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# Zebrafish optomotor response to second-order motion illustrates that age-related changes in motion detection depend on the activated motion system

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## ABSTRACT

Various aspects of visual functioning, including motion perception, change with age. Yet, there is a lack of comprehensive understanding of age-related alterations at different stages of motion processing and in each motion system. To understand the effects of aging on second-order motion processing, we investigated optomotor responses (OMR) in younger and older wild-type (AB-strain) and acetylcholinesterase (*ache*<sup>sb55/+</sup>) mutant zebrafish. The mutant fish with decreased levels of acetylcholinesterase have been shown to have delayed age-related cognitive decline. Compared to previous results on first-order motion, we found distinct changes in OMR to second-order motion. The polarity of OMR was dependent on age, such that second-order stimulation led to mainly negative OMR in the younger group while older zebrafish had positive responses. Hence, these findings revealed an overall aging effect on the detection of second-order motion. Moreover, neither the genotype of zebrafish nor the spatial frequency of motion significantly changed the response magnitude. Our findings support the view that age-related changes in motion detection depend on the activated motion system.

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## 1. Introduction

Age-related impairments in vision can have major consequences on the well-being of older population. Accordingly, identifying changes in visual functioning during adulthood and revealing associated neural mechanisms have become an important line of aging research (Owsley, 2011, 2016). In particular, visual motion has become one of the most studied aspects of vision (Burr and Thompson, 2011; Nakayama, 1985; Nishida, 2011) since motion perception is crucial for survival in a dynamic environment. Age-related

impairments in motion perception have been recently identified as a risk factor for motor vehicle crashes in real-world settings (Swain et al., 2021a, 2021b). Besides having importance for daily life situations, it has been proposed that previous research on visual motion provides a conceptual framework to investigate age-related changes at different levels of sensory/perceptual processing (Billino and Pilz, 2019). Studying age-related changes in this visual feature can provide a comprehensive understanding of perceptual aging and important implications for other cognitive abilities.

The existence of different motion systems has been identified by many studies. In particular, the human visual system is sensitive to different types of motion, including first-order (Fourier) and second-order (non-Fourier) motions (Cavanagh and Mather, 1989; Chubb and Sperling, 1988; Lu and Sperling, 1995). First-order motion is defined by spatiotemporal changes in the luminance of a retinal image, while second-order motion is characterized by variations in stimulus properties other than luminance, such as

Abbreviations: AD, Alzheimer's Disease; ANOVA, Analysis of Variance; CHRNA7, Alpha 7 Subunits of Cholinergic Nicotinic Receptor; FDR, False Discovery Rate; OMR, Optomotor Response; SE, Standard Error; SEM, Standard Error of Mean

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contrast, flicker, or spatial frequency (Chubb and Sperling, 1988; Smith, 1994). That is to say; a second-order motion is defined by a quality that does not result in an overall change in luminance or motion energy in the Fourier spectrum of the stimulus. The processing of first- and second-order stimulus can be explained within the framework of a receptive field (Baker, 1999). When the light and dark regions of a first-order image (a luminance-defined sine-wave grating) overlap with the excitatory and inhibitory areas of a simple cell receptive field, the cell strongly responds to such stimulus, and hence, a linear sum of the stimulation produces a strong response. However, for a second-order image, the excitatory and inhibitory regions of the receptive field receive equal amounts of luminance, and a linear summation of the stimulation does not produce a net response. Therefore, these kinds of stimuli are considered “second-order” in terms of the spatial scale of the cell’s receptive field and require more processing steps involving non-linear computations to be detected by the visual system. This also applies to dynamic moving stimuli, and the processing of first- and second-order motion has been associated with different motion systems. Our natural visual environment contains both types of stimuli frequently, suggesting that processing both types of information is essential in our daily lives (Johnson and Baker, 2004; Schofield, 2000). Mounting psychophysical evidence highlights distinct characteristics of associated motion systems. For instance, Nishida et al. (1997) showed that an increase in the thresholds for identifying the direction of a first- or second-order stimulus is observed only after adapting to the stimuli with the same motion type. Similarly, around threshold levels, the detection of first- and second-order stimuli is only facilitated by the background with the same type of manipulation (Schofield and Georgeson, 1999). Smith and Ledgeway (1998) also reported that the temporal characteristics of contrast sensitivity to each motion type are distinct such that the sensitivity function for first-order motion peaks at medium temporal frequencies while the function for second-order motion is low-pass, peaking at lower frequency levels.

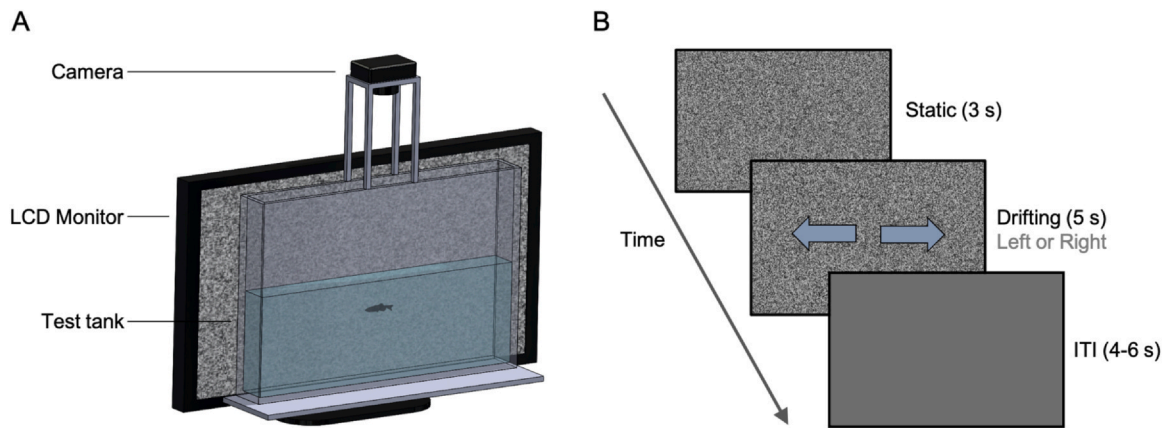
The processing of second-order stimuli requires additional steps and more complex computations than those for first-order stimuli. Therefore, age-related impairments (e.g., increase in perceptual thresholds) in second-order processing are expected to be more severe (Faubert, 2002). Consistent with these predictions, Habak and Faubert (2000) showed that the increase of contrast thresholds in older individuals is higher for the second-order stimuli than those of the first-order stimuli. In addition, age-related sensitivity decline was observed at low and high temporal frequencies for the first-order motion. On the other hand, sensitivity loss was evident across all temporal frequencies for the second-order motion. Tang and Zhou (2009) also reported that the age-related decay in contrast sensitivity for both static and moving stimuli starts earlier for the second-order stimuli compared to the first-order stimuli. Yet, the rate of decline is relatively slower. These findings support the view that these 2 motion types may show distinct patterns of alterations throughout aging. In line with this view, Billino et al. (2008) suggested distinct patterns of changes during aging for different types of motion. Based on the findings of several studies, they further proposed that these motion-specific alterations might not necessarily depend on the complexity level of the motion as Faubert (2002) suggested, but rather may be the product of having different susceptibilities to age-related physiological deterioration in the specialized neural mechanisms processing different types of motion. Although psychophysical studies generally report more profound age-related changes in the perception of second-order stimuli than first-order stimuli, careful consideration should be given to the stimulus parameters and stimulation types used in the studies to reach more comprehensive conclusions (see also Allard et al., 2013; Billino et al., 2011). Notably, similar to first-order motion, possible

neurobiological underpinnings of the perceptual deficits seen in second-order motion processing remain to be explored.

Interestingly, previous research indicated that non-mammals such as larval Zebrafish and *Drosophila* can detect and perceive second-order motion (Orger et al., 2000; Theobald et al., 2008). Zebrafish have become an appealing model for studying both neurobiological changes and cognitive processes during aging. Like mammals, zebrafish have an integrated nervous system with basic vertebrate brain organization (Wullimann et al., 1996) and exhibit age-related deteriorations such as cognitive decline (Yu et al., 2006) and gradual senescence during aging (Arslan-Ergul et al., 2016; Kishi et al., 2003). Regarding the availability of genetic tools and transgenic approaches in zebrafish, disease models have been developed, making them a valuable vertebrate system to study the underlying mechanisms of age-related alterations (Celebi-Birand et al., 2018; Celebi-Birand et al., 2021). Previous research revealed that zebrafish also experience motion illusions commonly used in human studies, such as reverse-phi, motion aftereffect, and rotating snakes illusions (Gori et al., 2014; Najafian et al., 2014; Orger et al., 2000). Similar to other animal models of vision, there are populations of neurons specialized for different motion features and distinct stages of motion processing have been identified in the zebrafish visual system (e.g., Duchemin et al., 2022; Wang et al., 2020; Yildizoglu et al., 2020). Other studies on the visual system and age-related cognitive decline in zebrafish provide evidence that this organism can be an important model for studying age-related alterations in visual motion processing (Adams and Kafaligonul, 2018; Baier, 2000; Rosa Salva et al., 2004).

In our previous study (Karaduman et al., 2021), we investigated first-order motion detection of aging zebrafish using optomotor responses (OMR). In these behavioral measurements, we also utilized a mutant zebrafish line with genetically altered cholinergic neurotransmission. Particularly, heterozygous mutants in this line (*ache<sup>sb55/+</sup>*) have been characterized by significantly decreased brain levels of acetylcholinesterase activity without inducing developmental, locomotor, or morphological defects (Behra et al., 2002; Ninkovic et al., 2006). A previous study indicated that *ache<sup>sb55/+</sup>* mutants at older ages showed comparable performance with the younger groups in some cognitive domains, including entrainment to temporal-spatial cues, learning performance in conditioned place preference tests, and flexibility of the learning strategies, while these cognitive domains declined with aging in wild-type control zebrafish (Yu et al., 2006). By including heterozygous *ache<sup>sb55/+</sup>* mutants in the experimental design, we aimed to further evaluate these mutants in the domains of visual perception and motion detection. We found that adult zebrafish mainly exhibit a negative OMR to the first-order motion (i.e., position shift in the opposite direction of motion) and the magnitude of this response was significantly dependent on low-level stimulus properties (e.g., contrast level and spatial frequency), highlighting stimulus-driven nature of this reflexive behavior. Rather than an overall aging effect, the findings revealed age and genotype interaction in the contrast domain. At only high contrast levels, the older wild-type group had smaller OMR than the corresponding younger group. In addition, compared to the older wild-types, the *ache<sup>sb55/+</sup>* older group showed an improvement at high contrast levels. These findings are in line with the recent studies on humans indicating that age-related changes in motion detection/discrimination depend on stimulus characteristics and criterion content. Moreover, they are also consistent with the neurophysiological evidence that the cholinergic modulations become dominant in the contrast domain (Disney et al., 2007; Soma et al., 2012).

To date, there is no systematic investigation on the second-order motion detection of adult zebrafish. It remains unknown whether adult zebrafish exhibit stimulus-driven OMR to second-order motion. In the present study, we first aimed to address this scientific



**Fig. 1.** (A) Behavioral setup for measuring zebrafish optomotor response. The test tank was in front of an LCD monitor, and the camera was placed above the tank to record fish movements. During the actual tests, there were white shields on the empty sides of the tank to prevent any external visual stimulation (not shown here). A computer controlled the timing of visual stimuli and camera recordings. (B) The second-order motion and the timeline of stimulation for each trial. On each trial, the random dots were shown for 3 seconds, and then flicker-frequency-modulated grating drifted in a specific direction (rightward or leftward) for 5 seconds. The spatial frequency of grating was varied across trials.

gap. As mentioned above, we frequently encounter second-order information in our daily lives. Therefore, understanding how aging affects the processing of second-order stimuli and neural mechanisms underlying age-related changes are important as well (Johnson and Baker, 2004; Schofield, 2000). Previous findings on age-related alterations in second-order motion perception are not conclusive as to whether the vulnerability to aging is comparable with that of first-order motion since the results highly depend on the type and parameters of the stimuli used (Billino et al., 2008, 2011; Tang and Zhou, 2009). A psychophysical study on humans emphasized the importance of testing age-related changes in second-order motion perception as a function of varying spatial frequency values (Reynaud et al., 2019). Accordingly, using OMR of different age groups (younger vs. older zebrafish), we wanted to identify age-related changes in the detection of second-order motion across different spatial frequencies. Age-related changes in second-order motion processing seem to be present across a wider range of spatial frequencies and also more profound compared to the effects of aging on first-order motion processing, which have been shown to be mostly restricted to a certain range of motion parameters (Allard et al., 2013; Habak and Faubert, 2000; Reynaud et al., 2019). Therefore, we particularly anticipated that age-related changes in the responses to second-order motion could be observed in the spatial frequency domain. Moreover, previous research on the effects of cholinergic neurotransmission on motion perception typically focused on first-order stimulation (e.g., Thiele et al., 2012), and how modulations of cholinergic neurotransmission affect the detection of second-order motion remains unclear. To test whether the cholinergic alteration would interact with age-related changes in the detection of second-order motion, we included both wild-types and *ache<sup>sb55/+</sup>* mutants in our measurements.

## 2. Methods

### 2.1. Animal husbandry

A total of 41 adult zebrafish (younger: 7–10 months, older: 24–43 months) that met the criteria of inclusion (i.e., the zebrafish that did not show abnormal behaviors during a testing session) were used in the study (see also [Data processing](#)). Thus, the data of 19 wild-types (AB strain: 9 younger and 10 older) and 22 mutant (*ache<sup>sb55/+</sup>*: 9 younger and 13 older) zebrafish were used in the final analyses. Age ranges were determined with respect to the progression of age-

related cognitive decline in zebrafish (Yu et al., 2006). The *ache<sup>sb55/+</sup>* line was obtained from the European Zebrafish Resource Center-Karlsruhe Institute of Technology and grown in the Zebrafish Facility at Bilkent University. All zebrafish were raised and maintained in a controlled and recirculating housing system, ZebTec (Techniplast, Italy), enabling a constant temperature of 28.5 °C and stable water quality parameters, with a 14:10 hour light:dark cycle. In the standard system conditions, zebrafish were fed twice a day with dry flakes (Sera, Germany) and once with Artemia (Techniplast, Italy). The stocking densities were kept as approximately 10 fish in 4-liter tanks, and the fish with the same birthdates were housed in the same tank. Animals were maintained with minimal disturbance to prevent any unnecessary stress. Two fish were taken from the facility system each week and kept together in an 8.5-liter holding aquarium (Petstore, Türkiye) for a week during the experiments. All the experimental procedures in the current study were approved by the local Animals Ethics Committee at Bilkent University with an approval date: August 5, 2015, and no: 2015/44.

### 2.2. Behavioral setup, visual stimulation, and testing procedure

To measure OMR to visual stimulation, we used a behavioral setup described previously (Karaduman et al., 2021). In brief, the setup consisted of an elongated test tank located in front of a monitor, a video camera, and a computer to control stimulus presentation and recording (Fig. 1A). The test tank (4 × 30 × 20 cm) was filled with 10 cm of water, and white shields were attached to the empty sides to exclude possible stimulation other than visual motion. The camera (Logitech HD Pro Webcam C920, 60 Hz) was placed above the test tank to record the behavioral activity of zebrafish. Visual stimuli were presented on the 18.5" LCD display (HP V196, 1366 × 768 pixel resolution, 60 Hz refresh rate). The stimulus presentation, video recordings, and timing were controlled using MATLAB 2016a (The MathWorks, Natick, MA, USA) with the Psychtoolbox 3.0 extension (Brainard, 1997; Kleiner et al., 2007; Pelli, 1997). A SpectroCAL photometer (Cambridge Research Systems, UK) was used for luminance calibration and gamma correction of the display. As in Orger et al. (2000), visual angles were calculated based on a 1.5 cm distance from the LCD screen.

As described in previous studies, there are different ways of generating second-order motion (Cavanagh and Mather, 1989; Chubb and Sperling, 1988). In the present study, flicker-frequency-modulated second-order motion was chosen to avoid any possible

first-order components induced by artifacts due to contrast/luminance values (O'Keefe and Movshon, 1998; Smith et al., 1998). Moreover, larval zebrafish exhibit the strongest OMR to flicker-defined motion compared to other types of second-order stimuli, including contrast-modulated and texture-defined motions (Orger et al., 2000). The stimuli consisted of two-dimensional random binary noise (i.e., random dots/pixels) whose temporal structure is modulated by a drifting square wave (half-wave rectified, 50% duty cycle). In each frame, the noise sample is replaced in the flickering parts but not in the static parts to form a frequency-defined grating (see Supplementary Fig. S1 for a space–time plot). A smooth motion was produced by incrementing the phase of the modulating square wave to move the boundaries between flickering (dynamic) and static parts (see Supplementary Video 1 for an example). This type of stimulation is free from luminance artifacts since it contains the same two luminance levels [i.e., white (72 cd/m<sup>2</sup>) and black (1.5 cd/m<sup>2</sup>) pixels with equal probability] in both the static and flickering parts and the expected mean luminance within an arbitrary space–time window is uniform. The spatial frequency values of moving/drifting stimuli were 0.01, 0.05, 0.1, 0.2, 0.4, or 0.8 c/deg. At the beginning of each trial, static random dots were displayed for 3 seconds. Then, second-order motion in a specific direction (rightward or leftward) is presented for 5 seconds with a speed of 20 deg/s. The next trial started after a variable (4–6 seconds) intertrial interval (Fig. 1B). The background luminance was 20 cd/m<sup>2</sup> during the intertrial interval.

The zebrafish have a diurnal (circadian) rhythm like humans, and the data were collected between 9:30 AM and 4:00 PM, corresponding to the active phase of fish (Zhdanova et al., 2008). Each fish was tested separately in the test tank and completed a main experimental session consisting of 180 trials (6 spatial frequencies × 30 trials). Before the main experimental session, a practice session lasting around 40 minutes, including second-order stimulation, was given to familiarize the fish with the testing environment and stimuli. It is worth mentioning that the zebrafish population used here also took part in our previous research on first-order motion (Karaduman et al., 2021). Therefore, each zebrafish was also familiar with/got exposed to first-order drifting gratings at a different time during the testing week.

### 2.3. Genomic DNA extraction and genotyping

After the behavioral measurements, the heterozygous *ache*<sup>sb55/+</sup> mutants and their wild-type siblings were genotyped. Following the euthanization with submersion in cold system water, tail tissue was separated from the trunk. Genomic DNA was extracted from the tail samples using standard procedures described previously (Avci et al., 2018; Karaduman et al., 2021). The tail tissues were incubated in 200 µL of DNA extraction buffer (containing 100 mM Tris-HCl pH:8.2, 200 mM NaCl, 10 mM EDTA, 0.5% SDS, 200 µg/mL proteinase K) at 55 °C overnight. Following this process, proteinase K was deactivated by heating the samples at 95 °C for 20 minutes. The DNA pellet was precipitated by adding 175 µL of isopropanol and centrifugation at 13,000 rpm for 20 minutes at 4 °C. The supernatants were discarded carefully, and pellets were air-dried for 20 minutes. The DNA pellets were re-suspended in 20 µL of nuclease-free water (ThermoFisher, Paisley, UK: AM9937), and their concentrations were determined by NanoDrop 2000 (Thermoscientific, Rockford, IL, USA). For further quantitative-PCR (q-PCR) experiments, 100 µg/µL of genomic DNA was used. Genotyping was carried out using q-PCR with allele-specific primers to distinguish the *ache* heterozygous mutants from the wild-type siblings. Two forward primers recognizing wild-type sequence (S) and point mutation existing in heterozygous mutants (N) were utilized during this process (Avci et al., 2018). Each sample was tested with both primers in duplicates, and mutants were

**Table 1**  
Primer sequences for genotyping experiments

|                        | Forward primer sequence | Reverse primer sequence |
|------------------------|-------------------------|-------------------------|
| S (wild-type sequence) | ACACGTGCCATATTGCAGAG    | CTGCTCCAGGGAAGAAGCTTG   |
| N (mutant sequence)    | ACACGTGCCATATTGCAGAA    | CTGCTCCAGGGAAGAAGCTTG   |

determined based on the amplification difference between these two primers (Table 1).

### 2.4. Data processing

We utilized OMR to evaluate motion detection and direction perception of adult zebrafish. OMR is a position-stabilizing reflex that has been widely used to quantify various visual functions of different species (Kalueff et al., 2013; Orger and de Polevieja, 2017). The paradigms based on OMR have been well-established for studying visual motion processing in various species. In particular, OMR provides reliable behavioral metrics for different types of visual stimulation associated with distinct motion systems such as first- and second-order motion and for investigating different aspects of visual motion processing (Krauss and Neumeyer, 2003; Maaswinkel and Li, 2003; Najafian et al., 2014; Orger and Baier, 2005; Orger et al., 2000). Moreover, the OMR testing procedures provide important advantages for studying motion processing in adult zebrafish. The OMR procedures are easy to apply since they allow the animal to move freely in the testing arena and acquire the necessary amount of oxygenation by the water flow from their gills which is hard to achieve when restrained in a small dish. This is particularly important to minimize stress in older animals and to identify abnormal behaviors during offline data analyses.

The video recordings of zebrafish activity were analyzed offline via MATLAB Video Processing Toolbox (The MathWorks, Natick, MA) and our custom scripts written in MATLAB. The processing steps were the same as those in Karaduman et al. (2021). For preprocessing, the video recordings for each trial were first converted to grayscale, and the average of the whole trial was computed for a representative background model. The inside of the test tank was cropped based on the background model, and then the background subtraction was applied for each frame. The determinant of the Hessian was used for blob detection, and the locations of blob centers in each frame were recorded as horizontal and vertical positions of fish in the test tank (Xu and Cheng, 2017). As a final step, a fifth-order median filter was applied for smooth movement pattern estimations. In the end, these processing steps led to accurate tracking of adult zebrafish position during each trial (Supplementary Video 2).

The position shift of fish along the longer side of the tank was used to quantify OMR. For a specific experimental condition of each zebrafish, we first referenced the horizontal position values based on the physical motion direction in each trial. The positive and negative values corresponded to a position shift in the same and opposite direction to that of the drifting grating, respectively. These values were then averaged across all trials of a specific condition, leading to an average position estimate of individual zebrafish throughout the presentation of an experimental condition (3 seconds of stationary and 5 seconds of drifting grating stimulation). To obtain OMR in centimeters, the mean position of the fish within the whole 5 seconds of drifting grating stimulation was calculated and the mean position within the last 2 seconds of the stationary period (i.e., baseline position level) was subtracted from this value. Some basic locomotor properties (e.g., speed) can affect the raw position shifts in centimeters. For instance, a faster swimming speed can lead to



larger position shifts within a fixed amount of time. Following previous research (e.g., Pix et al., 2000), we employed a common normalization procedure to restrict the contribution of such potential confounds. To normalize the responses, for each zebrafish, the baseline-corrected position shifts of all conditions were divided by the difference between the maximum and minimum of these values [i.e., (max–min) in the observed position shift range of an individual fish]. This basic approach allowed us to circumvent potential confounding factors and have a reliable comparison across different zebrafish groups.

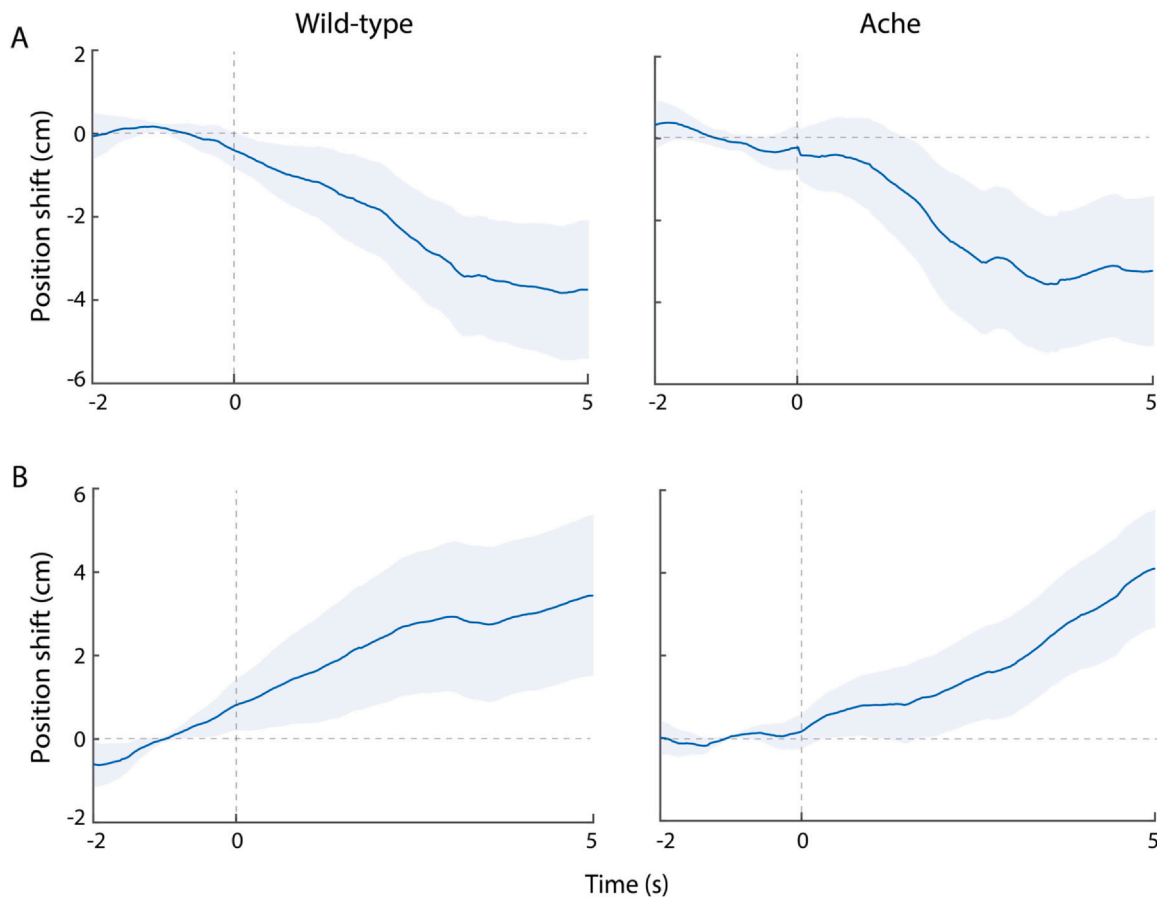
Even though the behavioral setup and testing procedure were designed to minimize stress-related behaviors and other similar confounds, several zebrafish showed specific behaviors that have been associated with stress/anxiety or escape response, such as the increased speed of movement, diving, rapid directional changes, or freezing (Kalueff et al., 2013). Before the calculation of OMR, we excluded these trials and even complete session of some fish that had included these behaviors throughout the measurements. In particular, we eliminated the trials in which the fish were swimming in circles at one of the corners of the test tank. This behavior was likely to be associated with stress but removing such trials also helped us to eliminate any possible bias in swimming direction. On average, 95.94% of the trials Standard Error of the Mean (SEM = 1.23%) were retained per behavioral testing session. The experimenter was blind to the conditions and zebrafish groups in all

phases of the data analyses. Further statistical tests were performed using SPSS (version 25, IBM SPSS Statistics, Armonk, NY) and R Statistical Software (v1.3.1093, RStudio Team, 2020).

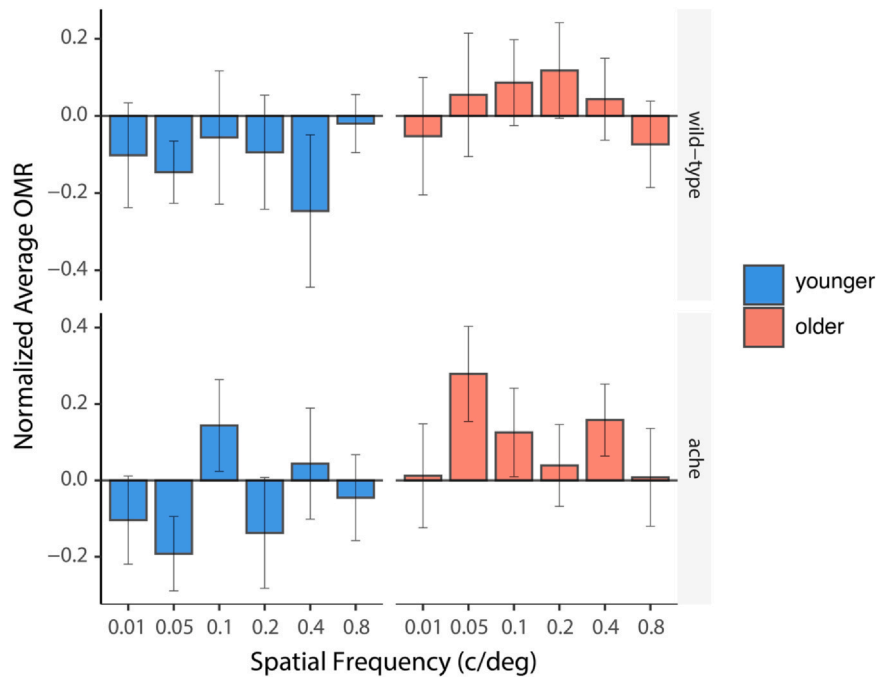
### 3. Results

We observed both positive and negative OMR to second-order motion. Fig. 2 displays sample trajectories of individual fish. These trajectories were along the longer side of the test tank parallel to the motion stimulation on display. The position shifts in the same and opposite direction of physical motion correspond to the positive and negative OMR, respectively. The visual motion elicited (mean) position shifts up to 4 cm. As mentioned above, these raw values may be confounded with basic locomotor properties such as swimming speed. Therefore, normalized OMR values were used to compute group-averaged responses for each condition and in further statistical tests.

The sample sizes were unbalanced across groups, and Levene's test showed that the variances for the spatial frequency of 0.05 c/deg were not equal across groups ( $F_{3,37} = 3.61, p = 0.022$ ). Thus, the homogeneity of variance assumption needed for Analysis of Variance (ANOVA) was violated. These factors would result in mixed-ANOVA not yielding accurate results. Accordingly, the mixed-effects model procedure was used for the statistical analyses since this procedure successfully deals with unbalanced data with heterogeneous variances (Heck et al., 2013). The model included the main effects and



**Fig. 2.** Sample position trajectories of individual younger (A) and older (B) zebrafish. The data of wild-type and *ache*<sup>sb55/+</sup> zebrafish are displayed on the left and right plots, respectively. In each plot, baseline-corrected but raw (i.e., not normalized) position shifts are shown as a function of time. The random dots were first static (–2 to 0 seconds) and started drifting at 0 second. The positive and negative values correspond to position shifts in the same (positive OMR) and opposite (negative OMR) direction to the physical motion. The thick blue curve indicates the mean position values, and the shaded area corresponds to the standard error ( $\pm$ SE) across trials. Since both younger and older zebrafish exhibited robust responses at the spatial frequency of 0.05 c/deg, these sample trajectories were selected from this condition (see also Figs. 3, 4A).



**Fig. 3.** Normalized average OMR of wild-type (up) and *ache*<sup>sb55/+</sup> (down) zebrafish to second-order motion. Each plot separately displays the values from the younger (left) and older (right) groups for each spatial frequency. The positive and negative values correspond to position changes in the same and opposite directions of the physical motion, respectively. The error bars correspond to  $\pm$ SE. Abbreviations: OMR, optomotor responses.

**Table 2**  
The outcome of linear-mixed-effects model

| Source                 | $df_{Num}$ | $df_{Den}$ | F     | p            |
|------------------------|------------|------------|-------|--------------|
| (Intercept)            | 1          | 41         | 0.041 | 0.840        |
| genotype               | 1          | 41         | 1.096 | 0.301        |
| age                    | 1          | 41         | 5.024 | <b>0.030</b> |
| sf (spatial frequency) | 5          | 205        | 0.642 | 0.668        |
| genotype * age         | 1          | 41         | 0.009 | 0.926        |
| genotype * sf          | 5          | 205        | 0.619 | 0.686        |
| age * sf               | 5          | 205        | 1.121 | 0.350        |
| genotype * age * sf    | 5          | 205        | 0.553 | 0.736        |

The numerator ( $df_{Num}$ ), and denominator degrees of freedom ( $df_{Den}$ ), F, and p values are shown in separate columns. The threshold for significance was set at  $p < 0.05$ , and significant p values are highlighted in bold.

interactions of age, genotype, and repeated measurement of spatial frequency as fixed effects. The model also had a subject-specific random intercept to account for intra-individual correlation among the measurements collected from a specific fish (Brauer and Curtin, 2018; Schumann et al., 2010). A linear mixed-effects model analysis was performed on the normalized responses (Fig. 3). The model showed no significant main effect of genotype ( $F_{1,41} = 1.096$ ,  $p = 0.301$ ) and spatial frequency ( $F_{5, 205} = 0.642$ ,  $p = 0.668$ ) or any interaction among factors ( $p > 0.05$ , Table 2). Interestingly, the mixed-effects analyses revealed that the age of zebrafish (younger vs. older) significantly alters responses ( $F_{1,41} = 5.024$ ,  $p = 0.03$ ).

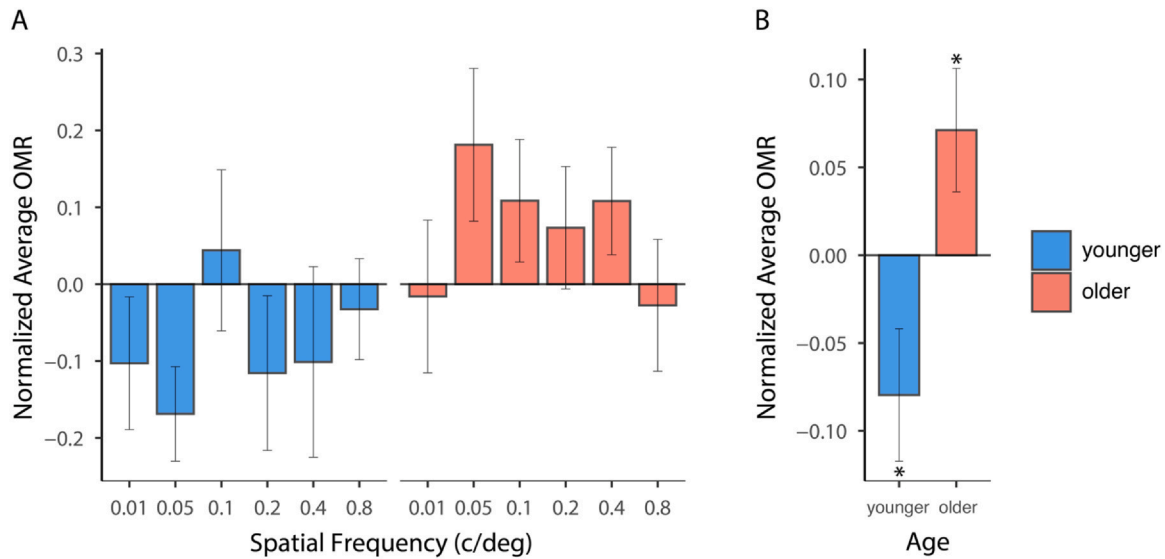
To further understand this dependency of OMR on age, the data were combined across different genotype groups (Fig. 4). The polarity of OMR was significantly different in age groups such that younger zebrafish swim in the opposite direction of second-order motion ( $M = -0.08$ ,  $SE = 0.049$ ), while older zebrafish swim in the same direction ( $M = 0.066$ ,  $SE = 0.043$ ). Two-sided one-sample permutation tests (sampling permutation distribution 5k) on the combined dataset (Fig. 4B) revealed significant deviations of OMR values from the baseline zero level for both age groups False Discovery Rate (FDR corrected,  $p_{adj} < 0.05$ ).

## 4. Discussion

In the current study, we investigated age-related changes in the detection of second-order motion using zebrafish OMR. We manipulated the spatial frequency of visual motion and our design included both adult wild-type and *ache*<sup>sb55/+</sup> zebrafish. Our analyses did not reveal any effect of spatial frequency and genotype. However, there was a significant effect of age. Interestingly, the findings indicated age-dependent polarity shift in OMR to second-order motion such that the younger zebrafish exhibited negative OMR while the older zebrafish had positive OMR (i.e., position shifts in the opposite and same direction of visual motion, respectively). Compared to our previous research on first-order motion, these findings highlight distinct characteristics of age-related alterations in second-order motion detection.

### 4.1. Age- and genotype-related changes in motion detection

In our previous study on first-order motion, we only found a three-way interaction among contrast, genotype, and age rather than an overall aging or genotype effect (Karaduman et al., 2021). The age-related alterations in negative OMR values depended on the contrast level of motion and zebrafish group. Compared to the younger wild-types, the older wild-types had smaller OMR at high contrast levels, suggesting a performance decrease in the detection of first-order motion. With regards to the *ache*<sup>sb55/+</sup> mutants, the older group exhibited strong OMR at high contrast levels, and there was a performance increase in this group compared to the older wild-types. On the other hand, there was no similar improvement in the younger group. Furthermore, the magnitude of negative OMR was significantly dependent on the spatial frequency of first-order drifting gratings. We observed a U-shaped spatial frequency dependency of negative OMR, which was qualitatively consistent with zebrafish sensitivity functions estimated with optokinetic reflexive eye movements. However, there were no main effects of age, genotype, or interaction in the spatial



**Fig. 4.** (A) The combined optomotor responses across different genotype groups for each spatial frequency condition are shown on the left. (B) The data were further combined across different spatial frequency levels on the right to better visualize the overall aging effect. The error bars correspond to  $\pm$ SE. Significant deviations from the baseline zero level were marked with an asterisk sign (\*  $p < 0.05$ ).

frequency domain. To have a comparison in the spatial frequency domain, we used the same spatial frequency values in the current study. The OMR to the second-order motion indicated distinct characteristics. First, the OMR values were not significantly dependent on the spatial frequency of second-order drifting gratings. More importantly, there was only an overall effect of aging rather than an interaction. The polarity of OMR (i.e., whether zebrafish shows OMR in the same or in the opposite direction to the visual motion) was dependent on the age of zebrafish. The second-order motion resulted in negative OMR in younger zebrafish, as commonly observed for the first-order motion. On the other hand, the polarity of OMR was reversed for the older zebrafish such that the second-order motion led to OMR in the same direction. Our findings revealed distinct properties of second-order processing in older zebrafish and suggest that the patterns of age-related changes are different for the first- and second-order motion systems. Previous research on humans indicated that the effect of aging on second-order motion processing is more consistent across a range of stimulus parameters such as temporal and spatial frequencies, unlike first-order motion (Habak and Faubert, 2000; Reynaud et al., 2019). Moreover, a recent notion argues that age-related alterations may differ at different levels of motion processing and motion systems engaged by different stimulus profiles and parameters (Billino and Pilz, 2019). Building on these findings, we expected distinct changes in the detection of second-order motion during aging, and our findings here on adult zebrafish are in line with this prediction. Studies including various visual tests also highlight the diverse nature of age-related alterations in visual processing (Garobio et al., 2023; Shaqiri et al., 2019). As opposed to evidence suggesting a common factor underlying cognitive changes in healthy aging, these studies indicated only weak correlations between visual tests in both younger and older adults and did not reveal any evidence for a common factor. That is to say, age-related deficits in one visual function did not imply deficits in other visual functions.

A strong relationship between the cholinergic system and motion perception has been reported in many species. Previous studies indicated that the upregulation of cholinergic signaling is associated with improvements in the detection and discrimination of motion direction while decreasing noise correlations (e.g., Thiele et al., 2012; Rokem and Silver, 2010, 2013). It has been proposed that these cholinergic contributions in visual processing can mimic attentional

modulations because attention similarly increases sensitivity in visual cortices and decreases noise correlations (Cohen and Maunsell, 2009; Goard and Dan, 2009; Thiele et al., 2009). As mentioned above, modulation of cholinergic signaling in mutants induced by the chronic reduction of acetylcholinesterase activity increased the magnitude of negative OMR and hence the detection of first-order motion in the older group when the contrast level of motion was high. On the other hand, there was no interaction in the spatial frequency domain. These results have been found to be meaningful since an increase in the contrast level leads to a direct increase in the motion energy and the signal-to-noise ratio of stimulation. Based on these findings on first-order motion, we hypothesized that the cholinergic alteration would interact with age-related changes in the detection of second-order motion. On the other hand, our results did not reveal any main effect of genotype or interaction with other factors. Contrary to first-order motion, the second-order motion does not contain spatiotemporal changes in luminance contrast (Hutchinson and Ledgeway, 2006; Johnson and Baker, 2004; Ledgeway and Smith, 1994). Besides minimizing the role of contrast, we only measured the OMR at different spatial frequency values in the present study. A lack of genotype effect or interaction with aging may be due to no direct manipulation of contrast level and signal-to-noise ratio in the second-order motion system. Previously, Kunchulia et al. (2019) examined the effects of age and genotype on the direction discrimination of humans using genetic variations in the alpha 7 subunits of cholinergic nicotinic receptor (CHRNA7). They found evidence of an age-related decline in (first-order) motion perception and a strong relationship between perceptual performance and a genetic variation of the CHRNA7. However, there was no interaction between the two factors, and the effects of genotype were not age-dependent. Together with these previous findings, our results on the second-order motion detection of zebrafish suggest that the relationship between the cholinergic system and motion processing during aging may depend on the type and parameters of motion stimulation and the activated motion system.

A subset of behavioral impairments during normal and pathological aging like Alzheimer's disease (AD) are associated with perturbations in the cholinergic system. Disruptions in the visual information processing can occur in AD, but also in the preclinical stages of AD (Krajcovicova et al., 2017). One particular interest in AD

research is to decipher the pattern of task-dependent changes in functional brain activity and the behavioral performance occurring at the early onset before AD progression. Of interest, using neural activities elicited by visual motion, [Javitt et al. \(2023\)](#) have successfully identified early amyloid deposition among older individuals without observable neurocognitive impairments. Visual motion processing can be further investigated to anticipate vulnerabilities and distinguish the preclinical stages of AD. Systematic investigations on motion perception within the context of healthy and pathological aging are still required, and animal models such as zebrafish (e.g., *ache*<sup>sb55/+</sup> mutants that have chronic depletion of acetylcholinesterase) can be a powerful tool to distinguish the behavioral phenotype in different tasks and visual motion types. Further systematic investigations of visual processing in *ache*<sup>sb55/+</sup> mutants may be informative for behavioral phenotypes in various tasks and understanding the preclinical stages of pathological aging such as AD.

It is important to note that the type and time range of cholinergic intervention vary across different studies. In particular, compensatory perceptual and/or cholinergic changes that alter the response and performance might occur at older ages. Another important point is the duration of cholinergic modulation. In studies using pharmacological interventions targeting cholinergic neurotransmission, impacts and exposure are relatively short-term and acute ([Chamoun et al., 2017](#); [Rokem and Silver, 2010](#)). The long-term effects of altered cholinergic signaling on behavioral and cognitive parameters are not well described. The *ache*<sup>sb55/+</sup> mutants are an example of life-long reduction in acetylcholinesterase activity. In these mutants, it is likely to observe adaptational responses to maintain homeostasis after the long-term manipulation of the cholinergic system, and the respective alterations in the behavioral performance might be blunted in less demanding perceptual tasks. The lack of an overall genotype effect in both of our studies on different motion types may be due to these adaptational and compensatory mechanisms in cholinergic transmission.

#### 4.2. Zebrafish optomotor response

There have been only few studies in the literature that investigated second-order motion detection of zebrafish. Previous studies showed that zebrafish exhibit both OMR to second-order motion ([Orgner et al., 2000](#); [Roeser and Baier, 2003](#); [Yildizoglu et al., 2020](#)), yet all of these studies used larval zebrafish. Larval zebrafish have been shown to exhibit positive OMR to different types of second-order motion, including flicker-defined motion, similar to the one used in the current study ([Orgner et al., 2000](#)). Compared to other types of second-order stimuli including contrast-modulated and texture-defined motions, zebrafish larvae had the strongest OMR to flicker-defined motion. [Orgner et al. \(2000\)](#) further reported that in larval zebrafish, contrast-modulated second-order motion elicits weaker OMR than luminance-modulated first-order motion at high spatial frequencies while it induces somewhat stronger responses at lower spatial frequency levels. This is consistent with the findings that the spatial resolution of second-order motion processing is lower than that of the first-order motion system ([Hutchinson and Ledgeway, 2006](#); [Reynaud et al., 2014](#)). To our knowledge, our study is the first research extending second-order motion perception of zebrafish to adult groups. Interestingly, our findings indicated that adult zebrafish can exhibit both negative and positive OMR to second-order motion and suggested that the polarity change in OMR is age-dependent. Similarly, previous research revealed that larval zebrafish have positive OMR to the first-order motion. On the other hand, investigations of adult behavior pointed out both positive and negative OMRs to visual motion. [Maaswinkel and Li \(2003\)](#) reported that the proportion of trials in which either positive or negative OMR

is observed depends on the speed, spatial and temporal frequencies of first-order visual motion. Although the findings by [Karaduman et al. \(2021\)](#) mainly indicated negative OMR to first-order motion, specific combinations of contrast level, spatial frequency, and zebrafish groups led to weak positive OMR. Together with the results of second-order motion here, research on this reflexive behavior emphasizes the polarity of OMR depends on specific motion parameters, motion type (i.e., the activated motion system), and age.

Although these behavioral findings provide stimulus-driven nature of OMR polarity, the neural mechanisms underlying polarity change in OMR still remain unknown. The optic tectum and pretectum provide further processing stages of motion beyond the retina in the zebrafish visual system. Previous research on larval zebrafish revealed that optic tectum and pretectum have distinct properties and extract different motion stimulus features. Compared to tectal neurons, pretectal neurons have large receptive fields for global motion properties and mainly drive the OMR through the hindbrain ([Bollman, 2019](#); [Naumann et al., 2016](#)). Moreover, imaging studies indicate parallel pathways for motion extraction and the subsequent reflexive OMR ([Wang et al., 2019](#); [Wang et al., 2020](#)). Notably, a recent study used various motion stimuli with or without the first Fourier component ([Duchemin et al., 2022](#)). Besides Fourier energy, the optic tectum responded to the different features of the stimulus, including texture, contrast, and edges. However, the pretectal activity was limited to the Fourier energy. Based on these findings, [Duchemin et al. \(2022\)](#) argued that in the case of second-order motion (i.e., in the absence of Fourier content), the optic tectum rather than pretectum may control the OMR in larval zebrafish ([Orgner et al., 2000](#)). Our findings extend these studies to adult zebrafish. Together with our previous research ([Karaduman et al., 2021](#)), our findings may reflect different features of neural sites processing visual motion and controlling the subsequent OMR. Interestingly, our behavioral results further suggest that the age-related alterations (and/or compensatory mechanisms) in these sites and associated processing pathways may be differential, and the optic tectum control of OMR may even lead to polarity change in younger and older zebrafish behavior. On the other hand, an ablation study suggested removal of the optic tectum in larval zebrafish had no role in second-order processing ([Roeser and Baier, 2003](#)). [Yildizoglu et al. \(2020\)](#) proposed that the pretectum gets input from directional selective neurons in the retina and integrates/refines visual motion signals to represent more complex higher-order motion cues. According to this view, the pretectum may underlie OMRs to second-order motion stimuli; thus, our behavioral results might indicate differential changes within the pretectum. Given that our second-order stimulation is large, the direction tuning properties of motion detectors in pretectum may alter during aging and hence, result in a change in OMR polarity. Further research combining adult zebrafish behavior and neural activities will be informative to test these alternatives and identify age-related changes in neural sites leading to different OMR polarities.

Lastly, one may argue that the observed distinct features of OMR to second-order motion (e.g., lack of spatial frequency dependency) might stem from using higher spatial frequency values than those the adult zebrafish visual system can detect for second-order motion. However, this explanation seems unlikely since the spatial frequency values used in this study cover a wide range of values commonly used in previous studies on the zebrafish visual system. In a similar spatial frequency range, it was found that shown to larval zebrafish exhibit strong OMR ([Orgner et al., 2000](#)). Furthermore, [Orgner et al. \(2000\)](#) provide systematic comparisons across OMRs to two motion types. The comparisons in the same spatial frequency range indicated that larval zebrafish exhibit weaker OMR to second-order motion than first-order motion, suggesting some different features of OMR elicited by second-order stimulation. A future



systematic investigation including different zebrafish groups and motion types will be informative to further characterize OMR and address whether a similar polarity reversal in OMR exists during the developmental stages of zebrafish (Bak-Coleman et al., 2015).

## 5. Conclusions

In conclusion, the present study revealed an overall aging effect on the second-order motion detection of adult zebrafish. We found that aging can alter the polarity of OMR to this motion type, and the patterns of age-related changes are different for first- and second-order motion processing. These results provide evidence that aging can have distinct influences on different stages of motion processing and the activated motion system. Overall, our findings contribute to a comprehensive understanding of age-related alterations in motion processing/perception, which is an important aspect of our daily experience. They also contribute to evaluate a promising aging model (i.e., zebrafish) within the conceptual framework of visual motion.

## Disclosure statement

The authors have no actual or potential conflicts of interest.

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## CRedit authorship contribution statement

**Aysenur Karaduman:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Validation; Visualization; Writing - original draft. **Elif Tugce Karoglu-Eravsar:** Methodology; Resources; Validation; Writing - review & editing. **Utku Kaya:** Formal analysis; Methodology; Visualization; Writing - review & editing. **Alaz Aydin:** Software; Visualization; Writing - review & editing. **Michelle M. Adams:** Conceptualization; Resources; Supervision; Writing - review & editing. **Hulusi Kafaligonul:** Conceptualization; Formal analysis; Methodology; Visualization; Funding acquisition; Supervision; Writing - review & editing.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.neurobiolaging.2023.06.005](https://doi.org/10.1016/j.neurobiolaging.2023.06.005).

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