and peripheral vasoconstriction. In the framework proposed by Noble and Hochman [66], the connection between NTS and LC modulates anxiety, arousal, and attention through extensive projections to central autonomic structures. In the case of the pupil, our results show a change in autonomic balance during DR. In principle, this could be provoked by a reduction in LC output, which would directly affect the modulation of the sympathetic pathway, and that could indirectly release the inhibition acting upon the Edinger-Westphal nucleus.

Despite the high likelihood that the DR could affect pupil diameter fluctuations as originally hypothesized, our experiment also produced an unexpected result.

Complex Autonomic Interplay During the Diving Reflex

Our study reveals a significant shift in autonomic control during the diving reflex, reflected by changes in LF and HF components in pupillometry data. This shift challenges the traditional view of reciprocal sympathetic-parasympathetic regulation. Specifically, the increase in entropy and reallocation of power towards higher frequencies in the Wet condition suggests a heightened parasympathetic response, alongside a more complex interplay of excitatory and inhibitory mechanisms within the pupillary control brain nuclei during apnea.

This increase in ApEn in the Wet condition is particularly noteworthy. While many biological signals, including pupil diameter, exhibit non-stationary and non-linear behavior[27], making their underlying determinism challenging to identify, measures of signal complexity such as ApEn provide deeper insights beyond the linear assumptions of Fourier analysis. Unexpectedly, the entropy of pupil diameter fluctuations increased during the Wet condition, when the diving reflex (DR) is presumed to be stronger. If we assume that reflexes are among the most hardwired (i.e., less plastic) neural and behavioral processes, this result was counterintuitive since we would have expected less erratic behavior of the pupil when recruited by the DR.

Indeed, higher entropy in apnea contrasts with findings in Schumann et al. [18], who reported that during deep breathing tests, both the pupillary unrest index and RMSSD of heart rate variability increased proportionally, but the complexity of pupillary dynamics decreased. Their results suggest that deep breathing, stimulating the parasympathetic system, makes fluctuations more predictable, therefore less complex. Despite absence of breathing, our results show that strengthening the parasympathetic component relative to the sympathetic one is associated with higher complexity instead. This discrepancy with our results might be due to different experimental settings and the unique nature of DR, which involves the co-activation of sympathetic and parasympathetic systems for multiple body functions [32,33]. On the contrary, such co-activation is perhaps negligible during breathing. Indeed, with cold water face immersion, some authors have proposed that the strong and simultaneous activation of both limbs of the autonomic nervous system can prime an 'autonomic conflict,' which may account for cardiac arrhythmias in healthy volunteers [65].

In line with Montano et al. [75], who demonstrated that low-frequency and high-frequency oscillations in HRV reflect dynamic interactions between sympathetic and parasympathetic systems, our findings suggest that these rhythms in pupillometry share a similarly mixed origin and interact continuously, rather than acting as purely antagonistic forces. This supports Malliani et al.'s [76] perspective that autonomic regulation is not a simple opposition between sympathetic (LF) and parasympathetic (HF) influences, but rather a complex, integrated system in which both work in tandem to maintain physiological equilibrium.

Moreover, since the LC exerts a phasic control over pupil fluctuations, and that the shift in autonomic balance could be due to a modulation of its activity, a weaker phasic input to downstream nuclei could be responsible for the higher complexity we observed, for example, by being effective only at random intervals. Another finding seems to contrast with the current results. Laurino et al. [46] observed decreased entropy in the cardiac system by analyzing heart rate variability during apnea, which is at odd with our increase in the pupil.

To reconcile this with our finding, we propose that the DR may exert differential, hierarchical control across various body functions: While the cardiac system becomes more regulated and ordered, reducing entropy, the pupillary system shows a more disorganized response, increasing entropy instead. This would confirm the hypothesis that the DR can prioritize the regulation of vital functions like the heartbeat over others with minor roles as a form of allostatic control.

Limitations and future directions. The accommodative demand might have influenced pupillary dynamics [71, 72, 73], particularly in the Wet condition, causing a significant hyperopic shift. Despite this, Turnbull et al. [10] found no correlation between accommodative demand and the pupil dilation ratio or hippus energy ratio, suggesting refractive error is not a major factor in the study of the parasympathetic component of hippus. However, future studies should include larger and more diverse samples to explore individual variability in response to the DR.

The use in this work of controlled-breathing condition (0.1 Hz, 6 breaths per minute) may lead to an underestimation of the real differences with apneic conditions. This slow breathing pattern significantly affects the PNS, maximizes heart rate variability, and dramatically enhances cardiorespiratory synchronization [66], therefore elevating power in the parasympathetic range. Consequently, the true impact of the DR on autonomic responses and pupillary dynamics might be even more pronounced than what we observe. Normal-, as opposed to controlled-, breathing may thus be a valid condition for future comparisons as it could reduce such inter-systemic synchronization.

Also, recent research highlights the relevance of high-frequency pupillary dynamics in assessing affective states and autonomic correlates [21], underscoring the need to consider emotional states when studying pupillary fluctuations. Incorporating psychometric tests in future studies could explore the emotional and cognitive aspects of the DR. Indeed, some authors have identified changes in neural processing of emotional stimuli and brain oscillations due to prolonged apnea [62, 63], further underscoring the complex interplay between autonomic regulation and pupillary behavior in apneic conditions.

Conclusions. Our study indicates that the DR is a valuable model for investigating autonomic responses through pupillary dynamics. Indeed, we observed significant shifts in pupillary dynamics during the DR, characterized by reduced low-frequency power. With a stronger DR, a significant shift in the parasympathetic-sympathetic equilibrium influencing the pupil occurs. This shift is accompanied by an increased complexity of hippus. To fully understand the biological significance of these findings, future research should explore how autonomic, emotional, and cognitive dynamics correlate with pupillary fluctuations under challenging apnea conditions. Integrating multiparametric acquisitions, such as ECG, EEG, and psychometric tests, will be thus crucial for advancing our understanding of the interactions between autonomic regulation, emotional states, and pupillary behavior.

Authors contribution

RV conceived the study; RV designed and performed the experiments; MR, ML, BGMH and LM analysed the data; RV and MR drafted a first version of the manuscript; BY and YD provided the pupillometer and developed the software; ML, LM, BY, YD, LG, VL and GA revised the draft and provided guidance; GA supervised the study. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring that questions related to accuracy or integrity are appropriately investigated and resolved.

Acknowledgments

The authors wish to thank Y-40 and Instructor Marco Mardollo for their guidance and logistical support in the apnea experiment. Additionally, the authors would like to express their gratitude to *Medicina Subacquea e Iperbarica "Piergiorgio Data"*, *Scuola Superiore Sant'Anna - Istituto di Fisiologia Clinica*, and *Area di Ricerca del CNR di Pisa* for their valuable support.

Competing interests

Authors declare no competing interests.

Fundings

None.

References

1 Loewenfeld IE. *The Pupil: Anatomy, Physiology, and Clinical Applications*. Detroit, MI: Wayne State University Press (1993).

2 McDougal DH, Gamlin PD. Autonomic control of the eye. *Compr Physiol.* (2015) 5:439–73. doi: 10.1002/cphy.c140014

3 Mathôt S. Pupillometry: Psychology, Physiology, and Function. J Cogn. 2018 Feb 21;1(1):16. doi: 10.5334/joc.18. PMID: 31517190; PMCID: PMC6634360.

4 Mathôt, S., & Van der Stigchel, S. (2015). New light on the mind's eye: The pupillary light response as active vision. *Current Directions in Psychological Science*, 24(5), 374–378. DOI: 10.1177/0963721415593725

5 Einhäuser, W. (2017). The pupil as marker of cognitive processes In: Zhao, Q. (Ed.), *Computational and Cognitive Neuroscience of Vision*, 141–169. Signapore: Springer; DOI: 10.1007/978-981-10-0213-7_7

6 Friedman, D., Hakerem, G., Sutton, S., & Fleiss, J. L. (1973). Effect of stimulus uncertainty on the pupillary dilation response and the vertex evoked potential. *Electroencephalography and Clinical Neurophysiology*, 34(5), 475–484. DOI: 10.1016/0013-4694(73)90065-5

7 Kahneman, D., & Beatty, J. (1966). Pupil diameter and load on memory. *Science*, 154(3756), 1583–1585. DOI: 10.1126/science.154.3756.1583

8 Gilzenrat, M. S., Nieuwenhuis, S., Jepma, M., & Cohen, J. D. (2010). Pupil diameter tracks changes in control state predicted by the adaptive gain theory of locus coeruleus function. *Cognitive, Affective, & Behavioral Neuroscience*, 10(2), 252–269. DOI: 10.3758/CABN.10.2.252

9 Mathôt, Sebastiaan. (2018). Pupillometry: Psychology, Physiology, and Function. Journal of Cognition. 1. 10.5334/joc.18.

10 Turnbull, Philip RK, et al. "Origins of pupillary hippus in the autonomic nervous system." Investigative ophthalmology & visual science 58.1 (2017): 197-203.

11 McLaren JW, Erie JC, Brubaker RF. Computerized analysis of pupillograms in studies of alertness. Invest Ophthalmol Vis Sci. 1992; 33: 671–676.

12 Centeno M, Feldmann M, Harrison NA, et al. Epilepsy causing pupillary hippus: an unusual semiology. Epilepsia. 2011; 52: e93–e96.

13 Steinhauer, S. R., Siegle, G. J., Condray, R., & Pless, M. (2004). Sympathetic and parasympathetic innervation of pupillary dilation during sustained processing. International Journal of Psychophysiology, 52(1), 77–86. DOI: 10.1016/j.ijpsycho.2003.12.005

14 Wang, C., & Munoz, D. P. (2015). A circuit for pupil orienting responses: implications for cognitive modulation of pupil size. Current Opinion in Neurobiology, 33, 134–140. DOI: 10.1016/j.conb.2015.03.018

15 Szabadi, Elemer. "Functional organization of the sympathetic pathways controlling the pupil: light-inhibited and light-stimulated pathways." Frontiers in Neurology 9 (2018): 1069.

16 Rosenberg ML, Kroll MH. Pupillary hippus: an unrecognized example of biologic chaos. J Biol Syst. 1999; 7: 85–94.

18 Schumann, Andy, et al. "Sympathetic and parasympathetic modulation of pupillary unrest." Frontiers in Neuroscience 14 (2020): 178.

19 Bouma H, Baghuis LC. Hippus of the pupil: periods of slow oscillations of unknown origin. Vision Res. 1971; 11: 1345–1351.

20 Breeden A. L., Siegle G. J., Norr M. E., Gordon E. M., Vaidya C. J. (2017). Coupling between spontaneous pupillary fluctuations and brain activity relates to inattentiveness. Eur. J. Neurosci. 45 260–266. 10.1111/ejn.13424

21 Onorati, Francesco, et al. "Characterization of affective states by pupillary dynamics and autonomic correlates." Frontiers in neuroengineering 6 (2013): 9.

22 Bradley MM, Miccoli L, Escrig MA, Lang PJ. The pupil as a measure of emotional arousal and autonomic activation. Psychophysiology. 2008 Jul;45(4):602-7. doi: 10.1111/j.1469-8986.2008.00654.x. Epub 2008 Feb 11. PMID: 18282202; PMCID: PMC3612940

23 Calcagnini, G., Censi, F., Lino, S., and Cerutti, S. (2000). Spontaneous fluctuations of human pupil reflect central autonomic rhythms. Methods Inf. Med. 39, 142–145

24 Nobukawa, Sou, et al. "Pupillometric complexity and symmetricity follow inverted-U curves against baseline diameter due to crossed locus coeruleus projections to the Edinger-Westphal nucleus." *Frontiers in Physiology* 12 (2021): 614479.

25 Fink, L., et al. "From pre-processing to advanced dynamic modeling of pupil data. PsyArXiv." (2021).

26 L. Mesin. Estimation of complexity of sampled biomedical continuous time signals using approximate entropy. *Front Physiol.*, 9:710, 2018.

27 L Mesin, A Monaco, and R Cattaneo. Investigation of nonlinear pupil dynamics by recurrence quantification analysis. *Biomed Res Int.*, 2013:420509, 2013.

28 Borgdorff, P. (1975). Respiratory fluctuations in pupil size. Am. J. Physiol. Legacy Content 228, 1094–1102.

29 Butler, Patrick J., and David R. Jones. "Physiology of diving of birds and mammals." Physiological reviews 77.3 (1997): 837-899.

30 Lindholm, Peter, and Claes EG Lundgren. "The physiology and pathophysiology of human breath-hold diving." Journal of Applied Physiology 106.1 (2009): 284-292.

31 Michael Panneton, W. "The mammalian diving response: an enigmatic reflex to preserve life?." Physiology 28.5 (2013): 284-297.

32 Foster, G. E., and A. W. Sheel. "The human diving response, its function, and its control." *Scandinavian journal of medicine & science in sports* 15.1 (2005): 3-12.

33 Panneton WM, Gan Q. The Mammalian Diving Response: Inroads to Its Neural Control. Front Neurosci. 2020 Jun 5;14:524. doi: 10.3389/fnins.2020.00524. PMID: 32581683; PMCID: PMC7290049

34 Schagatay, Erika, Johan PA Andersson, and Bodil Nielsen. "Hematological response and diving response during apnea and apnea with face immersion." European journal of applied physiology 101 (2007): 125-132.

35 Shamsuzzaman, A., Ackerman, M. J., Kuniyoshi, F. S., Accurso, V., Davison, D., Amin, R. S., & Somers, V. K. (2014). Sympathetic nerve activity and simulated diving in healthy humans. Autonomic Neuroscience: Basic and Clinical, 181, 74-78.

36 Peter Kyriakoulis. *The efficacy of cold facial immersion and the diving response in treating panic disorder*. PhD thesis, Swinburne University of Technology, 2019.

37 Zénon, Alexandre. "Eye pupil signals information gain." *Proceedings of the Royal Society B* 286.1911 (2019): 20191593.

38 D Naranjo, R Cattaneo, and L Mesin. Development of a prototype for the analysis of multiple responses of the autonomic nervous system. *Biomedical Signal Processing and Control*, 70:102994, 2021.

39 F Onorati, LT Mainardi, F Sirca, V Russo, and R. Barbieri. Nonlinear analysis of pupillary dynamics. *Biomed Tech (Berl).*, 61:95–106, 2016.

40 K Ohtsuka, K Asakura, H Kawasaki, and M Sawa. Respiratory fluctuations of the human pupil. *Exp Brain Res*, 71:215–217, 1988.

41 Winfried Neuhuber and Falk Schr¨odl. Autonomic control of the eye and the iris. *Autonomic Neuroscience*, 165(1):67–79, 2011.

42 J Reimer, MJ McGinley, Y Liu, and et al. Pupil fluctuations track rapid changes in adrenergic and cholinergic activity in cortex. *Nature Communications*, 7:13289, 2016.

43 SM. Pincus. Approximate entropy as a measure of system complexity. *Proc. Natl. Acad. Sci. U.S.A.*, 88:2297–2301, 1991.

44 Valic, Zoran, et al. "Muscle oxygen supply during cold face immersion in breath-hold divers and controls." Aviation, space, and environmental medicine 77.12 (2006): 1224-1229.

45 Gooden, Brett A. "Mechanism of the human diving response." Integrative physiological and behavioral science 29 (1994): 6-16

46 Laurino, Marco, et al. "Mind-body relationships in elite apnea divers during breath holding: a study of autonomic responses to acute hypoxemia." Frontiers in neuroengineering 5 (2012): 4.

47 Vega, Jose L. "Edmund Goodwyn and the first description of diving bradycardia." Journal of applied physiology 123.2 (2017): 275-277.

48 Liu, He, and Mary Vagula. "Cardiovascular Changes in Human Diving Reflex Based on Student-Collected Data in a Physiology Lab Course." HAPS Educator 25.1 (2021): 45-50.

49 Yamashita, Jumpei, et al. "Pupillary fluctuation amplitude before target presentation reflects short-term vigilance level in Psychomotor Vigilance Tasks." PLoS One 16.9 (2021): e0256953.

50 Rajkowski J., Kubiak P., Aston-Jones G. (1994). Locus coeruleus activity in monkey: phasic and tonic changes are associated with altered vigilance. Brain Res. Bull. 35 607–616.

51 Aston-Jones G., Rajkowski J., Cohen J. (1999). Role of locus coeruleus in attention and behavioral flexibility. Biol. Psychiatry 46 1309–1320. 10.1016/S0006-3223(99)00140-7

52 Aston-Jones, G., & Cohen, J. D. (2005). An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. *Annual Reviews Neuroscience*, 28, 403–450. DOI: 10.1146/annurev.neuro.28.061604.135709

53 Freeman RL. Noninvasive evaluation of heart rate: time and frequency domains. In: Low PA, Benarroch EE, editors. Clinical Autonomic Disorders. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2008. pp. 185–197

54 Wang, Chin-An, et al. "Arousal effects on pupil size, heart rate, and skin conductance in an emotional face task." *Frontiers in neurology* 9 (2018): 1029.

55 Ferretti, G. (2001). Extreme human breath-hold diving. European Journal of Applied Physiology, 84(4), 254-271. https://doi.org/10.1007/s004210000377

56 Lüdtke H, Wilhelm B, Adler M, Schaeffel F, Wilhelm H. Mathematical procedures in data recording and processing of pupillary fatigue waves. Vision Res. 1998; 38: 2889–2896.

57 Prasad B, Choi YK, Weaver TE, Carley DW. Pupillometric assessment of sleepiness in narcolepsy. Front Psychiatry. 2011; 2: 35.

58 Ukai K, Tsuchiya K, Ishikawa S. Induced pupillary hippus following near vision: increased occurrence in visual display unit workers. Ergonomics. 1997; 40: 1201–1211.

59 Warga M, Lüdtke H, Wilhelm H, Wilhelm B. How do spontaneous pupillary oscillations in light relate to light intensity? Vision Res. 2009; 49: 295–300.

60 Larson MD. Mechanism of opioid-induced pupillary effects. Clin Neurophysiol. 2008; 119: 1358–1364.

61 Bokoch MP, Behrends M, Neice A, Larson MD. Fentanyl, an agonist at the mu opioid receptor, depresses pupillary unrest. Auton Neurosci. 2015; 189: 68–74.

62 D. Menicucci, F. Artoni, R. Bedini, A. Pingitore, M. Passera, A. Landi, et al. Brain responses to emotional stimuli during breath holding and hypoxia: an approach based on the independent component analysis. *Brain Topography*, 27:771–785, 2014.

63 F. Steinberg, N. H. Pixa, and M. Doppelmayr. Electroencephalographic alpha activity modulations induced by breath-holding in apnoea divers and non-divers. *Physiology & Behavior*, 179:90–98, 2017.

64 Calcagnini, G., et al. "Baroreceptor-sensitive fluctuations of heart rate and pupil diameter." 2001 Conference Proceedings of the 23rd Annual International Conference of the IEEE Engineering in Medicine and Biology Society. Vol. 1. IEEE, 2001.

65 Shattock MJ, Tipton MJ. 'Autonomic conflict': a different way to die during cold water immersion? J Physiol. 2012 Jul 15;590(14):3219-30. doi: 10.1113/jphysiol.2012.229864. Epub 2012 Apr 30. PMID: 22547634; PMCID: PMC3459038.

66 Noble, Donald J., and Shawn Hochman. "Hypothesis: pulmonary afferent activity patterns during slow, deep breathing contribute to the neural induction of physiological relaxation." *Frontiers in physiology* 10 (2019): 1176.

67 Brown, R. P., and Gerbarg, P. L. (2005). Sudarshan Kriya yogic breathing in the treatment of stress, anxiety, and depression: part I-neurophysiologic model. J. Altern. Complement. Med. 11, 189–201. doi: 10.1089/acm.2005.11.189

68 Jerath, R., Edry, J. W., Barnes, V. A., and Jerath, V. (2006). Physiology of long pranayamic breathing: neural respiratory elements may provide a mechanism that explains how slow deep breathing shifts the autonomic nervous system. Med. Hypotheses 67, 566–571. doi: 10.1016/j.mehy.2006.02.042

69 Jerath, R., Barnes, V. A., Dillard-Wright, D., Jerath, S., and Hamilton, B. (2012). Dynamic change of awareness during meditation techniques: neural and physiological correlates. Front. Hum. Neurosci. 6:131. doi: 10.3389/fnhum.2012.00131

70 Nordine, Michael, et al. "The human dive reflex during consecutive apnoeas in dry and immersive environments: Magnitude and synchronicity." Frontiers in Physiology 12 (2022): 725361.

71 Campbell, F. W., J. G. Robson, and G. Westheimer. "Fluctuations of accommodation under steady viewing conditions." The Journal of Physiology 145.3 (1959): 579.

72 Stark, Lawrence R., and David A. Atchison. "Pupil size, mean accommodation response and the fluctuations of accommodation." Ophthalmic and Physiological Optics 17.4 (1997): 316-323.

73 Collins, Michael, Brett Davis, and Joanne Wood. "Microfluctuations of steady-state accommodation and the cardiopulmonary system." Vision research 35.17 (1995): 2491-2502.1 Loewenfeld IE. *The Pupil: Anatomy, Physiology, and Clinical Applications*. Detroit, MI: Wayne State University Press (1993).

74 Yellin D, Berkovich-Ohana A, Malach R. Coupling between pupil fluctuations and resting-state fMRI uncovers a slow build-up of antagonistic responses in the human cortex. Neuroimage. 2015 Feb 1;106:414-27. doi: 10.1016/j.neuroimage.2014.11.034. Epub 2014 Nov 20. PMID: 25463449

75 Montano, Nicola, et al. "Power spectrum analysis of heart rate variability to assess the changes in sympathovagal balance during graded orthostatic tilt." *Circulation* 90.4 (1994): 1826-1831.
76 Malliani, A., Pagani, M., Montano, N., & Mela, G. S. (1998). Sympathovagal balance: a reappraisal. *Circulation*.