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RESEARCH NOTE

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 [1,2], Gerald Quinlan[2], Ceren Ordas[2] and Jeanne M. Serb [2]

[1]*Department of Zoology, University of São Paulo, Rua do Matão, Travessa 14, n. 101, São Paulo, Brazil; and*
[2]*Department of Evolution, Ecology and Organismal Biology, Iowa State University, 2200 Osborn Dr., Ames IA, USA*

Correspondence: J.A. Audino; e-mail: jorgeaudino@ib.usp.br

Ethanol-preserved specimens represent one of the most common resources for biological research. However, little is known of how ethanol preservation may change tissue morphology and impact the interpretation of trait quantification in structures, such as eyes. While scallop eyes are an interesting system for investigating eye evolution and visual adaptations, cross-species comparisons mainly depend on museum specimens. Therefore, to test whether ethanol-preserved specimens serve as accurate indicators of natural eye morphology, we investigated the effects of preservation on selected traits, such as eye and pupil diameter, in the scallop *Argopecten irradians*. We also compared ethanol-preserved eyes to paraformaldehyde (PFA)-fixed eyes to investigate possible impacts on retinal morphology. Our results demonstrate that eye size does not change with short-term preservation, whereas pupil size becomes significantly larger, likely due to the contraction of actin fibers during dehydration. When comparing measurements among eyes and treatments, eye size correlates to pupil size, but is not correlated to body size. We found that ethanol-preserved eyes provide close estimates of retinal traits, with similar photoreceptor spacing distance and number of photoreceptor cells, compared to samples fixed in PFA. These findings might also be applicable in the context of other mollusks, especially bivalves and gastropods, with delicate visual systems. Our study provides evidence that ethanol-preserved eyes exhibit tissue-specific differences that should be acknowledged in morphological studies. For example, pupil size should be investigated while accounting for post-preservation effects. Other traits, such as lens shape, are inconsistent and severely impacted by preservation. Finally, eye size and some photoreceptor cell measurements can be helpful to describe natural morphology.

Natural history museums and other scientific collections are a critical repository for biological diversity and serve as a primary source for phylogenetic, morphological and evolutionary analyses (Card *et al.*, 2021). However, when accessing biological information from historical specimens, one must critically ask how accurately the preserved materials represent natural conditions. Fluid preservation historically includes an initial fixation step with some cross-linking fixative agents, such as aldehydes (e.g. formalin), and subsequent storage in ethanol. More recently, museum samples have been subjected to direct preservation in ethanol for DNA preservation, but this can result in dehydration of tissues, poor tissue penetration, changes in biomass, deformation, loss of pigmentation and dilution of the ethanol preservative (Simmons, 2014). Surprisingly, there has been little investigation about how ethanol preservation impacts specific tissues and organs (e.g. Martinez, Berbel-Filho & Jacobina, 2013; Sotola *et al.*, 2019; Ziegler & Sagorny, 2023), especially those that are fluid-filled, delicate and unprotected by an exoskeleton, such as eyes (Thomas *et al.*, 2020).

Eyes are a useful trait to examine adaptation and museum specimens are paramount to study these evolutionary patterns. Parameters such as eye aperture, eye size and retinal organization are important components to understand visual functions of sensitivity and resolution. To determine if preserved eyes provide reliable estimates of natural eye conditions, we conducted a study with the common bay scallop *Argopecten irradians* (Lamarck, 1819) to quantify the effects of 95% ethanol preservation on ocular features. While it is known that these delicate organs are prone to preservation-induced artefacts (Speiser *et al.*, 2016) and pupil diameter naturally varies due to constricting musculature in living animals (Miller *et al.*, 2019), the impact of preservation methods on these tissues has never been examined in scallops. To address the possible preservation effect, we examine the same freshly dissected eyes after ethanol preservation to determine whether after treatment (1) eye and pupil sizes change, (2) eye size continues to correlate with pupil size and (3) eye size and pupil size change with body size. We expect that all eye components shrink uniformly after dehydration and the same amount of shrinkage in all animals, regardless of individual size. Finally, although ethanol is not a proper fixative for fine anatomical study, we expect reliable estimates from histological sections produced by ethanol-preserved samples regarding simple morphological measurements, such as photoreceptor spacing and number.

We examined 12 individuals of *A. irradians* obtained from the Gulf Specimen Marine Lab (Panacea, FL, USA). To investigate eye external morphology before preservation, mantle margins containing eyes were dissected from 10 individuals. Left and right mantle strips were attached to cardboard using entomological pins, submersed in the same saltwater the scallops were kept in and immediately examined under the stereomicroscope. Photographs were taken of five eyes on the ventral region of both left and right sides for each specimen (10 eyes per animal), for a total of 100 sampled eyes. Saltwater was drained and replaced with 95% ethanol.

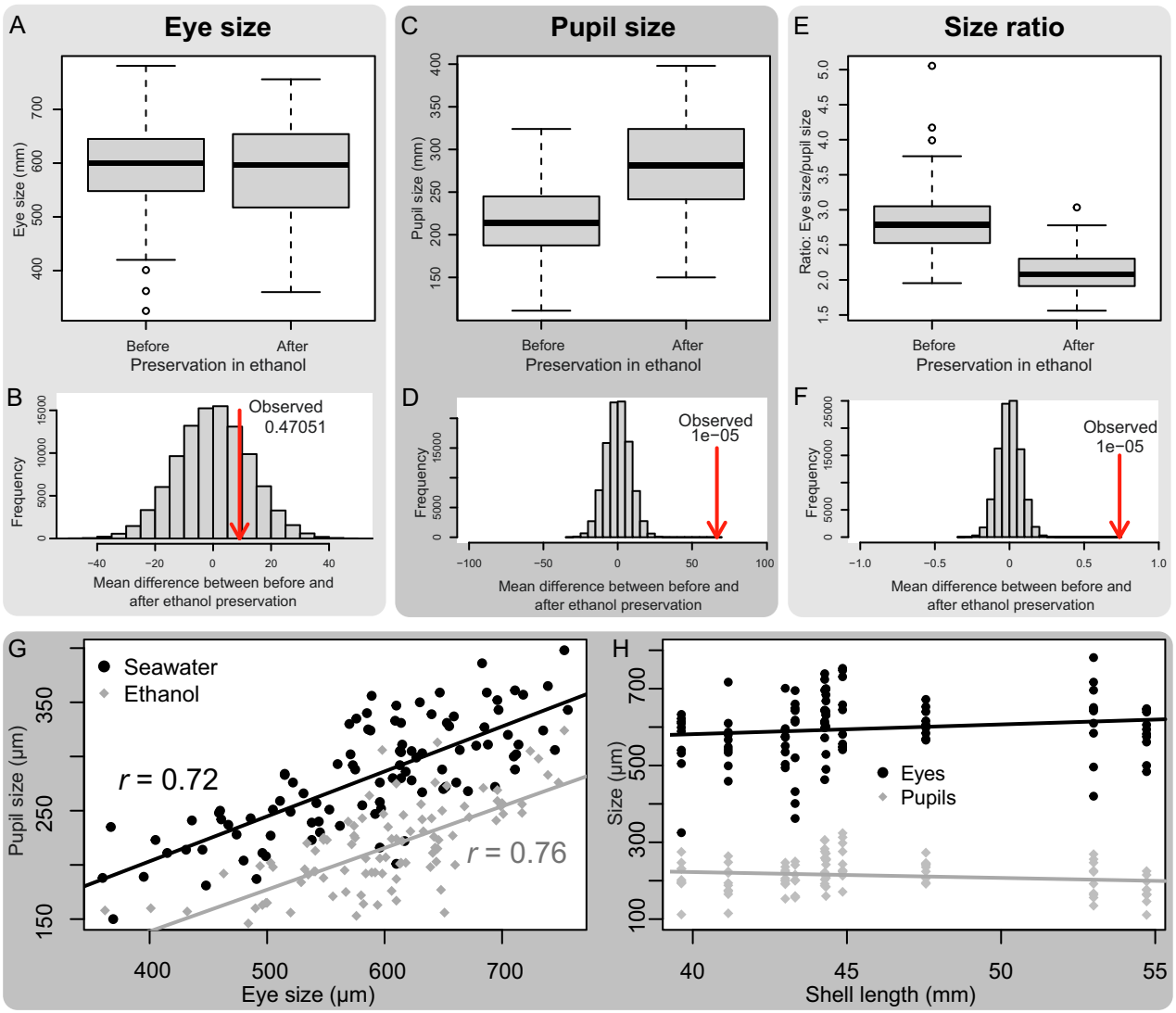

Figure 1. Comparison of eye and pupil size in the scallop *Argopecten irradians* before and after preservation in 95% ethanol. We generated a permutation-based empirical sampling distribution after 100,000 iterations to test the null hypothesis of no size difference before and after ethanol preservation (**B**, **D**, **F**). **A.** Boxplots of eye diameter. **B.** Differences in eye diameter lie within the expected distribution under the null hypothesis (i.e. the difference is insignificant). **C.** Boxplots of pupil diameter. **D.** Pupil diameter significantly changes after ethanol preservation. **E.** Boxplots of the ratio between eye and pupil diameters. **F.** The ratio between eye and pupil sizes also changes after ethanol preservation. **G.** Regression of pupil diameter and eye diameter before and after ethanol preservation. Pearson correlation coefficient (*r*). **H.** Regression of eye/pupil and body size, measured as shell length, highlighting lack of scaling relationship.

After 24 h of dehydration, the same scallop eyes were photographed based on previous mapping of positions. Images of eyes pre- and post-preservation were analysed in ImageJ v. 1.54f (12) to determine eye and pupil diameter. A size ratio between these two diameters was calculated for each eye and means were obtained for each treatment. We tested for differences in eye size, pupil size and size ratio after ethanol preservation using a parametrical approach (Student's *t*-test) and a permutation-based resampling method. We also assessed the statistical significance of the association between eye and pupil diameter and their relationship to shell length as a proxy for body size. All analyses were conducted in R v. 4.3.2 (R Core Team, 2021), and codes are available as Supplementary Material.

We also compared ethanol-preserved eyes to those fixed in PFA, following standard protocols for histology (Audino *et al.*, 2015). Two scallop individuals were anesthetized in 7.5% $MgCl_2$ for 2 h. Eyes were dissected and fixed in 4% PFA in PBS 0.1 M for 3 h. Three ethanol-fixed eyes and three PFA-fixed eyes were embedded in LR White resin for histology and sectioned in a Leica UC6 ultramicrotome. Histological images of the central region of the retina were measured using ImageJ to compare photoreceptor spacing and rhabdom length (five measurements per eye), as well as photoreceptor number based on nuclei count.

Surprisingly, eye size is not affected by ethanol preservation, as no significant change in eye diameter occurred after dehydration (Fig. 1A; Student's *t*-test: $P = 0.4$). By comparing the same eyes between treatments, the mean diameter of dissected eyes in seawater (595.72 ± 83.21 μm) and ethanol-preserved eyes (586.58 ± 95.29 μm) is statistically the same, as supported by permutation-based resampling methods (Fig. 1B). This is a particularly welcoming finding considering that the size of soft organs is substantially affected by varying degrees of contraction after dehydration, as observed in bivalve siphons (Sartori *et al.*, 2008). Because the eyes of *A. irradians* do not shrink, we argue that measurements from preserved specimens can provide reliable estimates of eye

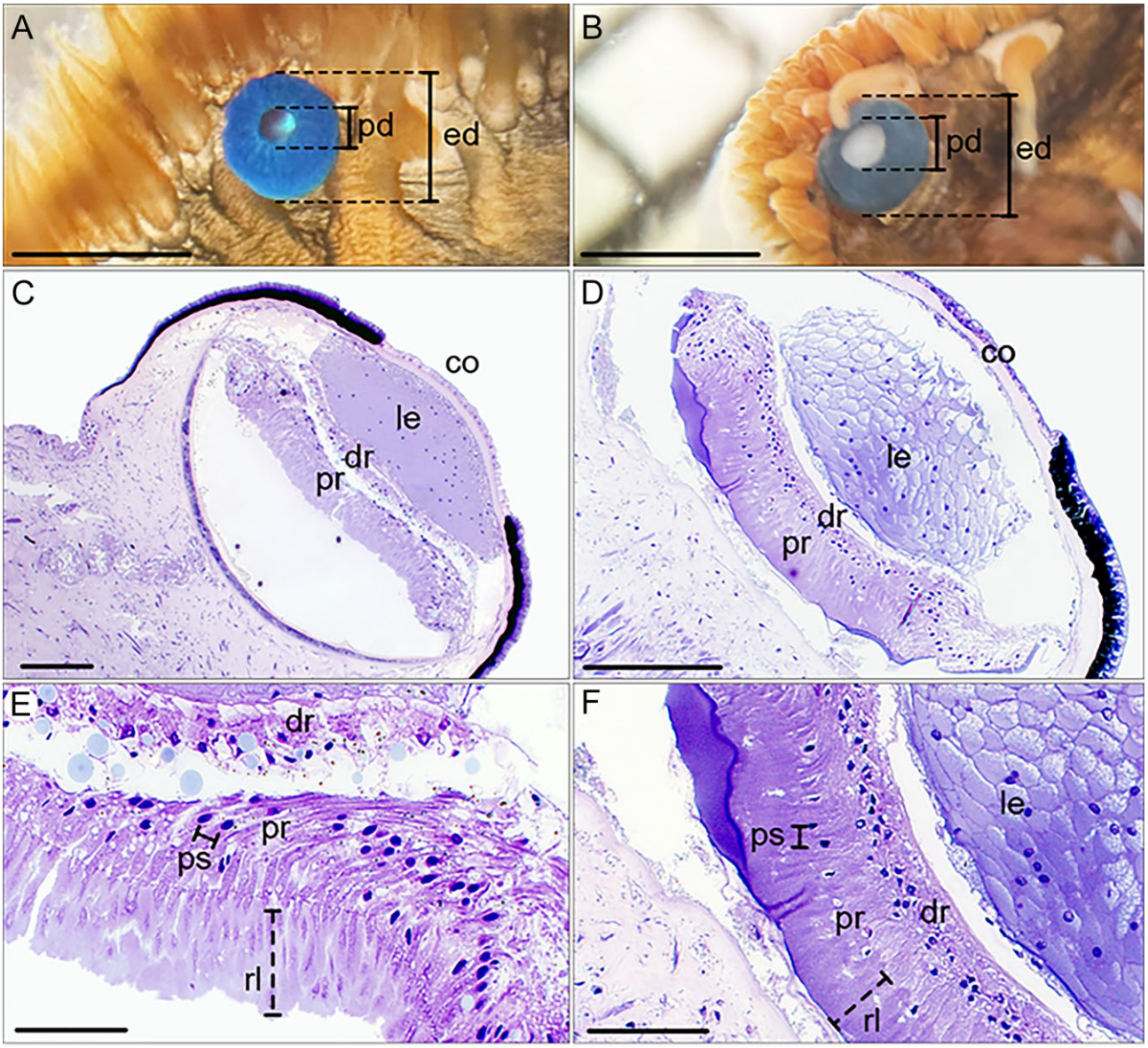

Figure 2. Eyes of the scallop *Argopecten irradians* under stereomicroscope (**A**, **B**) and in 3 μm histological sections stained with Azure B (**C**–**F**). **A.** A single eye was photographed after dissection under seawater. **B.** The same eye after 24 h of fixation in 95% ethanol. **C.** Section through the central region of an eye fixed in 4% PFA. **D.** Section through the central region of an eye preserved in ethanol. **E.** Detail of the double-retina (sample fixed in 4% PFA). **F.** Detail of the double-retina (sample fixed in ethanol). Relevant measurements are indicated with lines between bars. Abbreviations: co, cornea; dr, distal retina; ed, eye diameter; le, lens; pd, pupil diameter; pr, proximal retina; ps, photoreceptor spacing; rl, rhabdom length. Scale bars: **A** = 1.0 mm; **B** = 1.0 mm; **C** = 100 μm; **D** = 100 μm; **E** = 40 μm; **F** = 40 μm.

size, maximizing morphological information that can be obtained from historical materials for cross-species comparisons. In contrast, pupil size remarkably changes after dehydration (Fig. 1C; Student's *t*-test: $P = 6.68 \times 10^{-18}$). The pupil's diameter (213.95 ± 44.32 μm) becomes significantly larger when preserved in 95% ethanol (280.67 ± 51.6 μm), as corroborated by permutation-based resampling methods (Fig. 1D). Contrary to our initial hypothesis that eye components shrink jointly, the change in the size ratio of eye and pupil emphasizes that only the pupil diameter changes after dehydration (Student's *t*-test: $P = 3.97 \times 10^{-29}$; Fig. 1E, F). Interestingly, scallops are known for having radial and circular actin fibres associated with the cornea, likely acting as fine muscles that dilate and constrict the pupil (Miller *et al.*, 2019). Severe dehydration caused by ethanol might cause muscle fibres to contract, explaining the extension of the pupil diameter. In pupillary response experiments, a previous study documented that the pupil's area of *A. irradians* can dilate up to 60% under the brightest experimental conditions (Miller *et al.*, 2019). Our results show that the pupil's diameter increased by 24% on average after preservation in ethanol. Accordingly, an increase of 24% in the radius is expected to cause an increase of 53.76% in the area of a circle, a % close to the experimental dilation of 60% previously identified. Therefore, ethanol-preserved eyes have pupils at maximum size, representing the maximum light intake into the eye. Even though pupil size must be cautiously considered in preserved specimens, it offers the possibility to predict a natural morphological condition that optimizes light sensitivity.

Table 1. Morphological variables related to optical properties measured from histological sections of *Argopecten irradians* eyes preserved in 95% ethanol ($n = 3$) and 4% paraformaldehyde in phosphate-buffered saline (PFA; $n = 3$).

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 ± SD	**196.46 ± 15.24**	**411.27 ± 68.89**	**5.41 ± 0.38**	**22.63 ± 2.23**	**118.33 ± 12.22**
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 ± SD	**281.93 ± 16.51**	**509.31 ± 45.15**	**5.37 ± 0.33**	**30.79 ± 2.35**	**130 ± 37.24**

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.

Next, we tested whether pupil diameter can be explained by eye diameter after ethanol preservation. Our results show that, indeed, larger pupils are correlated to larger eyes ($P = 2.2 \times 10^{-16}$; Fig. 1G), and such a relationship is maintained after ethanol preservation (Fig. 1G). These data are important to the interpretation of eye function. For example, many vertebrates, such as anurans, show increased relative eye sizes and pupillary proportions to maximize visual sensitivity (Thomas *et al.*, 2020). Scallops provide a nonvertebrate example of animals that use a pupillary mechanism associated with light sensitivity and resolution (Miller *et al.*, 2019). Our results prove that scaling relationships and size can be accurately examined in ethanol-preserved scallops. We then tested for an allometric relationship between eye and body sizes, a frequent scaling association among animals (Caves, Sutton & Johnsen, 2017). Our results show that neither eye diameter nor pupil diameter scale with shell length, used here as a proxy of body size. Unlike most animals that show positive eye-body allometry (Thomas *et al.*, 2020), our findings suggest that a fixed range of eye size occurs in *A. irradians*, regardless of the size of the individuals. Nevertheless, we expect eye investment to vary greatly across scallop species, considering the increased ecological diversity and variation in other optical traits, such as eye number (Speiser & Johnsen, 2008; Audino, Adams & Serb, 2022).

Histological data reveal that ethanol-preserved eyes can help describe and estimate some traits of optical relevance (Fig. 2). Despite differences in eye size, ethanol-preserved retinas reveal a similar photoreceptor spacing distance and a similar number of photoreceptor cells compared to retinas preserved in 4% PFA (Table 1). Even though photoreceptor cell number is not expected to vary with the preservation method, we demonstrate that the trait, a potential measure of visual investment, can be estimated from ethanol-preserved samples. Rhabdom length is slightly shorter in ethanol-preserved retinas than in those prepared using histological standard fixation, possibly because of the size difference between eyes or abrupt dehydration with shrinkage (Table 1). In the comparative anatomy of scallop species, retinal data of *A. irradians*, fixed in buffered 4% formaldehyde, suggest similar rhabdom length of the proximal retina (Speiser & Johnsen, 2008). However, this previous study sampled larger eyes, which is reflected in larger measurements of internal diameter (670 ± 40 μm) and photoreceptor spacing (5.8 ± 0.2 μm) for the species (Speiser & Johnsen, 2008). Not surprisingly, lenses across scallop species have shown great variation in shape, including globular, flat and fusiform, regardless of the preservation method. The same was observed among our samples (Fig. 2). Considering the delicate nature of this tissue, fixation and dehydration likely cause unpredictable changes, making chemically preserved lenses an inconsistent predictor of scallop eye morphology. Other artifacts were noted, such as how the lens has moved away from the cornea in some cases for both treatments (Fig. 2). In addition, PFA-preserved eyes show a large artefactual spacing between the retina and the mirror layer (Fig. 2).

Overall, histological information should always be carefully interpreted because of the possibility of preservation-induced artefacts. Here, we used PFA-preserved samples for comparisons, but future work using other methods, such as ultrarapid fixation via high-pressure freezing, will be relevant to compare and obtain a more natural morphology. In addition, preservation time could be explored since long-term preservation is another factor causing unpredictable morphological changes, e.g. as observed in cephalopod specimens from museum collections (Voight, 2001). Knowing the limitations and expected artefacts of eye preservation is crucial to the functional morphology and optics of any visual system.

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

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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