Quantifying the Ontogeny of Optokinetic and Vestibuloocular Behaviors in Zebrafish, Medaka, and Goldfish

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Beck, James C., Edwin Gilland, David W. Tank, and Robert Baker. Quantifying the ontogeny of optokinetic and vestibuloocular behaviors in zebrafish, medaka, and goldfish. J Neurophysiol 92: 3546–3561, 2004. First published July 21, 2004; doi:10.1152/jn.00311.2004. We quantitatively studied the ontogeny of oculomotor behavior in larval fish as a foundation for studies linking oculomotor structure and function with genetics. Horizontal optokinetic and vestibuloocular reflexes (OKR and VOR, respectively) were measured in three different species (goldfish, zebrafish, and medaka) during the first month after hatching. For all sizes of medaka, and most zebrafish, Bode plots of OKR (0.065–3.0 Hz, ±10°/s) revealed that eye velocity closely followed stimulus velocity (gain > 0.8) at low frequency but dropped sharply above 1 Hz (gain < 0.3 at 3 Hz). Goldfish showed increased gain proportional to size across frequencies. Linearity testing with steps and sinusoids showed excellent visual performance (gain > 0.8) in medaka almost from hatching; but zebrafish and goldfish exhibited progressive improvement, with only the largest equaling medaka performance. Monocular visual stimulation in zebrafish and goldfish produced gains of 0.5 versus <0.1 for the eye viewing a moving versus stationary stimulus pattern but 0.25 versus <0.1 in medaka. Angular VOR appeared much later than OKR, initially at only high accelerations (>200°/s at 0.5 Hz), first in medaka followed by larger (8.11 mm) zebrafish; but it was virtually nonexistent in goldfish. Velocity storage was not observed except for an eye velocity build-up in the largest medaka. In summary, a robust OKR was achieved shortly after hatching in all three species. In contrast, larval fish seem to be unique among vertebrates tested in their lack of significant angular VOR at stages where active movement is required for feeding and survival.

INTRODUCTION

The vertebrate oculomotor system has aided survival by maintaining visual acuity during self and visual world motion (Land 1999; Walls 1962) in nearly the same basic form for over 450 million years of evolution (Baker and Gilland 1996). Since the oculomotor system is so universal, lower vertebrate systems are now utilized to link oculomotor behavior with its genetic underpinnings. One animal in particular, the zebrafish, Danio rerio, is an established developmental vertebrate model (Fishman 2001), undergoing genome sequencing (Vogel 2000). Initial work in zebrafish utilized oculomotor behaviors such as the optokinetic response (OKR) to screen for defects in the retina (Brockerhoff et al. 1995, 1997) as well as early components of the visual pathway (for review see Baier 2000; Neuhauss 2003; Rick et al. 2000; Roesser and Baier 2003). However, the oculomotor system has been largely a tool and not the primary focus of these investigations.

Normally, the visual and vestibular systems work in concert to produce reflexive compensatory eye movements. In the laboratory, however, these sensory motor systems can be studied individually (Robinson 1968). The visual surround (e.g., striped drum) can be rotated en bloc to produce an OKR as the visual system strives to minimize retinal slip (Collewijn 1991). In contrast, the vestibular system lacks any inherent means of feedback in the absence of vision (dark), thus operating in an open-loop mode. Vestibuloocular reflexes (VOR) are evoked by stimulation of two separate, but complementary, sensory components: the otoliths detecting linear (L) acceleration and the semicircular canals detecting angular (A) acceleration (Angelaki et al. 1995; Hess and Angelaki 1993). These two vestibular reflexes can also be examined separately. Tilting the head to a fixed angle produces a static change in eye position, while a dynamic LVOR is generated by translating the head linearly (Paige 2002). Rotating the animal with the head centered in the axis of rotation stimulates the semi-circular canals while minimally affecting the otolith organs, thereby creating an eye movement entirely related to the AVOR (Wilson and Melvill Jones 1979).

To date, only two groups have sought to address the development of oculomotor function in larval zebrafish. Moorman et al. (1999, 2002; Riley and Moorman 2000) focused on the development and function of the static LVOR in zebrafish at 5 days postfertilization (dpf). Easter and Nicola (1996, 1997) examined the onset and development of optokinetic and angular vestibular behaviors in larval zebrafish aged 2–4 dpf. The first reflexive eye movements appeared at 3 dpf, as slow phase responses to a drum rotating constantly at 2.4°/s. Fast phase resets appeared shortly thereafter and OKR gain (ratio of eye to stimulus velocity) was noted to reach 0.9 by 4 dpf. Horizontal angular VOR, measured with a presumptive ganzfeld (a featureless visual surround), was reported just after the onset of OKR. Spontaneous saccades appeared prior to 4 dpf and reached adult velocities by 5 dpf. Easter and Nicola (1997) concluded that early larval spontaneous and reflexive optokinetic behaviors approached adult levels of performance with vestibuloocular behaviors following nearly the same ontogenetic time course.

Zebrafish represent just 1 of 23,000 extant teleost species (Eschmeyer 1998); thus to ensure that they are representative...
examples of vertebrate oculomotor development as a whole as well as to provide a more complete understanding of oculomotor function in general, two additional fish species were selected: goldfish (*Carassius auratus*) and medaka (*Oryzias latipes*). Like zebrafish, goldfish are cyprinids and the two share many traits unique to that group. Adult goldfish are extensively used as a neurophysiological structure/function model system. OKR (Easter 1972; Keng and Anastasio 1997; Marsh and Baker 1997) and VOR (Pastor et al. 1992; Schairer and Bennett 1986a,b) have been shown to exhibit robust performance and adaptation. Purkinje cell activity has been recorded during normal and learned oculomotor behaviors (Marsh 1998; Pastor et al. 1997), showing roles for cerebellar pathways during the learning, acquisition, and memory phases of oculomotor plasticity (McElligott et al. 1998; Pastor et al. 1994a). Nearly all key oculomotor related subnuclei have been anatomically and physiologically identified, establishing functional contributions to short-term memory of eye position and eye velocity (Aksay et al. 2001; Beck et al. 2001; Graf et al. 1997; Pastor et al. 1991, 1994b; Suwa et al. 1999). The oculomotor behavior of the adult goldfish thus represents a developmental endpoint by which to compare the progress of larval oculomotor development.

Unlike goldfish, medaka are distant relatives to zebrafish. Members of the beloniforms, a group more derived than cyprinids, medaka are separated from zebrafish by over 110 million years of evolution (Nelson 1994). Medaka serves as a model system for sex determination, environmental toxicity, as well as developmental studies and is the subject of a current genome sequencing project (Ishikawa 2000; Wittbrodt et al. 2002). From an early age, medaka exhibit superb visual acuity and optomotor tracking capabilities (Carvalho et al. 2002) and hence provide another attractive candidate for genetic investigations of oculomotor behavior.

A general outline of the ontogeny of oculomotor behaviors has been provided for larval zebrafish (Easter and Nicola 1997); however, the examination was limited to only the first few days after hatching. In addition, that study lacked the quantification needed to effectively search for subtle differences in mutations that would permit genetic dissection of oculomotor functions. Here, a quantitative and ontogenetic assessment of the oculomotor system is presented in wild-type zebrafish, goldfish, and medaka from 5–35 dpf.

Part of this work has appeared elsewhere in abstract form (Gilland et al. 2002).

**METHODS**

**Animals (Supplemental Movie 1)***

Animals tested were wild-type zebrafish (*D. rerio*), goldfish (*C. auratus*), and medaka (*O. latipes*). Adult goldfish and fertilized eggs were obtained from Hunting Creek Fisheries (Thurmont, MD); zebrafish and medaka adults were obtained from Aquatic Research Organisms (Hampton, NH) and larvae spawned in the laboratory. Adult goldfish 10–13 cm in length (tip to peduncle) were reared and tested at 18°C. Larval goldfish were reared at room temperature (22°C) in small beakers and transferred to small aquaria at ~15 dpf. Zebrafish and medaka were raised in an incubator maintained at 28°C. After 10–15 dpf they were transferred to an Aquatic Habitats (Ap-
Methods for recording eye movements from adult goldfish with a scleral search coil have been extensively described (Aksay et al. 2000; Marsh and Baker 1997; Pastor et al. 1992). Larval eye positions were measured and recorded using a real-time video system designed for tracking the horizontal eye movements of larval animals (Beck et al. 2004). The specimen holder containing the animals was placed into the mounting stage and illuminated from below with an infrared LED (Fig. 1C). An initial image was captured (Fig. 1C1, Movie 2) with a standard, infrared sensitive CCD camera attached to a video frame grabber under the control of custom software written in National Instruments’ (Austin, TX) LabVIEW programming environment. A region of interest (ROI) was drawn around the animal (Fig. 1C2, Movie 2), the eyes (Fig. 1C3, Movie 2), and a fixed point on the body (Fig. 1C4, Movie 2).

Once the real-time video processing began, the two ROIs selected around the eyes and along the body axis were binary thresholded and inverted, producing white eyes (Fig. 1D5, Movie 2) and body reference (Fig. 1D6, Movie 2). The center of mass of the body reference was then calculated (Fig. 1D7, Movie 2). To select only the eyes within the thresholded ROI (Fig. 1D5, Movie 2) and to reject pigments (melanophores) in the head around the eyes, the size of each object in the eye ROI was computed. The two largest objects in the eye ROI (Fig. 1C3, Movie 2) were the eyes, and the center of mass of each eye was determined (Fig. 1D8, Movie 2). The center of each eye served as the starting point for a pixel seeding operation (“magic
that smoothly filled in the entire shape of each eye by selecting neighboring pixels that were of similar intensity (Fig. 1E9, Movie 2). The equivalent ellipse major and minor axes of each filled eye (Fig. 1E10, Movie 2), and the body axis was determined (Fig. 1E11, Movie 2). The body axis connected the midpoint between the eyes to the center of the thresholded body reference (Fig. 1D7, Movie 2). Eye positions (Fig. 1E12, Movie 2) were calculated as the angle of the major axis of each eye (Fig. 1E10, Movie 2) relative to the body axis (Fig. 1E11, Movie 2). Left and right directions were established by evaluating the vector cross product of the eye and body axis. Eye positions, computed with an accuracy $\pm 0.1^\circ$, were converted on-line at a rate of 60 Hz to voltages (16 bit) using D/A converters on a National Instruments computer card (PCI-6052E).

**Data acquisition and analysis**

Eye position with a 60-Hz temporal resolution plus drum and table position, including the visible light LED voltage (for timing of light ON-OFF) were digitized using Axoscope and a Digidata 1200B (Axon Instruments; Union City, CA) at 200 Hz. Data processing was accomplished using custom routines in MATLAB (MathWorks, Natick, MA) as previously described (Aksay et al. 2000; Major et al. 2004; Mensh et al. 2004). Briefly, eye positions were filtered and digitally differentiated to generate eye velocities. Fast phases in eye velocity were interactively removed by differentiating eye velocity to acceleration and setting an acceleration threshold for each behavioral trial recorded. Acceleration peaks above a manually set threshold, corresponding to individual fast phases, as well as 0.15 s before and 0.2–0.4 s after each peak were removed by computer. Blinks (Pastor et al. 1991), previously called stretches (Easter 1971), and irregular eye movements were manually removed and excluded from analysis. For video measurements, timing differences due to processing delays (38 ms) were removed between eye position records and other traces.

The amplitude and phase of eye velocity in response to sinusoidal rotation of the drum and table were quantified in MATLAB by least squares fitting of eye velocity, over several stimulus cycles, to a rotation of the drum and table were quantified in MATLAB by least squares fitting of eye velocity, over several stimulus cycles, to a rotation of the drum and table. Saccade analysis used the slope of a linear regression (least absolute deviation) fit to the maximum saccadic eye velocity (unfiltered) versus the saccadic position amplitude for one eye. Leftward saccades were positive and rightward saccades were negative. For one eye, this slope calculation was functionally equivalent to the use of absolute values of velocity and amplitude and regressing through the origin ($<1\%$ difference).

Statistical tests were performed using Igor Pro 4.0 (WaveMetrics, Lake Oswego, OR). ANOVA was used to examine differences in performance from sinusoidal frequency and linearity testing as well as during velocity steps. Multiple group and individual comparisons were made, post hoc, among all size classes for each treatment, i.e., specific stimulus frequency/amplitude, with the Student-Newman-Keuls test (Norman and Streiner 2000; Zar 1996). A Student’s $t$-test was used to compare eye velocity gains from initial/build-up and sustained parts of velocity steps (1st and 2nd halves of a step). Significance was determined for $P < 0.05$. Unless otherwise stated, results are reported as ±SE.

**Quantitative measurements of oculomotor performance** were categorized according to fish length. Recent work has concluded that size not age is the best metric of fish maturation (Fuiman et al. 1998; Higgs et al. 2002). Since many variables may affect fish growth (temperature, light cycle, and/or husbandry; Davis et al. 2002; Kamler 2002), reporting fish length permits a more effective comparison of results between experiments. To measure animal size, photographs were taken through a dissecting scope with a 3.34-megapixel digital camera (Nikon 990). Total animal length was measured in pixels against a calibrated standard using the public domain ImageJ program (National Institutes of Health). A size distribution histogram was used to group animals based on clusters of similarly sized individuals (see Table 1).

**RESULTS**

**General considerations**

In total, the oculomotor performance of over 300 zebrafish, goldfish, and medaka larvae, from shortly after hatching (5, 7, and 10 dpf, respectively) to the juvenile-larval transition (~35 dpf), were examined during preliminary and final experiments. Larvae utilized for testing were chosen from age-specific community tanks and were 1) healthy in appearance (e.g., inflated swim bladder, active feeding, robust evasive response) and 2) of average size relative to peers. Some of the larvae prepared for testing (20%) were excluded from analysis when they either failed to perform fast phases during an initial low-frequency (0.065 Hz) optokinetic stimulus (~10%) or exhibited a sudden drop in performance during testing, also usually an inability to make fast phases (~10%). Based on

**TABLE 1.**  
**Class number and larval body length for OKR, constant velocity saccade, performance, and VOR plots**

<table>
<thead>
<tr>
<th>Size/age class</th>
<th>Mean length, mm</th>
<th>Range, mm</th>
<th>Mean Age, dpf</th>
<th>n</th>
<th>Mean length, mm</th>
<th>Range, mm</th>
<th>Mean Age, dpf</th>
<th>n</th>
<th>Mean length, mm</th>
<th>Range, mm</th>
<th>Mean Age, dpf</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.91 ± 0.02</td>
<td>3.81–4.01</td>
<td>6.4 ± 1.0</td>
<td>11</td>
<td>5.95 ± 0.05</td>
<td>5.76–6.08</td>
<td>9.83 ± 1.2</td>
<td>6</td>
<td>5.00 ± 0.06</td>
<td>4.57–5.42</td>
<td>10.7 ± 0.45</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>4.22 ± 0.03</td>
<td>4.10–4.42</td>
<td>12.7 ± 1.7</td>
<td>14–15</td>
<td>6.25 ± 0.03</td>
<td>6.17–6.36</td>
<td>13.3 ± 2.5</td>
<td>7</td>
<td>6.21 ± 0.06</td>
<td>5.88–6.46</td>
<td>17.2 ± 0.88</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>4.73 ± 0.03</td>
<td>4.53–4.93</td>
<td>15.9 ± 1.5</td>
<td>16</td>
<td>6.56 ± 0.04</td>
<td>6.41–6.75</td>
<td>16.3 ± 1.9</td>
<td>14–16</td>
<td>7.65 ± 0.10</td>
<td>6.85–8.15</td>
<td>22.5 ± 1.0</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>5.25 ± 0.05</td>
<td>5.06–5.47</td>
<td>21.7 ± 1.7</td>
<td>6–7</td>
<td>7.08 ± 0.05</td>
<td>6.91–7.26</td>
<td>30.1 ± 2.7</td>
<td>8</td>
<td>8.69 ± 0.08</td>
<td>8.45–8.88</td>
<td>31.7 ± 1.7</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>5.98 ± 0.06</td>
<td>5.73–6.15</td>
<td>24.2 ± 1.5</td>
<td>6</td>
<td>7.64 ± 0.06</td>
<td>7.36–7.97</td>
<td>31.7 ± 2.7</td>
<td>11</td>
<td>9.29 ± 0.08</td>
<td>9.09–9.46</td>
<td>31.3 ± 1.3</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>6.86 ± 0.08</td>
<td>6.42–7.35</td>
<td>26.4 ± 0.7</td>
<td>11</td>
<td>8.78 ± 0.24</td>
<td>8.29–9.34</td>
<td>23.8 ± 3.6</td>
<td>4</td>
<td></td>
<td></td>
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<tr>
<td>7</td>
<td>8.11 ± 0.29</td>
<td>7.62–8.94</td>
<td>33.8 ± 1.3</td>
<td>4</td>
<td></td>
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Values are means ± SE. OKR, optokinetic reflexes; VOR, vestibuloocular reflexes.
preliminary experiments and comparison with otherwise identical age peers, it was clear that these animals were not merely below average in performance but were acutely compromised, possibly from the stress of agarose embedding. From this remaining pool (80%), 71 zebrafish, 53 goldfish, and 51 medaka were selected for analysis (Table 1).

**Optokinetic velocity steps**

Velocity steps are a means of assessing oculomotor performance by testing the linearity of the optokinetic system with stimuli of increasing amplitude (10–50°/s). Figure 2 shows representative responses of zebrafish (goldfish were similar) and medaka to bi-directional drum velocity steps at 0.1 Hz. Previous studies of the vertebrate visuomotor system separated the optokinetic reflex into early components (1st 200 ms) and indirect (delayed) components for analysis (Cohen et al. 1977; Collewijn 1969). The temporal resolution of the video tracking system (17 ms) limited accurate quantification of the latency and acceleration during the early component. Therefore analysis focused on eye velocity during the longer, delayed component (Collewijn 1969; Lisberger et al. 1981). The delayed component has been divided into two parts: 1) an early rise in eye velocity that gradually increases, termed “build-up”, that plateaus during the course of a step into 2) a sustained level of eye velocity (Cohen et al. 1977). The gain (ratio of eye velocity to stimulus velocity) of the initial rise and build-up in eye velocity in the first 2.5 s of the step was calculated and compared with the gain during the sustained component of eye velocity, here, the last 2.5 s of the step (see Fig. 2F).

At low step velocity (10°/s, Figs. 2, A and B, and 3), the initial and sustained gains of both small and large animals were quite similar. However, as step velocity increased, two trends were clear among the cyprinids: 1) initial gains were greater than sustained gains and 2) overall performance increased with size (Figs. 2, C and D, and 3, A–E). OKR step performance improved for zebrafish ≥5.98 mm and for goldfish ≥7.08 mm. For these larger animals, initial gains were significantly greater than the smaller sized animals (20–50°/s; ANOVA, *P* < 0.05), and initial and sustained gains were nearly of equal amplitude (paired *t*-test; Fig. 3, D and E, *). In addition, there appeared to be a difference in fast phase resets between small and large cyprinids. At 10°/s (Fig. 2, A and B), small animals made fewer, larger amplitude (position change) resets than larger animals, although both exhibited comparable eye velocity gains. With increasing velocity (Figs. 2, C and D, and 3, A–E), the fast phase amplitude in smaller zebrafish and goldfish could not match that of larger animals and, sometimes, resets failed to occur (Fig. 2C, *). Even though larger animals made more fast phases, eye velocity could not always be maintained throughout the step (Fig. 2D, arrows).

Medaka, in contrast, showed almost no disparity between initial and sustained gains or increased performance with size.

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**FIG. 2.** Optokinetic velocity steps. Optokinetic reflex (OKR) performance in small (A) and large (B) zebrafish visually stimulated with drum velocity steps of ±10°/s at 0.1 Hz. Vertical dashed lines in position traces show that the number of resetting fast phases was greater for the larger animal. C: decreased OKR eye velocity gain with increased step amplitude (40°/s, same animal as A). *Resets sometimes failed to occur. D: larger zebrafish were not able to maintain eye velocity during the course of a 50°/s step (arrows), but both small (E) and large (F) medaka closely followed drum velocity. Arrows show that sustained eye velocity in the latter was greater toward the end of the step than during early/build-up eye velocity. In each record, the top traces show left (black) and right (gray) eye position, and the bottom traces show left (black) and right (gray) eye velocity and stimulus velocity (dashed line). Vertical dashed lines in the eye position records are fast phases and have been removed from the velocity traces. Velocity and position scale bars are to the left. ° represents eye position perpendicular to the body axis. Leftward eye movements are up, rightward down. Gain values are average of entire step; time scale in F applies to all panels.
Even with large amplitude steps (±50°/s), medaka of all sizes maintained an eye velocity that closely matched drum velocity throughout the step, and all animals exhibited a high-frequency of fast resetting phases (Figs. 2, E and F, and 3C). Like some zebrafish, larger medaka showed a build-up in eye velocity during the step with the sustained gain greater than the initial gain (Figs. 2F and 3C, arrows), a signature of an active velocity storage mechanism (Cohen et al. 1977; Collewijn 1991).

**Optokinetic after-nystagmus**

The observation of increased eye velocity during the course of the velocity steps, i.e., sustained > initial gain, suggested the development of a central neural mechanism for velocity storage (Cohen et al. 1977). A more direct indicator of velocity storage is the persistence of eye velocity after the cessation of stimulation in the form of optokinetic after-nystagmus (OKAN). During OKAN in adult goldfish, eye velocity and fast phases continue after the light is extinguished with velocity decaying exponentially to 0°/s with a time constant (τ) ≈ 75 s (Marsh and Baker 1997), with τ the measure of velocity storage (Cohen et al. 1977). To measure τ in larval fish, the drum, operating at a constant velocity (20°/s) was illuminated for 20–30 s, and then the light was extinguished. There was no observable OKAN in any larval animal tested, and τ was consistently ≈ 0.5 s for all three species (Fig. 4, data not shown). Even among the largest animals that showed increased sustained eye velocity with bi-directional steps (e.g., Fig. 2F), only slight increases in τ were observed when quantified with exponential fitting (Fig. 4C).

**Saccade metrics**

The apparent difference in both the frequency and amplitude of saccades between small and large animals, notably in zebrafish and goldfish, prompted a closer assessment of saccade performance (Fig. 5). The frequency of spontaneous saccades was examined under both light and dark conditions. Spontaneous activity in the light was higher in zebrafish than in goldfish or medaka and increased almost fourfold with maturity (Fig. 5A, filled points). Goldfish saccade frequency in the
light remained relatively constant and was generally lower than medaka until a large increase at size class 6. Saccade frequency in the dark was slightly lower than in the light for both cyprinids at all sizes (data not shown). Initially, almost no saccades were made by larval medaka in the dark; however, by the end of the study period, the largest medaka (class 5) were making saccades only slightly less frequently (0.12 ± 0.02 Hz) than in the light. The frequency of resetting fast phases, driven with an optokinetic stimulus (30°/s velocity step), roughly quadrupled over spontaneous levels in zebrafish and increased over eightfold in goldfish and medaka (Fig. 5A, open symbols).

The increase in resetting frequency with size largely paralleled that seen for spontaneous saccades. Larval goldfish did not make as many spontaneous saccades as adults (Fig. 5A, lone squares); but when optokinetically stimulated, the largest larvae made a comparable, and sometimes greater, number of resets as adults.

Although frequency is important, fast phase resets need to be of sufficient amplitude and velocity to reposition the eyes in the orbit to maintain slow phase eye velocity and minimize loss in vision (Carpenter 1991; Collewijn 1970; Walls 1962). Because saccade velocity is generally proportional to the change in eye position (saccade amplitude), the slope of maximum velocity versus amplitude (Collewijn 1970; Easter 1975; Fuchs 1967; Mensh et al. 2004; Zuber et al. 1965) provides another useful metric for saccade performance. Saccade velocity-amplitude (VA) slopes were calculated for both spontaneous and OKR behaviors (Fig. 5B). In zebrafish and goldfish, saccade VA slopes during OKR were not significantly higher than during spontaneous saccades until the largest sizes. In contrast, all medaka had steeper VA slopes during optokinetic resets than during spontaneous saccades. Increases in saccadic VA slope with size were modest for zebrafish and medaka but much greater for goldfish (Fig. 5B). In particular, the sigmoid-shaped size-VA relationship for larval goldfish almost doubled by size class 3, preceding improvements in velocity step gain, and increased again at class 6. Adult goldfish spontaneous saccadic performance (Easter 1975; Mensh et al. 2004) was similar to that of the largest larval goldfish but exhibited a steeper saccade VA slope during optokinetic resets (Fig. 5B).

Optokinetic sinusoids (Supplemental Movie 3)

Smaller larval animals were often unable to maintain a constant, peak eye velocity during the 5-s period of the drum velocity step (Figs. 2C and 3, A–E). Consequently, sinusoids of increasing amplitude (10–50°/s at 0.125 Hz; Fig. 6) were chosen as another means of assessing the linearity of the optokinetic system because stimulus velocity varies with time and maximum velocities are reached for only brief periods. Behavioral performance was characterized by both eye velocity gain as well as the phase of eye velocity relative to the stimulus (Fernandez and Goldberg 1971; Melvill Jones and Milsum 1971; Robinson 1981). As seen with velocity steps, all three species could follow low-amplitude (10°/s) sinusoids with high eye velocity gain and minimal phase shifts (Fig. 7). With increasing stimulus amplitude, performance for both cyprinids dropped (decreased gain, leads in phase) but later improved with size (Figs. 6, A–C and 7, A and B; Movie 3).
the smallest cyprinids, the eyes would occasionally lock in an extreme position but did so much less frequently than during velocity steps. Overall, sinusoidal linearity improved steadily with maturity, with statistically significant differences principally between the smallest and largest sized animals. Eye velocity gain from 30–50°/s and phase leads from 40–50°/s of small zebrafish (classes 1–3) were significantly lower (ANOVA, $P < 0.05$) compared with the largest size class (Fig. 7A). Mid-sized zebrafish (classes 4–6) showed improved linearity with size and were only significantly less than the largest zebrafish at 50°/s. Goldfish linearity showed the most change with size (Fig. 7B). The largest size class performed significantly better than classes 1–2 at 20 and 30°/s and significantly better than classes 1–3 at 40 and 50°/s (ANOVA, $P < 0.05$). The phase lead of the smallest goldfish was significantly greater than the others across all stimulus velocities.

In contrast to zebrafish and goldfish, all medaka followed the sinusoidal drum stimulus extremely well with gains $>0.8$ for all stimulus velocities (Fig. 6, B and D, and 7C). Although the phase lead of the smallest medaka was significantly greater than larger medaka above 10°/s (classes 2–5), this phase difference was minimal compared with that observed for small zebrafish and goldfish. Overall, medaka was more linear in both gain and phase than either goldfish or zebrafish at all sizes. As with steps, only the largest cyprinids were able to perform as well as small medaka.

**Optokinetic frequency bandwidth (Supplemental Movies 4, 5, and 6)**

Bode frequency plots are a useful way to characterize the input-output relationship of the oculomotor system using sinusoidal stimuli of varying frequency at a constant velocity amplitude (Robinson 1981), here, 0.065–3.0 Hz and ±10°/s. It was at this amplitude that larvae of all sizes exhibited nearly equal performance during sinusoidal linearity testing. Representative tests showing differences in performance between the smallest and largest animals as well as between species are shown (cf. Fig. 8, A vs. B vs. C; also see Movie 4, Movie 5). The overall change in frequency bandwidth is summarized in Bode plots made of eye velocity gain and phase versus frequency for each size class in Fig. 9.

In general, all three species were able to follow low-frequency drum oscillations with high gain within the first few days after hatching (Fig. 8, A–C, smallest sizes). Eye velocity gain and phase were largely steady from 0.065 to 1.0 Hz; higher stimulus frequencies yielded decreasing gain with increasing phase lag. At low stimulus frequencies, zebrafish and medaka exhibited minimal improvement in OKR with increased size while goldfish showed a progressive increase. However, at 3 Hz, the highest frequency tested, both gain and phase improved significantly during zebrafish maturation from the smallest size (Figs. 8G and 9A; ANOVA, $P < 0.05$). Goldfish performance qualitatively improved with size, exhibiting significant improvements at most frequencies (Figs. 8, B, E, and H, and 9B; 4/7; ANOVA, $P < 0.05$), with post hoc analysis showing the gain in the two smallest size classes to be significantly less than the larger groups at 3 Hz (Figs. 8H and 9B). Eye velocity gain and phase of even the smallest medaka surpassed those of the largest cyprinids at most frequencies. At 3 Hz, phase shifts were similar among the larger animals of all three species, though eye velocity gain in goldfish was nearly double that of zebrafish and medaka (Fig. 9). Another behavioral feature that was observed in the digitized records and confirmed by visual observation was the appearance of jerky eye movements among the smaller sizes of all species and often in goldfish and medaka (Fig. 8, B and F, insets; Movie 6).

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**FIG. 6.** Sinusoidal linearity testing. OKR performance of small and large zebrafish (A and C) and medaka (B and D) at the highest stimulus velocity tested, 50°/s at 0.125 Hz (Movie 3). Vertical dashed lines in A show fast resetting phases where eye velocity decreased in the small zebrafish. Trace markings and calibrations are as in Fig. 2.
Monocularity (Supplemental Movie 7)

During each of the preceding oculomotor tests, all three species exhibited extremely conjugate eye movements. To assess the independence of eye motion, stripes were removed from one-half of the rotating optokinetic drum (180°) to reveal stripes on the stationary outer drum. As a result, one eye was presented with a stationary, patterned stimulus and the other with a patterned stimulus that rotated sinusoidally at 0.125 Hz and ±20°/s (Fig. 10A; Movie 7). The eye following the moving stimulus showed no obvious nasal/temporal asymmetry in eye velocity while the other eye remained relatively still (Fig. 10A; Movie 7). Saccades were of the same direction but of differing velocity while the other eye remained relatively still (Fig. 10A, B; Movie 7). Zebrafish exhibited a significant decrease in monocular gain with maturity (ANOVA, *P* < 0.05). An unexplained drop occurred in goldfish size class 2, which included animals (*n* = 4) from two spawns and three separate recording sessions. For all species, the gain of the stationary eye was low (<0.1, open markers, Fig. 10B). The phase difference was near 0° for the moving eye in cyprinids, but small medaka exhibited a phase lead of 15° that steadily decreased toward 0° with maturation (data not shown). In small zebrafish, the stationary stripes were replaced with a featureless, gray ganzfeld and the moving drum rotated as before (Fig. 10C, *n* = 3, 2, 3). The velocity of the moving eye was not significantly different in the smallest zebrafish compared with binocular stimulation but decreased significantly in larger fish (ANOVA, *P* < 0.05). Eye velocity of the ganzfeld viewing eye remained unchanged.

Angular VOR (Supplemental Movies 8, 9, and 10)

Angular VOR was evoked with horizontal sinusoidal and step rotation in the dark. The earliest compensatory eye movements were observed in about one-half (6/15) of the smallest sized medaka. With a table stimulus of 1 Hz, ±120°/s (±680°/s²), medaka eye velocity was very small (gain = 0.19 ± 0.05 and phase lead of 31 ± 5° not shown). Surprisingly, neither the smallest sized zebrafish (Movie 8) nor goldfish exhibited an AVOR when tested with sinusoids or steps with accelerations >500°/s². In comparison, the gain of AVOR in adult goldfish averaged 0.9 at 0.125 Hz, ±16°/s—12.6°/s² peak acceleration (Pastor et al. 1992; Schairer and Bennett 1986a). Notably, none of the larval animals <14 dpf exhibited any observable low-frequency AVOR, e.g., at 0.125 Hz, ±12.5°/s². In addition, eye movements in all but the largest cyprinid larvae were generally of such low amplitudes as to be imperceptible by visual observation of the live video image. Although changes in eye position could be measured with the eye tracking algorithm (Fig. 11A), least squares fitting of the expected sinusoid to the eye velocity traces mostly yielded unreliable Pearson coefficients (*r*² < 0.5). As cyprinids grew in size, AVOR appeared capriciously, and eye velocity gain was never >0.25 and rarely >0.10 at any frequency and/or amplitude during the study period (max, 1 Hz, ±120°/s sinusoids or 0.5 Hz ±60°/s² steps). The best performing cyprinid was a zebrafish shown in Fig. 11B. An intermediate acceleration was chosen for analyzing maturation of AVOR in larval medaka (table stimulus of 0.5 Hz, ±60°/s, ±190°/s²). A plot of AVOR eye velocity gain and phase versus size showed eye velocity increased from zero in the smallest animals to a gain and phase in the largest that were comparable to adult goldfish (Fig. 11, C and D; Movie 9).

The vestibular responses to table velocity steps in the different medaka size classes paralleled the sinusoidal responses. During table steps of ±40°/s (peak 190°/s²), small- to medium-sized larvae generally showed either small or no transient response (10–15°/s amplitude; Fig. 12A). These small step responses were generally seen in cases that also exhibited low eye velocity gain evoked by sinusoidal stimuli. High gain eye velocity responses to head velocity steps were only seen in the larger-sized medaka. Progression from minimal responses to eye velocity pulses briefly reaching above unity gain are shown for medaka 7.2–8.8 mm in length (Fig. 12, A–C). For intermediate-sized animals, eye velocity gradually increased during the first few successive steps of table movement (Fig. 12B, arrows). Even for the largest animals with a strong compensatory response (Fig. 12, C and D; Movie 10), eye velocity was
FIG. 8. OKR sinusoidal frequency series for different-sized larval zebrafish (A, D, and G), goldfish (B, E, and H), and medaka (C, F, and I). Representative examples are shown during visual stimulation with sinusoidal drum rotation at 0.065 (A–C, Movie 4), 0.5 (D–F), and 3.0 Hz (G–I, Movie 5), ±10°/s. Horizontal arrows in A show that, at low frequency (A–C), small animals made large excursions in eye position and larger animals exhibited a more limited range (Movie 4). Some small animals (B and F, insets) showed jerky eye movements (Movie 6). The same animals are shown for all 3 frequencies with the time scaling located on the right for each frequency.
of short duration relative to the step length. Stimulated with a peak acceleration /H11022 600°/s² (Fig. 12 D, same animal as C), eye velocity quickly returned to 0 in /H11021 500 ms, showing the short vestibular time constant typically observed in this study.

DISCUSSION

In contrast to the results and conclusions of Easter and Nicola (1997), quantitative analysis of the eye movements of developing zebrafish revealed distinct differences in oculomotor performance that varied not only with maturity; and, as shown here with goldfish and medaka, these differences also extend across species. Notably, optokinetic performance was robust from just after hatching both in the cyprinids and medaka, while AVOR was entirely absent until many days later. Visual tracking in the two cyprinids showed progressive improvement, whereas that of medaka remained relatively unchanged from the early, robust performance. Angular VOR, absent at first in all fish, matured progressively and appeared first in the medaka, followed significantly later in zebrafish and goldfish.

**Visual performance**

Bode plots of larval optokinetic bandwidth, performed with the use of sinusoids, showed that all three species exhibited relatively high eye velocity gains with minimal phase shifts at low frequencies (Fig. 9); however, as frequency increased, gain decreased and phase lagged. These low-pass filtering characteristics were not unexpected as they are common to all vertebrate optokinetic systems (Collewijn 1991). The jerky eye movements observed in small animals (Fig. 8, B and F, insets) may be a common developmental feature related to the recruitment of a limited number of motor units. OKR frequency performance increased with maturity, gradually in goldfish (Fig. 9B), more rapidly in zebrafish (Fig. 9A), but almost immediately in medaka (Fig. 9C). Both visual motion processing as well as optical properties of the eye could play a role in performance bandwidth (Carpenter 1991). However, shortly after hatching, both larval medaka and zebrafish exhibit comparable visual acuity of ∼5°, which is much less than the 15° stripes used here (Carvalho et al. 2002; Clark 1981). Nevertheless, a possibility that was not systematically tested in this study was that maturation of visual motion sensitivity may proceed at different rates for different target spatial frequencies.

The bandwidth in larval animals compared favorably to other afoveate, lateral-eyed animals, such as rabbit (Collewijn 1969), rat (Hess et al. 1985), and mouse (van Alphen et al. 2001) and was equal to or greater than the adult goldfish (Keng and Anastasio 1997; Marsh and Baker 1997; Schairer and Bennett 1986b). A comparison of eye velocity at a single frequency (0.125 Hz) showed that larval eye velocity at either 10 or 20°/s was almost double that reported for adult goldfish at 16°/s (Marsh and Baker 1997; Schairer and Bennett 1986b: gains of 0.45 and 0.41, respectively). Perhaps, the reason for the difference is the high-contrast, striped drum used for larval visual stimuli. Adult goldfish eye velocity was also higher (gain = 0.6; Keng and Anastasio 1997) when tested with a stripe drum than with a planetarium projecting spots of light (Marsh and Baker 1997; Schairer and Bennett 1986b), although still less than larval animals. It is therefore possible this difference in eye velocity reflects a true age-dependent decrease in OKR gain performance concomitant with the subsequent acquisition of AVOR. With both visual and vestibular components of the oculomotor system fully functional in the adult, OKR gain may need not be so high to generate compensatory eye movements.

**Velocity steps and OKAN**

Optokinetic steps reveal two well-studied components of visual behavior: an early, short latency pathway (presumed pretectum to brain stem) and a delayed, longer time-course pathway that builds-up eye velocity (presumed to include brain stem-cerebellar pathways, Cohen et al. 1977; Fuchs and Mus-
decrease in sustained eye velocity (Fig. 2). Larval fish showed either no build-up (Fig. 2) or an actual decrease in sustained velocity is reached (Cohen et al. 1977; Collewijn et al. 1980; Marsh and Baker 1997). Instead of a gradual increase, most fish exhibit a relatively slow build-up in eye velocity until a sustained eye velocity is reached (Cohen et al. 1977; Collewijn et al. 1980; Waespe et al. 1985) as well as the velocity integrator which is purported to comprise the remaining portion of the velocity step and exhibits a relatively slow build-up in eye velocity until a sustained velocity is reached (Cohen et al. 1977; Collewijn et al. 1980; Marsh and Baker 1997). Instead of a gradual increase, most larval fish showed either no build-up (Fig. 2) or an actual decrease in sustained eye velocity (Fig. 2, arrows). Evidence for slow build-up in the indirect pathway was only seen in the largest medaka that exhibited a step response indicative of velocity storage (Fig. 2, arrows).

The presence of a short-term memory of eye velocity (Cohen et al. 1977) after a prolonged velocity step is a direct measure of the velocity storage neural integrator, which is purported to aid spatial orientation during tilt and translation (Fuchs and Mustari 1993; Wearne et al. 1999). While velocity storage has been readily observed from adult goldfish (Marsh and Baker 1997) to primates (Cohen et al. 1977), evidence of storage in the zebrafish and goldfish, but not medaka. However, the most parsimonious explanation, suggested by the results, is that the frequency and amplitude of saccadic resets significantly limits maintenance of high eye velocity in zebrafish and goldfish, but not medaka.

Optokinetic linearity and saccades

At low stimulus velocities (10°/s), zebrafish, goldfish, and medaka of all sizes showed a comparable ability to follow visual stimuli (Figs. 3 and 7). As velocity amplitude increased (20–50°/s), medaka exhibited a linear response in oculomotor performance with eye velocity gain remaining unchanged. In contrast, zebrafish and goldfish showed a nonlinear decrease in eye velocity gain with increasing stimulus amplitude. Performance with higher velocity stimuli improved steadily with increasing size in the cyprinids, especially with sinusoidal stimulation (Fig. 7, A and B). The improving ability to maintain a high eye velocity might depend on several factors. Since even the smallest cyprinids could initially follow high-amplitude stimuli (e.g., Fig. 2C), it is unlikely that the ability to generate high velocity is a limiting factor. Decreased vestibular activity could also play a role as VIIIth nerve lesions affect the ability to adequately follow step stimuli for several months (Collewijn 1976). However, the most parsimonious explanation, suggested by the results, is that the frequency and amplitude of saccadic resets significantly limits maintenance of high eye velocity in zebrafish and goldfish, but not medaka.

Table 2. Larval class number and n for monocular testing plot

<table>
<thead>
<tr>
<th>Size/Age Class</th>
<th>Zebrafish</th>
<th>Goldfish</th>
<th>Medaka</th>
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<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>4</td>
<td>6</td>
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</table>
The relationship between saccade velocity and amplitude (Collewijn 1970; Easter 1975; Fuchs 1967; Mensh et al. 2004; Zuber et al. 1965) yields a slightly different view of the influence saccadic maturation has on optokinetic tracking. The increase in goldfish OKR gain (Figs. 3B and 7B) corresponded with the rise in VA slope at size classes 3–6, while a correlation in zebrafish was less evident. Interestingly, only medaka and the largest larval cypri- rinids were able to increase resetting amplitude-velocity in response to an optokinetic stimulus (Fig. 5B), which, in part, may have contributed to the improved eye velocity gain and phase achieved by these animals at high stimulus amplitudes. Overall, goldfish showed different developmental profiles than zebrafish or medaka for both measures of saccade performance. This contrasts with the general similarity in maturation of OKR linearity performance in zebrafish and goldfish relative to medaka (Figs. 3 and 7) and suggests that the performance contributions of saccade frequency versus saccade VA slope differ between the three species.

Monocularity

All of the animals tested showed conjugate eye movements in response to bilateral presentation of visual stimuli. In lateral-eyed animals, the visual fields of the two eyes do not extensively overlap; the visual motion perceived by each eye can be...
combined centrally to generate binocular eye movements. Experiments in mammals (Simpson et al. 1979) and pigeon (Gioanni et al. 1983) have localized one site for this central neural processing of horizontal visual slip to the accessory optic system and nucleus of the optic tract in the pretectum (for review, see Simpson et al. 1988). Zebrafish and goldfish showed an ability to almost completely dissociate the motion of the two eyes during monocular stimulation with rotating stripes and a stationary pattern, while medaka were less adept at this task. Unlike observations in other developing animals (Braddick and Atkinson 1983; van Hof-van Duin 1978; Wallman and Velez 1985), a strong temporal-nasal preference to eye velocity gain was not observed. Interestingly, eye movements became less decoupled with maturity in zebrafish when tested with either a ganzfeld or stationary pattern. In light of recent work on CNS asymmetries in zebrafish (Concha et al. 2000; Essner et al. 2000; for review, see Halpern et al. 2003; Liang et al. 2000), monocular eye movements may offer a suitable way to correlate structural changes in neural asymmetry with behavior.

**AVOR**

Previous investigations of angular VOR in larval fish reported a robust compensatory VOR in larval zebrafish as early as 4 dpf using low acceleration stimuli (±12°/s² peak; Easter and Nicola 1997). However, in that investigation, AVOR was measured in the light, and visual feedback was not eliminated. Here, when AVOR of small larvae was tested in the dark, no eye movements were observed at low accelerations. Moreover, a significant AVOR was observed only in larger animals with high acceleration canal stimuli (>180°/s²). Medaka were the first to consistently produce an AVOR, and they showed progressive increase in gain with size (Fig. 11D). Zebrafish developed a significant AVOR only in the largest sizes (Fig. 11B), and almost no AVOR was observed in any goldfish tested under these rearing conditions. Among these species, adult AVOR performance has only been measured fully in goldfish, which, unlike larval animals, exhibit a robust eye velocity in response to angular vestibular stimulation even at low accelerations (Pastor et al. 1992; Schairer and Bennett 1986a).

Based on existing anatomical and physiological data of inner ear development in zebrafish (Haddon and Lewis 1996; Whitterfield et al. 2002) as well as the nearly ubiquitous presence of an AVOR in the juveniles of other vertebrates, e.g., human (Finocchio et al. 1991), kitten (Flanderin et al. 1979), and chick (Wallman et al. 1982), the total lack of an AVOR in early larval fish was unexpected. The semicircular canals are formed, replete with hair cells in the cristae, by 3 dpf (Haddon and Lewis 1996; Waterman and Bell 1984). By 5 dpf, the canals have attained their basic configuration (Bever and Bekete 2002), and afferent fibers from the cristae have entered the larval brain stem (Haddon and Lewis 1996; Raible and Kruse 2000). Likewise, vestibulo-oculomotor projections also appear at these early stages (Suwa et al. 1996). Whether the hair cells from the canal cristae are functional or not is unknown; nevertheless, hair cells from zebrafish lateral line neuromasts are operational by 5 dpf (Nicolson et al. 1998). Otolith hair cells and afferents must be active if static VOR behavior, as measured in the light, in 5 dpf zebrafish (Moorman et al. 1999) can also be shown in the dark; in addition, proper otoconial activity is important both for survival (Riley and Moorman 2000) and posture (Nicolson et al. 1998; Riley and Moorman 2000) of young larvae. Despite the presence of all the constitutive parts, the absence of behavioral manifestations of canal activity may be due to an inability of inertial forces to drive endolymph flow within the canals of larval animals.

This hypothesis is consistent with theoretical studies of vertebrate semicircular canal morphology (Muller 1994, 1999; Rabbitt 1999; Rabbitt et al. 2003). Endolymph movement within the canals, and hence acceleration sensitivity, is significantly constrained by the canal arc radius as well as frictional forces induced by the canal lumen diameter. Indeed, acceleration sensitivity drops as the square root of lumen diameter; thus, for example, the canal sensitivity of larval carp has been calculated to be 160 times less than that of an adult guppy, Lebistes (Muller 1999). If canal lumen diameter is the correct explanation for the low AVOR gains, it appears that, even in the largest cyprinid larvae studied, endolymph flow continues to be significantly less than that of the adult animals. Nevertheless, when the canals do become sensitive to acceleration stimuli as in large medaka (Fig. 11, C and D), the vestibular time constant was found to be short (<1s; Fig. 12D). In contrast, the vestibular time constant of adult animals is several seconds in length: 5 s in toadfish (Boyle and Hightstein 1990), 3 s in goldfish (Hartmann and Kline 1980), and 6 s in primates (Fernandez and Goldberg 1971). In addition to vestibular sensitivity, modeling studies have suggested that size also affects the length of the vestibular time constant, with τ proportional to the area of the cupula (Rabbitt et al. 2003).

Thus it seems possible that the lack of an AVOR in larval fish has less to do with genetic expression of the necessary central neurons and more to do with simply the very small size of newly hatched larvae. This hypothesis is testable as activation of the angular vestibular system, either through mechanical or chemical stimulation, and the measured behavioral response could provide evidence that the central connectivity is present and operational despite the apparent lack of endolymph flow. Alternatively, the big ears zebrafish mutant identified in the Tübingen screen with large semicircular canals (Whitterfield et al. 1996) or another teleost species, such as rainbow trout (Oncorhynchus mykiss), that hatches at a larger size might present unique opportunities to establish the correlation between canal size and acceleration sensitivity.

In summary, the precocious optokinetic responses and near total lack of angular vestibular activity in larval fish suggests that evolutionary pressures have selected for a highly functional visual system over the requirement to sensitively detect angular acceleration. Because the animals tested exhibit differing lifestyles from highly kinetic zebrafish to slowly swimming goldfish with medaka intermediate in behavior, it is possible the small variations in the behaviors observed could be due to slight differences in their ontogenetic timing. Indeed, one might hypothesize that any small, free swimming larvae, no matter how primitive or derived, would follow a similar pattern of oculomotor development. Therefore zebrafish and medaka offer excellent models for genetic dissection of the neuronal components underlying oculomotor behavior.