

Research Article

Total Outflow Facility in Live C57BL/6 Mice of Different Age

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What Is It about?

Mice have an aqueous drainage system that is similar in organization and function to that of primates. Live mice and ex vivo mouse eyes are increasingly being used as glaucoma research models to better understand aqueous physiology, dissect out pathophysiology, and test new therapies. We measured total outflow facility in vivo across the adult lifespan of C57BL/6 mice, a common background strain for engineered mice. Our data provides outflow facility reference information for live C57BL/6 mice that may be used as background controls in age-related glaucoma studies.

Keywords

Trabecular meshwork · Outflow facility · Glaucoma · C57BL/6 · Perfusion apparatus

Abstract

Purpose: To characterize total outflow facility across the live adult mouse lifespan as a reference for mouse glaucoma studies and the common C57BL/6 background strain. **Methods:** Microperfusion was performed by single-needle cannulation and feedback-controlled coupling of pressure and flow to maintain a constant pressure in the anterior chambers of live C57BL/6NCRl mice aged 3–4 months ($n = 17$), 6–9 months ($n = 10$), and 23–27 months ($n = 12$). This mouse age range represented an equivalent human age range of young adult to elderly. We characterized the following across age groups in vivo: (1) outflow facility based on constant pressure perfusion in a pressure range of 15–35 mm Hg, (2) perfusion flow rates, and (3) anterior segment tissue histology after perfusion. Thirty-nine live mice underwent perfusion. **Results:** Pressure-flow rate functions were consistently linear for all age groups (all $R^2 >$

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0.96). Total outflow facility in mice aged 3–4, 6–9, and 23–27 months was 0.0066, 0.0064, and 0.0077 $\mu\text{L}/\text{min}/\text{mm Hg}$, respectively. Facility was not significantly different between age groups (all $p > 0.4$). The groups had closely overlapping frequency distribution profiles with right-sided tails. Post hoc estimates indicated that group facility differences of at least 50% would have been detectable, with this limit set mainly by inherent variability in the strain. A trend toward higher perfusion flow rates was seen in older mice aged 23–27 months, but this was not significantly different from that of mice aged 3–4 months or 6–9 months ($p > 0.2$). No histological disruption or difference in iridocorneal angle or drainage tissue structure was seen following perfusion in the different age groups. **Conclusion:** We did not find a significant difference in total outflow facility between different age groups across the live C57BL/6 mouse adult lifespan, agreeing with some human studies. The possibility that more subtle differences might exist ought to be judged with respect to the heterogeneity in facility at different ages. Our findings provide reference data for live perfusion studies pertaining to glaucoma involving the C57BL/6 strain.

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Introduction

Mice have an aqueous drainage system that is similar in organization and function to that of primates. Live mice and ex vivo mouse eyes are increasingly being used as glaucoma research models to better understand aqueous physiology, dissect out pathophysiology, and test new therapies [1–14]. The mouse also offers practical advantages for studying age-related glaucoma as its lifespan and period of ageing are relatively short, spanning a period of up to 2 years [15] compared with equivalent ageing in nonhuman primates of 20–30 years [16–19].

We have established a 1-needle anterior chamber microperfusion approach for live mouse eyes [1, 14] that is suited to tiny mouse anterior chambers compared with 2-needle approaches traditionally used in larger primates [20, 21]. A feedback control system coupling pressure and perfusion flow rate permits reliable constant-pressure perfusion of the mouse anterior chamber through a single needle. Pressure transduction in this system is accurate, flow measurements are stable and reproducible [1, 14], and outflow facility values agree with other reports [4–6].

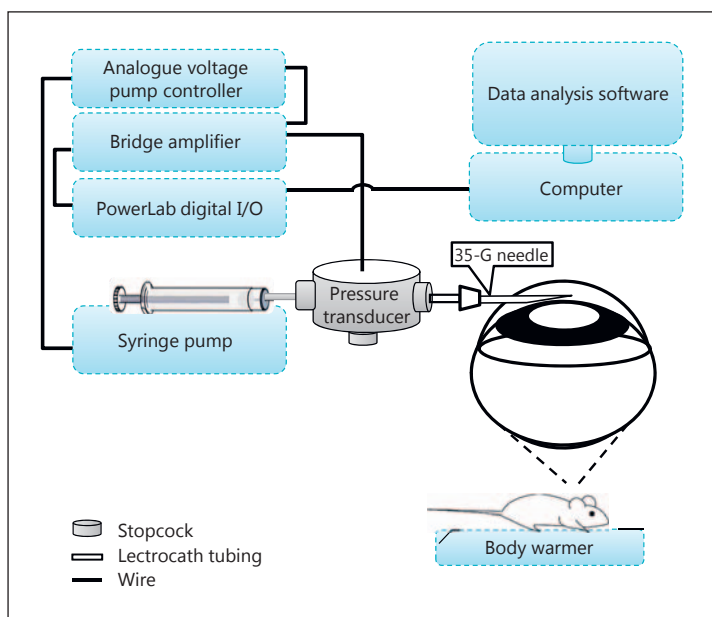
We measured total outflow facility in vivo in C57BL/6 mice, a common background strain for engineered mice. Perfusion studies were performed in mice aged 3–4 months, 6–9 months, and 2 years to measure total outflow facility across the adult mouse lifespan equivalent to young adult to elderly humans [15].

Methods

Animal Husbandry, Anesthesia, and Perfusion Apparatus

Mouse experiments were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research. Institutional Animal Care and Use Committee (IACUC) approval was obtained. C57BL/6NcrJ mice aged 3–4 months and 6–9 months were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice 23–27 months were aged from the pool of mice obtained originally at 6–9 months. The study group comprised 20 male and 19 female mice. The mice were raised and housed in air-filtered clear cages with a bedding of pine shavings, subject to a 12-h light/dark cycle, and fed ad libitum.

Fig. 1. Schematic diagram of experimental setup for feedback-controlled single-needle constant pressure anterior chamber perfusion in live mice. A 35-G needle for live mouse anterior chamber cannulation was connected via rigid tubing to a pressure transducer and then microsyringe pump, bridge amplifier, and digital I/O unit integrated with analytical software on a computer. The pump controller provided a feedback loop between the pressure transducer, pump, and amplifier.



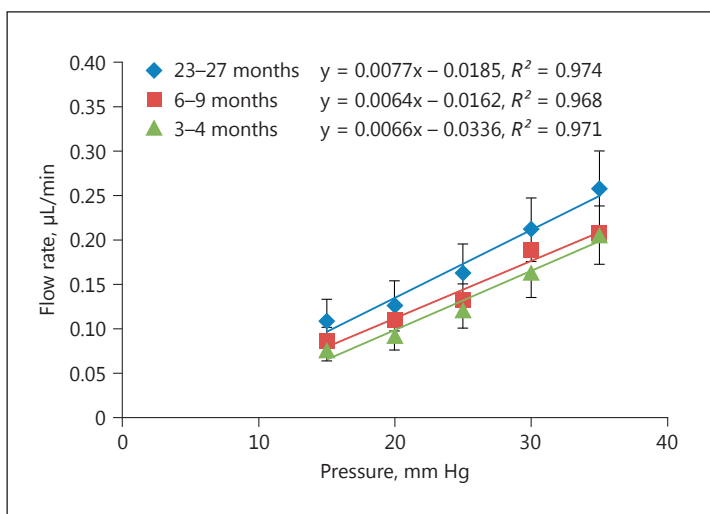
Mice were anesthetized by intraperitoneal injection of a mixture of ketamine (60–85 mg/kg), xylazine (6–8.5 mg/kg), and acepromazine (1.5–2.5 mg/kg), and titrated based on observed mouse movement as well as moment-to-moment monitoring for irregular flow rate and pressure as traced by perfusion recording software (LabChart 7.3.4; ADInstruments, Colorado Springs, CO, USA).

Anterior chamber cannulation was performed with a 35-gauge needle (Medicom, Lachine, QC, Canada) connected to a perfusion apparatus with a calibrated glass microsyringe (50 μ L, Hamilton 1705TLL; Hamilton Inc., Reno, NV, USA), microperfusion pump (PHD Ultra; Harvard Apparatus, Holliston, MA, USA), and a computer (Fig. 1), as previously described [1]. The needle insertion site on the cornea was monitored for leakage as judged by external pooling of leaking aqueous, alteration of fluorescein dye applied to the cannulation site in which clear fluid appeared with the leakage, or disruption of a silicone grease smear across the external corneal needle insertion site. With correct cannulation, it was rare to see leakage around the needle entry site. The sharp needle afforded easy corneal penetration during cannulation without tissue contusion. Needle blockage did not occur, as evidenced by the sharply increased outflow rate typically seen with needle removal. Needles were not reused.

Perfusion Protocols and Analysis

For constant pressure perfusion, the anterior chamber was perfused to achieve a stable constant pressure for at least 3 min during which flow and pressure were recorded. This process was repeated for constant pressure perfusions at physiologically relevant pressures of 15, 20, 25, 30, and 35 mm Hg. To determine total outflow facility of each animal, pressure and flow rate data were extracted from the software at a rate of 1 per 10 ms for 15,000 consecutive data points for each pressure condition. Perfusion flow rate represented the physiological outflow rate and perfusion pressure represented intraocular pressure. The relationship between pressure and flow rate for each animal was analyzed in scatter plots and modeled by regression analysis. Measurement accuracy, control algorithm, and flow rate variability at each pressure was reported in a previous publication [1].

Fig. 2. Linear relationship between flow and pressure over a perfusion pressure range of 15–35 mm Hg in mice aged 3–4 months (triangles; $n = 17$; slope 0.0066), 6–9 months (squares; $n = 10$; slope: 0.0064), and 23–27 months (diamonds; $n = 12$; slope: 0.0077). Analysis of differences in total outflow facility between the age groups revealed no significant differences (all $p > 0.5$). Error bars: standard error of the mean.



Live mouse total outflow facility (C ; $\mu\text{L}/\text{min}/\text{mm Hg}$) was determined as the slope of the flow rate (F ; $\mu\text{L}/\text{min}$) versus pressure (P ; mm Hg) regression function. Frequency distribution analysis of outflow facility in the different groups was performed and compared. Group data were compared using the nonparametric Mann-Whitney U test with $p < 0.05$ considered significant. Data was log-transformed where necessary for analysis of variance (ANOVA) and t tests. Mean flow rates for equivalent pressures were determined and analyzed for differences between age groups by ANOVA. Histological analysis (hematoxylin and eosin) of formalin-fixed paraffin-embedded sections of the outflow tissues from mice of different age was performed to ascertain if tissue disruption occurred during perfusions.

Results

Thirty-nine live C57BL/6NCrI mice underwent constant pressure anterior chamber perfusion within a perfusion pressure range of 15–35 mm Hg. Mean (\pm SD) body weight was 27 ± 1.6 , 27 ± 1.5 , and 42 ± 14 g for mice aged 3–4 months ($n = 17$), 6–9 months ($n = 10$), and 23–27 months ($n = 12$), respectively. Mice aged 23–27 months, having gained weight during ageing, were heavier than 3- to 4-month-old ($p = 0.004$) and 6- to 9-month-old mice ($p = 0.003$). Mice 3–4 months and 6–9 months were similar in weight ($p = 0.6$).

A consistently linear relationship between perfusion flow rate and pressure in a pressure range of 15–35 mm Hg was seen across all age groups (all $R^2 > 0.96$). Total outflow facility in mice aged 3–4 months, 6–9 months, and 23–27 months was 0.0066, 0.0064, and 0.0077 $\mu\text{L}/\text{min}/\text{mm Hg}$, respectively, as shown in Figure 2.

The pressure-flow relationship curve for mice aged 23–27 months had a slight vertical offset compared with mice aged 3–4 months and 6–9 months, as seen in Figure 2. A trend toward higher perfusion flow rates for a given perfusion pressure was seen in mice aged 23–27 months compared with mice aged 3–4 months and 6–9 months, but apparent differences were not significant (all $p > 0.2$; Table 1).

Total outflow facility in mice aged 3–4 months was not significantly different from mice aged 6–9 months ($p = 0.94$) or 23–27 months ($p = 0.44$), as shown in Figure 3. Total outflow facility in mice aged 6–9 months and 23–27 months was also not significantly different ($p = 1.0$).

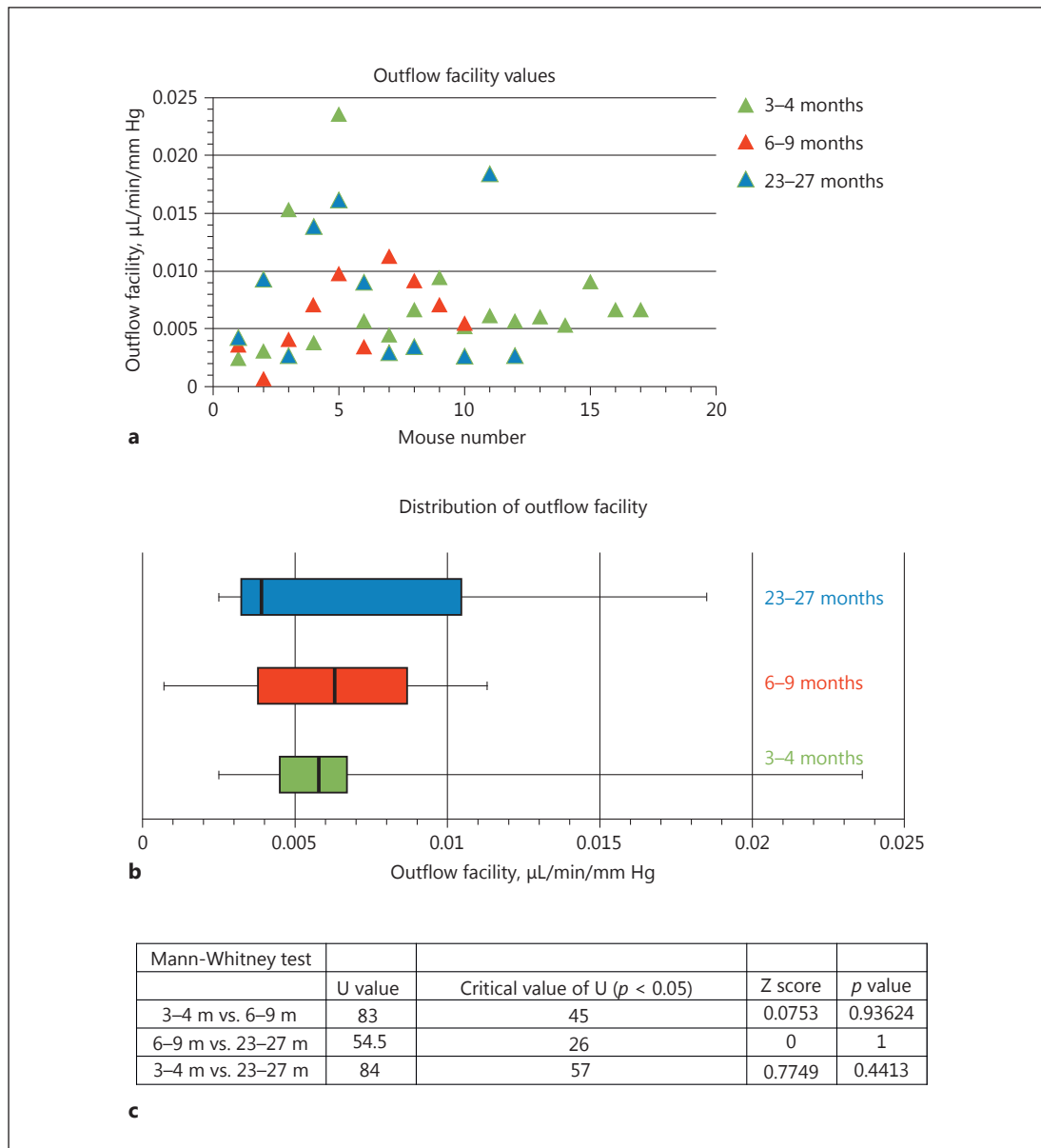


Fig. 3. Outflow facility measurements in different age groups: scatter plot (a), box and whisker plot (b), and significance testing using the Mann-Whitney U test (c). Green: 3–4 months. Red: 6–9 months. Blue: 23–27 months. Box and whisker plots show minimum, 1st quartile, median, 3rd quartile, and maximum outflow facility for each age group.

Table 1. Comparison of perfusion flow rates ($\mu\text{L}/\text{min}$) at different perfusion pressures in mice aged 3–4 months, 6–9 months, and 23–27 months

Age	15 mm Hg	20 mm Hg	25 mm Hg	30 mm Hg	35 mm Hg
3–4 months	0.08±0.05	0.09±0.07	0.12±0.08	0.16±0.11	0.21±0.13
6–9 months	0.09±0.06	0.11±0.08	0.13±0.08	0.19±0.09	0.21±0.12
23–27 months	0.11±0.08	0.13±0.09	0.16±0.11	0.21±0.12	0.26±0.14

Values are presented as means \pm standard deviation. $p > 0.2$ for comparisons between age groups.

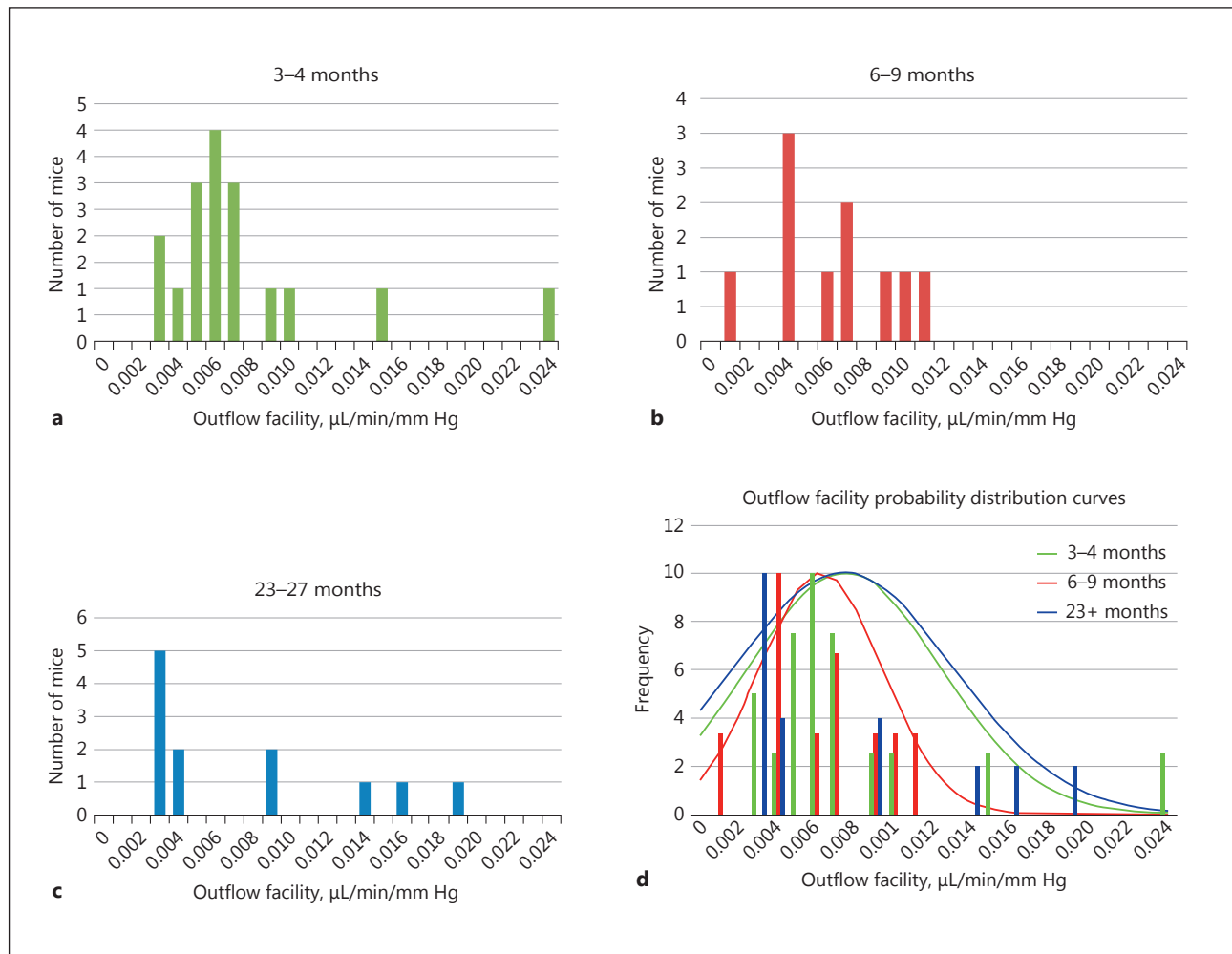


Fig. 4. Outflow facility frequency distribution analysis for mice aged 3–4 months (a), 6–9 months (b), and 23–27 months (c). Distribution analysis is shown as superimposed curves (d) for the different age groups identified as green: 3–4 months, red: 6–9 months, and blue: 23+ months.

Outflow facility frequency distributions for the different age groups were non-Gaussian with a right tail, as shown in Figure 4. The nonparametric Mann-Whitney U test did not show significant differences between the age groups, as reported in Figure 3.

Histological analysis showed no morphological differences in the drainage tissues or iridocorneal angle structures after perfusion, as shown in Figure 5.

Discussion

We measured total outflow facility in C57BL/6 mice of different ages representing sampling across a significant portion of the adult mouse lifespan: mice aged 3–4 months represented young adults, mice aged 6–9 months were middle-aged, and mice 23–27 months represented elderly mice. Typical human ages over which primary open-angle glaucoma develops fall within this range [15]. Total outflow facility measurements in our live C57BL/6 mice were similar to those reported following constant pressure perfusion in enucleated [4]

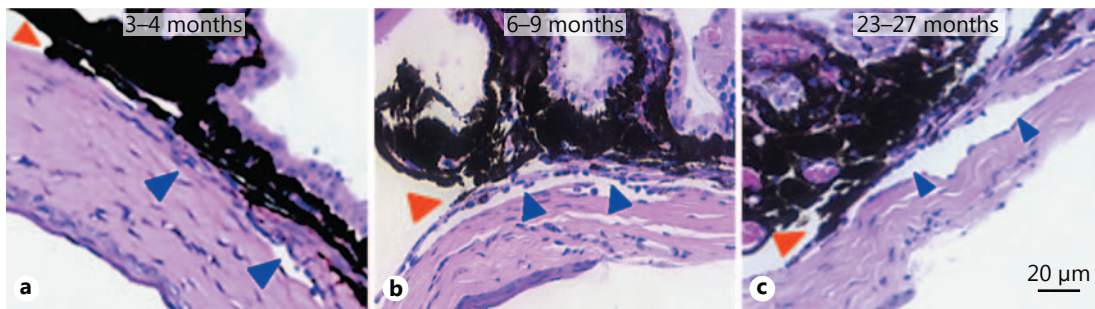


Fig. 5. Representative hematoxylin and eosin-stained paraffin sections of perfused mouse anterior segments and drainage tissues at ages 3–4 months (**a**), 6–9 months (**b**), and 23–27 months (**c**). Red arrowheads indicate the anterior edge of the iridocorneal angle. Blue arrowheads indicate the location of Schlemm’s canal and overlying trabecular meshwork.

Table 2. Mean outflow facility in the different mouse age groups

Age	Mean outflow facility, $\mu\text{L}/\text{min}/\text{mm Hg}$	Group SD	ANOVA SEM
3–4 months	0.0074	0.0051	0.0012
6–9 months	0.0062	0.0031	0.0015
23–27 months	0.0074	0.0059	0.0014
Method	SEM	Minimum detectable difference	% change
Pairwise contrast in 3-group ANOVA	0.00185	0.0037	50%

Mean facility values tabulated here were calculated by averaging individual outflow facility of mice within each age group. These values are expected to differ slightly from the values obtained by linear regression of each group’s average flow rates with reference to perfusion pressure [1, 3], as shown in Figure 2. SD, standard deviation; SEM, standard error of the mean.

and live C57BL/6 eyes [1], as well as other strains of live mice, such as NIH Swiss white (0.0051 $\mu\text{L}/\text{min}/\text{mm Hg}$) [5].

We did not find a significant difference in outflow facility between the age groups. Sample size calculation was based on our prior published data using the same perfusion system and mouse strain (baseline outflow facility of 0.006 $\mu\text{L}/\text{min}/\text{mm Hg}$; standard deviation of 0.002 $\mu\text{L}/\text{min}/\text{mm Hg}$) [1, 14]. Estimated age-related facility decline was extrapolated from data covering an equivalent lifespan in nonhuman primates [17] (~50%, to 0.003 $\mu\text{L}/\text{min}/\text{mm Hg}$). Post hoc analysis to test group differences when modeling all 3 age groups together by ANOVA estimated that the minimum detectable difference in mean facility between the oldest and youngest age groups for our samples was 50% (Table 2). Our analysis of outflow facility frequency distributions showed a non-Gaussian distribution with right tail of values that was reproducible across all age groups. The lack of significant change was associated with marked overlap in facility values, as seen in superimposed group scatterplots and frequency distributions in Figures 3 and 4. It should be kept in mind that the 90% power level of our sample calculation, while reasonably stringent, carries a 10% chance of β error (type II; false negatives). Also, our study, which was powered to detect a difference of at least 50%, may have missed smaller facility differences. The clinical significance of this possibility would have to

be judged with respect to intraocular pressure, which has been reported as unchanged or slightly increased over a 1- to 2-year age range in C57BL/6 mice [22, 23].

Our observation that total outflow facility did not change with age in live C57BL/6 mice agrees with perfusion studies of enucleated eyes of C57BL/6 mice [4]. Our study extended the upper age limit previously reported for live mouse facility [22] to over 2 years, and still we did not observe a change in outflow facility. A recent live C57BL/6 mouse perfusion study showed a trend, but not significant age-related increase, of outflow facility in mice aged up to 14 months [22]. Rhesus monkeys show a decline of outflow facility with age [16, 17]. Human studies report reduced or unchanged outflow facility with age [24–33].

We observed a slight offset of the flow-pressure function in the presence of unchanging slope in mice aged 23–27 months relative to other age groups. While this suggests a trend toward increased perfusion flow rate across perfusion pressures in our oldest mice, apparent differences between age groups were not significant for any of the perfusion pressures we used (15, 20, 25, 30, or 35 mm Hg; all $p > 0.2$). True increased perfusion flow with unchanged outflow facility in this scenario may reflect a concurrent increase in outflow rate (e.g., uveoscleral) or decrease in inflow (pseudofacility or aqueous formation) with age and during perfusions [34, 35]. In primates, uveoscleral outflow, aqueous formation, and pseudofacility are reported to decline with age [18, 25, 33, 36–39], episcleral venous pressure is steady with age, and ocular rigidity increases with age [25, 33, 39–41].

We did not study other mouse aqueous dynamics parameters, which have been reported [5, 7, 42, 43]. It is possible that the separate outflow components of conventional (trabecular) and unconventional (uveoscleral) facility change relative to each other with age. Establishing whether mouse conventional facility changes with age requires specific methodology adapted for mice to study whether other factors such as scleral rigidity, pseudofacility, and uveoscleral outflow also change with age in mice [16–19, 24, 25, 44].

In summary, our data provides outflow facility reference information for live C57BL/6 mice that may be used as background controls in age-related glaucoma studies. Our post hoc estimates indicated that a facility change of at least 50% would have been detectable using our methodology in the animals we tested. We did not find a significant difference in total outflow facility across age groups covering the C57BL/6 mouse adult lifespan. This agreed with a number of human observations [30–33], but not other studies in humans [24–29] or primates [16, 17]. It is possible that subtler facility differences existed, which to ascertain requires testing of significantly more animals. The practicality of embarking on this should be judged by the heterogeneity in our facility data at the different mouse ages and cost and time needed to age the animals.

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Disclosure Statement

The authors report no conflicts of interest.

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