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Assessing Pupil-Linked Changes in Locus Coeruleus-Mediated Arousal Elicited by Trigeminal Stimulation

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DIPARTIMENTO TRASLAZIONALE E DELLE NUOVE TECNOLOGIE IN MEDICINA E CHIRURGIA SPERIMENTALI

Sede di Via S. Zeno 31, 56127 Pisa (Italy) - Tel. +39-050-2213500 - Fax +39-050-2213527

Pisa 11/06/2019

Dear Dr Myers

with the present letter I submit to Jove on behalf of my colleagues, the revised version of the research article, originally titled:

“**How to assess trigeminal effects on cognitive performance and the stink of the central noradrenergic system.**”

by Maria Paola Tramonti Fantozzi, Tommaso Banfi, Vincenzo De Cicco, Massimo Barresi, Enrico Cataldo, Davide De Cicco, Luca Bruschini, Paola d’Ascanio, Gastone Ciuti, Ugo Faraguna and myself.

As requested by referee2, the title has been modified and now it sounds:

How to assess pupil-linked changes in Locus Coeruleus mediated arousal elicited by trigeminal stimulation.

I hope that the performed revision may fulfil the requests of both editor and referees.

Sincerely yours,

Diego Manzoni

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TITLE:

Assessing Pupil-Linked Changes in Locus Coeruleus-Mediated Arousal Elicited by Trigeminal Stimulation

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SUMMARY:

To verify whether trigeminal effects on cognitive performance involve locus coeruleus activity, two protocols are presented that aim to evaluate possible correlations between the performance and task-related pupil size changes induced by chewing. These protocols may be applied to conditions in which locus coeruleus contribution is suspected.

ABSTRACT:

Current scientific literature provides evidence that trigeminal sensorimotor activity associated with chewing may affect arousal, attention, and cognitive performance. These effects may be due to widespread connections of the trigeminal system to the ascending reticular activating system (ARAS), to which noradrenergic neurons of the locus coeruleus (LC) belongs. LC neurons contain projections to the whole brain, and it is known that their discharge co-varies with pupil size. LC activation is necessary for eliciting task-related mydriasis. If chewing effects on cognitive performance are mediated by the LC, it is reasonable to expect that changes in cognitive performance are correlated to changes in task-related mydriasis. Two novel protocols are presented here to verify this hypothesis and document that chewing effects are not attributable to aspecific motor activation. In both protocols, performance and pupil size changes observed during specific tasks are recorded before, soon after, and half an hour following a 2 min period of either: a) no activity, b) rhythmic, bilateral handgrip, c) bilateral chewing of soft pellet, and d) bilateral chewing of hard pellet. The first protocol measures level of performance in spotting target numbers displayed within numeric matrices. Since pupil size recordings are recorded by an appropriate pupillometer that impedes vision to ensure constant illumination levels, task-related mydriasis is evaluated during a haptic task. Results from this protocol reveal that 1) chewing-induced changes in performance and task-related mydriasis are correlated and 2) neither performance nor mydriasis are enhanced by handgrip. In the second protocol, use of a wearable pupillometer allows measurement of pupil size changes and performance during the same task, thus allowing even stronger evidence to be obtained regarding LC involvement in the trigeminal effects on cognitive activity.

INTRODUCTION:

In humans, it is known that chewing quickens cognitive processing[1,2] and improves arousal[3,4], attention[5], learning, and memory[6,7]. These effects are associated with shortening of the latencies of cortical event-related potentials[8] and an increase in the perfusion of several cortical and subcortical structures[2,9].

Within cranial nerves, the most relevant information sustaining cortical desynchronization and arousal is carried by trigeminal fibres[10], likely due to strong trigeminal connections to the ascending reticular activating system (ARAS)[11]. Among ARAS structures, the LC receives trigeminal inputs[11] and modulates arousal[12,13], and its activity covaries with pupil size[14–18]. Although the relation between LC resting activity and cognitive performance is complex, task-related enhancement of LC activity leads to arousal-associated[19] pupil mydriasis[20] and enhanced cognitive performance[21]. There is reliable covariation between LC activity and pupil size, and the latter is currently considered a proxy of central noradrenergic activity[22–26].

Asymmetric activation of sensorimotor trigeminal branches induces pupil asymmetries (anisocoria)[27, 28], confirming the strength of the trigemino-coerulear connection. If the LC participates in the stimulating effects of chewing on cognitive performance, it may affect parallel task-related mydriasis, which is an indicator of LC phasic activation during a task. It may also affect performance, so a correlation can be expected between chewing-induced changes in performance and mydriasis. Moreover, if trigeminal effects are specific, chewing effects should be larger than those elicited by another rhythmic motor task. In order to test these hypotheses, two experimental protocols are hereby presented. They are based on combined measurements of cognitive performance and pupil size and performed before and after a short period of chewing activity. These protocols utilize a test consisting in finding target numbers displayed in numeric attentive matrices[29], alongside with non-target numbers. This test verifies attentive and cognitive performance.

The overall goal of these protocols is to illustrate that trigeminal stimulation elicits specific changes in cognitive performance, which cannot be ascribed aspecifically to the generation of motor commands and are related to pupil-linked changes in LC-mediated arousal. Applications of the protocols extend to all behavioral conditions in which performance can be measured and involvement of the LC is suspected.

PROTOCOL:

All steps follow the guidelines of the Ethical Committee of the University of Pisa.

1. Participant recruitment

1.1. Recruit a subject population according to the specific goal of the study (i.e., normal subjects and/or patients, male and/or females, young people and/or elders).

2. Material preparation

2.1. Prepare a soft pellet; use commercially available chewing gum (**Table of Materials**; initial hardness = 20 Shore OO).

2.2. Prepare a hard pellet; use silicon rubber pellets (**Table of Materials**; constant hardness = 60 Shore OO)[30].

2.3. Prepare an anti-stress ball for a handgrip task. Use a polyurethane foam-made ball (**Table of Materials;** constant hardness = 30 Shore OO)[30].

2.4. Prepare a tangram puzzle (**Table of Materials**; number of pieces = seven) for performing the haptic task.

3. Flowchart of the experiment

3.1. Flowchart of protocol 1:

3.1.1. Evaluate baseline performance (see section 4.1) in the cognitive (matrices) test (T0, control).

3.1.2. Evaluate pupil size (see section 4.2) at rest (no activity requested from the subject) (T0, control).

3.1.3. Evaluate pupil size during a haptic task based on tangram (T0, control).

3.1.3.1. Remove one of the pieces from the puzzle and place it in the subject’s hand.

3.1.3.2. Ask the subject to put the piece back into the puzzle, without looking at the puzzle.

3.1.4. Ask each subject to perform three specific activities for 2 min or to rest for 2 min, according to steps 3.1.4.1−3.1.4.4. Ask the subjects to perform these activities in separate sessions occurring on different days (2−3 days between sessions).

3.1.4.1. Ask the subject to chew a self-administered soft pellet for 2 min, letting him/her spontaneously choose both the rate of chewing and side of the mouth on which to chew. After 1 min of chewing, ask him/her to change the chewing side (and the pellet).

3.1.4.2. Ask the subject to chew a self-administered hard pellet for 2 min. After 1 min, ask him/her to change the chewing side (but not the pellet).

3.1.4.3. Ask the subject to perform a rhythmic squeezing of an anti-stress ball (handgrip exercise) for 2 min at the rate and on the hand of their choosing. After 1 min, ask the subject to switch hands.

3.1.4.4. Ask the subject to rest (no activity) for 2 min.

3.1.5. Just after the end of each step (3.1.4.1−3.1.4.4), evaluate performance in the matrices test and pupil size at rest and during the haptic task (T7).

NOTE: The term “at rest” means that the subject during the pupil size measurement is relaxing. The term “during haptic task” means that the subject during the pupil size measurement is performing the task based on tangram.

3.1.6. Thirty minutes following the end of each step (3.1.4.1−3.1.4.4), evaluate performance and pupil size at rest and during the haptic task (T37).

3.2. Flowchart of protocol 2:

3.2.1. Evaluate pupil size while the subject is resting (T0, control; see section 4.3).

3.2.2. Evaluate baseline performance in the cognitive (matrices) test while simultaneously testing pupil size (T0, control).

3.2.3. Ask each subject to perform three specific activities for 2 min or to rest for 2 min, according to steps 3.2.3.1−3.2.3.4. Ask the subjects to perform these activities in separate sessions occurring on different days (2−3 days between sessions).

3.2.3.1. Ask the subject to chew a self-administered soft pellet for 2 min, letting him/her spontaneously choose both the rate of chewing and side of the mouth on which to chew. After 1 min of chewing, ask him/her to change the chewing side (and the pellet).

3.2.3.2. Ask the subject to chew a self-administered hard pellet for 2 min. After 1 min, ask him/her to change the chewing side (but not the pellet).

3.2.3.3. Ask the subject to perform a rhythmic squeezing of an anti-stress ball (handgrip exercise) for 2 min at the rate and on the side of their choosing. After 1 min, ask the subject to switch hands.

3.2.3.4. Ask the subject to relax (no activity) for 2 min.

3.2.4. Just after the end of each step (steps 3.2.3.1−3.2.3.4), evaluate pupil size at rest and both performance and pupil size in the matrices test (T7).

3.2.5. Thirty minutes following the end of each step (steps 3.2.3.1−3.2.3.4), evaluate pupil size at rest and both performance and pupil size in the matrices test (T37).

4. Measured variables in protocols 1 and 2

4.1. Cognitive performance

NOTE: In both protocols 1 and 2, measure cognitive performance using a test based on a modified version of the Spinnler-Tognoni numeric matrices test[29].

4.1.1. Display three numerical matrices (10 x 10) printed on paper to the subject. Then, ask the subject to sequentially scan the matrix lines, while ticking with a pencil as many of the target numbers as possible (60 targets out of 300 total displayed numbers) indicated above each matrix (**Figure 1**) within 15 s.

4.1.2. Utilize matrices with different positions of target numbers at T0, T7, and T37 to avoid the introduction of confounders related to learning processes.

4.1.3. Evaluate offline the performance index (PI), scanning rate (SR), and error rate (ER) as follows: PI = (target numbers underlined in 15 s)/15; SR = (target + non-target numbers scanned in 15 s)/15; ER = (target numbers missed + non-target numbers underlined in 15 s)/15.

4.2. Pupil size in protocol 1

4.2.1. Prepare the subject for the pupil size measurement with a corneal topographer-pupillographer (**Table of Materials**), which prevents vision of the environment, using one of the following two acquisition procedures.

4.2.1.1. Record a single camera shot of the pupil (**Figure 2A,B**) with a constant illumination level of 40 lux, pressing the specific button on the corneal topographer-pupillographer. Maintain an optimal working distance of 56 mm between the camera and pupil.

NOTE: A single measurement is sufficient, due to the low level of variability of pupil size measured at constant illumination.

4.2.1.2. Perform a continuous recording of the pupil (sampling rate = 5 Hz; **Figure 2C,D**) in the continuous acquisition modality. Discard the first 20−50 measurements (4−10 s), since during this lapse of time, the pupil diameter is growing (the acquisition starts together with pupil illumination at 40 lux). Average the remaining measurements.

4.2.2. Record pupil size of the left and right eyes separately at rest (steps 3.1.2, 3.1.5, and 3.1.6).

4.2.3. Record pupil size during the haptic task (steps 3.1.3, 3.1.5 and 3.1.6; left and right separately). When using the single shot modality (step 4.2.1.1), acquire the photo during the second of two task repetitions, at the beginning of puzzle surface exploration. In the continuous mode of recording (step 4.2.1.2), start the acquisition when the piece of the puzzle has been placed in the hand of the subject.

4.2.4. Evaluate offline left and right pupil size at rest and during the haptic task by direct acquisition of the values (in mm) displayed by the software. Calculate the task-related mydriasis by subtracting the pupil size at rest from pupil size during the haptic task, and obtain all average left-right values.

4.3. Pupil size in protocol 2

4.3.1. Prepare the subject for the pupil size measurement using a wearable pupillometer/eye tracker (**Figure 3A**), endowed with a 3D-printed glass frame structure, using the following procedure.

4.3.1.1. Have the subject wear the wearable pupillometer. Adjust the position of the two infrared cameras **(Figure 3A-2,3)** mounted on bars stemming from the frame (**Table of Materials**), so that the eyes are within the field of view of the cameras and in focus.

4.3.1.2. Acquire images of the pupil (sampling rate = 120 Hz), which are processed online by the software supplied with the wearable pupilometer and provides pupillary diameter (in mm) using a geometrical model of the "average" human ocular. Disregard blink artifacts.

4.3.1.3. Record continuously the environmental illumination level using a calibrated logarithmic light sensor mounted on the wearable pupillometer frame. Use a frontal RGB camera mounted on the wearable pupillometer **(Figure 3A-1)** to record the subject field of view (sampling rate = 30 Hz) useful to study gaze behaviour.

4.3.2. Record simultaneously the size of the two pupils at rest for 20 s (**Figure 3B**).

4.3.3. Record the size of the pupils while the subject performs the Spinnler-Tognoni test, so to have pupil size and cognitive performance simultaneously recorded (steps 3.2.2, 3.2.4, and 3.2.5).

4.3.4. Evaluate offline left and right pupil size at rest and during the haptic task by averaging acquired values (n = 2,400) for each pupil. Calculate the task-related mydriasis by subtracting the pupil size at rest from the pupil size during the task, then all the average left-right values.

4.4. Gaze position

NOTE: Reconstruct online the fixation point using the images of the two pupils obtained from section 4.3. Process the acquired frames in real-time and estimate the gaze fixation point using a previously calculated transfer function[31] specific for each subject wearing the eye tracker.

4.4.1. If necessary, when performing protocol 2, reconstruct gaze position from the pupil images. To do this, add four computer detectable vision markers (ArUco or AprilTag libraries of the instrument software) to four corners of the matrices sheet used in section 4.1.

4.4.2. Allow the calibration system (embedded in the eye tracker software as for the pupil headset used) to acquire the data, and evaluate the parameters of the transfer function that map the fixation point, starting from images of the two pupils. As an example, ask the subject to gaze at a predefined sequence of points that are shown in his/her field of view (i.e., the four corners of the matrices sheet and at the center of the sheet itself), which are recorded simultaneously by the additional RGB camera mounted on the frame and facing the field of view.

4.4.3. Record pupil size during the matrices test.

4.4.4. Calculate offline gaze position that appears as a mark on every frame of the subject's field of view. Use the four markers to track gaze position over the matrices across frames.

5. Statistical analysis

5.1. Analyze pupil size at rest and during the task, task-induced mydriasis, PI, SR, and ER under four conditions (no activity, handgrip, soft pellet, hard pellet) for 3x (T0, T7, T37) using repeated measures ANOVA and statistics software package.

5.2. Analyze changes in variables with respect to baseline values (T0) under four conditions, (no activity, handgrip, soft pellet, hard pellet) for 2x (T7, T37) using repeated measures ANOVA.

5.3. When running ANOVA, if the software indicates that the data distribution is not spherical, take the p-value corresponding to the Greenhouse-Geisser ε correction from the outputted statistic table.

5.4. Correlate the changes in performance (PI, SR, ER) at T7 and T37 with those observed in task-related mydriasis by linear regression analysis.

REPRESENTATIVE RESULTS:

Figure 4 shows a representative example of the results obtained when protocol 1 was applied to a single subject (46 years old, female). PI was increased soon after having chewed (T7) both a hard (from 1.73 numb/s to 2.27 numb/s) and soft pellet (from 1.67 numb/s to 1.87 numb/s) (**Figure 4A**). However, 30 min later (T37), the increased performance persisted only for the hard pellet. On the other hand, both a lack of activity and the handgrip exercise had a negative effect on performance, which dropped from 1.73 numb/s to 1.67 numb/s and from 1.6

numb/s to 1.53 numb/s, with a tendency to recover recorded 30 min later, during the last experimental evaluation.

As observed in **Figure 4B**, qualitatively similar changes were observed for the task-related mydriasis. In this instance, measurements consisted of individual samples taken randomly when the subject was resting. During the haptic task, two samples were recorded, but the first was discarded. Alternatively, in the continuous acquisition mode of the instrument, 100 samples were recorded in 20 s, with the first 20–50 measurements disregarded, and the remaining were then averaged following the removal of blink artefacts (**Figure 3**). Individual samples closely reflect the average value, due to the fact that pupil size reaches a very stable level 2–5 s following the start of eye illumination at a constant level (**Figure 3**). Data illustrated in **Figure 4** and **Figure 5** have been replicated in a population of 30 subjects, and both the chewing- and handgrip-induced changes were statistically confirmed. On the other hand, when the subjects were not involved in any activity, there were no modifications in cognitive performance and mydriasis[30] both at T7 and T37.

Despite the fact that 1) performance and mydriasis were recorded in different tasks and 2) the 12 experimental points illustrated in **Figure 5A,B** were recorded on 4 separate days, it is remarkable that a strong correlation was observed between performance and task-related mydriasis ($r = 0.939$, $p < 0.0005$, $y = 1.166x - 0.417$). As can be inferred from **Figure 5A**, this relation was due to the modifications induced by chewing hard and soft pellets. Even more surprisingly, a correlation was evident also when the corresponding changes with respect to baseline values were considered ($r = 0.924$, $p < 0.001$, $y = 1.210x + 0.101$; **Figure 5B**).

Among the 30 subjects analyzed in the study of Tramonti Fantozzi et al.[30], PI and mydriasis were significantly correlated in 26 of them, with slopes of the corresponding regression lines ranging from 0.310–1.327 numb/s/mm. The corresponding changes were significantly correlated in 22 subjects (range of slopes: 0.390–1.408).

Even stronger evidence of LC involvement in the stimulating effects of chewing on cognitive performance can be obtained by correlating the chewing-induced changes in PI with the change in mydriasis observed only during the execution of the matrices test. This can be achieved under the more natural conditions of protocol 2, in which subjects perform the matrices test while pupil size is simultaneously recorded (**Figure 6**).

FIGURE LEGENDS:

Figure 1: Example of Spinnler-Tognoni numerical matrices. The test consists in identifying the target numbers indicated above each matrix, which have been ticked by the subject.

Figure 2: Example of pupil size recordings from a single subject in protocol 1. (**A**) Recording of pupil size at rest, single shot. (**B**) Recording of pupil size during haptic task, single shot. (**C**) Continuous recording of pupil size at rest for 20 s. (**D**) Continuous recording of pupil size during haptic task for 20 s. Arrows indicate blinking artefacts. In (C) and (D), data taken from time 0 to time 4 s are discarded from the analysis.

Figure 3: Example of pupil size recordings in protocol 2. (A) Photo of a subject wearing the pupillometer. The numbers 1−3 indicates the position of the three cameras, which allow behaviour (1) and pupil size (2–3) recordings. (**B**) Top trace: level of the environmental lightening. Middle and bottom traces: left and right pupil size during performance of the Spinnler-Tognoni matrices test.

Figure 4: Changes in performance and task-related mydriasis induced by different sensorimotor activities in protocol 1. (**A**) Changes in PI. (**B**) Changes in task-related mydriasis. In (A) and (B), dots, black squares, circles and white squares represent data relative to chewing hard pellet, chewing soft pellet, handgrip, and no activity, respectively. Each activity was performed for 2 min from time 5 min to time 7 min.

Figure 5: Relation between PI and task-related mydriasis. (**A**) PI values obtained at different times during the different activities illustrated in **Figure 4** are plotted as a function of the corresponding values of task-related mydriasis. (**B**) Changes in PI with respect to time zero (evaluated as a difference) have been plotted as a function of the corresponding changes in task-related mydriasis. In (A) and (B), dots, black squares, circles, and white squares represent data relative to chewing hard pellet, chewing soft pellet, handgrip, and no activity, respectively. Dashed lines are regression lines of all the data points.

Figure 6: Simultaneous recording of performance and task-related mydriasis. Single frame view of a subject performing the attentive matrices test, taken from the camera mounted on the pupilometer frame. The inset on the right upper corner shows the simultaneous images of both pupils. The green circle represents the fixation point. The red spot and circles drown on the pupil are the pupil centre and contour, as evaluated by the tracking system operating on the eye's videos.

DISCUSSION:

The protocols presented in this study address the acute effects of sensorimotor trigeminal activity on cognitive performance and the role of the LC in this process. This topic has some relevance, considering that 1) during aging, the deterioration of masticatory activity correlates with cognitive decay[32–34]; people that preserve oral health are less prone to neurodegenerative phenomena; 2) malocclusion and teeth extraction induces neurodegenerative effects in animals at hippocampal and cortical level[35–39]; 3) the LC exerts trophic action on the brain, regulates neurovascular coupling, and inhibits neuroinflammation and accumulation of beta-amyloid[11,40]; 4) there is evidence that neurodegenerative diseases can be triggered by neurodegenerative processes at the LC level[11,40].

Protocol 1 allows the defining of specific effects of chewing with respect to a) learning processes elicited by successive repetitions of the task and b) other kinds of ordinary motor activity. Moreover, it establishes the presence/absence of a correlation between changes in performance and mydriasis, with the latter considered an indicator of phasic LC activation during task. This evidence strongly suggests involvement of the LC in the effects of sensorimotor trigeminal activation. Such a protocol has been successfully applied by Tramonti Fantozzi et al.[30]. As seen in the results section, it may also be utilized to assess the degree of dependence of performance on pupil changes linked to LC-mediated arousal at the level of individual subjects. Gaining this measurement (performance/LC activation) represents a new and important neuropsychological variable that can be studied in relation to gender, age, drug administration, and any behavioral condition.

The main limitation of protocol 1 is that pupil size measurements are performed at constant lightening, impeding vision and precluding the assessment of mydriasis elicited during matrices scanning. This obliges the recording of mydriasis during a different task. This problem is resolved by performing protocol 2, in which a wearable pupillometer endowed with a light sensor is introduced. In this way, it is possible to contextually record both cognitive performance and mydriasis during the same task, providing even more compelling evidence about the effects of sensorimotor activity on LC and performance. This also helps to address studies aimed at relating LC activation to behavioral conditions. For a correct application of protocol 2, care must be taken to keep a constant level of environmental lighting and preliminary calibration of wearable instruments.

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DISCLOSURES:
The authors have nothing to disclose.

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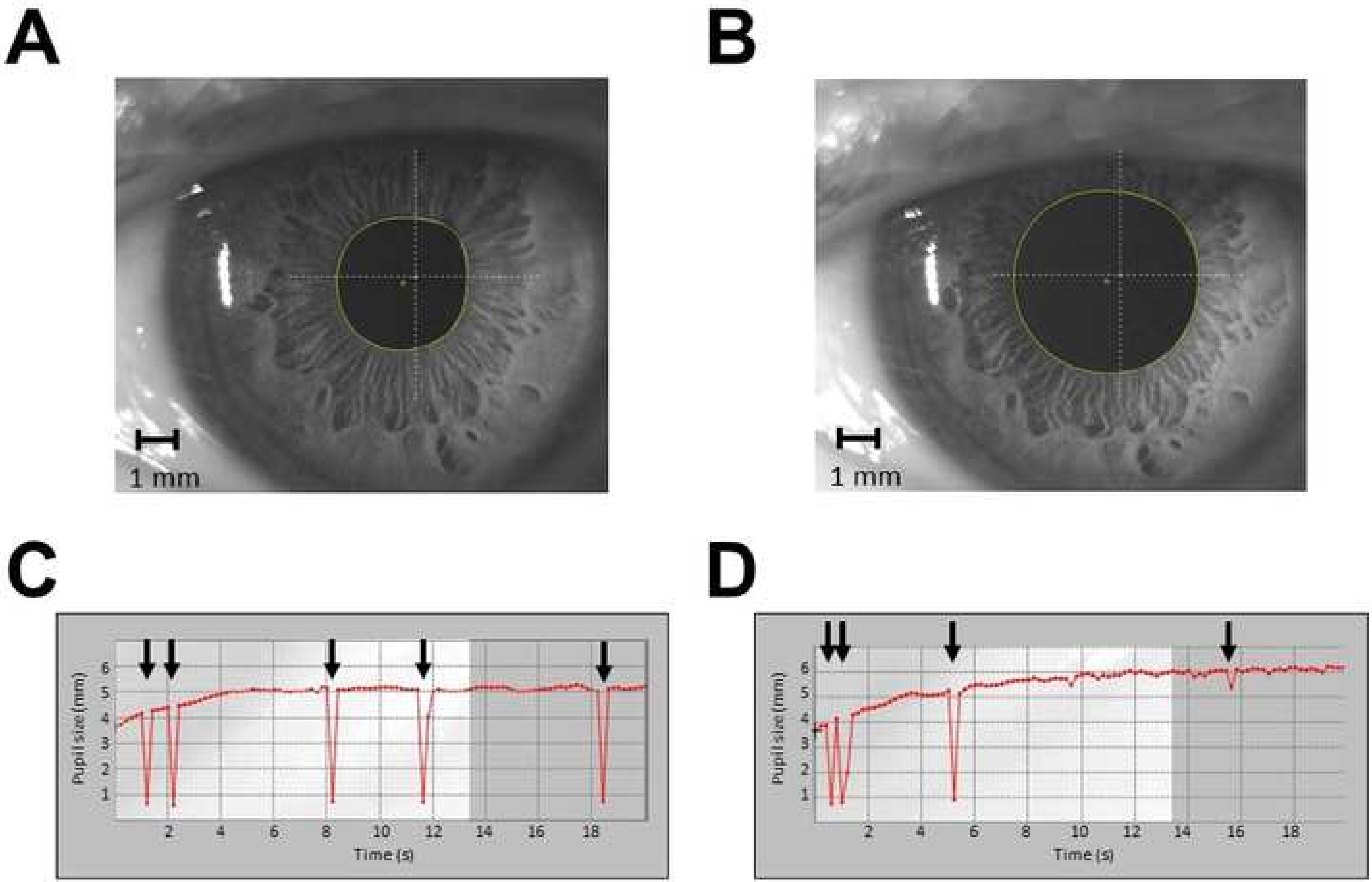
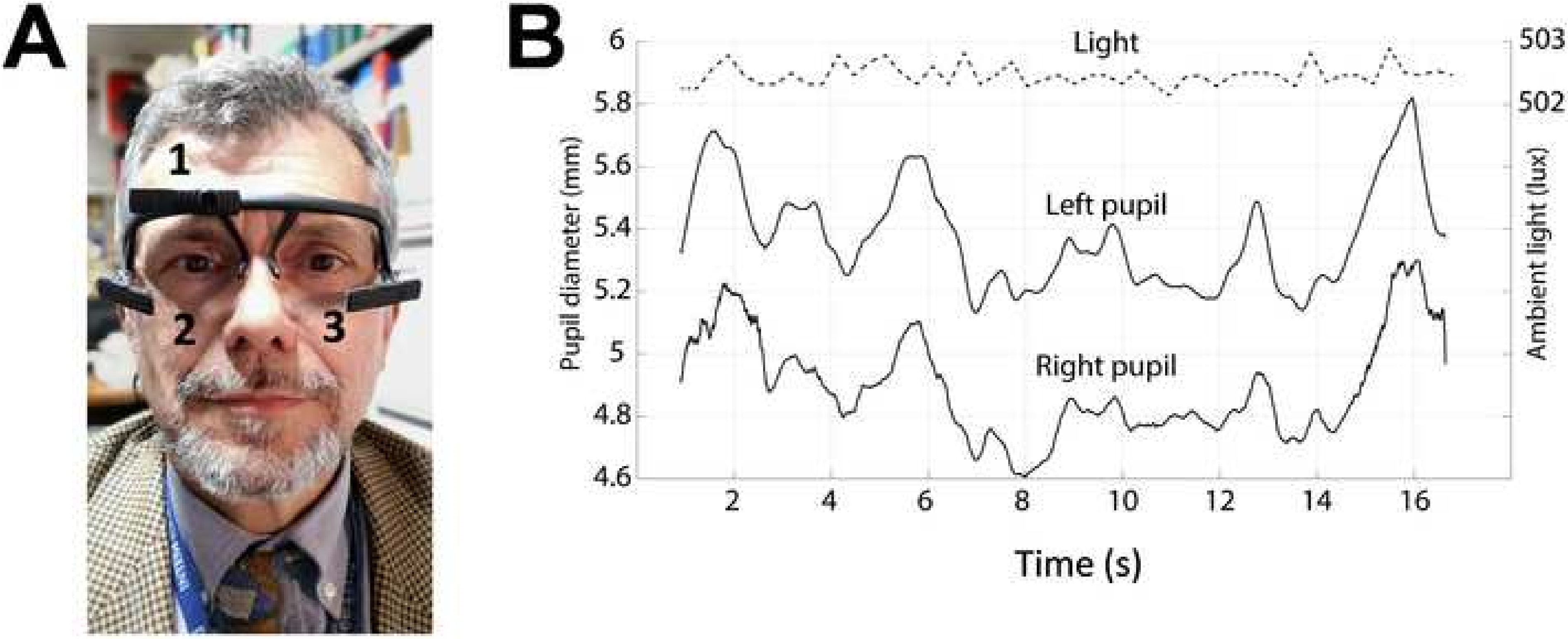
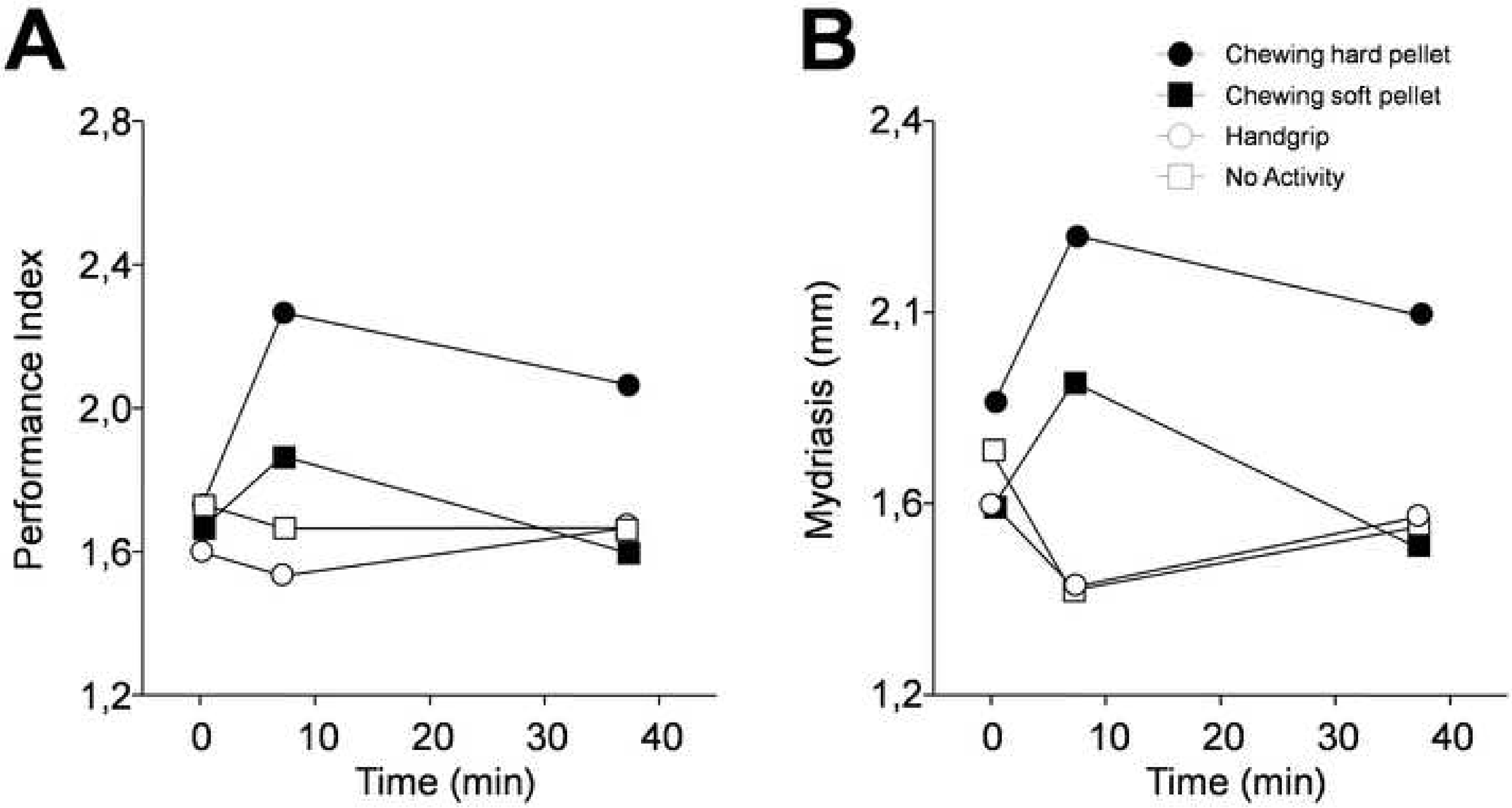
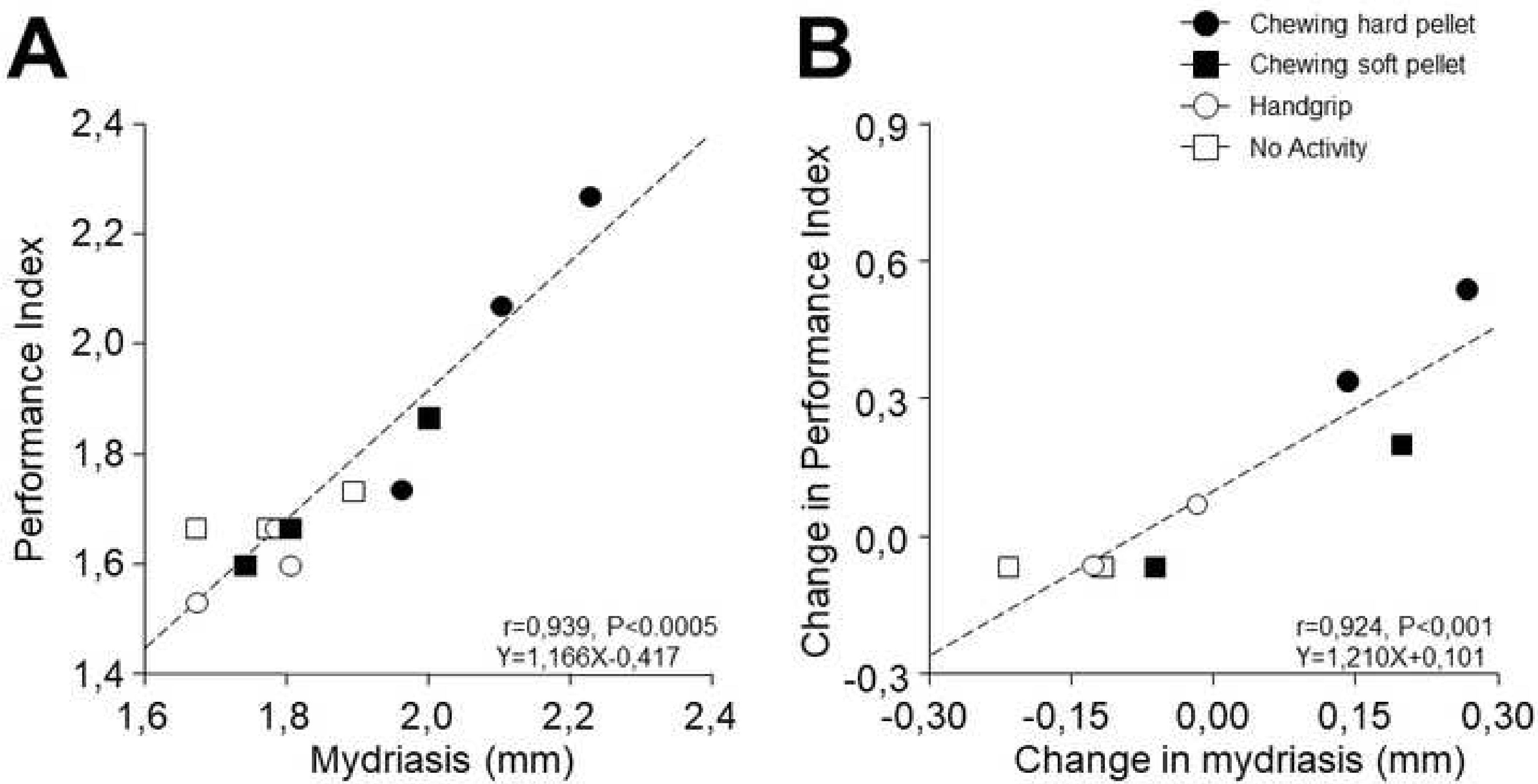
-----------5-----------	---------6/8---------	--------1/4/9-------
1 4 7 3 5 8 2 9 6 0	1 4 7 3 5 6 2 9 8 0	8 1 7 4 3 2 5 9 0 6
7 2 1 4 9 3 1 6 8 4	6 4 7 3 8 2 5 9 0 1	7 5 1 6 4 2 3 9 8 0
5 1 7 9 6 8 2 3 8 7	7 2 6 4 9 3 1 0 8 4	5 1 7 4 6 8 3 8 9 7
8 3 6 9 5 2 1 4 0 7	1 6 5 9 4 0 7 0 2 8	1 0 6 4 7 3 5 9 2 8
2 8 4 9 7 3 6 0 1 3	2 6 4 9 7 5 8 0 1 3	8 1 3 4 6 9 5 2 0 7
1 6 5 9 4 0 7 8 5 6	3 1 2 6 5 4 0 8 7 9	2 8 1 7 4 3 6 0 9 3
8 4 7 3 2 5 9 0 1 9	7 6 5 3 8 1 4 9 2 0	3 1 2 4 8 5 6 9 7 0
2 6 5 8 7 4 1 3 2 4	2 6 5 8 7 4 1 3 0 4	0 3 1 7 4 3 9 6 2 8
3 5 2 1 8 5 6 9 7 9	5 1 7 9 6 2 8 3 0 7	2 1 6 4 8 7 9 3 0 5
8 7 5 3 1 4 6 2 0 3	1 3 6 7 5 2 1 4 8 5	0 6 1 5 4 3 7 9 8 2

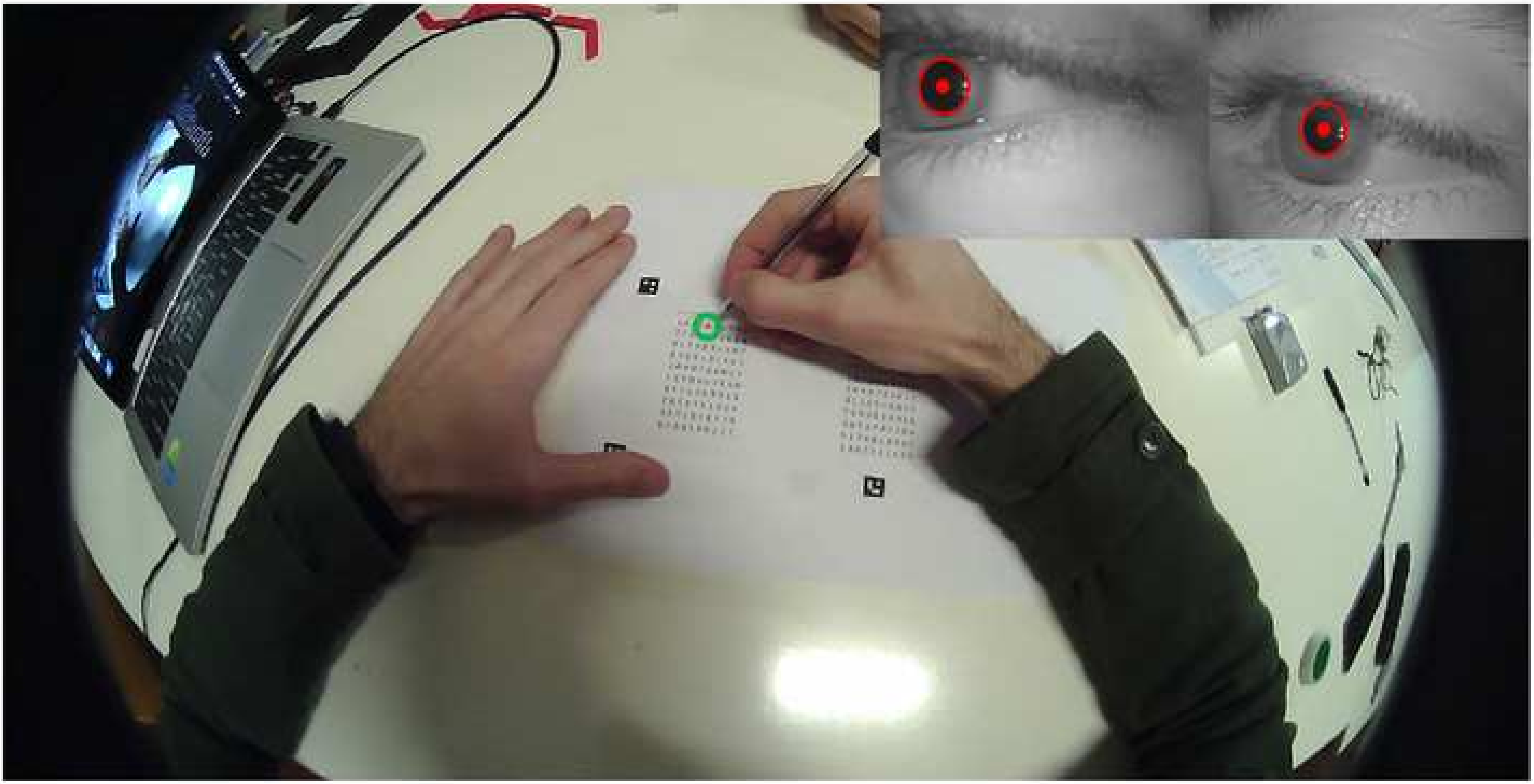
Figure




A
1
2
3
B
Light
Left pupil
Right pupil
Pupil diameter (mm)
Ambient light (lux)
6
5.8
5.6
5.4
5.2
5
4.8
4.6
503
502
2
4
6
8
10
12
14
16
Time (s)


A
B
Chewing hard pellet
Chewing soft pellet
Handgrip
No Activity
Performance Index
2,8
2,4
2,0
1,6
1,2
0
10
20
30
40
Time (min)
Mydriasis (mm)
2,4
2,1
1,6
1,2
0
10
20
30
40
Time (min)


A
Performance Index
2,4
2,2
2,0
1,8
1,6
1,4
1,6
1,8
2,0
2,2
2,4
Mydriasis (mm)
r=0,939, P<0.0005
Y=1,166X-0,417
B
Chewing hard pellet
Chewing soft pellet
Handgrip
No Activity
Change in Performance Index
0,9
0,6
0,3
0,0
-0,3
-0,30
-0,15
0,00
0,15
0,30
Change in mydriasis (mm)
r=0,924, P<0,001
Y=1,210X+0,101



Name of Material/ Equipment	Company
Anti-stress ball	Artengo, Decathlon, France
Chewing gum	Vigorsol, Perfetti, Italy
Infrared Camera-Wearable pupillometer	Pupil Labs, Berlin, Germany
Pupillographer	CSO, Florence, Italy
Silicon rubber	Prochima, Italy
Software for pupil detection - wearable pupillometer	Pupil Labs, Berlin, Germany
Tangram Puzzle	Città del Sole srl, Milano, Italy
Wearable pupillometer	Pupil Labs, Berlin, Germany

Catalog Number	Comments/Description
TB600	
Commercially available product	
Pupil Labs headset	
MOD i02, with chin support	
gls50	
Pupil Labs headset	
Tangram Puzzle	
Pupil labs model	Dimension of the frame: 13.5 x 15.5cm

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Author(s): TRAMONTI FANTOZZI MP; BANFI T; DE CICCO V; BARRESI M; CATALDO E; DE CICCO D; BRUSCHINI L; CIUTI G; FARAGUNA U; MANZONI D

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2. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See some comments marked in the attached manuscript.
We have added to the description of the steps all the information requested within the editor comments

3. Please address specific comments marked in the attached manuscript.
Done

4. After you have made all the recommended changes to your protocol section, please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.
Done. The flowchart of the experiments is represented by the highlighted steps from 2.1 to 3.2.5. The further highlighted steps (4.1.1-4.3.4) are those reported in 2.1 - 3.2.5. Please note that the experiment that has to be filmed is only that one referring to step 3.1.4.1 (chewing soft pellet). For this reason, the step 3.1.5 has to be changed from:
"3.1.5. Just after the end of each step (3.1.4.1–3.1.4.4), evaluate performance in the matrices test and pupil size at rest and during haptic task (T7). "
To:
"3.1.5. Just after the end of step 3.1.4.1, evaluate performance in the matrices test and pupil size at rest and during haptic task (T7)."

5. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.
Steps 2.1-2.3.5 form a continuous sequences. Steps 4.1.1-4.3.4 have to be inserted within flowchart where indicated (see steps 3.1.1, 3.1.2, 3.2.1)

6. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
Done

7. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the materials alphabetically by material name.
Done

-

Comments in the text

Lines 41-46. Please shorten it to no more than 50 words (abstract)

Done. The abstract has been changed as it follows "To verify whether trigeminal effects on cognitive performance involve the Locus Coeruleus activity, we propose two protocols aimed to evaluate a possible correlation between the performance and the task-related pupil size changes induced by chewing. These protocols may be applied to conditions where the Locus Coeruleus contribution is suspected."

Line 133. The editor added this. Is this correct? 3.1.1.

"Evaluate baseline performance (see section 4.1) in the cognitive (matrices) test (T0, control)."

OK

Line 136. The editor added this. Is this correct?

"3.1.2. Evaluate pupil size (see section 4.2) at rest (no activity requested to the subject) (T0, control)."

OK

Lines 145-147. This is unclear. Is each step below considered a session? Do you mean that subject comes on day 1 and perform activity 3.1.4.1, then on day 3 or 4 and perform activity 3.1.4.2, etc.? Or does the subject perform different activities in different sessions that are completed in one day?

"3.1.4. Ask each subject to perform three specific activities for 2 min or to have 2 min of rest, according to steps 3.1.4.1−3.1.4.4. Go through these steps in separate sessions (2−3 days between sessions)."

The same subject have to come in different days. The text changed to "3.1.4. Ask each subject to perform three specific activities for 2 min or to have 2 min of rest, according to steps 3.1.4.1−3.1.4.4. Ask the subjects to perform these activities in separate sessions occurring in different days (2−3 days between sessions)." See lines 143-145 of the revised version

In the same manner also the 3.2.3 point was changed in "Ask each subject to perform three specific activities for 2 min or to have 2 min of rest, according to steps 3.2.3.1−3.2.3.4. Ask the subjects to perform these activities in separate sessions occurring in different days (2−3 days between sessions)." See lines 177-179 of the revised version.

Lines 222-275. The editor broke the paragraphs into steps and reorganized sections. Please review for accuracy.

(4.2. Pupil size in protocol 1)

Changes to the text proposed by the editors have been performed, mainly for answering to the specific editorial comments. They are indicated below.

-Lines 224-225 have been changed to:" 4.2.1. Prepare the subject for the pupil size measurement with a corneal topographer-pupillographer (Table of Materials) (which prevents vision of the environment) using one of the following two acquisition procedures." See lines 222-224 of the revised version.

-Line 227 has been changed to: "4.2.1.1. Perform single camera shot of the pupil (Figure 2A,B), with constant illumination level (40 lux), pressing the specific button on the corneal

topographer-pupillographer. Keep the optimal working distance of 56 mm between the camera and the pupil. See lines 226-228 of the revised version
-Lines 237-238 have been changed to: "4.2.2. Record pupil size at rest (steps 3.1.2, 3.1.5 and 3.1.6), left and right separately. "See line 238.
-Lines 240-241 have been changed to:"4.2.3. Record pupil size during the haptic task (steps 3.1.3, 3.1.5 and 3.1.6), left and right separately." See lines 240-241 of the revised version.
Lines 248-249 have been changed to "by subtracting the pupil size at rest from the pupil size during the haptic task and obtain all the average left-right values". See lines 248-249 of the revised version.
-Lines 253-254 have been changed to: ".3.1. Prepare the subject for the pupils size measurement using a wearable pupillometer /eye tracker (Pupil Labs, see Figure 3A), endowed with a 3D printed glass frame structure, using the following procedure. Let the subject wear the wearable pupillometer. Adjust the position of the two infrared cameras (Figure 3A-2,3) mounted on bars stemming from the frame (Table of Materials), so that the eyes are within the field of view of the cameras and in focus."See lines 253-258.
-Lines 256-259 have been changed to: "4.3.1.1. Acquire pupil's images (sampling rate: 120 Hz), which are processed online by the software supplied with the wearable pupilometer (Table of Materials), which provides pupillary diameter in millimetres using a geometrical model of the "average" human ocular and disregard blink artifacts." See lines 260-263 of the revised version.
-Lines 261-263 have been modified to: "4.3.1.2. Record continuously the environmental illumination level by using a calibrated logarithmic light sensor mounted on the wearable pupillometer frame. Use frontal RGB camera mounted on the wearable pupillometer (Figure 3A-1) to record the subject field of view (sampling rate: 30 Hz) useful to studying gaze behaviour." See lines 265-268 of the revised version.
-Line 267 has been changed to: "Record the size of the pupils while the subject performs the Spinnler-Tognoni test, so to have" See lines 272-274 of the revised version.
-Lines 270-271 have been erased.

Line 227. What is the distance between the subject and the camera? Is this done by pressing a button on the corneal topographer-pupillographer? Please include these details.
Details have been added to the text, at lines 226-228 of the revised version.

Line 248. The editor rephrased this sentence. Please review for accuracy.
Done. The phrase now sounds "Calculate the task-related mydriasis by subtracting the pupil size at rest from the pupil size during <u>the haptic</u> task and obtain all the average left-right values". See lines 247-249.

Line 254. Is this custom made? Please specify the dimensions of the frame in the Table of Materials.
No, the 3D printed structure is produced by Pupil Labs and has dimensions of about 13.5 x 15.5cm. These data have been added to the table of materials. The phrase has been changed to " Prepare the subject for the pupils size measurement using a wearable pupilometer/eye tracker (Pupil Labs, see Figure 3A), endowed with a 3D printed glass frame structure, using the following procedure".See lines 253-255 of the revised version

Line 256. Please include product information in the Table of Materials. Are they part of the pupil diameter?
Yes. Pupil Labs headset, Pupil Labs. Added in the table of materials

Line 256.Please specify the positions (positions 2 and 3 in Figure 3A?)
Yes, the editor is correct. The information has been added in the text

Line 257. Convert to what and how?
Pixels in mm. The phrase has been simplified as it follows: "Acquire pupil's images (sampling rate: 120 Hz), which are processed online by the software supplied with the wearable pupilometer (Table of Materials), which provides pupillary diameter in millimetres using a geometrical model of the "average" human ocular and disregard blink artifacts. " See lines 260-263 of the revised version.

Line 259. What software? Please include it in the Table of Materials.
Software has been added to the table of materials.

Line 262. Which instrument? It is clear. Where is this camera located? It is unclear. Please consider adding a step that describes the setup of all instrument/cameras. Please specify the position of this camera? Position 1 in Figure 3A? Please also include camera information in the Table of Materails.
The instrument is the wearable pupilometer. The phrase has been clarified as it follows. "Record continuously the environmental illumination level by using a calibrated logarithmic light sensor mounted on the wearable pupillometer frame. Use frontal RGB camera mounted on the wearable pupillometer (Figure 3A-1) to record the subject field of view……………… ". See lines 265-268. Information about the camera has been added to the table of materials.

Lines 270-271. Does this apply to both protocol 1 and protocol 2? If so, add this as step 4.2.5 for protocol 1. Please specify the position of this camera? Position 1 in Figure 3A? Please also include camera information in the Table of Materials.
No, this does not apply to protocol 1. The camera is that mentioned in the answer to the previous comment.

Line 279.-280. Can this be simplified to "obtained from section 4.3"?
Yes, the phrase has been changed accordingly. See lines 282-283.

Lines 280-282. Please describe how or provide a relevant reference. When does the subject wear the eye tracker? It is not mentioned in previous steps.
The requested reference has been added. The eye tracker is the wearable pupilometer: this point has been specified at line 253-254 of the revised version. Lines 279-282 have been changed to: "NOTE: Reconstruct online the fixation point using the images of the two pupils obtained from section 4.3. Process the acquired frames in real time and estimate the gaze fixation point by

using a previously calculated transfer function[31] specific for each subject wearing the eye tracker." See lines 282-285 of the revised version.

Lines 285-286. Please include material information in the Table of Materials. Do you mean matrics sheet used in section 4.1?

The requested information has been added to the Table of Materials. We clarified that the matrix sheet was that one utilised in step 4.1. Lines 284-286 have been changed to: "4.4.1. If necessary, when using protocol 2, reconstruct gaze position from pupil images. For this purpose, add four computer detectable vision markers (ArUco or AprilTag libraries of the instrument software) to the four corners of the matrices sheet already used instep 4.1." See lines 287-289 of the revised version.

Line 288. Can you provide an example? "4.4.2. Ask the subject to gaze at a predefined sequence of points that are shown and tracked in his/her field of view. Simultaneously record the field of view by the additional RGB camera mounted on the frame."

We added a procedural example. Lines 288-293 have been changed to: "4.4.2. Let the calibration system (embedded in the eye tracker software as for the Pupil headset used) acquire the data and evaluate the parameters of the transfer function that maps the fixation point starting from the images of the two pupils. As an example, ask the subject to gaze at a predefined sequence of points that are shown in his/her field of view (the four corners of the matrices sheet and at the centre of the sheet itself) and recorded simultaneously by the additional RGB camera mounted on the frame and facing the subject field of view." See lines 291-297 of the revised version.

Line 292. What calibration system? It is unclear.

This point has rephrased and merged with the preceding one. See lines 291-297 of the revised version.