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Research article

Lack of Williams syndrome-associated genes alters quantity discrimination in zebrafish

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ABSTRACT

The ability to discriminate sets of items based on their numerosity is alleged to be an evolutionary conserved mechanism in all vertebrates. People with Williams syndrome (WS), a rare multigenic condition, show altered number and quantity cognition abilities. Assessing the contribution of specific genes to WS using animal models could help understand the basis of numerical impairments. Here, we assessed the quantitative abilities of juvenile zebrafish (*Danio rerio*) with loss of function of two of the genes affected in WS using a group size preference behavioural assay. The selected genes were: *baz1b*, implicated in neural crest development; and *fzd9b*, associated with neuronal functioning. The contrasts studied were 2 versus 5, 2 versus 4 and 2 versus 3. While group-level comparisons did not reveal statistically significant genotype differences, single-sample tests suggested a reduced preference for larger shoals in some contrasts among mutants. These trends were more apparent when the total number of items likely exceeded working memory capacity (i.e., 6 or more items), while performance on small numerosity contrasts remained relatively intact. These data agree with previous analyses of humans with WS and offer preliminary evidence that specific genes may influence quantity discrimination. Our research also supports the use of zebrafish as model organisms in which to characterise the neurobiological basis of dyscalculia in WS and associated disorders.

1. Introduction

Strong evidence suggests that the ability to discriminate the number of items in a group relies on an evolutionary conserved mechanism with a shared genetic and neurobiological basis across vertebrates [1–3]. The adaptive nature of this non-symbolic quantity discrimination ability becomes apparent in different everyday scenarios such as intra- and inter-specific interplay (e.g., maternal care, predators vs. preys) and environmental interactions (e.g., foraging) [4]. Dyscalculia, a congenital impairment in number and arithmetic learning, has been well documented in humans [5,6]. The prevalence of cases of dyscalculia within families and in certain genetic conditions further emphasize the importance of genetics on the evolution of numerical skills, although the genes involved are currently unknown [6].

Williams syndrome (WS; OMIM number 194050; also known as Williams-Beuren syndrome) is a rare multigenic condition caused by hemi-deletion of some 28 genes on chromosome 7q11.23. WS leads to multi-systemic deficits, including distinct morphophysiological, behavioural and cognitive phenotypes. Of relevance are the characteristic differential cognitive abilities which makes WS particularly interesting for the study of numerical learning disabilities. For instance, individuals with WS often show relatively strong verbal and social skills but exhibit

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marked deficits in visuospatial tasks. Although their IQ is regarded as normal in around half of the cases, impairments in mathematical abilities are prevalent within WS, along with increased social predisposition (hypersociability) and anxiety proneness. Interestingly, their math difficulties seem to be of nonverbal origin, related to deficiencies in basic numerical and/or visuospatial processing [7–9]. Importantly, WS individuals present altered processing of both discrete and continuous numerosity-related aspects [10], which makes them of special interest for studying the connections between 'number sense' and other magnitude processing parameters encompassed by the broader term of 'quantity'.

However, there are severe discrepancies in the literature regarding the number-related capacities affected in WS. Previous studies have shown impairments in the ability of WS individuals to accurately estimate up to eight objects [11] and in the ontogeny of estimation skills of up to 11 dots [12]; while others found no deficits in the range of small number of objects (between 2 and 3) but impaired large number discrimination (between 8 and 16) [13]. One study on adolescents with WS showed that approximate numerosities (non-symbolic number processing abilities) were severely impaired while the use of verbal number estimation using number words (symbolic number processing) was largely conserved [14]. In contrast, another study supported a conserved ability to discriminate small numerosities in WS while reporting deficits in number representations [15]. Despite these discrepancies, there is strong evidence that people with WS typically show poor number processing abilities.

These WS-related findings are consistent with the proposed existence of two different systems: the object tracking system (OTS), the ability to keep track of a few individual objects at once, involved in the representation of small (<4) numerosities; and the approximate number system (ANS), the ability to estimate and compare quantities without counting, involved in the representation of large (>5) numerosities. However, establishing genotypic-phenotypic correlations in WS is challenging as many genes are affected within 7q11.23. The combinational effect of multiple genes and their differential penetrance might be behind the discrepancies in the existing literature.

Among WS-affected genes, there are some with known roles in neurodevelopment as well as others with a postulated impact [16]. For example, *BAZ1B* has been implicated in neural crest development, craniofacial morphology, and social cognition [17,18], while *FZD9* plays a role in synaptic plasticity through Wnt signalling pathways [19–21]. Other genes frequently discussed in the WS literature include *LIMK1*, associated with visuospatial deficits [22], *GTF2IRD1*, linked to motor coordination and cognition [23], and *CLIP2*, involved in neuronal microtubule function [24]. In this regard, atypical small 7q11.23 deletions can help draw direct correlations [9]. However, these correlations are complicated by variability in the size and content of the deleted region, gene dosage effects, and the differential penetrance of individual genes. Nonetheless, individuals with WS may rely on different cognitive strategies to solve number-related scenarios than typically developing individuals, which might render wrong interpretations of their performance in such tasks [7]. Therefore, using animal models allows researchers to study the effects of individual WS genes under controlled conditions, helping to clarify which genes contribute to numerical difficulties in WS and how they affect the brain mechanisms involved.

Studies of individual genes have increasingly helped to shed light on how specific molecular pathways contribute to core cognitive processes. For example, *FOXP2* has been linked to language and symbolic reasoning, which are related to numerical reasoning [25]; *GRIK1* and *GRIK2*, involved in glutamatergic signalling and synaptic plasticity, have been associated with attentional and memory functions [26]; and *DYRK1A* to cognitive impairments associated with Down syndrome, including number-related deficits [27]. These examples highlight how studying individual genes can help unravel the biological basis of higher cognitive functions, although these studies often face challenges in separating specific cognitive effects from broader developmental issues. Nevertheless, no study to date has directly examined the contribution of specific genes to non-symbolic quantity discrimination, a foundational component of numerical cognition across species.

Zebrafish (*Danio rerio*) are a powerful model in which to assess different aspects of translational neuroscience [28,29], including quantitative skills [4,30–32]. Recent research has shown a consistent preference for greater numerosity during zebrafish's early brain development, supporting the idea that numerical abilities might be hardwired in vertebrates [30,33]. On this matter, we have developed a robust group size preference (GSP) assay which allows rapid evaluation of the quantitative discrimination abilities of zebrafish at an early age [34], an adaptation of the confined stimulus approach developed for angelfish (*Pterophyllum scalare*) by Gomez-Laplaza and Gerlai [35].

Using this GSP assay we [34] have previously shown that working memory, together with attentional constraints, determine how these zebrafish represent sets of items. Juvenile zebrafish do not seem to use the OTS and the ANS as two differently specialised systems for small (represented as discreet entities) and large (approximate numerical magnitude) numerosity respectively [36]. Instead, juvenile zebrafish preferentially use the OTS as a gating mechanism to infer numerosity unless the number of items at which they need to pay attention exceeds their working memory and attention capabilities. If this limit is overcome, juvenile zebrafish can represent number quantity through the ANS, provided the numerical ratio between shoals is sufficiently large (e.g., 1:2), as shown in previous studies [34].

Recently, we have established and confirmed loss-of-function zebrafish lines for two of the genes affected in WS with known neurodevelopmental role and relevance to WS-associated cognitive features. These genes were selected for this study based on their documented contribution to neurological function, their involvement in behavioural phenotypes, and the availability of stable, well-characterised zebrafish lines. On the one hand, zebrafish lacking functional *baz1b*, a gene coding for a chromatin remodeller implicated in neural crest development, show mild neurocristopathy (abnormal development of neural crest-derived tissues), reduced stress- and anxiety-related behaviours and altered ontogeny of social behaviours [18]. On the other hand, zebrafish lacking *fzd9b*, encoding a transmembrane Wnt receptor, have altered stress-reactivity at larval stages and anxiety-driven responses later in life [21]. These lines provide an ideal platform to assess the contribution of specific WS genes to early-developing cognitive traits, such as quantity discrimination.

In the present investigation we sought to use our GSP behavioural assay to assess the contribution of these WS-related genes to the quantitative abilities of juvenile zebrafish, in the context of a well-characterised cognitive phenotype linked to WS. By addressing the concept of 'quantity' rather than 'number', both considered magnitude-related abilities with a shared cognitive mechanism [37], we aimed to assess the contribution of these genes to numerosity, regardless of the continuous and/or discrete variables involved.

2. Materials and methods

2.1. Subjects

All animal procedures in this study were reviewed and approved by the Queen Mary University of London (QMUL) ethics committee (AWERB) following consultation of the ARRIVE guidelines (NC3Rs, UK) and conducted in accordance with the Animals (Scientific Procedures) Act, 1986 and Home Office Licenses.

Approximately 4200 fish were used for this study: 1736 as test fish (~27–30 per genotype and contrast, see individual experiments for specific N number) and the rest as stimuli. All zebrafish were 30–35 days post-fertilization (dpf) from the fish facility at QMUL. Zebrafish used in this study originated from a Tübingen background line as the wild-type (WT) strain. The two loss-of-function zebrafish lines tested in this study, *baz1b*^{44bpDEL} and *fzd9b*^{52bpDEL}, also originated from the same WT strain

as reported previously [18,21]. For each gene, this line was selected as the primary mutant line and is presented in the main figures based on the stronger or more consistent phenotypes reported in the original publications. The predicted molecular consequences of these mutations are shown in Supplementary Figure 1, with full validation described in the original publications [18,21]. All subjects returned to the facility after testing and were used to maintain the breeding stock.

To obtain the juvenile fish required for this study, adult breeders of known genotypes were placed in designated breeding tanks (with perforated floors to isolate the eggs) at evening and eggs collected the following morning. Eggs were incubated in groups of no more than 50 per Petri dish at 28°C until 5 dpf. From 6 dpf onwards, subjects were kept in groups of ~50 fish per tank (unknown sex) in a recirculating system at a constant temperature of 28 ± 2°C and fed twice daily with commercial fry food (Gemma 75/150; Skretting, USA) and life paramecium/brine shrimp, depending on their age.

2.2. Power calculations

Sample size was determined based on our previous study [34] to N = 30 fish for each comparison. However, there was a ceiling effect in our current data that we did not anticipate from our previous research. A ceiling effect can limit the sensitivity of the measurement potentially masking variability and, additionally, makes our data non-normally distributed. Accordingly, we used again the data from our previous research to estimate sample size required using a non-parametric post hoc power analysis. To do so, we employed G*Power 3.1.9.7 software [38] with the following parameters: test type of exact – proportions using inequality, two independent groups (unconditional); options as *t*-test; and type of analysis post hoc computation of achieved power. The input parameters were: one-tailed test (as experimental test is expect to perform in one direction –better than chance level); difference between proportions (p1 - p2) of 0.42574142 (based on 31–33 days post-fertilization subjects from the contrast 2 versus 5 from our previous publication); proportion in group 2 (p2) of 0.5 (chance level); significance level (α) of 0.05; sample size for group 1 of 8; and sample size for group 2 of 30.

The outputted parameters were: an achieved power (1 - β) of 0.7979235, which indicates that the test has a fairly strong probability of detecting a true effect (a power of around 0.8 is considered acceptable) thus meaning that the test is relatively reliable, but there is still a 20.21 % chance of missing a true effect; and an actual α of 0.0506390, which suggests that the test has a slightly higher risk of making a Type I error than the typical 0.05 threshold, although this difference is minimal so the test's risk of falsely detecting an effect when there isn't one is still within an acceptable range.

2.3. Experimental design

Experiments were devised to test whether there was a differential ability to discriminate the number of conspecifics between WT and loss-of-function zebrafish using a GSP assay that we previously developed [34]. Our zebrafish experiments build on previous research using other fish species, such as archerfish (*Toxodes* sp.), which points towards the existence of an approximate magnitude system in fish [39] and the influence of non-numerical magnitudes on their choices [40]. In fact, the GSP assay that we use is a zebrafish adaptation to the experimental apparatus previously developed by Gómez-Laplaza and Gerlai using angelfish (*Pterophyllum scalare*) in which the stimulus fish were confined in small compartments to restrict swimming [35].

Contrasts (see 2.4.) were chosen based on their increased ratio of difficulty and our previous data on age matched juvenile WT. To mitigate any potential side bias, the side at which the larger shoal was allocated was first randomly determined and then counterbalanced across trials.

For each trial of each loss-of-function line (Fig. 1A), test fish were obtained by sibling breeding ≥ 5 pairs/trios (1 male, 2 females) of known genotypes: WT (WT x WT), homozygous (HOM x HOM) and heterozygous (HET, obtained from WT x HOM crossings). Breeding was done over three consecutive days per test trial (using different fish each day) so each test trial could be performed during three consecutive days. For each day, the number and sex of HOM and WT fish used to obtain the HETs were counterbalanced to avoid any possible parental imprinting. After breeding, eggs were collected and pooled together according to genotype. To avoid any 'dish/tank effect', at least 5 Petri dishes of ~50 fertilized eggs each were selected per genotype per day and reared separately. At 6 dpf, at least three tanks (~50 juvenile fish each) of each genotype were set to rear until testing at 30–35 dpf.

On the day of the GSP assay, ≥ 2 tanks of juvenile fish were used as test fish for each genotype. The order of testing varied across days such that each day started with a different genotype with a pseudorandom order of testing each trial. Although experimenters were not blind to genotype, they were blinded to their contrast allocation.

Stimuli fish originated from the same WT background as loss-of-function lines. They were reared in similar manner as the test fish but, to ensure there were enough subjects for all contrasts tested, 8–10 Petri dishes of ~50 fertilized eggs each were selected each day and 5–7 tanks set to rear until testing day. Since our previous research demonstrated an influence of the overall space on the ability to discriminate small and large numerosity [34], we did not perform overall space correction in the present study.

Within each corresponding holding tank, both test and stimuli subjects were chosen at random (excluding those that did not look healthy or were malformed) and gently placed into their corresponding part of the assay ('test arena' and 'stimulus compartment' respectively, see below).

2.4. Group size preference (GSP) assay

Each test fish was used only once and matched for age and size to stimuli fish. All fish used were fed one hour prior to experimentation, which took place between 10 am to 1 pm. Behavioural testing was conducted inside an illuminated DanioVision Observation Chamber, recorded with an overhead Daniovision larval tracking system camera and analysed with the Ethovision software (Noldus Information Technology, UK). Two DanioVision Observation Chambers, with corresponding cameras, were run in parallel each day of testing, each one manipulated by a different experimenter and under the conditions explained above (see 2.3.).

The GSP assay was performed as described previously (see "experiment (i)" within [34]). Briefly, the testing chamber (Fig. 1B) consisted of a single 'test arena' of 8 cm long (clear viewing ends) x 6 cm wide (opaque) with two 'stimulus compartments' attached at the clear ends. The 'stimulus compartments' and the 'test arena' were completely sealed to avoid transmission of vibration/chemical cues between compartments. Each 'stimulus compartment' was further divided into eight 1.5 cm long and 1 cm wide opaque sections, except the view to the 'test arena' (1 cm) which remained clear. Thus, stimuli could only visually interact with test subjects. For analysis, the area of the 'test arena' was divided in three compartments: a central zone and two 1 cm zone bands along the 8 cm ends, right in front of stimulus compartments (close to stimuli fish).

Five minutes before a trial, stimuli fish were introduced into each section of the two 'stimulus compartments'. Depending on the discrimination studied, between 2 and 5 stimuli fish were assigned to each side, with the position of the larger group counterbalanced across trials. Contrasts tested were 2 versus 5 (2 v 5, ratio: 0.4), 2 versus 4 (2 v 4, ratio: 0.5) and 2 versus 3 (2 v 3, ratio: 0.67). During this period, the viewing window between 'test arena' and each 'stimulus compartment' was blocked with an opaque removable barrier.

At the beginning of the trial, a test fish was introduced into the 'test arena' and allowed to explore for 5 min. After this time, the test fish was

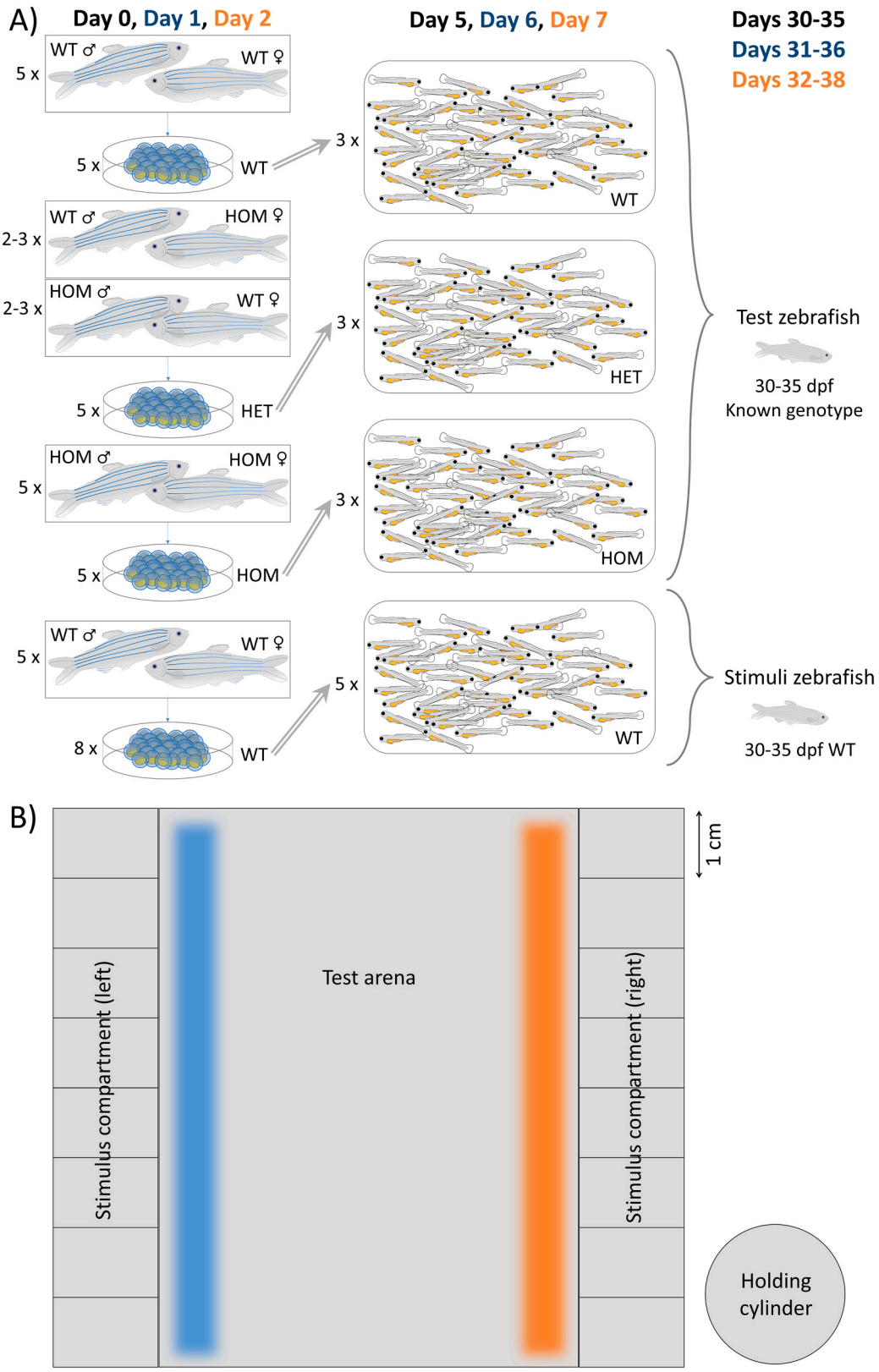

Fig. 1. Experimental plan and group size preference (GSP) assay. A) Breeding strategy for each trial. Numbers (2–5 x) show minimal numbers used for each step. Each trial consisted of three consecutive breeding days (day 0: first day of breeding, in black; day 1: second day of breeding, in blue; day 3: third day of breeding, in orange). At the corresponding 5 days post-fertilization (dpf), fry were transferred to a recirculating system and maintained until 30–35 dpf for testing (same colour code as breeding days). B) Diagram of the testing chamber and the holding cylinder. Note that there are two stimulus compartments, one at the left and one at the right of the test arena, which are further subdivided in smaller compartments to singly-house stimulus fish. During each GSP test, Ethovision system is used to record the time spent by the test zebrafish close to the stimulus compartments, the two 1 cm bands along the 8 cm ends of the test arena (blue and orange for left and right respectively).

constrained into a removal transparent cylinder (2 cm diameter) located at the centre of the 'test arena' and barriers were lifted to reveal the stimulus fish. After one minute during which the test fish was able to view both 'stimulus compartments', the holding cylinder was removed and the position of the test fish recorded over a 5 min period. Any fish that did not visually inspect both sides of the arena while in the holding cylinder (operationally defined as failing to orient its body towards both stimulus compartments) was excluded from the analysis.

Setting the recording time of each assay to 5 min allowed us to screen sufficient animals within the experimentation time at the stated age (see above). Although others have used a similar procedure recording for a period of up to 10 min or 15 min in adult [35] and larval [41] zebrafish, we recorded for 5 min as our previous work and that of collaborators used similar timing for larval zebrafish which was enough to reveal significant differences [34,42]. Furthermore, previous studies using adult zebrafish demonstrated that social preference decreases after 5 min [43]. Similarly, Xiccato and colleagues, when assessing quantitative abilities in adult guppies (*Poecilia reticulata*), showed that fish made a significant preference in the first 5 min only [44].

The preference for a larger group was assessed using the following GSP index: time spent near larger group / (time spent near larger group + time spent near smaller group). A subject was considered to be near a shoal when it entered a 1 cm wide 'region of interest' adjacent to that shoal chamber. Resulting GSP indexes for the lines studied can be found in Supplementary Table 1.

2.5. Statistics

All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 25.0. As with our previous article [34], data deviated from normality (Kolmogorov–Smirnov test, $p < 0.05$) and thus we used non-parametric tests. Kolmogorov–Smirnov test was also used to investigate whether the distributions differed. Significant differences between groups were also assessed with Kruskal–Wallis tests followed by pairwise comparisons with Bonferroni correction for multiple comparisons, and one-sample Wilcoxon signed rank test was used to differentiate a significant preference from chance (0.50). All statistical tests were two tailed. Figures were generated using GraphPad Prism 9.0.2 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). To transparently convey interindividual variability, individual data points are displayed as dots in all behavioural graphs. We represent group performance graphically using mean ± standard error of the mean (SEM) to facilitate visual comparison and clarity for the reader. Nonetheless, descriptive statistics in the Results section are reported as mean and standard deviation (SD) to balance statistical rigour with clarity in data presentation.

3. Results

Statistical analysis of the *baz1b*^{44bpDEL} line (Fig. 2) did not find overall significant differences in their distributions ($p > 0.05$ in all cases). Similarly, there were no differences in the preference for the larger group between genotypes across all contrasts ($x^2_2 = 0.581$, $p = 0.748$). In addition, analysis of the preference for each contrast for all genotypes found there was no significant difference between contrasts ($x^2_2 = 4.548$, $p = 0.103$) and pairwise comparisons with Bonferroni correction found no significant differences between genotypes per contrast. However, one sample Wilcoxon signed rank tests showed there was a significant preference for the larger shoal in the 2 versus 5 contrast in both WT and HET (WT: mean (m) = 0.67, standard deviation (SD): 0.34, N = 27, p = 0.019, score (S) = 91.5; HET: m = 0.68, SD = 0.31, N = 28, p = 0.009, s = 89) while HOM failed to show significance at this contrast (m = 0.59, SD = 0.36, N = 28, p = 0.284, s = 156). Interestingly, no genotype showed a preference for the larger group in the 2 versus 4 contrast (WT: m = 0.62, SD = 0.4, p = 0.185, s = 134; HET: m = 0.55, SD = 0.36, N = 27, p = 0.414, s = 155; HOM: m = 0.62, SD = 0.37, N = 30, p = 0.098, s = 152) or the more difficult contrast of 2 versus 3 (WT: m = 0.57, SD = 0.38, N = 30, p = 0.348, s = 187; HET: m = 0.55, SD = 0.34, N = 28, p = 0.439, s = 169; HOM: m = 0.5, SD = 0.41, N = 29, p = 0.846, s = 208.5). It is important to note that a significant deviation from chance does not imply a significant difference between genotypes; thus, although single-sample tests suggest that WT and HET may succeed in some contrasts, these results should be interpreted with caution, as no significant differences between genotypes were found through group-level comparisons.

A number of zebrafish remained near one of the stimuli for the entire duration of the assay across all contrasts and genotypes (GSP index = 1: WT: 2 v 5: 3/27; 2 v 4: 4/27; 2 v 3: 5/30; HET: 2 v 5: 1/28; 2 v 4: 2/27;

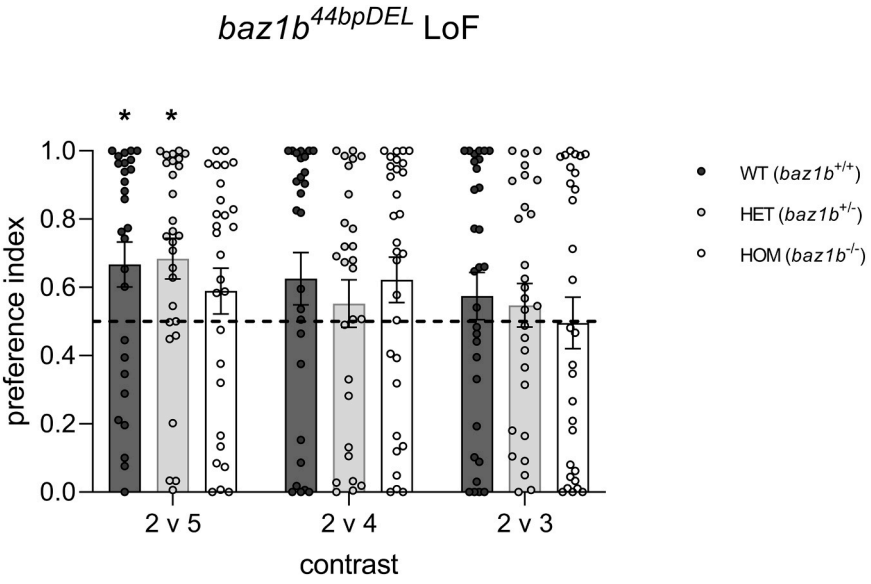

Fig. 2. Lack of functional *baz1b* impairs zebrafish quantitative discrimination in the GSP assay. WT and *baz1b*$^{+/-}$ zebrafish showed a preference for the larger group when confronted with an easier contrast of 2 versus 5 (2 v 5, ratio: 0.4), whereas *baz1b*$^{-/-}$ could not. None of the zebrafish were able to discriminate the more difficult contrasts of 2 versus 4 (2 v 4, ratio: 0.5) and 2 versus 3 (2 v 3, ratio: 0.67). Each dot represents a subject, allowing for visualisation of interindividual variability; bars show mean ± Standard Error Mean (SEM). Dashed line designates chance level. * $p < 0.05$ vs. chance.

2 v 3: 2/28; HOM: 2 v 5: 2/28; 2 v 4: 3/30; 2 v 3: 1/29; GSP index = 0: WT: 2 v 5: 1/27; 2 v 4: 3/27; 2 v 3: 4/30; HET: 2 v 5: 0/28; 2 v 4: 1/27; 2 v 3: 1/28; HOM: 2 v 5: 2/28; 2 v 4: 2/30; 2 v 3: 3/29). However, since the Kolmogorov–Smirnov test did not reveal significant differences in their distributions (see above), and the assay includes an initial confinement period within a transparent cylinder to ensure the fish assess both sides before being released [34], these individuals were retained in the analysis.

Similar statistical analysis was carried out for the *fzd9b*^{52bpDEL} line (Fig. 3). When examining all contrasts together, we did not find a significant difference in preference for the larger shoal between genotypes ($x_2^2 = 1.160$, $p = 0.560$). Similarly, there were no significant differences in their distributions ($p > 0.05$ in all cases) and analysis of the difference in preference between contrasts for all three genotypes grouped also showed there was no significant preference for the larger shoal overall ($x_2^2 = 3.018$, $p = 0.221$). Pairwise comparisons with Bonferroni correction found no significant differences between genotypes per contrast. Nonetheless, when we performed the one sample Wilcoxon signed rank test, WT showed a significant preference for the larger shoal in the 2 versus 5 and 2 versus 4 contrasts (2 v 5: m = 0.72, SD = 0.37, N = 28, p = 0.034, s = 110; 2 v 4: m = 0.74, SD = 0.38, N = 28, p = 0.022, s = 91; 2 v 3: m = 0.63, SD = 0.41, N = 28, p = 0.078, s = 126). In opposition, HET fish only showed a significant preference for the larger group in the more difficult contrast of 2 versus 3 while no preference was found when confronted with the easier contrasts of 2 versus 5 and 2 versus 4 (2 v 5: m = 0.69, SD = 0.42, N = 27, p = 0.112, s = 123; 2 v 4: m = 0.51, SD = 0.43, N = 27, p = 0.469, s = 179.5; 2 v 3: m = 0.68, SD = 0.38, N = 27, p = 0.015, s = 88.5). Finally, HOMs did not show any significant preference for the larger shoal in any contrast (2 v 5: m = 0.7, SD = 0.42, N = 28, p = 0.057, s = 120; 2 v 4: m = 0.57, SD = 0.46, N = 28, p = 0.760, s = 180; 2 v 3: m = 0.62, SD = 0.43, N = 28, p = 0.200, s = 147). As with the *baz1b* line, these patterns suggest possible trends, but the lack of significant genotype differences in group-level analyses calls for a cautious interpretation.

In the *fzd9b* study, similarly to the *baz1b* assessment, there was a number of zebrafish that remained near one of the stimuli for the entire duration of the assay across all contrasts and genotypes (GSP index = 1: WT: 2 v 5: 5/27; 2 v 4: 7/27; 2 v 3: 7/27; HET: 2 v 5: 4/27; 2 v 4: 5/27; 2 v 3: 7/27; HOM: 2 v 5: 6/28; 2 v 4: 8/28; 2 v 3: 6/28; GSP = 0: WT: 2 v 5: 3/27; 2 v 4: 2/27; 2 v 3: 2/27; HET: 2 v 5: 3/27; 2 v 4: 3/27; 2 v 3: 1/27; HOM: 2 v 5: 4/28; 2 v 4: 7/28; 2 v 3: 3/28).

Although we previously generated two loss-of-function lines for each gene of interest in earlier publications [18,21], only one of each is reported in the present study. However, the second loss-of-function lines yielded partially overlapping results (see Supplementary Figures 2 and 3).

4. Discussion

We have assessed the quantitative skills of juvenile zebrafish with loss-of-function for either *baz1b* or *fzd9b*, two of the genes affected in WS. These two genes were chosen based on their known developmental roles, predicted importance for the neurological features of WS and availability of the zebrafish mutants within our facility [18,21]. For each line, homozygous, heterozygous, and closely related wildtype fish were assessed using our previously developed GSP assay at 30–35 dpf [34]. Our data supports an involvement of both *BAZ1B* and *FZD9* in the altered numerical abilities of WS.

In both cases, loss-of-function fish failed to show a preference for the larger group of conspecifics at one or more of the contrasts when compared to WT. Our previous data using the same assay suggests that the quantity discrimination in juvenile zebrafish is the result of an interplay between different cognitive skills including attentional and memory-related competences [34]. Thus, our current data suggests that lack of functional *baz1b* and *fzd9b* reduces these capabilities in juvenile zebrafish, particularly their ability to approximate large numerosities.

Although all contrasts involve the number 2, what determines whether zebrafish rely on the OTS or the ANS is the total number of conspecifics presented in the assay. In our previous study using this same task in juvenile zebrafish (30–35 days post-fertilisation), we found that performance declines when the total number of items exceeds five, suggesting a working memory limit at this developmental stage [34]. Accordingly, in contrasts such as 2 versus 5, the total number of conspecifics (7) exceeds the working memory limit of zebrafish (around 5 items) and thus is more likely to recruit ANS processing, while 2 versus 3 (5 fish total) and 2 versus 4 (6 fish total) may still fall within the OTS range. In line with this interpretation, our data suggest that whenever the number of items exceeded that of their working memory capabilities,

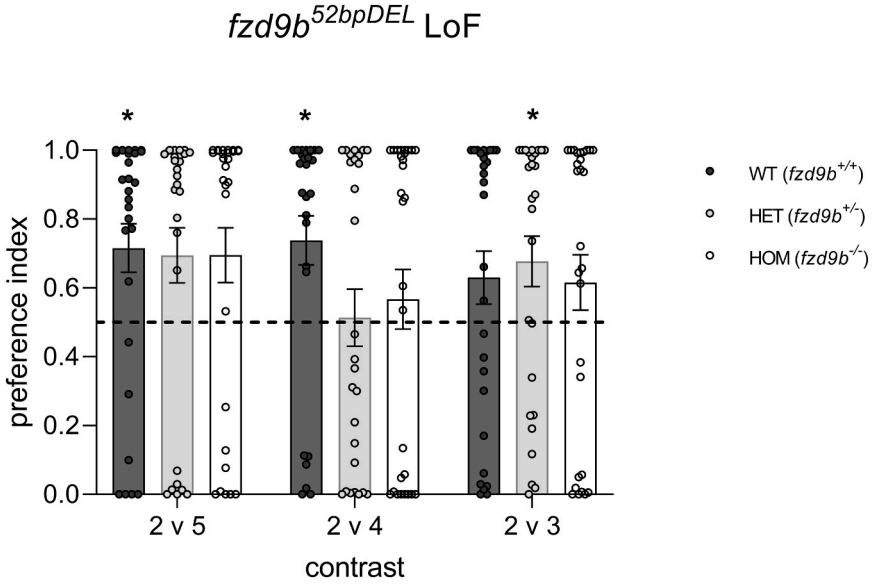

Fig. 3. Dosage of functional *fzd9b* influences zebrafish quantitative discrimination abilities. WT zebrafish demonstrate a significant preference for the larger group when confronted with both contrasts 2 versus 5 (2 v 5, ratio: 0.4) and 2 versus 4 (2 v 4, ratio: 0.5). Strikingly, *fzd9b*$^{+/-}$ fish were able to show a preference for the larger group at the most difficult ratio of 2 versus 3 (2 v 3, ratio: 0.67) while neither WT nor *fzd9b*$^{-/-}$ showed a preference. Each dot represents a subject, allowing for visualisation of interindividual variability; bars show mean ± SEM. Dashed line designates chance level. * $p < 0.05$ vs. chance.

mutants from both lines failed to represent numbers as quantities through the ANS and thus failed to discriminate between shoals.

In the case of *baz1b*, heterozygous fish, similar to wildtypes, preferred the larger shoal in the easier contrast of 2 versus 5, while the homozygous did not. However, this apparent 'dose-dependent effect' was not statistically supported by direct comparisons between genotypes and was not consistently replicated across lines. Moreover, it is possible that wildtype performance in the 2 versus 5 contrast approached a ceiling, which may have limited our ability to detect more subtle genotype differences. Therefore, while the data suggest a possible dosage-related effect of *baz1b* on numerosity discrimination, this remains tentative and requires further validation.

A similar impairment in the preference for the larger shoal was observed in the *fzd9b* line when confronted with the contrast of 2 versus 5. However, interestingly, heterozygous fish for this gene showed a preference for the larger group in the most difficult contrast of 2 versus 3 while wildtype fish did not show this preference. This raises the possibility that lack of *fzd9b* affects zebrafish's ability to approximate number magnitudes rather than to represent small numerosities. It appears that *fzd9b* heterozygous fish were able to discriminate the contrast in which the total number of items is the lowest (2 versus 3, the contrast farthest from the upper limit of their working memory capabilities), even outperforming wildtypes. These data would agree with previous research in humans with WS which showed that, while their ability to approximate larger numerosities was severely impaired, there was a conserved ability to discriminate the range of small numerosities [13,14].

The preserved ability of *fzd9b* (main line) and *baz1b* (supplementary line) mutant lines to discriminate small numerosities (e.g., 2 versus 3) is consistent with the idea that the observed impairments are not due to a general cognitive deficit but reflect a specific disruption in large quantity processing. Small-number comparisons are typically mediated by the OTS, which depends on working memory and attention [2]. The fact that mutants succeed in these contrasts indicates that core cognitive functions remain intact. In contrast, impaired performance in 2 versus 5, more likely to recruit the ANS, points to a selective deficit. This pattern mirrors findings in WS, where ANS-based processing is impaired despite preserved small-number discrimination [13,14].

Therefore, our research suggests that both *baz1b* and *fzd9b* may contribute to the non-symbolic number processing of larger numerosities. Considering the postulated nonverbal origin of WS math difficulties [7–9], our research sheds some light onto the genetic basis of the mathematical impairments associated with this syndrome. According to our data, WS individuals' mathematical difficulties would stem from their diminished ability to distinguish ratios and/or spatial cognition rather than their working memory. Nonetheless, due to the limitations of this animal model, we could not assess the potentially additional impairment of symbolic number representation, which has previously been reported deficient in WS [15]. Similarly, the impact of other genes affected within 7q11.23, their combinatorial effect and/or, as our data suggests, their dosage might also influence the overall number processing abilities of WS.

Although our results point towards specific quantity impairments, we have only assessed quantity discrimination via a single assay at a single stage; thus, we must acknowledge potential influences of non-quantitative factors and/or masked variability, due to the assay's potential bimodal effects and/or the lines themselves (e.g., confronting mutants with WTs which might differ in morphology, sociability and/or stress-reactivity). Importantly, while statistical outcomes guide interpretation, significance reflects not only central tendency but also the consistency of responses: wildtype fish may have exhibited lower variability, thereby increasing statistical power, whereas greater variability among mutants could have obscured true group differences. To better characterise these effects, we report variability and effect sizes in the Supplementary Materials to contextualise trends that may lack statistical significance but remain biologically meaningful. For this reason, displaying individual data points helps to convey group-level patterns more transparently. Nevertheless, a limitation of our GSP assay is that tested fish are compelled to make a choice, even if random, as it is unlikely for them to remain in the central open space of the arena. In this regard, we observed some fish that stayed entirely in proximity to one shoal throughout the test, regardless of whether it was the larger or smaller one. This behaviour could indicate either an inability to distinguish between shoal sizes or simple indifference to size of the shoals. However, considering these individual 'behavioural personalities', genuine indifference would likely manifest either as a preference for one shoal (proactive fish) or as repeated movement between both (reactive fish) [45].

Importantly, the two alleles tested for each gene did not produce fully overlapping results. Specifically, *baz1b* insertion mutants (supplementary line) performed better than deletion mutants (main line) in the 2 versus 3 contrast, while the two *fzd9b* mutant lines diverged in the 2 versus 5 condition. For each gene, we selected one mutant line as the primary line (presented in the main figures) based on the stronger or more consistent phenotypes previously reported in the original characterisation of these alleles [18,21]. The second line for each gene, presented in the Supplementary Figures, was used for replication and comparison purposes. Therefore, these discrepancies could reflect differences in the strength or penetrance of each mutation, or other allele-specific effects, and should be considered a limitation when interpreting the findings. Nonetheless, the number of fish that remained near a single shoal for the entire test duration did not significantly differ between contrasts or genotypes, nor did their overall distribution. Additionally, we have previously shown that neither *baz1b* nor *fzd9b* influences sociability once it has been established [18,21], reinforcing the interpretation that their inability to distinguish shoal size is not a result of altered social behaviour. Furthermore, our GSP assay includes an initial phase in which fish are confined within a transparent cylinder before making a choice [34], ensuring that they can observe both shoals before making a decision.

Similarly, anxiety-like states could be considered as potential factors influencing the outcome of the GSP assay [46]. While *baz1b* mutants exhibit a reduced anxiety-like phenotype [18], *fzd9b* mutants display an increased anxiety-like phenotype [21]. Nevertheless, since both mutants exhibit similar impairments, regardless of potential differences in this behavioural trait, anxiety-like states can likely be ruled out as a confounding factor. Given the strong salience of sociability in this assay, it is plausible that social preference overrides any potential interference from anxiety.

Furthermore, we have specifically addressed the concept of 'quantity', which might include continuous and discrete variables. Nonetheless, evidence suggests a shared cognitive mechanism for both [37]. Thus, zebrafish may have based their behaviour on the activities of their companions, as previously reported in other species [40]. Importantly, our test chambers had sealed compartments, which eliminates potential olfactory or somatosensory input influencing zebrafish's behaviour. Evidence of impairments in processing dynamic signals in humans with developmental dyscalculia [47] further support our observations.

Given that the mechanisms underlying quantity representation in humans change with context and age, it seems possible that there are similar changes in zebrafish over the course of development [34,48]. Therefore, our findings highlight the importance to further characterise these and related genes in different numerical assays (e.g., operant conditioning paradigms [31]) and at different developmental stages.

After having used our GSP assay to rapidly screen lines in which quantity discrimination is altered, further studies on the role of those genes would serve to unravel the genetic basis of dyscalculia in WS and associated disorders. For instance, functional imaging techniques to assess developmental differences in the innate neural circuits for quantities in zebrafish [4,49] would help to elucidate the role of such genes on the development of dyscalculia. Similarly, assessing the ability of adult zebrafish to integrate other types of numerical-related information such as ordinal and spatial cues [50] might prove useful to

understand the interactions with other cognitive abilities.

CRediT authorship contribution statement

Torres-Perez Jose Vicente: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sofia Anagianni:** Visualization, Software, Formal analysis, Data curation. **Eva Sheardown:** Methodology, Formal analysis. **Maria Elena Miletto-Petrazzini:** Writing – review & editing, Investigation, Formal analysis, Data curation, Conceptualization. **Scott E. Fraser:** Writing – review & editing, Funding acquisition. **Brian Butterworth:** Writing – review & editing, Visualization, Funding acquisition. **Giorgio Vallortigara:** Writing – review & editing, Visualization, Supervision, Funding acquisition, Conceptualization. **Caroline H. Brennan:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Inclusion and diversity statement

We support inclusive, diverse, and equitable conduct of research.

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Declaration of Competing Interest

Authors have no conflict of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2025.115860.

Data Availability

Individual preference indexes used for this manuscript can be found in Supplementary Materials and raw data and statistical outputs can be kindly requested to the corresponding authors.

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