Intraocular Pressure in Zebrafish: Comparison of Inbred Strains and Identification of a Reduced Melanin Mutant with Raised IOP

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PURPOSE. The goal was to establish an accurate method for measuring intraocular pressure (IOP) in living adult zebrafish and to determine the average IOPs of genetically different strains and mutants. The overall purpose was to develop the zebrafish system for studies of IOP and glaucoma. Elevated IOP and anterior segment dysgenesis are two known risk factors for glaucoma. Because albino mammals have increased IOP and are prone to anterior segment anomalies, the study was focused on a reduced melanin zebrafish mutant, brass.

METHODS. Servo-null electrophysiology was used to measure IOP in anesthetized adult zebrafish. A pulled-glass microelectrode was used to penetrate the cornea into the anterior chamber, and pressure transduction was recorded. IOP, histology, and visual behavioral assays were used to assess the brass mutant.

RESULTS. Significant differences in IOP were detected between genetically distinct zebrafish strains. IOP averages were highest in the long fin (LF) strain (20.5 ± 1.2 mm Hg) and lowest in the Oregon AB (AB) strain (10.8 ± 0.3 mm Hg). In brass mutants, which show a reduced melanin phenotype, IOPs were elevated and mild iris hypoplasia was noted. No defects were observed within the retina or in visual function.

CONCLUSIONS. In the present study, an accurate method was developed for measuring IOP in adult zebrafish. Both the ability to measure IOP in the genetically powerful zebrafish model and the conservation of elevated IOP and anterior segment anomalies between albino mammals and hypopigmented fish are important. They strongly support the use of zebrafish for studying the complex genetics of elevated IOP and glaucoma.

Glaucoma is a genetically heterogeneous group of conditions broadly characterized by progressive optic nerve damage.1,2 Elevated intraocular pressure (IOP) is a common risk factor for the development and progression of glaucoma. Although familial inheritance of glaucoma is well documented and a few disease-causing genes have been found, the multifactorial nature of glaucoma has limited identification of causative genes and molecules in most cases. One strategy for identifying the genetic relationships between causative and modifier genes in glaucoma is to use a forward genetics approach. With this approach, mutagenized animals are selected for specific phenotypes (for example abnormal IOP or retinal ganglion cell degeneration), and the affected genes are identified through standard genetic and molecular procedures. Phenotype selection and pedigree analysis requires a large number of animals. Genetic studies in mice have been extremely valuable for characterizing the role of specific genes in glaucoma and for experimentally testing candidate genes and gene–gene interactions in promoting glaucoma related phenotypes.3–5 However, the use of rodents in forward genetic screens is severely limited by the substantial time and expense needed to generate large populations of mutagenized animals and then to construct phenotyped pedigrees. This is especially true of age-related, progressive phenotypes such as elevated IOP and glaucoma. Recently, the zebrafish—with short generation times, large clutch sizes, and simple housing requirements—has emerged as a leading model for conducting forward genetic studies in vertebrates and in particular for analyzing complex diseases traits.6 Indeed, several large-scale chemical and insertion mutagenesis screens have already been conducted. From these screens, several valuable models of human diseases have been established.7,8 With the zebrafish genome sequence nearly complete, the time needed to link a mutant phenotype to a specific chromosomal locus has been greatly reduced.

To lay the groundwork for using zebrafish to study the genetics of elevated IOP and its relationship to other glaucoma phenotypes and to extend our previous work with mice,9–11 we sought to establish and validate a procedure for measuring IOP in zebrafish. The small size that makes zebrafish advantageous for genetic studies, makes physiological experiments challenging. For example, the anterior segment, in which IOP is measured, is on average 1.4 mm in diameter and has an average maximum depth of 0.25 mm at the peripheral angle. The small surface area and relative thickness of the zebrafish cornea (42–58 μm at the region adjacent to the angle) preclude the use of instruments such the handheld tonometer (TonoPen; Mentor, Norville, OH). To measure IOPs in the small eyes of zebrafish, we used servo-null electrophysiology to quantify the hydrostatic pressure in the anterior chamber. With this technique, pressures of aqueous solutions are measured by maintaining in dynamic equilibrium the conductivity at the tip of a sensing electrode. This is accomplished by integrating external positive and negative air pressure sources with a servo-null amplifier, piezoelectric valve, and pressure transducer (Fig. 1). Servo-null electrophysiology is well established for measuring pressures in rat kidney tubules, small-diameter blood vessels, and the chick embryonic heart.12,13 More recently, a servo-null method has been used to measure IOP in the mouse eye.14 Here, we report a reliable servo-null ap-
Materials and Methods

Animals

All experiments and fish maintenance protocols were approved by the Animal Care and Use Committee at each institution. Wild-type zebrafish from five genetic backgrounds maintained as inbred lines (brass) were used to validate the servo-null system for measurement of IOP. All fish were maintained in a shared-water, multirack system within 10-L tanks (Aquatic Habitats, Apopka, FL). Fish were kept at an approximate density of two to three fish per liter. The environment was regulated with a 14-hour light period at 28.5°C (pH 7.2) and a salinity of 600 μS. All fish were fed twice daily with newly hatched brine shrimp and a vitamin-enriched dry mix. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Design

Adult zebrafish, 10 to 14 months old and 24 to 34 mm in length, were used for measuring IOP. Eye diameters measured from 1.8 to 2.1 mm. Fish were anesthetized in 400 mL of tricaine (0.16 mg/mL, ethyl 3-aminobenzoate methanesulfonate) for approximately 2 minutes. Each fish was anesthetized one at a time until they were unconscious as judged by lack of response to physical stimuli. IOP was measured as soon as the fish failed to respond to physical stimuli. If necessary, anesthesia was maintained during IOP measurement by applying tricaine directly to the gills with a Pasteur pipette.

When anesthesia was obtained, the fish was placed on top of a sterile sponge that rested in a dish of tricaine. The sponge acted to keep the protective mucosal coating of the fish moist and to maintain anesthesia. The AgCl reference electrode was then placed underneath the zebrafish. A drop of tricaine was placed on the eye, and a micro-manipulator was used to advance the recording electrode into the drop of tricaine. Once the recording electrode was positioned above the eye, the null meter on the servo-null device was zeroed by setting the resistance at the tip of the electrode to 8 to 10 kΩ. The micromanipulator was then used to advance the recording electrode through the cornea overlying the pupil and into the anterior chamber. The position of the electrode was visualized under a dissecting microscope, and care was taken to ensure that the shank did not cause corneal deformation and that the electrode tip was not in contact with the lens.

Once the position of the electrode was verified in the anterior chamber, the junction between the electrode and cornea was further sealed with a drop of cyanoacrylate adhesive (Elmer’s Products, Inc., Columbus, OH). This rapidly formed a hardened barrier around the junction of the electrode and corneal surface. The IOP was then recorded at a rate of 240 Hz for a period greater than 30 seconds. The needle was then quickly removed from the eye and the recording electrode was returned to the tricaine bath to ensure that the null meter remained zeroed. The polymerized adhesive was then removed from the eye with a pair of number 5 student forceps, and the fish was transferred and revived in a holding tank of fresh fish water.

Instrumentation

Electrodes. Recording electrodes were made with borosilicate glass capillaries (TW100 to 4, 1.0 outer diameter [OD]/0.75 inner diameter [ID]; World Precision Instruments, Sarasota, FL) pulled with a pipette puller (Flaming Brown P-85; Sutter Instruments, Novato, CA). The electrodes were designed to come to a fine point very quickly to create a short, strong shank that could cleanly penetrate the zebrafish cornea. The tips of the electrodes were broken by hand with forceps to an approximate ID of 10 μM. Electrodes were back filled with 5 M KCl, by using 35-gauge microfill needles (World Precision Instruments). These electrodes displayed a resistance of 0.8 to 0.1 MΩ when introduced into the tricaine bath solution.

Servo-null Micropressure System. The recording electrode was connected to a commercially available servo-null system (Model 900A, Micropressure System; World Precision Instruments). This system consists of a recording electrode, a reference electrode, a pressure transducer, and a servo-null amplifier. The servo-null system constantly measures the resistance to electrical flow at the tip of the KCl-filled glass electrode. When the recording electrode is inserted into the anterior chamber, hydrostatic forces within the eye push aqueous humor into the electrode tip. As the aqueous humor/KCl interface moves within the electrode, the servo-null device measures the change in resistance and responds via the pressure transducer and piezoelectric vacuum/pressure pump. This device creates a counter pressure that is equal to the hydrostatic pressure within the anterior chamber. The analog output from the servo-null device was converted to a digital signal with a data acquisition device (DI-700; Dataq Instruments, Akron, OH). Pressures were constantly displayed on a computer monitor. All data were recorded and analyzed with waveform recording software (WinDaq: Dataq Instruments).

Criteria for Inclusion of Data Points

The following conditions were necessary for a trial to be considered successful and included in the final data set of IOPs:

1. The recording electrode must zero with the null detector when introduced into tricaine bath solution, both before and after injection.
layer from five central retinal fields of view (500 cell (RGC) layer were quantified by counting nuclei within the RGC thickness was measured from the edge of the outer nuclear layer to the pupil to the base, where the iris forms the angle with cornea. Retinal data point. Iris thickness was measured halfway from the tip at the proximity to the optic nerve. These were averaged and constituted one point.

To assess iris thickness, measurements were taken from the three most fish and five mutant fish were obtained to cut central ocular sections.

**Histologic Analysis and Morphometrics**

Eyes were removed from adult fish after terminal anesthesia and fixed overnight at 4°C in 2.5% gluteraldehyde/1% paraformaldehyde in phosphate-buffered sucrose (pH 7.4). The next morning, eyes were dehydrated and infiltrated with Epon/Araldite. Transverse sections 1 to 2 μm thick were heat mounted on glass slides and stained with 1% methylene blue/1% azure blue II in 1% borax.

For ocular morphometric measurements, eyes from five wild-type fish and five mutant fish were obtained to cut central ocular sections. To assess iris thickness, measurements were taken from the three most central, sagittal sections, as judged by maximum lens diameter and proximity to the optic nerve. These were averaged and constituted one data point. Iris thickness was measured halfway from the tip at the pupil to the base, where the iris forms the angle with cornea. Retinal thickness was measured from the edge of the outer nuclear layer to the edge of the inner limiting membrane. Cells within the retinal ganglion cell (RGC) layer were quantified by counting nuclei within the RGC layer from five central retinal fields of view (500 μm in width). A 40× objective was used for all morphometric analysis.

**Visual Behavioral Assays**

**Optokinetic Reflex Assay.** The optokinetic reflex (OKR) assay was performed as previously described. Briefly, 7-day-old fish were placed in a small glass beaker containing 4% methyl cellulose to partially immobilize the larvae. The beaker was then placed within a rotating drum which contained alternating black and white vertical stripes on the inside (15° wide). For each larva, the drum was rotated in both directions for 15 seconds each, and the number of saccades during a 1-minute trial was scored.

**Startle-Response Assay.** The startle-response (SR) assay was performed as previously described. Briefly, adult fish were placed in transparent rectangular containers and allowed to acclimatize for 30 minutes before testing. When fish faced the observer, black paper was passed in front of their field of view, a stimulus that normally elicits an escape response. The number of times the fish displayed a rapid-escape response during 10 startles was scored. The time required for testing varied from 3 to 8 minutes per fish. All tests were performed in 200-lux broad-spectrum illumination.

**RESULTS**

**Validation Assays**

To ensure that our micropressure system was capable of accurately measuring pressures over the expected range of IOPs, we obtained measurements from a calibrated water column from 0 to 74 mm Hg. Reported IOPs in various vertebrate species vary from 8 mm Hg (sea lions, Zalophus californianus) to 25 mm Hg (zebras, Equus bruchelli). Over this range, using the standard pressure column, a tight linear relationship was noted between the measured and actual pressures (Fig. 2A).

To determine whether we could measure IOP over the expected range of pressures, we performed a test that had been used to validate pressure measurements in the mouse eye. In this assay, a terminally anesthetized zebrafish had a pressure-inducing micropipette inserted into the temporal portion of the anterior chamber and a recording electrode inserted into the nasal region (Fig. 2B). Pressure to the inducing micropipette was supplied by a microsyringe driver capable of delivering 0 to 75 mm Hg. After both pipettes were inserted, pressure from the syringe driver was increased in a stepwise manner from the...
genetic basis of phenotype variability.3,4 For the strains tested, microsatellite markers have been invaluable for positional cloning projects to identify the genetic loci. This degree of nucleotide polymorphism has been shown to be highly variable across the genome based on a panel of microsatellite markers.

Overall, we measured IOPs of 14 fish per family from the five inbred strains (AB), Long Fin (LF), Ekkwill (EK), Tübingen (TU), and Tübingen Long Fin, (TL) (Fig. 4). These strains are approximately 70% percent genetically different across the genome. A representative IOP recording from zebrfish is shown in Figure 2C. The following strains, Oregon AB (AB), Long Fin (LF), Ekkwill (EK), Tübingen (TU), and Tübingen Long Fin (TL) were tested to determine whether differences could be detected between genetically different strains of zebrafish. In the mouse, various inbred strains have been shown to have significantly different IOPs.5,10 A representative IOP recording from zebrafish is shown in Figure 2C. The following strains, Oregon AB (AB), Long Fin (LF), Ekkwill (EK), Tübingen (TU), and Tübingen Long Fin (TL) were analyzed for average IOP (Fig. 3). Overall, we measured IOPs of 14 fish per family from the five strains (Fig. 4). These strains are approximately 70% percent different across the genome based on a panel of microsatellite repeat sequences. This degree of nucleotide polymorphism has been invaluable for positional cloning projects to identify the genetic basis of phenotype variability.3,4 For the strains tested, average IOPs varied from 10.8 to 20.6 mm Hg (Table 1). Statistical analysis indicated that pair-wise comparisons between most strains showed significant differences in IOPs (P < 0.0001, Student’s t-test; Table 1). This twofold difference is very similar to that reported between distinct mouse strains and to the range of IOPs in the normal human population.10,18

Twofold Difference in IOP between Distinct Inbred Zebrafish Strains

After validation of the procedure for measuring IOP in zebrafish, we tested whether differences could be detected between genetically different strains of zebrafish. In the mouse, various inbred strains have been shown to have significantly different IOPs.5,10 A representative IOP recording from zebrafish is shown in Figure 2C. The following strains, Oregon AB (AB), Long Fin (LF), Ekkwill (EK), Tübingen (TU), and Tübingen Long Fin (TL) were analyzed for average IOP (Fig. 3). Overall, we measured IOPs of 14 fish per family from the five strains (Fig. 4). These strains are approximately 70% percent different across the genome based on a panel of microsatellite repeat sequences. This degree of nucleotide polymorphism has been invaluable for positional cloning projects to identify the genetic basis of phenotype variability.3,4 For the strains tested, average IOPs varied from 10.8 to 20.6 mm Hg (Table 1). Statistical analysis indicated that pair-wise comparisons between most strains showed significant differences in IOPs (P < 0.0001, Student’s t-test; Table 1). This twofold difference is very similar to that reported between distinct mouse strains and to the range of IOPs in the normal human population.10,18

Raised IOP in a Reduced-Melanin Mutant

In mice, mutations that affect the melanin synthesis pathway can contribute to anterior segment dysgenesis.19 In addition, albino mice of strain C57BL/6j have higher IOPs than otherwise genetically identical but pigmented C57BL/6j mice housed in the same environment.10 In zebrafish, reduced melanin mutants in general have been shown to have decreased visual sensitivity.20 Thus, we investigated the IOP in a reduced-melanin zebrafish mutant, brass. Recessive brass mutations have been identified in several genetic screens for pigment abnormalities.21,22 The gene responsible for the brass phenotype has not been identified, but the genetic lesion has been linked to chromosome 13.23 The primary phenotype of the mutants is reduced melanocyte pigmentation, which does not affect pigment cell morphology or survival.24 For our analysis, we crossed brass homozygous mutants with AB wild-type fish, the strain in which the brass mutation was originally identified. The resultant heterozygous fish, all with normal pigment, were then raised and characterized as adults. Zebrafish that were homozygous wild-type, heterozygous, and homozygous white genetic clones were tested individually.

Because of the elevated IOP in brass mutants and the observations that pigmentation mutations in mice and in humans have been associated with developmental abnormalities in the anterior segment of the eye,19,25,26 we compared brass with control AB eyes by histology. In addition, to reduced pigmentation, an iris hypoplasia was noted and the iris stroma vasculature appeared reduced in homozygous brass mutants (Figs. 6A, 6B). No differences in ocular morphology were observed in heterozygous fish. Quantitative measurements revealed the brass iris was thinner than the iris from wild-type sibling fish (18.1 μm vs. 12.3 μm; Fig. 6C). Despite a thinning of the mutant iris, the overall morphology of the anterior segment appeared normal. In addition, no other ocular abnor-
malities were noted when comparing brass with wild-type eyes.

Finally, we performed behavioral assays to assess whether vision was compromised in brass mutants either as larvae or adults. To test vision in larvae, we used an assay to measure behavior dependent on vision, the OKR.15 When larval fish are presented a slow moving stimulus across the visual field (alternating black and white stripes on a rotating drum), they respond by following the stimulus with a smooth pursuit. Once the object has moved outside of the field of view, both eyes undergo a rapid saccade. This assay has been used successfully to isolate multiple visually compromised mutants.15,27 Under various lighting conditions, brass and wild-type larvae were indistinguishable in performance (Fig. 7A). This assay demonstrated that vision in brass mutants is established normally.

To assay whether there is visual deterioration with age in brass mutants, we used the SR assay and measured retinal thickness and cell numbers within the RGC layer.16 With the SR assay, a threatening object (large black bar), is presented to an isolated adult fish, which responds by performing a rapid escape turn. We performed this assay under medium lighting conditions (200 lux), as reduced pigment mutants have previously been found to perform less robustly on this assay under bright or dim lighting conditions, possibly because of photon scatter and altered visual sensitivity tuning responses, respectively.20 We found that both brass and wild-type fish displayed escape behavior of similar proportions in response to visual stimuli (Fig. 7B). Because retinal cell loss can often occur without measurable changes in visual behavior, we analyzed total retinal thickness and RGC layer cell density. No changes in either parameter were detected between wild-type and brass mutant eyes (Fig. 8).

### DISCUSSION

#### Toward the Use of Zebrafish to Study Glaucoma Phenotypes

A long-term goal of our studies is to use zebrafish to study the complexity of glaucoma phenotypes, as a powerful complement to studies being conducted in mice and other model systems. Multiple factors, including different genes, different mutations, and environmental variables are thought to promote and modify glaucoma. One major risk factor for this disease is elevated IOP. In this study, we validated a servo-null electrophysiological technique for measuring adult zebrafish IOP. We applied this technique to characterize the average IOPs of five inbred strains of zebrafish. Finally, we identified a hypopigmented mutant, brass, with elevated IOP and iris hypoplasia.

#### Very Similar IOP Distributions in Mammals and Zebrafish

An approximately twofold range of IOP exists in different mammalian species. In mice, IOP between strains ranges from approximately 10 to 20 mm Hg, with different strains having statistically significant differences in mean IOP.10 The mean IOP considering mice of all inbred strains included in a large study was 14.5 mm Hg.10 Similarly, within a nonselected sample of 5941 normal people, IOP varied from 10.5 to 20.7 mm Hg.28 Subsequent clinical studies have shown similar results,16 and human IOP differs between isolated populations.25 Herein, we report zebrafish IOP and show that in this nonmammalian vertebrate, both the range of IOP and mean IOP are remarkably similar to mammalian values. IOP ranges from 10.8 to 20.5 mm Hg between distinct fish strains. The strain with the lowest IOP was AB (10.8 ± 0.3 mm Hg), whereas the strain with the highest average IOP was LF (20.5 ± 1.2 mm Hg). The mean IOP across all reported zebrafish strains is 16.5 mm Hg. The striking similarity in normal IOP distributions between zebrafish and mammals supports the use of zebrafish for genetic analyses of IOP and other glaucoma phenotypes.

#### Effect of Pigmentation Mutations on IOP in Mammals and Fish

Analysis of IOP in brass mutants, which are hypopigmented, suggests further physiological conservation between zebrafish and mammals. We have shown that albino mice of strain C57BL/6J have higher IOPs than otherwise genetically identical

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**TABLE 1. IOP Averages and Statistical Comparisons**

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<th>EK (16.3 ± 1.0)</th>
<th>TL (18.4 ± 0.6)</th>
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<th>AB (10.8 ± 0.3)</th>
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* Mean IOP ± SEM (mm Hg); $n = 14$.
† Two-tailed Student's $t$-test.
but pigmented C57BL/6J mice housed in the same environment. Similarly, we show here that the average IOP of hypopigmented brass mutants (16.9 mm Hg) is higher than the mean value of non-mutant, strain matched fish (11.0 mHg). The observation of an intermediate phenotype in brass heterozygotes for IOP, but not for pigmentation, is interesting. This result suggests that the brass gene product may function in a dose-dependent fashion for IOP, whereas pigmentation is normal above a certain threshold of brass function. The eventual molecular identification of the brass gene is important for a better understanding of these results.

Because of their elevated IOP, brass mutants provide an important resource for future zebrafish studies for the study of glaucoma. The ability to measure IOP in zebrafish is the first step toward exploiting the zebrafish system for glaucoma research. Another important step is the generation of zebrafish models with pathologically elevated IOP and RGC death. Sensitized screens involve the use of mutant strains predisposed to a particular phenotype in order to identify other genes that can interact with the initial mutation to either exacerbate or suppress the phenotype. The brass mutation significantly elevates IOP, but does not cause RGC death. Thus, brass is a useful sensitizing mutation for use in mutagenesis screens. By crossing the brass mutation with mutagenized lines, mutations that interact with brass to cause pathologically elevated IOP and/or glaucomatous optic nerve degeneration may be identified.

**FIGURE 6.** Comparison of wild-type and brass iris thickness. (A) Wild-type iris and (B) brass mutant iris. Higher magnification inset shown for each. (C) Average iris thickness ± SEM for wild-type and brass zebrafish (n = 5 for each). *Significant difference (P < 0.001, Student’s t-test). Bar, 75 μm (22.5 μm inset). Iris stroma and pigmented and nonpigmented epithelium are circled. Arrows: annular ligament (AL) at the base of the cornea. The staining differences within corneal stroma reflect dorsal-ventral variability of the annular ligament and are due to slightly different planes of section. Observation of multiple sections did not indicate defects within the brass cornea.

**FIGURE 7.** Behavioral assays indicate vision is normal in brass mutants. (A) OKR assay. Number of saccades in brass and wild-type larvae in response to rotating stripes presented to the visual field. (B) SR assay. Percentage of escape responses in brass and wild-type adults when presented a visual threat. No significant differences were detected between wild-type and brass fish.
Effect of Pigmentation Mutations on Ocular Development in Mammals and Fish

In mice, mutations in the tyrosinase gene cause albinism and elevated IOP. We recently demonstrated that the tyrosinase gene participates in anterior segment development in mice. We found that in mice, tyrosinase mutations modify the severity of anterior segment abnormalities caused by mutations in the mouse orthologues of two human genes (CYP1B1 and FOXC1). Both of these loci contribute to glaucoma in humans. In human albinos, the prevalence of anterior segment dysgenesis is increased. In addition to tyrosinase-deficient individuals, anterior segment dysgenesis also occurs in tyrosinase-positive individuals who often have reduced pigmentation of the eyes, skin, and hair. This suggests that additional, yet to be identified pigmentation genes can affect glaucoma phenotypes. In the current study, the hypopigmented zebrafish with the brass mutation had iris hypoplasia, consistent with a role for the pigmentation pathway in modulating anterior segment formation in fish. Although the brass mutation has not been identified, linkage analysis indicates that the affected gene is not tyrosinase itself. It will be interesting to identify the genetic lesion in brass mutants and to test whether the brass mutation can modify other zebrafish anterior segment dysgenic phenotypes. The importance of pigmentation status for normal ocular development provides further evidence for conservation of molecular pathways between mammals and zebrafish.

In conclusion, by developing a technique to measure IOP in zebrafish, we have laid the foundation for studying the complex genetics of elevated IOP and glaucoma in this genetically attractive organism. We anticipate that zebrafish studies will complement studies in various species. The coupling of forward and reverse genetic approaches in zebrafish and mice provides a powerful and synergistic approach for unraveling the complexities and relationships of glaucoma phenotypes.

References


FIGURE 8. Cell density within the RGC layer and total retinal thickness in brass versus wild-type eyes. Histology of retina from wild-type (A) and brass mutants (B). Mean cell density within the RGC layer (C) and total retinal thickness (D) did not show differences between wild-type (●) and brass fish (□).


