
Ethanol-preserved eyes provide ocular and retinal predictors of natural morphological conditions in scallops: A case study with *Argopecten irradians* (Bivalvia: Pectinidae)

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10 4 **Ethanol-preserved eyes provide ocular and retinal predictors of natural morphological**
11 **conditions in scallops: A case study with *Argopecten irradians* (Bivalvia: Pectinidae)**
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35 16 **Short running head**
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37 17 RELIABILITY OF ETHANOL-PRESERVED EYES
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3 21 Ethanol-preserved specimens represent one of the most common resources for biological
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5 22 research. However, little is known of how ethanol preservation may change tissue morphology and
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7 23 impact the interpretation of trait quantification in structures, such as eyes. While scallop eyes are
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9 24 an interesting system for investigating eye evolution and visual adaptations, cross-species
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11 25 comparisons mainly depend on museum specimens. Therefore, to test whether ethanol-preserved
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13 26 specimens serve as accurate indicators of natural eye morphology, we investigated the effects of
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15 27 preservation on selected traits, such as eye and pupil diameter, in the scallop *Argopecten irradians*.
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17 28 We also compared ethanol-preserved eyes to paraformaldehyde-fixed eyes to investigate possible
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19 29 impacts on retinal morphology. Our results demonstrate that eye size does not change with **short-**
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21 30 **term** preservation, whereas pupil size becomes significantly larger, **likely due to the contraction of**
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23 31 **actin fibers during dehydration. When comparing measurements among eyes and treatments,** eye
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25 32 size correlates to pupil size, but is not correlated to body size. We found that ethanol-preserved
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27 33 eyes provide close estimates of retinal traits, with similar photoreceptor spacing distance and
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29 34 number of photoreceptor cells, compared to samples fixed in paraformaldehyde. **These findings**
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31 35 **might also be applicable in the context of other mollusks, especially bivalves and gastropods, with**
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33 36 **delicate visual systems. Our study provides evidence that ethanol-preserved eyes exhibit tissue-**
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35 37 **specific differences that should be acknowledged in morphological studies. For example, pupil size**
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37 38 **should be investigated while accounting for post-preservation effects. Other traits, such as lens**
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39 39 **shape, are inconsistent and severely impacted by preservation. Finally, eye size and some**
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41 40 **photoreceptor cell measurements can be helpful to describe natural morphology.**

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39 41 Natural history museums and other scientific collections are a critical repository for
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41 42 biological diversity and serve as a primary source for phylogenetic, morphological, and
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43 43 evolutionary analyses (Card *et al.*, 2021). However, when accessing biological information from
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45 44 historical specimens, one must critically ask how accurately the preserved materials represent
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47 45 natural conditions. Fluid preservation **historically** includes an initial fixation step with some cross-
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49 46 linking fixative agents, such as aldehydes (e.g., formalin), and subsequent storage in ethanol. More
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51 47 recently, museum samples have been subjected to direct preservation in ethanol for DNA
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53 48 preservation, but this can result in dehydration of tissues, poor tissue penetration, changes in
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55 49 biomass, deformation, loss of pigmentation, and dilution of the ethanol preservative (Simmons,
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57 50 2014). Surprisingly, there has been little investigation about how ethanol preservation impacts
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59 51 specific tissues and organs, e.g., (Martinez, Berbel-Filho & Jacobina, 2013; Sotola *et al.*, 2019;

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3 52 Ziegler & Sagorny, 2023), especially those that are fluid-filled, delicate, and unprotected by an
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5 53 exoskeleton, such as eyes (Thomas *et al.*, 2020).

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7 54 Eyes are a useful trait to examine adaptation and museum specimens are paramount to study
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9 55 these evolutionary patterns. Parameters such as eye aperture, eye size, and retinal organization are
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11 56 important components to understand visual functions of sensitivity and resolution. To determine if
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13 57 preserved eyes provide reliable estimates of natural eye conditions, we conducted a study with the
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15 58 common bay scallop *Argopecten irradians* (Lamarck, 1819) to quantify the effects of 95% ethanol
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17 59 preservation on ocular features. While it is known that these delicate organs are prone to
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19 60 preservation-induced artifacts (Speiser *et al.*, 2016) and pupil diameter naturally varies due to
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21 61 constricting musculature in living animals (Miller *et al.*, 2019), the impact of preservation methods
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23 62 on these tissues has never been examined in scallops. To address the possible preservation effect,
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25 63 we examine the same freshly dissected eyes after ethanol preservation to determine whether after
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27 64 treatment (I) eye and pupil sizes change, (II) eye size continues to correlate with pupil size, and
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29 65 (III) eye size and pupil size change with body size. We expect that all eye components shrink
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31 66 uniformly after dehydration and the same amount of shrinkage in all animals, regardless of
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33 67 individual size. Finally, although ethanol is not a proper fixative for fine anatomical study, we
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35 68 expect reliable estimates from histological sections produced by ethanol-preserved samples
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37 69 regarding simple morphological measurements, such as photoreceptor spacing and number.

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39 70 We examined 12 individuals of the common bay scallop *Argopecten irradians* obtained
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41 71 from Gulf Specimen Marine Lab (Panacea, Florida). To investigate eye external morphology
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43 72 before preservation, mantle margins containing eyes were dissected from ten individuals. Left and
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45 73 right mantle strips were attached to cardboard using entomological pins, submersed in the same
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47 74 saltwater the scallops were kept in, and immediately examined under the stereomicroscope.
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49 75 Photographs were taken of five eyes on the ventral region of both left and right sides for each
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51 76 specimen (10 eyes per animal), for a total of 100 sampled eyes. Saltwater was drained and replaced
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53 77 with 95% ethanol. After 24 h of dehydration, the same scallop eyes were photographed based on
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55 78 previous mapping of positions. Images of eyes pre- and post-preservation were analyzed in ImageJ
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57 79 version 1.54f (12) to determine eye and pupil diameter. A size ratio between these two diameters
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59 80 was calculated for each eye, and means were obtained for each treatment. We tested for differences
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61 81 in eye size, pupil size, and size ratio after ethanol preservation using a parametrical approach
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63 82 (Student's t-test) and a permutation-based resampling method. We also assessed the statistical

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3 83 significance of the association between eye and pupil diameter and their relationship to shell length
4 84 as a proxy for body size. All analyses were conducted in R version 4.3.2 (R Core Team, 2021), and
5 85 codes are available as Supplementary Material.

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9 86 We also compared ethanol-preserved eyes to those fixed in paraformaldehyde (PFA),
10 87 following standard protocols for histology (Audino *et al.*, 2015). Two scallop individuals were
11 88 anesthetized in 7.5% MgCl₂ for 2 h. Eyes were dissected and fixed in 4% PFA in PBS 0.1 M for 3
12 89 h. Three ethanol-fixed eyes and three paraformaldehyde-fixed eyes were embedded in LR White
13 90 resin for histology and sectioned in a Leica UC6 ultramicrotome. Histological images of the **central**
14 91 **region of the retina** were measured using ImageJ to compare photoreceptor spacing and rhabdom
15 92 length (**five measurements per eye**), as well as photoreceptor number based on nuclei count.

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21 93 Surprisingly, eye size is not affected by ethanol preservation, as no significant change in
22 94 eye diameter occurred after dehydration (Fig. 1A, Student's t-test: $P = 0.4$). **By comparing the same**
23 95 **eyes between treatments**, the mean diameter of dissected eyes in seawater (**$595.72 \pm 83.21 \mu\text{m}$**) and
24 96 ethanol-preserved eyes (**$586.58 \pm 95.29 \mu\text{m}$**) is statistically the same, as supported by permutation-
25 97 based resampling methods (Fig. 1B). This is a particularly welcoming finding considering that the
26 98 size of soft organs is substantially affected by varying degrees of contraction after dehydration, as
27 99 observed in bivalve siphons (Sartori *et al.*, 2008). Because the eyes of *A. irradians* do not shrink,
28 100 we argue that measurements from preserved specimens can provide reliable estimates of eye size,
29 101 maximizing morphological information that can be obtained from historical materials for cross-
30 102 species comparisons. In contrast, pupil size remarkably changes after dehydration (Fig. 1C,
31 103 Student's t-test: $P = 6.68 \times 10^{-18}$). The pupil's diameter (**$213.95 \pm 44.32 \mu\text{m}$**) becomes significantly
32 104 larger when preserved in 95% ethanol (**$280.67 \pm 51.6 \mu\text{m}$**), as corroborated by permutation-based
33 105 resampling methods (Fig. 1D). Contrary to our initial hypothesis that eye components shrink
34 106 jointly, the change in the size ratio of eye and pupil emphasizes that only the pupil diameter changes
35 107 after dehydration (Student's t-test: $P = 3.97 \times 10^{-29}$, Fig. 1E, F). Interestingly, scallops are known for
36 108 having radial and circular actin fibers associated with the cornea, likely acting as fine muscles that
37 109 dilate and constrict the pupil (Miller *et al.*, 2019). Severe dehydration caused by ethanol might
38 110 cause muscle fibers to contract, explaining the extension of the pupil diameter. **In pupillary**
39 111 **response experiments**, a previous study documented that the pupil's area of *A. irradians* can dilate
40 112 **up to 60% under the brightest experimental conditions** (Miller *et al.*, 2019). Our results show that
41 113 **the pupil's diameter increased by 24% on average after preservation in ethanol. Accordingly, an**

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3 114 increase of 24% in the radius is expected to cause an increase of 53.76% in the area of a circle, a
4 percent close to the experimental dilation of 60% previously identified. Therefore, ethanol-
5 115 preserved eyes have pupils at maximum size, representing the maximum light intake into the eye.
6 116 Even though pupil size must be cautiously considered in preserved specimens, it offers the
7 possibility to predict a natural morphological condition that optimizes light sensitivity.
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12 119 Next, we tested whether pupil diameter can be explained by eye diameter after ethanol
13 preservation. Our results show that, indeed, larger pupils are correlated to larger eyes ($P=2.2\times 10^{-}$
14 120 16 , Fig. 1G), and such a relationship is maintained after ethanol preservation (Fig. 1G). These data
15 121 are important to the interpretation of eye function. For example, many vertebrates, such as anurans,
16 122 show increased relative eye sizes and pupillary proportions to maximize visual sensitivity (Thomas
17 123 *et al.*, 2020). Scallops provide a non-vertebrate example of animals that use a pupillary mechanism
18 124 associated with light sensitivity and resolution (Miller *et al.*, 2019). Our results prove that scaling
19 125 relationships and size can be accurately examined in ethanol-preserved scallops. We then tested
20 126 for an allometric relationship between eye and body sizes, a frequent scaling association among
21 127 animals (Caves, Sutton & Johnsen, 2017). Our results show that neither eye diameter nor pupil
22 128 diameter scale with shell length, used here as a proxy of body size. Unlike most animals that show
23 129 positive eye-body allometry (Thomas *et al.*, 2020), our findings suggest that a fixed range of eye
24 130 size occurs in *A. irradians*, regardless of the size of the individuals. Nevertheless, we expect eye
25 131 investment to vary greatly across scallop species, considering the increased ecological diversity
26 132 and variation in other optical traits, such as eye number (Speiser & Johnsen, 2008; Audino, Adams
27 133 & Serb, 2022).
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40 135 Histological data reveal that ethanol-preserved eyes can help describe and estimate some
41 136 traits of optical relevance (Fig. 2). Despite differences in eye size, ethanol-preserved retinas reveal
42 137 a similar photoreceptor spacing distance and a similar number of photoreceptor cells compared to
43 138 retinas preserved in 4% PFA (Table 1). Even though photoreceptor cell number is not expected to
44 139 vary with the preservation method, we demonstrate that the trait, a potential measure of visual
45 140 investment, can be estimated from ethanol-preserved samples. Rhabdom length is slightly shorter
46 141 in ethanol-preserved retinas than in those prepared using histological standard fixation, possibly
47 142 because of the size difference between eyes or abrupt dehydration with shrinkage (Table 1). In the
48 143 comparative anatomy of scallop species, retinal data of *A. irradians*, fixed in buffered 4%
49 144 formaldehyde, suggest similar rhabdom length of the proximal retina (Speiser & Johnsen, 2008).
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3 145 However, this previous study sampled larger eyes, which is reflected in larger measurements of
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5 146 internal diameter ($670 \pm 40 \mu\text{m}$) and photoreceptor spacing ($5.8 \pm 0.2 \mu\text{m}$) for the species (Speiser
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7 147 & Johnsen, 2008). Not surprisingly, lenses across scallop species have shown great variation in
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9 148 shape, including globular, flat, and fusiform, regardless of the preservation method. The same was
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11 149 observed among our samples (Fig. 2). Considering the delicate nature of this tissue, fixation and
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13 150 dehydration likely cause unpredictable changes, making chemically preserved lenses an
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15 151 inconsistent predictor of scallop eye morphology. Other artifacts were noted, such as how the lens
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17 152 has moved away from the cornea in some cases for both treatments (Fig. 2). In addition, PFA-
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19 153 preserved eyes show a large artefactual spacing between the retina and the mirror layer (Fig. 2).

19 154 Overall, histological information should always be carefully interpreted because of the
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21 155 possibility of preservation-induced artifacts. Here, we used PFA-preserved samples for
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23 156 comparisons, but future work using other methods, such as ultrarapid fixation via high-pressure
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25 157 freezing (HPF), will be relevant to compare and obtain a more natural morphology. In addition,
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27 158 preservation time could be explored since long-term preservation is another factor causing
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29 159 unpredictable morphological changes, for example, as observed in cephalopod specimens from
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31 160 museum collections (Voight, 2001). Knowing the limitations and expected artifacts of eye
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33 161 preservation is crucial to the functional morphology and optics of any visual system.

33 162 Our results provide an empirical test to support that ethanol-preserved specimens can be
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35 163 helpful for collecting external and internal anatomical information about eyes, which is particularly
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37 164 valuable for rare specimens and samples for which ideal fixation is not feasible. We found support
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39 165 for our initial hypothesis that eye size and pupil size are correlated. In contrast, eye and pupil
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41 166 diameter are not correlated to body size, an interesting fact that should be investigated for other
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43 167 species. Most importantly, eye size does not vary after preservation in ethanol, whereas pupils
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45 168 become significantly larger. Consequently, ethanol-preserved eyes are helpful to investigate the
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47 169 diversity and evolution of visual investing in scallops based on eye size, pupil size, and retinal
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49 170 measurements. However, other investigations focused on optics and functioning will likely depend
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51 171 on living specimens or other preservation methods. Based on our results, we expected to see
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53 172 variation in the eye aperture of preserved mollusks with delicate camera-type eyes, such as
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55 173 gastropods, and suitable retina preservation in other types of bivalve eyes. Whenever possible, we
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57 174 encourage other research groups to evaluate the impacts of preservation methods and the reliability
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59 175 of morphological data from historical, preserved specimens.

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186 Conflict of Interest Statement

187 The authors have no conflicts of interest to declare.

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Table 1. Morphological variables related to optical properties measured from histological sections of *Argopecten irradians* eyes preserved in 95% ethanol (n=3) and 4% PFA (paraformaldehyde in phosphate-buffered saline, n =3). Values represent means obtained from five independent measurements from the same histological image. For the complete dataset, see Supplementary Material. Means and standard deviations for the two groups are indicated in bold. Photoreceptor spacing is measured as the distance between two nuclei of adjacent cells (see Fig. 2). Rhabdom length corresponds to the specialized portion of the photoreceptor cell (see Fig. 2). Photoreceptor number cover both distal and proximal retinas. Abbreviations: ID, internal diameter; PD, pupil diameter; PR, proximal retina; PS, photoreceptor spacing; RL, rhabdom length.

| Fixation method | PD (μm) | ID (μm) | PS in the PR (μm) | RL in the PR (μm) | Photoreceptor number |
|---------------------------|------------------------------|------------------------------|-----------------------------------|-----------------------------------|------------------------------|
| 95% Ethanol | 179.54 | 333.4 | 5.812 | 25.078 | 105 |
| 95% Ethanol | 200.73 | 436.12 | 5.396 | 22.11 | 121 |
| 95% Ethanol | 209.12 | 464.3 | 5.048 | 20.704 | 129 |
| Mean | 196.46 | 411.27 | 5.41 | 22.63 | 118.33 |
| \pmSD | \pm15.24 | \pm68.89 | \pm0.38 | \pm2.23 | \pm12.22 |
| 4% PFA | 298.27 | 561.42 | 5.044 | 32.148 | 87 |
| 4% PFA | 282.3 | 484.9 | 5.712 | 32.166 | 152 |
| 4% PFA | 265.24 | 481.61 | 5.362 | 28.072 | 151 |
| Mean | 281.93 | 509.31 | 5.37 | 30.79 | 130 |
| \pmSD | \pm16.51 | \pm45.15 | \pm0.33 | \pm2.35 | \pm37.24 |

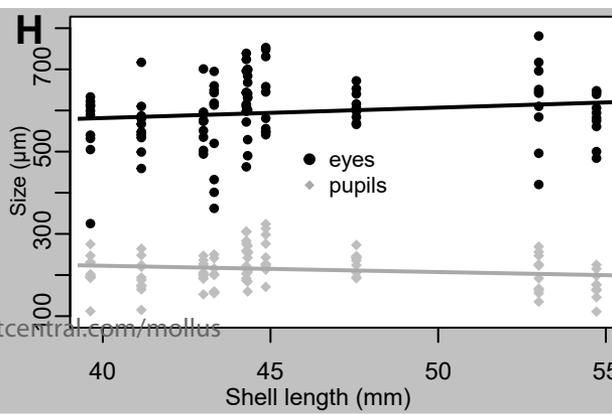
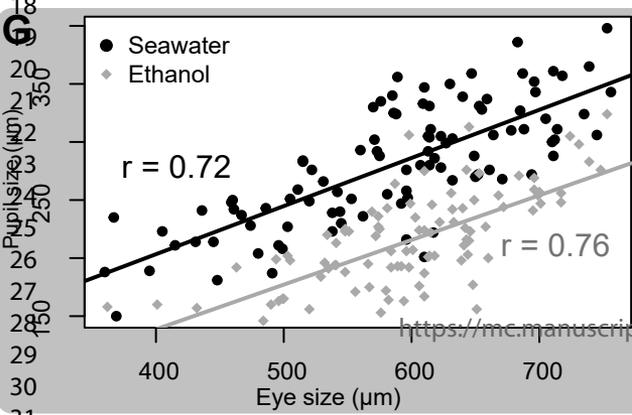
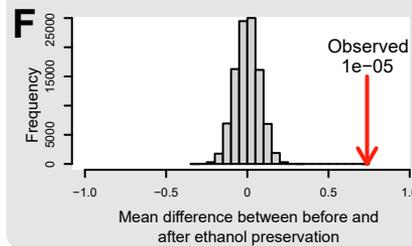
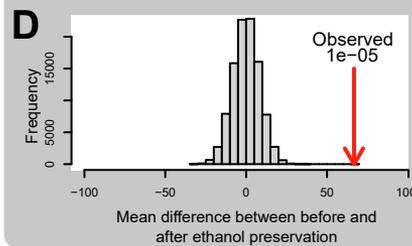
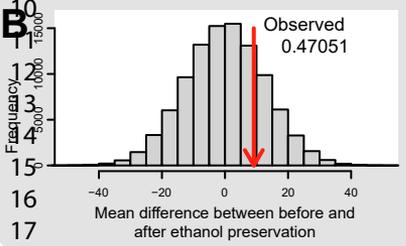
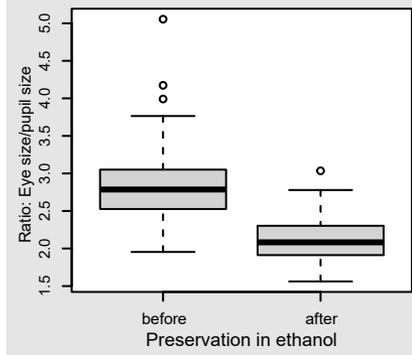
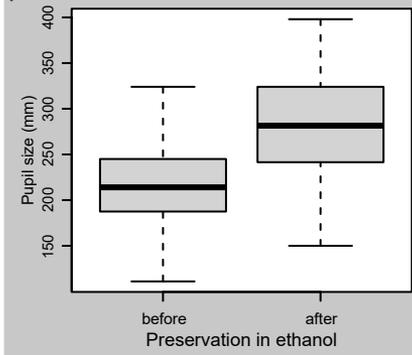
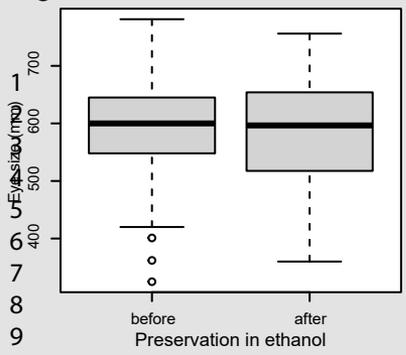
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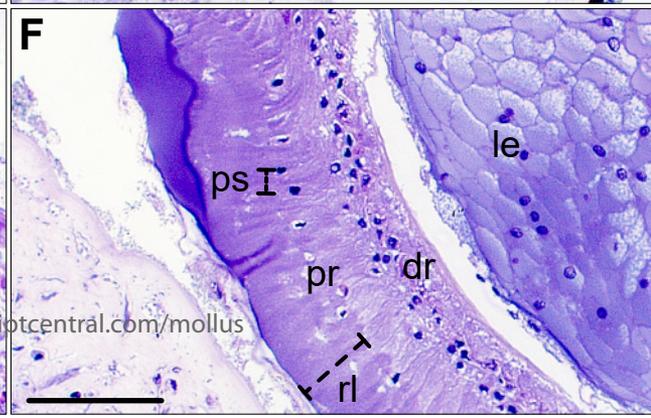
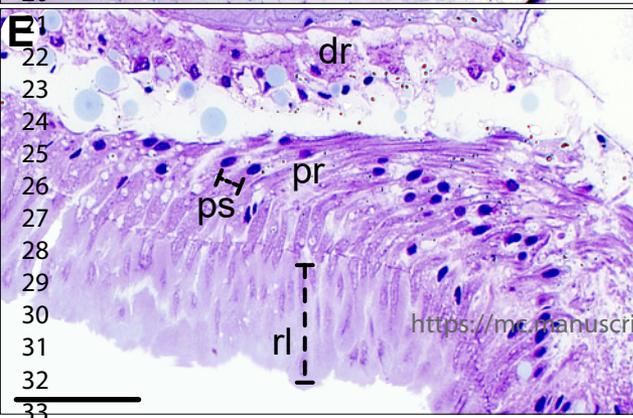
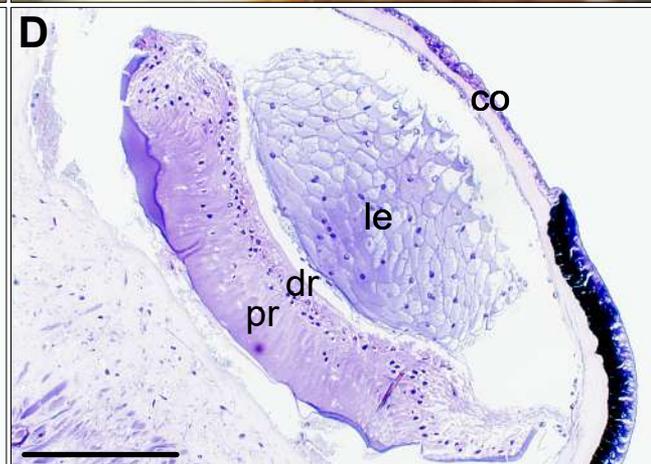
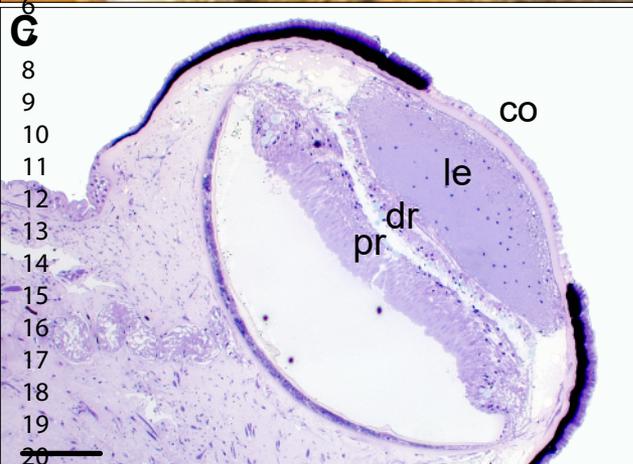
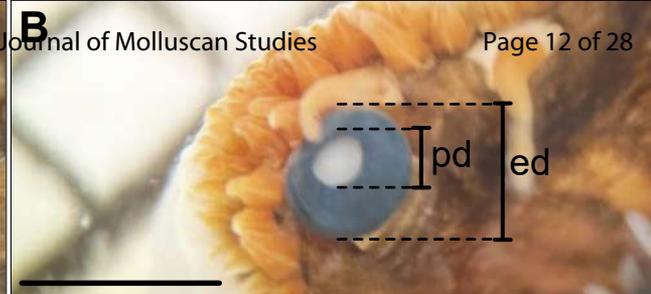
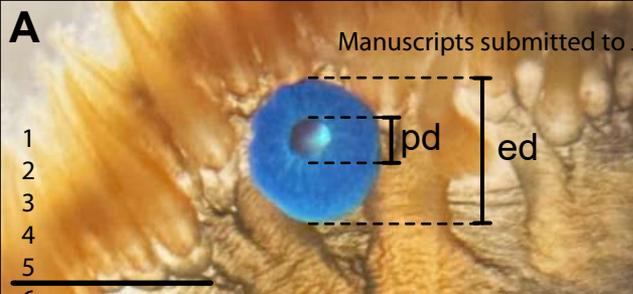
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8 **Figure 1.** Comparison of eye and pupil size in the scallop *Argopecten irradians* before and after
9 preservation in 95% ethanol. We generated a permutation-based empirical sampling distribution
10 after 100,000 iterations to test the null hypothesis of no size difference before and after ethanol
11 preservation (B, D, F). **A.** Boxplots of eye diameter. **B.** Differences in eye diameter lie within the
12 expected distribution under the null hypothesis, *i.e.*, the difference is insignificant. **C.** Boxplots of
13 pupil diameter. **D.** Pupil diameter significantly changes after ethanol preservation. **E.** Boxplots of
14 the ratio between eye and pupil diameters. **F.** The ratio between eye and pupil sizes also changes
15 after ethanol preservation. **G.** Regression of pupil diameter and eye diameter before and after
16 ethanol preservation. Pearson correlation coefficient (r). **H.** Regression of eye/pupil and body size,
17 measured as shell length, highlighting lack of scaling relationship.
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28 **Figure 2.** Eyes of the scallop *Argopecten irradians* under stereomicroscope (A-B) and in 3 μm
29 histological sections stained with Azure B (C-F). **A.** A single eye was photographed after dissection
30 under seawater. **B.** The same eye after 24h of fixation in 95% ethanol. **C.** Section through the
31 central region of an eye fixed in 4% PFA. **D.** Section through the central region of an eye preserved
32 in ethanol. **E.** Detail of the double-retina (sample fixed in 4% PFA). **F.** Detail of the double-retina
33 (sample fixed in ethanol). Relevant measurements are indicated with lines between bars.
34 Abbreviations: co, cornea; dr, distal retina; ed, eye diameter; le, lens; pd, pupil diameter; pr,
35 proximal retina; ps, photoreceptor spacing; rl, rhabdom length. Scale bars: A = 1.0 mm; B = 1.0
36 mm; C = 100 μm ; D = 100 μm ; E = 40 μm ; F = 40 μm .
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3 **SUPPLEMENTARY MATERIALS**
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12 **Title**

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14 **Ethanol-preserved eyes provide ocular and retinal predictors of natural**
15 **morphological conditions in scallops: A case study with *Argopecten irradians***
16 **(Bivalvia: Pectinidae)**
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21
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SUPPLEMENTARY MATERIALS

Table S1. Shell and eye measurements of *Argopecten irradians* individuals before and after preservation in 95% ethanol. Abbreviations: E., ethanol; ED, eye diameter; L., left side; PD, pupil diameter; R., right side; S., sea water.

Table S2. Morphological variables related to optical properties measured from histological sections of *Argopecten irradians* eyes preserved in 95% ethanol and 4% PFA (paraformaldehyde in phosphate-buffered saline). Abbreviations: ID, internal diameter; PD, pupil diameter; PR, proximal retina; PS, photoreceptor spacing; RL, rhabdom length.

R Codes. Script with all codes used for data analysis and plotting in R markdown format.

Table S1. Shell and eye measurements of *Argopecten irradians* individuals before and after preservation in 95% ethanol. Abbreviations: E., ethanol; ED, eye diameter;

L., left side; PD, pupil diameter; R., right side; S., sea water.

| Individual ID | Shell Height (mm) | Shell Length (mm) | S.L.ED (µm) | S.L.PD (µm) | S.R.ED (µm) | S.R.PD (µm) | E.L.ED (µm) | E.L.PD (µm) | E.R.ED (µm) | E.R.PD (µm) | S.L. Ratio (ED/PD) | S.L. Mean Ratio | SR Ratio (ED/PD) | S.R. Mean Ratio | E.L. Ratio (ED/PD) | D.L. Mean Ratio | E.R. Ratio (ED/PD) | E.R. Mean Ratio |
|---------------|-------------------|-------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------------|-----------------|------------------|-----------------|--------------------|-----------------|--------------------|-----------------|
| Scallop 1 | 43.92 | 44.86 | 556 | 171 | 748 | 276 | 753 | 398 | 586 | 325 | 3.25 | | 2.71 | | 1.89 | | 1.80 | |
| | | | 753 | 324 | 581 | 243 | 459 | 248 | 630 | 350 | 2.32 | | 2.39 | | 1.85 | | 1.80 | |
| | | | 548 | 223 | 731 | 298 | 739 | 365 | 683 | 386 | 2.46 | 2.60 | 2.45 | 2.51 | 2.02 | 1.92 | 1.77 | 1.87 |
| | | | 658 | 227 | 541 | 213 | 718 | 357 | 697 | 343 | 2.90 | | 2.54 | | 2.01 | | 2.03 | |
| | | | 645 | 313 | 548 | 225 | 647 | 359 | 573 | 292 | 2.06 | | 2.44 | | 1.80 | | 1.96 | |
| Scallop 2 | 54.84 | 54.72 | 594 | 163 | 582 | 164 | 562 | 236 | 714 | 311 | 3.64 | | 3.55 | | 2.38 | | 2.30 | |
| | | | 561 | 111 | 639 | 198 | 652 | 272 | 745 | 306 | 5.05 | | 3.23 | | 2.40 | | 2.43 | |
| | | | 645 | 215 | 575 | 225 | 711 | 288 | 694 | 272 | 3.00 | 3.73 | 2.56 | 3.16 | 2.47 | 2.32 | 2.55 | 2.46 |
| | | | 648 | 177 | 500 | 165 | 712 | 302 | 592 | 247 | 3.66 | | 3.03 | | 2.36 | | 2.40 | |
| | | | 484 | 146 | 606 | 176 | 431 | 214 | 491 | 187 | 3.32 | | 3.44 | | 2.01 | | 2.63 | |
| Scallop 3 | 53.32 | 53.00 | 651 | 156 | 717 | 256 | 650 | 270 | 632 | 267 | 4.17 | | 2.80 | | 2.41 | | 2.37 | |
| | | | 696 | 269 | 781 | 247 | 609 | 333 | 415 | 211 | 2.59 | | 3.16 | | 1.83 | | 1.97 | |
| | | | 496 | 163 | 642 | 226 | 553 | 251 | 632 | 303 | 3.04 | 3.31 | 2.84 | 2.95 | 2.20 | 2.21 | 2.09 | 2.10 |
| | | | 420 | 135 | 584 | 192 | 496 | 211 | 515 | 283 | 3.11 | | 3.04 | | 2.35 | | 1.82 | |
| | | | 610 | 167 | 646 | 223 | 735 | 324 | 649 | 288 | 3.65 | | 2.90 | | 2.27 | | 2.25 | |
| Scallop 4 | 42.90 | 44.32 | 632 | 242 | 529 | 184 | 653 | 331 | 614 | 331 | 2.61 | | 2.88 | | 1.97 | | 1.85 | |
| | | | 699 | 254 | 700 | 257 | 659 | 337 | 571 | 302 | 2.75 | | 2.72 | | 1.96 | | 1.89 | |
| | | | 668 | 254 | 641 | 211 | 615 | 311 | 480 | 204 | 2.63 | 2.60 | 3.04 | 2.92 | 1.98 | 1.93 | 2.35 | 1.97 |
| | | | 684 | 271 | 490 | 160 | 610 | 347 | 614 | 304 | 2.52 | | 3.06 | | 1.76 | | 2.02 | |
| | | | 631 | 256 | 601 | 207 | 655 | 328 | 576 | 335 | 2.46 | | 2.90 | | 2.00 | | 1.72 | |
| Scallop 5 | 40.33 | 41.15 | 567 | 175 | 610 | 224 | 367 | 235 | 531 | 266 | 3.24 | | 2.72 | | 1.56 | | 2.00 | |
| | | | 459 | 115 | 499 | 165 | 395 | 189 | 520 | 249 | 3.99 | | 3.02 | | 2.09 | | 2.09 | |
| | | | 580 | 172 | 548 | 188 | 623 | 305 | 522 | 276 | 3.37 | 3.05 | 2.91 | 2.87 | 2.04 | 1.88 | 1.89 | 1.96 |
| | | | 587 | 264 | 541 | 194 | 696 | 352 | 588 | 324 | 2.22 | | 2.79 | | 1.98 | | 1.81 | |
| | | | 534 | 220 | 717 | 248 | 570 | 330 | 505 | 251 | 2.43 | | 2.89 | | 1.73 | | 2.01 | |
| Scallop 6 | 38.82 | 39.63 | 610 | 275 | 614 | 202 | 614 | 280 | 538 | 239 | 2.22 | | 3.04 | | 2.19 | | 2.25 | |
| | | | 505 | 198 | 540 | 194 | 542 | 257 | 436 | 241 | 2.55 | | 2.78 | | 2.11 | | 1.81 | |
| | | | 633 | 231 | 325 | 112 | 661 | 276 | 596 | 276 | 2.74 | 2.60 | 2.90 | 2.83 | 2.39 | 2.23 | 2.16 | 2.03 |
| | | | 599 | 247 | 622 | 231 | 581 | 255 | 511 | 259 | 2.43 | | 2.69 | | 2.28 | | 1.97 | |
| | | | 589 | 193 | 532 | 196 | 618 | 286 | 467 | 237 | 3.05 | | 2.71 | | 2.16 | | 1.97 | |
| Scallop 7 | 45.01 | 44.28 | 644 | 203 | 598 | 306 | 613 | 305 | 461 | 242 | 3.17 | | 1.95 | | 2.01 | | 1.90 | |
| | | | 739 | 283 | 615 | 222 | 688 | 311 | 460 | 250 | 2.61 | | 2.77 | | 2.21 | | 1.84 | |
| | | | 724 | 305 | 610 | 187 | 560 | 293 | 575 | 288 | 2.37 | 2.65 | 3.26 | 2.59 | 1.91 | 1.96 | 2.00 | 1.86 |
| | | | 463 | 192 | 572 | 219 | 585 | 340 | 640 | 339 | 2.41 | | 2.61 | | 1.72 | | 1.89 | |
| | | | 696 | 259 | 643 | 276 | 711 | 361 | 589 | 356 | 2.69 | | 2.33 | | 1.97 | | 1.65 | |
| Scallop 8 | 42.04 | 43.00 | 574 | 236 | 701 | 247 | 448 | 181 | 360 | 188 | 2.43 | | 2.84 | | 2.48 | | 1.91 | |
| | | | 551 | 223 | 503 | 202 | 474 | 228 | 503 | 227 | 2.47 | | 2.49 | | 2.08 | | 2.22 | |
| | | | 494 | 199 | 574 | 227 | 613 | 292 | 445 | 214 | 2.48 | 2.84 | 2.53 | 2.71 | 2.10 | 2.18 | 2.08 | 2.21 |
| | | | 592 | 193 | 596 | 206 | 664 | 306 | 685 | 327 | 3.07 | | 2.89 | | 2.17 | | 2.09 | |
| | | | 576 | 153 | 535 | 191 | 627 | 299 | 596 | 216 | 3.76 | | 2.80 | | 2.10 | | 2.76 | |
| Scallop 9 | 42.46 | 43.32 | 649 | 250 | 660 | 200 | 610 | 201 | 756 | 343 | 2.60 | | 3.30 | | 3.03 | | 2.20 | |
| | | | 362 | 158 | 643 | 216 | 405 | 223 | 671 | 268 | 2.29 | | 2.98 | | 1.82 | | 2.50 | |
| | | | 520 | 156 | 401 | 160 | 545 | 230 | 486 | 243 | 3.33 | 2.83 | 2.51 | 2.92 | 2.37 | 2.34 | 2.00 | 2.23 |
| | | | 618 | 202 | 613 | 201 | 607 | 280 | 705 | 320 | 3.06 | | 3.05 | | 2.17 | | 2.20 | |
| | | | 695 | 241 | 432 | 157 | 596 | 258 | 623 | 278 | 2.88 | | 2.75 | | 2.31 | | 2.24 | |
| Scallop 10 | 46.35 | 47.56 | 616 | 246 | 569 | 240 | 499 | 208 | 710 | 300 | 2.50 | | 2.37 | | 2.40 | | 2.37 | |
| | | | 598 | 192 | 566 | 195 | 544 | 240 | 617 | 222 | 3.11 | | 2.90 | | 2.27 | | 2.78 | |
| | | | 603 | 235 | 584 | 206 | 538 | 223 | 515 | 284 | 2.57 | 2.66 | 2.83 | 2.70 | 2.41 | 2.24 | 1.81 | 2.36 |
| | | | 606 | 224 | 672 | 244 | 687 | 359 | 369 | 150 | 2.71 | | 2.75 | | 1.91 | | 2.46 | |
| | | | 653 | 273 | 640 | 243 | 678 | 310 | 597 | 252 | 2.39 | | 2.63 | | 2.19 | | 2.37 | |

Table S2. Morphological variables related to optical properties measured from histological sections of *Argopecten irradians* eyes preserved in 95% ethanol and 4% PFA (paraformaldehyde in phosphate-buffered saline). Abbreviations: ID, internal diameter; PD, pupil diameter; PR, proximal retina; PS, photoreceptor spacing; RL, rhabdom length.

| Eye | Preservation | PD (μm) | ID (μm) | PS in the PR (μm) | | | | Mean | SD | RL in the PR (μm) | | | | Mean | SD | Photoreceptor cell number | | |
|------------------|-----------------|--------------------------------|--------------------------------|--------------------------------|-----|-----|-----|------|---------------------------------|--------------------------------|------|------|------|------|------|-------------------------------|-----|--------------|
| 1 | EtOH | 179.5 | 333.4 | 6.2 | 5.1 | 6.5 | 5.3 | 6.0 | 5.8 | 0.6 | 25.1 | 23.0 | 24.8 | 26.5 | 26.0 | 25.1 | 1.3 | 105 |
| 2 | EtOH | 200.7 | 436.1 | 5.3 | 5.7 | 5.6 | 4.6 | 5.8 | 5.4 | 0.5 | 22.0 | 22.4 | 21.1 | 23.7 | 21.3 | 22.1 | 1.0 | 121 |
| 3 | EtOH | 209.1 | 464.3 | 5.3 | 5.6 | 4.4 | 4.7 | 5.2 | 5.0 | 0.5 | 22.1 | 23.9 | 19.7 | 17.5 | 20.3 | 20.7 | 2.4 | 129 |
| 1 | PFA | 298.3 | 561.4 | 5.8 | 6.6 | 4.5 | 4.6 | 3.7 | 5.0 | 1.1 | 31.9 | 30.5 | 32.4 | 32.5 | 33.6 | 32.1 | 1.1 | 87 |
| 2 | PFA | 282.3 | 484.9 | 5.7 | 5.7 | 5.1 | 5.8 | 6.3 | 5.7 | 0.4 | 31.9 | 34.0 | 32.0 | 33.2 | 29.7 | 32.2 | 1.6 | 152 |
| 3 | PFA | 265.2 | 481.6 | 6.7 | 4.3 | 5.2 | 5.3 | 5.3 | 5.4 | 0.8 | 24.4 | 31.7 | 30.1 | 25.6 | 28.6 | 28.1 | 3.0 | 151 |
| Mean | EtOH | 196.5 | 411.3 | | | | | | 5.4 | | | | | | | 22.6 | | 118 |
| SD | | 15.2 | 68.9 | | | | | | 0.4 | | | | | | | 2.2 | | 12.2 |
| Mean | 4% PFA | 281.9 | 509.3 | | | | | | 5.4 | | | | | | | 30.8 | | 130.0 |
| SD | | 16.5 | 45.2 | | | | | | 0.3 | | | | | | | 2.4 | | 37.2 |
| Reference | 4% formaldehyde | 400 \pm 30 | 670 \pm 40 | | | | | | 5.8 \pm 0.2 | | | | | | | 30 \pm 10 | | |

Speiser D, Johnsen S. 2008. Comparative Morphology of the Concave Mirror Eyes of Scallops (Pectinoidea). *Am. Malacol. Bull.* 26(1–2):27–33.

Ethanol-preserved eyes provide ocular and retinal predictors of natural morphological conditions in scallops: A case study with *Argopecten irradians* (Bivalvia: Pectinidae)

Jorge A. Audino, Gerald Quinlan, Ceren Ordas, and Jeanne M. Serb

July 15, 2024

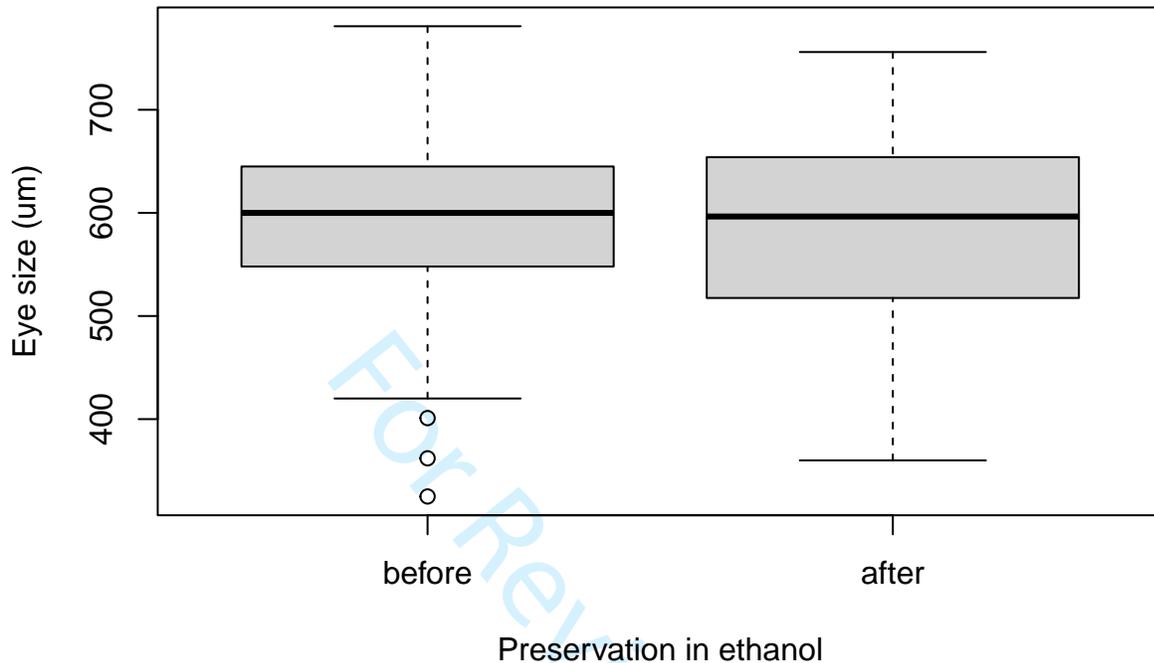
Read raw data: 10 eyes examined in 10 scallops

```
library(xlsx)
data <- read.xlsx("Supplemental_material_TableS1_revised.xlsx", sheetIndex = 1,
                 endRow = 51,
                 header = T)

# Organize dataset
# Sample size = 100 eyes before and after ethanol preservation
df <- data.frame(Eye.size = c(data$S.L.ED..um., data$S.R.ED..um.,
                             data$E.L.ED..um., data$E.R.ED..um.),
                 Pupil.size = c(data$S.L.PD..um., data$S.R.PD..um.,
                                data$E.L.PD..um., data$E.R.PD..um.),
                 Condition = rep(c('before', 'after'), each = 100),
                 Ratio = c(data$S.L..Ratio..ED.PD., data$SR.Ratio..ED.PD.,
                           data$E.L..Ratio..ED.PD., data$E.R..Ratio..ED.PD.),
                 Specimen = rep(c("scallop1", "scallop2", "scallop3", "scallop4",
                                 "scallop5", "scallop6", "scallop7", "scallop8",
                                 "scallop9", "scallop10"), each=5),
                 shell = rep(c(data$Shell.Length..mm.[complete.cases(data$Shell.Length..mm.)]), each = 100))
Condition <- as.factor(df$Condition)
Condition <- relevel(Condition, "before")
Specimen <- as.factor(df$Specimen)
```

EYE SIZE: testing whether eye size changes after ethanol preservation

```
# Plot eye size before and after ethanol preservation
plot(Condition, df$Eye.size, ylab = "Eye size (um)", xlab = "Preservation in ethanol")
```



```

31 # Using the difference between means as the test value
32 eye.size.means <- tapply(df$Eye.size, Condition, mean)
33 eye.size.sd <- tapply(df$Eye.size, Condition, sd)
34 obs <- eye.size.means[1]-eye.size.means[2]
35 # Permutation procedure
36 permute <- 99999
37 P <- 1
38 rand.vec.eye <- array(NA, (permute + 1))
39 rand.vec.eye[permute + 1] <- obs
40 for(i in 1:permute){
41   # Shuffle data
42   y.rand.eye <- sample(df$Eye.size) #breaking the association with condition
43   rand.vec.eye[i] <- diff(tapply(y.rand.eye, Condition, mean))
44 }
45 # Significance assessment
46 P.value.eye.size <- length(which(abs(rand.vec.eye) >= abs(rand.vec.eye[permute + 1])))/(permute + 1)
47 P.value.eye.size
48

```

```

49
50 ## [1] 0.46973
51

```

```

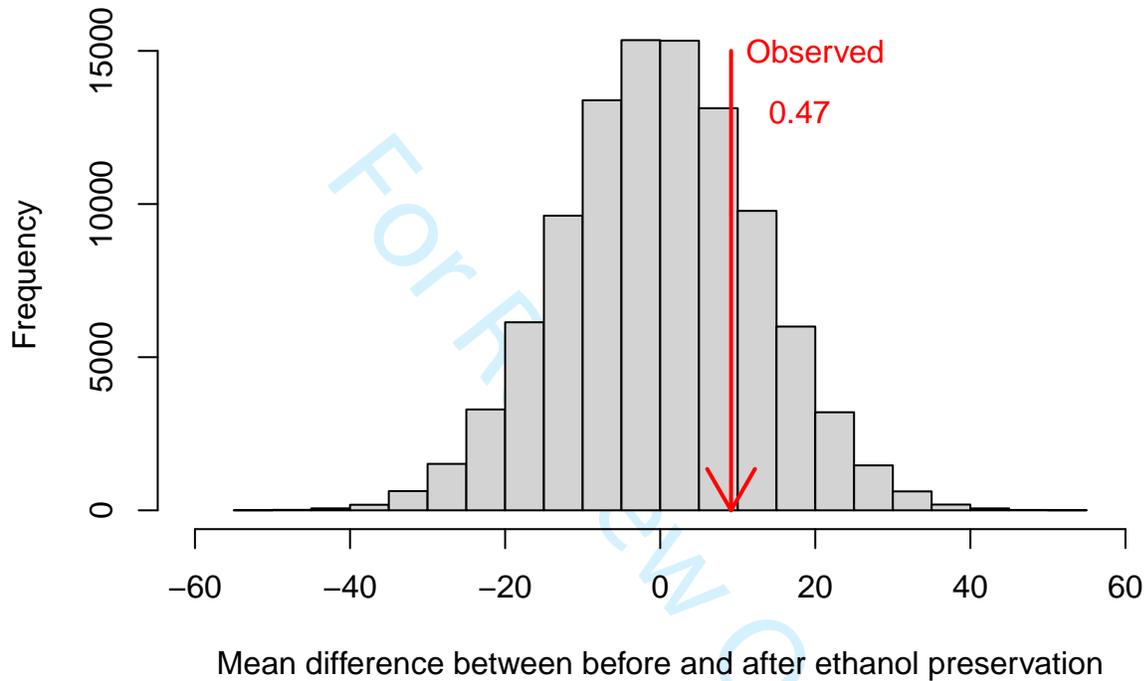
52 # Plot empirical sampling distribution
53 hist(rand.vec.eye, freq = T, col = "lightgray",
54      main = NULL,
55      xlab = "Mean difference between before and after ethanol preservation",
56      xlim= c(-60,60))
57
58
59

```

```

1
2
3
4 # Plot observed value
5 arrows(obs, 0, obs, 15000, col = "red", code = 1, lwd = 2)
6 text (20, 15000, col = "red", labels = "Observed", cex = 1)
7 text (18, 13000, col = "red", labels = c(round(P.value.eye.size, digits = 2)),
8     cex = 1 )
9
10
11
12
13

```



```

38 # Distribution centered on 0.0, as expected under the null hypothesis
39 # Student's t-Test: parametric approach
40 t.test.eye.size <- t.test(log(df$Eye.size) ~ df$Condition, data = df)
41 t.test.eye.size$p.value
42

```

```

43 ## [1] 0.4074486
44

```

```

45 # CONCLUSION: null hypothesis is not rejected. Eye size does not change
46 # significantly after preservation
47
48
49

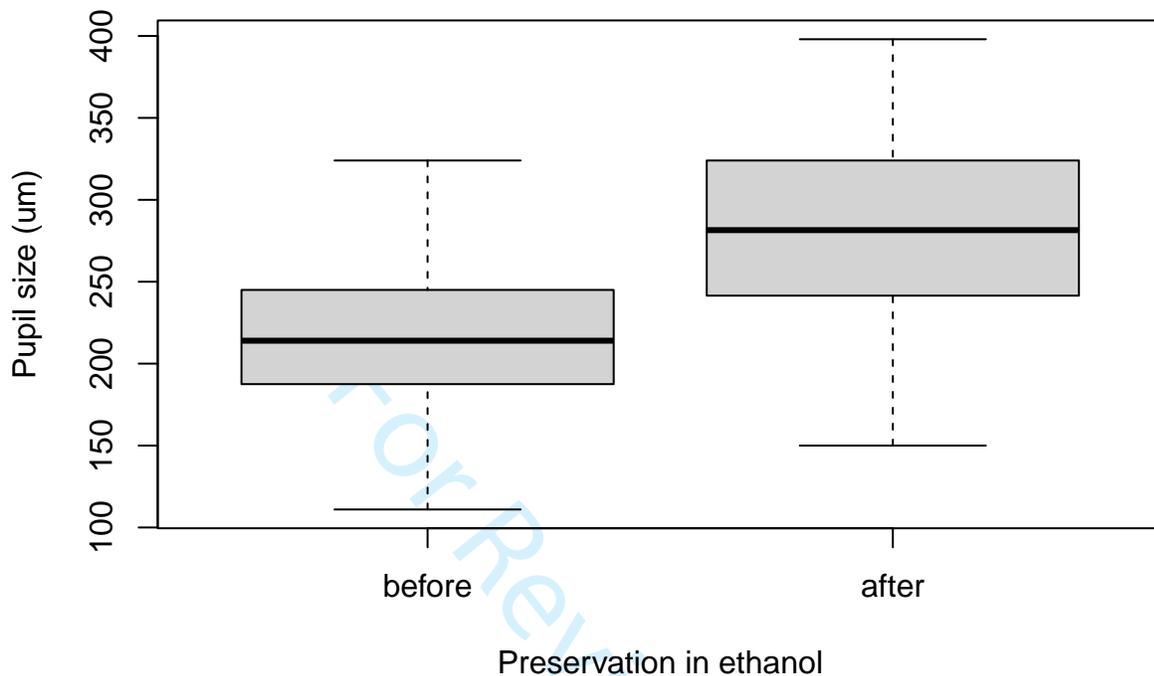
```

PUPIL SIZE: testing whether pupil size changes after ethanol preservation

```

51
52
53 # Plot pupil size before and after ethanol preservation
54 plot(Condition, df$Pupil.size, ylab = "Pupil size (um)", xlab = "Preservation in ethanol")
55
56
57
58
59
60

```



```

31 # Using the difference between means as the test value
32 pupil.means <- tapply(df$Pupil.size, Condition, mean)
33 pupil.sd <- tapply(df$Pupil.size, Condition, sd)
34 obs.pupil <- pupil.means[2]-pupil.means[1]
35 # Permutation procedure
36 permute <- 99999
37 P <- 1
38 rand.vec.pupil <- array(NA,(permute + 1))
39 rand.vec.pupil[permute + 1] <- obs.pupil
40 for(i in 1:permute){
41   # Shuffle data
42   y.rand.pupil <- sample(df$Pupil.size) #breaking the association with condition
43   rand.vec.pupil[i] <- diff(tapply(y.rand.pupil, Condition, mean))
44 }
45 # Significance assessment
46 P.value.pupil <- length(which(abs(rand.vec.pupil) >= abs(rand.vec.pupil[permute + 1])))/(permute + 1)
47 P.value.pupil

```

```
## [1] 1e-05
```

```

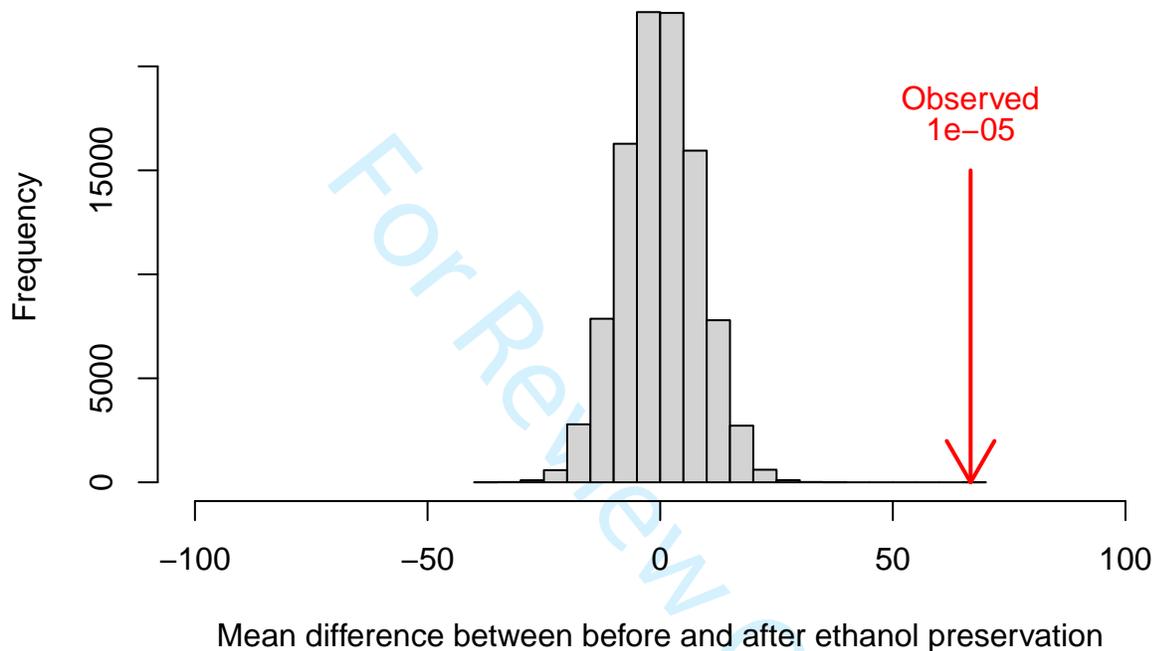
52 # Plot empirical sampling distribution
53 hist(rand.vec.pupil, freq = T, col = "lightgray",
54      main = NULL,
55      xlab = "Mean difference between before and after ethanol preservation",
56      xlim= c(-100,100))

```

```

1
2
3
4 # Plot observed value
5 arrows(obs.pupil, 0, obs.pupil, 15000, col = "red", code = 1, lwd = 2)
6 text (obs.pupil, 18500, col = "red", labels = "Observed", cex = 1)
7 text (obs.pupil, 17000, col = "red", labels = (P.value.pupil), cex = 1)
8
9
10
11
12
13

```



```

34
35
36
37 # Distribution centered on 0.0, as expected under the null hypothesis
38 # Testing using Student's t-Test
39 t.test.pupil.size <- t.test(log(df$Pupil.size) ~ df$Condition, data = df)
40 t.test.pupil.size$p.value
41
42

```

```

43 ## [1] 6.687134e-18
44

```

```

45 # CONCLUSION: null hypothesis is rejected, alternative hypothesis is accepted.
46 # Pupil size differs, getting bigger after preservation in ethanol.
47
48

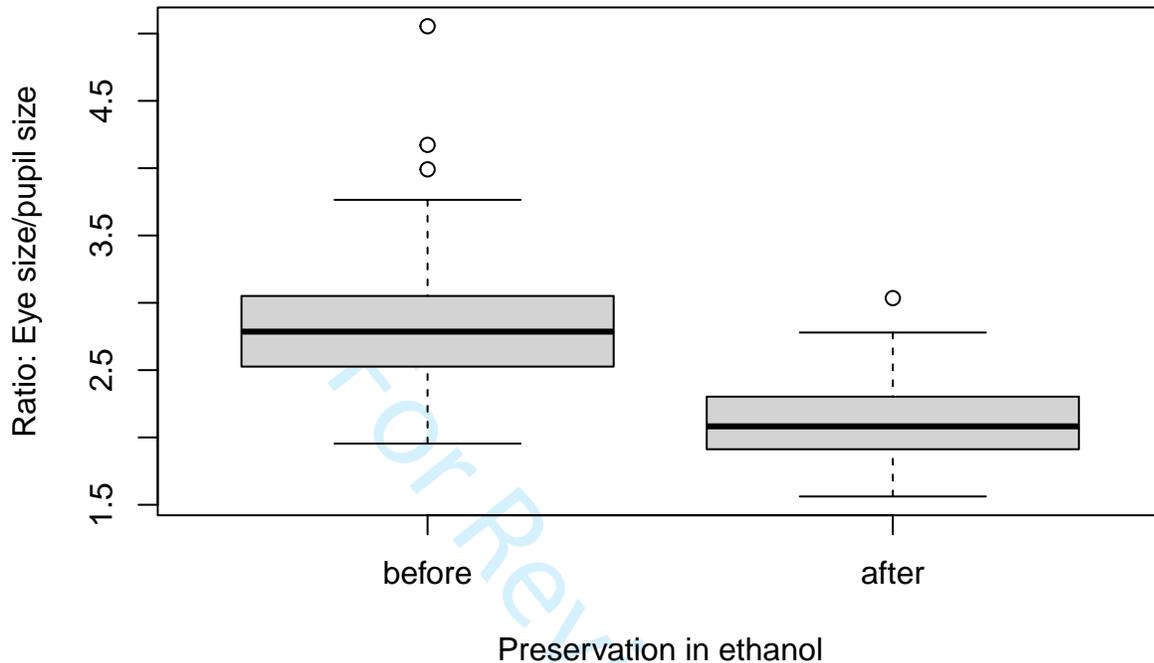
```

RATIO EYE/PUPIL: testing whether eye/pupil ratio changes after ethanol preservation

```

49
50
51
52 # Plot ratio before and after ethanol preservation
53 plot(Condition, df$Ratio, ylab = "Ratio: Eye size/pupil size", xlab = "Preservation in ethanol")
54
55
56
57
58
59
60

```



```

31 # Using the difference between means as the test value
32 ratio.means <- tapply(df$Ratio, Condition, mean)
33 obs.ratio <- ratio.means[1]-ratio.means[2]
34 # Permutation procedure
35 permute <- 99999
36 P <- 1
37 rand.vec.ratio <- array(NA,(permute + 1))
38 rand.vec.ratio[permute + 1] <- obs.ratio
39 for(i in 1:permute){
40   # Shuffle data
41   y.rand.ratio <- sample(df$Ratio) #breaking the association with condition
42   rand.vec.ratio[i] <- diff(tapply(y.rand.ratio, Condition, mean))
43 }
44 # Significance assessment
45 P.value.ratio <- length(which(abs(rand.vec.ratio) >= abs(rand.vec.ratio[permute + 1])))/(permute + 1)
46 P.value.ratio

```

```
## [1] 1e-05
```

```

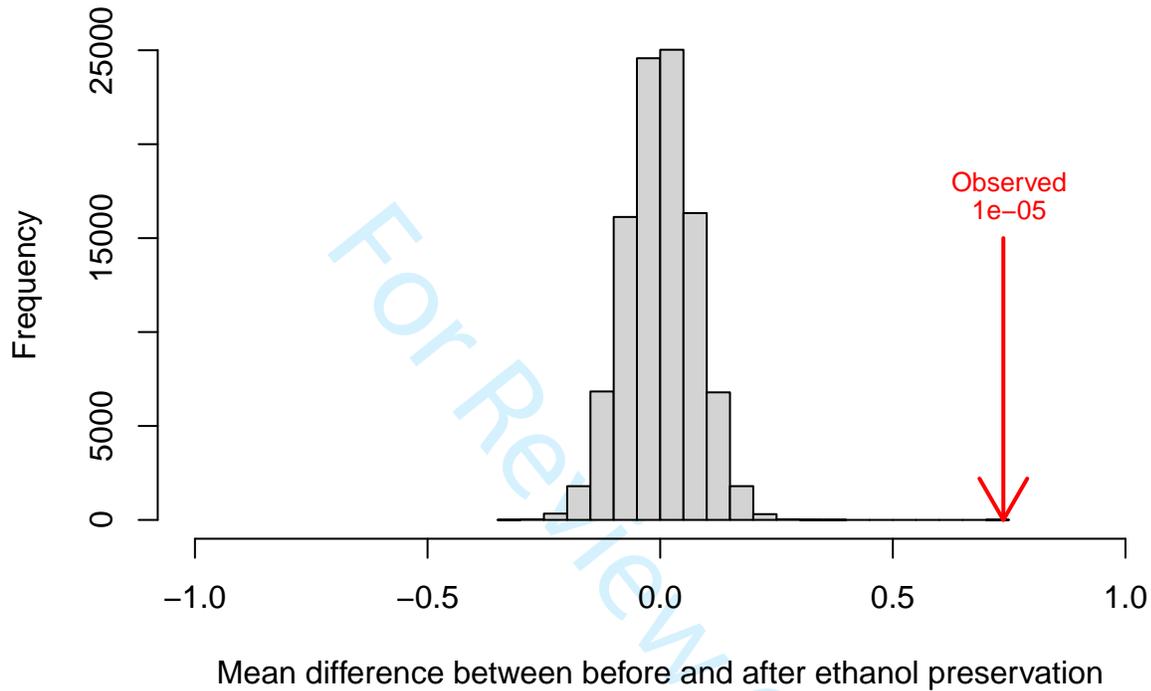
51 # Plot empirical sampling distribution
52 hist(rand.vec.ratio, freq = T, col = "lightgray",
53       main = NULL,
54       xlab = "Mean difference between before and after ethanol preservation",
55       xlim= c(-1,1))
56 # Plot observed value

```

```

arrows(obs.ratio, 0, obs.ratio, 15000, col = "red", code = 1, lwd = 2)
text (0.75, 18000, col = "red", labels = "Observed", cex = 0.8)
text (0.75, 16500, col = "red", labels = (P.value.ratio), cex = 0.8 )

```



```

# Distribution centered on 0.0, as expected under the null hypothesis
# Testing using Student's t-Test
t.test.eye.ratio <- t.test(df$Ratio ~ df$Condition, data = df)
t.test.eye.ratio$p.value

```

```
## [1] 3.974383e-29
```

```

# CONCLUSION: null hypothesis is rejected, alternative hypothesis is accepted.
# Ratio between eye and pupil size differs.
# The ratio gets smaller, which makes sense since pupil size gets larger.

```

REGRESSION: how pupil size correlates with eye size

```

# Fit a linear model: pupil size ~ eye size (seawater)
short.df <- df[1:100,] # sampling only dissected eyes in seawater
modell1 <- lm(short.df$Pupil.size ~ short.df$Eye.size)
summary(modell1) #provides regression coefficients

```

```

1
2
3
4  ## Call:
5  ## lm(formula = short.df$Pupil.size ~ short.df$Eye.size)
6  ##
7  ## Residuals:
8  ##      Min       1Q   Median       3Q      Max
9  ## -89.595 -18.519   0.672  21.062  91.173
10 ##
11 ## Coefficients:
12 ##              Estimate Std. Error t value Pr(>|t|)
13 ## (Intercept)   -15.19086    22.39007  -0.678   0.499
14 ## short.df$Eye.size  0.38465     0.03723  10.332 <2e-16 ***
15 ## ---
16 ## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
17 ##
18 ## Residual standard error: 30.82 on 98 degrees of freedom
19 ## Multiple R-squared:  0.5214, Adjusted R-squared:  0.5165
20 ## F-statistic: 106.8 on 1 and 98 DF,  p-value: < 2.2e-16

```

```

21
22 anova(model1) #provides model term tests
23

```

```

24
25 ## Analysis of Variance Table
26 ##
27 ## Response: short.df$Pupil.size
28 ##              Df Sum Sq Mean Sq F value    Pr(>F)
29 ## short.df$Eye.size  1 101432  101432  106.76 < 2.2e-16 ***
30 ## Residuals          98  93110     950
31 ## ---
32 ## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
33

```

```

34 # CONCLUSION: Eye size explain variation in pupil size.
35

```

```

36
37 # Fit a linear model: pupil size ~ eye size (ethanol-fixed eyes)
38 etoh.df <- df[101:200,] # sampling only ethanol-preserved eyes
39 model5 <- lm(etoh.df$Pupil.size ~ etoh.df$Eye.size)
40 summary(model5) #provides regression coefficients
41

```

```

42
43 ## Call:
44 ## lm(formula = etoh.df$Pupil.size ~ etoh.df$Eye.size)
45 ##
46 ## Residuals:
47 ##      Min       1Q   Median       3Q      Max
48 ## -89.379 -24.161   0.821  22.267  74.327
49 ##
50 ## Coefficients:
51 ##              Estimate Std. Error t value Pr(>|t|)
52 ## (Intercept)    37.48500    20.91017   1.793  0.0761 .
53 ## etoh.df$Eye.size  0.41458     0.03519  11.781 <2e-16 ***
54 ## ---
55 ## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
56 ##
57
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```

```
1
2
3 ## Residual standard error: 33.37 on 98 degrees of freedom
4 ## Multiple R-squared: 0.5861, Adjusted R-squared: 0.5819
5 ## F-statistic: 138.8 on 1 and 98 DF, p-value: < 2.2e-16
6
```

```
7 anova(model5)
```

```
8
9
10 ## Analysis of Variance Table
11 ##
12 ## Response: etoh.df$Pupil.size
13 ##           Df Sum Sq Mean Sq F value    Pr(>F)
14 ## etoh.df$Eye.size 1 154527 154527 138.79 < 2.2e-16 ***
15 ## Residuals      98 109111   1113
16 ## ---
17 ## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
18
19 # CONCLUSION: Eye size explain variation in pupil size.
```

```
20
21 # Check if variables are normally distributed
```

```
22 shapiro.test(short.df$Pupil.size) # we can assume a normal distribution
```

```
23
24
25 ##
26 ## Shapiro-Wilk normality test
27 ##
28 ## data: short.df$Pupil.size
29 ## W = 0.99123, p-value = 0.7628
```

```
30
31 shapiro.test(short.df$Eye.size) # we can assume a normal distribution
```

```
32
33
34 ##
35 ## Shapiro-Wilk normality test
36 ##
37 ## data: short.df$Eye.size
38 ## W = 0.97794, p-value = 0.09171
```

```
39
40 shapiro.test(etoh.df$Pupil.size) # we can assume a normal distribution
```

```
41
42
43 ##
44 ## Shapiro-Wilk normality test
45 ##
46 ## data: etoh.df$Pupil.size
47 ## W = 0.98972, p-value = 0.6419
```

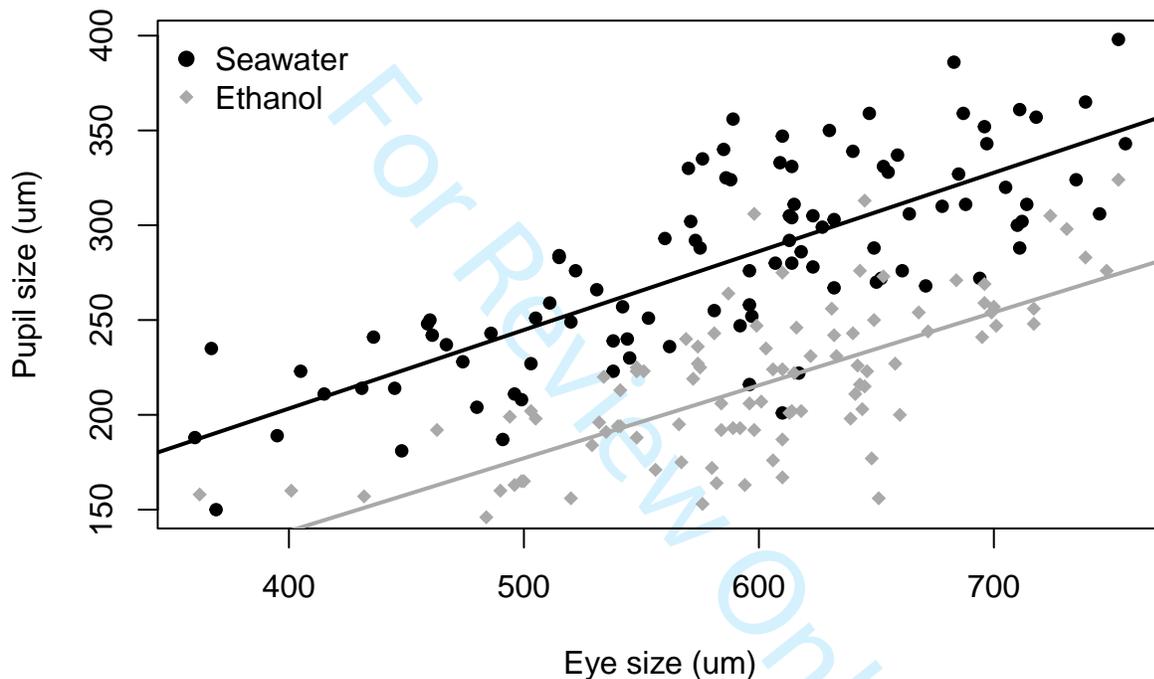
```
48
49 shapiro.test(etoh.df$Eye.size) # we can assume a normal distribution
```

```
50
51
52 ##
53 ## Shapiro-Wilk normality test
54 ##
55 ## data: etoh.df$Eye.size
56 ## W = 0.9748, p-value = 0.05201
```

```

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4 plot(etoh.df$Pupil.size ~ etoh.df$Eye.size, pch = 19, col = "black", cex=0.8,
5       xlab = "Eye size (um)", ylab = "Pupil size (um)")
6 points(short.df$Pupil.size ~ short.df$Eye.size, pch = 18, col = "darkgray", cex=1)
7 abline(coef(model5), col="black", lwd = 2)
8 abline(coef(model1), col="darkgray", lwd = 2)
9 legend("topleft", c("Seawater", "Ethanol"), col=c("black","darkgray"),
10        pch=c(19, 18), cex = 1, bty = "n")
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```



41 **ALLOMETRIC RELATIONSHIPS:** how eye & pupil sizes relate to body size

```

42
43
44 # Fit a linear model: eye size ~ body size
45 model2 <- lm(short.df$Eye.size ~ short.df$shell)
46 summary(model2) #provides regression coefficients
47
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```

```

##
## Call:
## lm(formula = short.df$Eye.size ~ short.df$shell)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -255.66  -43.59   5.43   50.71  166.52
##
## Coefficients:

```

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```
##           Estimate Std. Error t value Pr(>|t|)
## (Intercept)   480.431    82.184   5.846 6.63e-08 ***
## short.df$shell    2.529     1.794   1.410   0.162
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 82.8 on 98 degrees of freedom
## Multiple R-squared:  0.01988,    Adjusted R-squared:  0.009882
## F-statistic: 1.988 on 1 and 98 DF,  p-value: 0.1617
```

```
anova(model2)
```

```
## Analysis of Variance Table
##
## Response: short.df$Eye.size
##           Df Sum Sq Mean Sq F value Pr(>F)
## short.df$shell  1  13632  13631.5   1.9881 0.1617
## Residuals      98  671945   6856.6
```

```
# Eye size does not vary with body size.
```

```
# Fit a linear model: pupil size ~ body size
model3 <- lm(short.df$Pupil.size ~ short.df$shell)
summary(model3) #provides regression coefficients
```

```
##
## Call:
## lm(formula = short.df$Pupil.size ~ short.df$shell)
##
## Residuals:
##      Min       1Q   Median       3Q      Max
## -111.03  -28.96   -1.71    29.07   108.95
##
## Coefficients:
##           Estimate Std. Error t value Pr(>|t|)
## (Intercept)   283.4671    43.6539   6.494 3.48e-09 ***
## short.df$shell  -1.5250     0.9528  -1.601   0.113
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 43.98 on 98 degrees of freedom
## Multiple R-squared:  0.02548,    Adjusted R-squared:  0.01553
## F-statistic: 2.562 on 1 and 98 DF,  p-value: 0.1127
```

```
anova(model3)
```

```
## Analysis of Variance Table
##
## Response: short.df$Pupil.size
##           Df Sum Sq Mean Sq F value Pr(>F)
## short.df$shell  1   4956   4956.2   2.5619 0.1127
## Residuals      98 189587   1934.6
```

```
# Pupil size does not vary with body size.
```

```
# Plotting models together
```

```
plot(short.df$shell, short.df$Eye.size, pch=19, bg="black", cex=0.7,  
      xlab = "Shell length (mm)", ylab = "Eye and pupil size (um)", ylim = c(100, 800))  
abline(coef(model2), col="black", lwd = 2)  
points(short.df$shell, short.df$Pupil.size, pch=18, col="gray", cex=1)  
abline(coef(model3), col="darkgray", lwd = 2)  
legend("center", c("eyes", "pupils"), pch=c(19, 18), col=c("black", "darkgray"),  
       bg= c("black", "darkgray"), cex = 0.9, bty = "n")
```

