Automatic quantification of juvenile zebrafish aggression

Héctor Carreño Gutiérrez, Irene Vacca, Anna Inguanzo Pons and William HJ Norton *

Department of Neuroscience, Psychology and Behaviour, College of Medicine, Biological Sciences
and Psychology, University of Leicester, Leicester, LE1 7RH, UK

*Author for correspondence. Dr Will Norton, Department of Neuroscience, Psychology and
Behaviour, College of Medicine, Biological Sciences and Psychology, University of Leicester,
Leicester, LE1 7RH, UK. Email whjn1@le.ac.uk; tel. 0044 (0)116 252 5078; fax 0044 (0)116 252 3330.

21 **Abstract**

22 **Background**

23 Although aggression is a common symptom of psychiatric disorders the drugs available to treat it are
24 non-specific and can have unwanted side effects. The zebrafish is an ideal model for aggression
25 research. Zebrafish are small, amenable to genetic and pharmacological manipulation, and agonistic
26 behaviour can be measured reliably.

28 **New Method**

29 In this study we have established a novel setup to automatically quantify aggression and locomotion
30 in one-month old juvenile zebrafish, a stage at which fish exhibit adult-like behaviour but are small
31 so that one camera can film several animals.

33 **Results**

34 We have validated our novel software by comparison to manual quantification of behaviour,
35 characterised the aggression of one-month old fish, and demonstrated that we can detect
36 alterations to aggression caused by mutation or drug application.

38 **Comparison with other methods**

39 The ability to record up to 12 juvenile fish allows us to speed up and standardise data acquisition
40 compared to studies of single fish.

42 **Conclusions**

43 This setup appears to be suitable to screen for drugs that decrease zebrafish aggression as a first
44 step toward developing novel treatments for this behaviour.

46 **Keywords**

47 Zebrafish, aggression, behaviour, locomotion, drug treatment

1. Introduction

Aggression can be defined as behaviour that aims to harm or injure another living being that is motivated to avoid such treatment (Baron, 1994). Although animals use aggression to protect offspring, establish dominance hierarchies and compete for resources (Koolhaas and Bohus, 1992), uncontrolled expression of this behaviour can be maladaptive. In humans, aggression can be motivated by anger, irritation, frustration, fear or pleasure (Nelson and Trainor, 2007). Violent or hostile aggression is debilitating and can lead to antisocial behaviour, delinquency and crime (Gibbon et al., 2010). Aggression is also a symptom of several psychiatric disorders including attention-deficit/hyperactivity disorder (ADHD), bipolar disorder, conduct disorder, schizophrenia and substance abuse disorder (Nevels et al., 2010) and it is a frequent reason for children and adolescents to be referred to psychiatrists (Connor et al., 2006).

In humans, the link between aggression and psychiatric disorders is complex. Aggression is often seen as a nuisance rather than an actual target for medical intervention (Pappadopulos et al., 2006). However, chronic aggression is predictive of longer and more intensive treatment with poorer outcomes. At present, aggression is managed using a combination of behavioural, cognitive and pharmacological therapies (Vitiello and Stoff, 1997). Treatment is further complicated by different subtypes of aggression with a distinct genetic- and neurobiological basis (Blair, 2010; Kempes et al., 2005; Montoya et al., 2012; Nevels et al., 2010; Scotto Rosato et al., 2012; Vitiello and Stoff, 1997). Studies of both humans and other animals suggest that two broad subtypes exist: impulsive (reactive, offensive or emotionally labile) and instrumental (pre-planned, defensive, unemotional or predatory) aggression (Heinz et al., 2011; Nelson and Trainor, 2007; Steiner et al., 2011). In humans each subtype is associated with different psychiatric disorders and may respond to different drugs (Pappadopulos et al., 2006). Furthermore, some human patients display a combination of both subtypes complicating management of this behaviour.

The drugs that are used to treat aggression show limited efficacy and may have unwanted side effects (Wernicke et al., 2003; Polzer et al., 2007; Nevels et al., 2010; Scotto Rosato et al., 2012). It is clear that novel anti-aggression drugs with increased potency and a more selective mechanism of action are needed.

It is difficult to develop drugs for psychiatric disorders. Between 1994 and 2001 only 7% of drugs targeting the central nervous system were successful in clinical trials (Pangalos et al., 2007). Several recent studies have shown that whole-animal behaviour-based screens may constitute a novel approach to identify such drugs (MacRae and Peterson, 2015; Rihel and Schier, 2012). Compared to single target in vitro screens, whole organism paradigms maintain the complex architecture of the brain and are ideal to examine drugs that act at multiple receptors (Rihel et al., 2012; Rihel and Schier, 2013). Zebrafish are an ideal model for this type of research due to their short generation time, small size and the ease of applying drugs by immersion. Zebrafish are also transparent until larval stages allowing neural circuits to be manipulated in the intact brain (Fetcho and Liu, 1998). A large number of identified mutant lines, genetic tools such as CRISPR/Cas9 (Prykhodzhiy et al., 2017) and optogenetics (Del Bene and Wyart, 2012.), and techniques to monitor neural activity (Higashijima et al., 2003) have already been established. Furthermore, standardised tests for both larval and adult zebrafish behaviour have already been developed (Norton and Bally-Cuif, 2010; Norton, 2013). Zebrafish may thus provide a compromise between more expensive rodent models (with high genetic- and physiological similarity to humans) and smaller non-vertebrates that have a less-conserved behavioural repertoire.

Zebrafish have already been used to examine the genetic and pharmacological basis of aggression (Jones and Norton, 2015). Agonistic behaviour is expressed as a series of stereotypic body postures which include rapid movements of the caudal fin and bouts of swimming and biting directed against an opponent (Gerlai et al., 2000). Aggression can be measured by observing the dyadic interaction of

two fish in a tank (Oliveira et al., 2011) or by recording the interaction of a single fish with its own mirror image (Gerlai et al., 2000). Although mirror-induced stimulation does not recapitulate all aspects of an interaction with another animal (Desjardins and Fernald, 2010; Oliveira et al., 2005; Teles et al., 2013), mirror fights and dyadic fights trigger a similar level of aggression (Teles et al., 2013). Mirror-induced aggression is simple to perform in the lab and has been used to characterise the genetic and neurobiological basis of this behaviour (Gutiérrez et al., 2017; Norton et al., 2011; Teles et al., 2013). This suggests that mirror-induced stimulation is suitable to identify novel drugs that can alter aggression.

The aim of this study was to establish a novel behavioural setup that permits efficient, standardised measurement of zebrafish aggression and locomotion. We used 1 month-old juvenile fish in these experiments because their small size permitted behaviour to be recorded in several animals at the same time. Furthermore, pallium development is complete at this stage (Dirian et al., 2014) and juvenile zebrafish are able to perform complex behaviours (Valente et al., 2012). We developed software to automatically detect aggression and compared it to manual quantification of the same films. We validated the software by recording mirror-induced aggression in the dark, as well as comparing mean aggression levels as the fish develop, during the day and throughout the year. Using this method, we detected changes to agonistic behaviour in *fibroblast growth factor receptor 1a* mutants that exhibit heightened aggression (Norton et al., 2011) and following ethanol or lithium treatment. The methods we present here suggest that juvenile zebrafish are a valid model for drug screens, permitting novel compounds that alter this behaviour to be identified.

2. Materials and Methods

2.1. Zebrafish strains, care and maintenance

Adult (3 month-old) and juvenile (28 day-old) zebrafish were maintained at the University of Leicester using standard protocols and in accordance with institutional guidelines for animal welfare. All work was conducted under a UK Home Office licence. The following strains were used: AB wild-type and *spiegeldanio/fibroblast growth factor receptor 1a* mutants (*fgfr1a*^{t3R705H/t3R705H} (Rohner et al., 2009)). *fgfr1a* mutants show increases in aggression, novel-object interaction and exploration and were used to validate our experimental protocol. Adult fish were mated overnight and fertilised eggs were collected the next morning. Eggs were kept in embryo medium in a 28.5°C incubator with a 14-hour day/night light cycle. On day 6, fry were transferred to the aquarium facility in groups of 20. All fish examined in a single experiment were the progeny of group incross of male and female fish. Juveniles were randomly sorted into the groups of 20, reducing the possibility that a batch effect might affect the data we obtained. Fish were fed twice a day. Fish younger than 21 days post fertilisation (dpf) were fed with GEMMA Micro 75 (Skretting). From 21 dpf to 28 dpf fish were fed with GEMMA Micro 150 (Skretting) and live artemia nauplii. Adult fish were fed with GEMMA Micro 300 (Skretting) and live artemia nauplii. The age, sex and size of fish in a group was carefully matched in all experiments. Fish were maintained in groups of 20 until analysis. One day before the experiment fish were transferred to an isolated room with the same temperature and light cycle as the aquarium facility.

2.2. Experimental apparatus and design

2.2.1. Apparatus to measure juvenile aggression

All juvenile behaviour experiments were carried out in a novel setup designed and built in collaboration with ViewPoint Behavior Technology (Lyon, France). The setup consisted of a large cubical (66 x 78 x 108 cm) permitting the behaviour of twelve 28-day old juvenile zebrafish to be measured in parallel. Ambient lighting was provided by an array of LED bulbs attached to the ceiling (150 Lux) and the temperature was maintained at 28°C. Infrared lighting was provided using a

custom-made light source that formed the floor of the apparatus, and behaviour was recorded using three cameras (Grasshopper 3, Point Grey research) mounted in the ceiling of the cubical (Fig. 1).

Locomotion and aggression were measured in 12 identical small plastic tanks (9 x 4.2 x 4 cm) that were similar to the adult mirror-induced aggression tank described in Norton et al., (2011). Three sides of the tank were covered with an opaque white material and the fourth side was left clear. The tanks were arranged in groups of four and an opaque white plastic divider was used to cover the transparent side before the experiment started. A mirror was placed outside the tank at an angle of 22.5° permitting the fish to interact with its own reflection once the divider had been removed (Gerlai et al., 2000) (Fig. 1). The tanks were filled with 100 ml water from the main zebrafish facility. These tanks were also used to habituate fish to the aggression setup and to apply drugs by immersion.

2.2.2. Software to quantify juvenile aggression and locomotion

Behaviour was recorded using video cameras mounted in the ceiling of the cubicle. The cameras were connected to a computer running an extension of ZebraLab software developed by ViewPoint Behavior Technology. This software simultaneously detected locomotion and aggression directed against the mirror. Locomotion was recorded by videotracking. The software detected aggression by using two values that form the basis of a normal trajectory analysis. The first value ("ratio") is the ratio between the distance a fish swims between the start and end point of ten images, and the sum total of inter frame distance moved during the same time. If the fish swam straight forwards the value was 1, whereas disordered movements reduced this value towards 0. The second value ("angle") was the arctangent of the fish's trajectory when facing the mirror. These values were used to calculate an index (H) that was heuristically adjusted to maximise the separation between two behaviours (aggression / non-aggression): $H = \text{angle} \cdot \log(\text{ratio}) / 2$. The H index was compared to a threshold that can be set by the user, and was calibrated by comparison to manual quantification of

the same films. The threshold can be recalibrated depending upon the age and size of the fish used in the experiment.

Additional filters were applied to remove false positives. No aggressive behaviour was detected if the fish was more than one body length away from the mirror ("threshold distance to the mirror"); and if a fish was aggressive ($H < \text{threshold}$) for more than 40% of a 0.5 second segment the whole time-period was recorded as aggression. The final results presented here represent the ratio between the sum of the time spent in aggression and the total time multiplied by 100. Aggression was displayed in "Aggression Units" (AU), an arbitrary unit that corresponds the H index and represents the total amount of time spent fighting the mirror image. Locomotion was displayed as "Locomotion Units" (LU) based upon the total number of pixels moved during an experiment. Results were exported into Microsoft Excel.

2.2.3. Procedure to measure juvenile aggression

Fish were fed with dry food on the morning before aggression was recorded. On the day of the experiment fish were placed into individual tanks using a wide-tipped Pasteur pipette. Each experiment lasted twelve minutes. In the first two minutes juveniles were allowed to habituate to the tank following transfer. During the next five minutes locomotion was recorded without visual access to the mirror with the opaque white plastic divider in place. The divider was then removed and aggressive interaction with the mirror was recorded for five minutes. All experiments were carried out between 11.00 and 15.00 to reduce circadian influences on behaviour unless indicated otherwise. In some cases, manual quantification of aggression was performed in LabWatcher (ViewPoint Behavior Technology). Each film was quantified by two independent observers blind to the genotype being scored. The mean value of both results was calculated and compared to automatic quantification.

2.2.4. Drug treatment

Juvenile aggression was compared with- and without drug treatment. The concentration of drug used was adapted from previous studies (Ethanol, Fontana et al., 2016; Lithium, Nery et al., 2014) taking into account the small size of juvenile zebrafish. A toxicity test was first performed for novel compounds. A single juvenile fish was immersed in the highest concentration of drug for thirty minutes and its behaviour was monitored. Alterations to behaviour (such as a lack of movement or a loss of balance) were taken as a sign of toxicity. If this occurred, fish were immediately removed from the drug and euthanised. The test was then repeated using a lower concentration of drug. The toxicity test is a refinement that reduced potential harm to the fish and identified the concentration of drug to use in subsequent experiments.

Juvenile zebrafish were immersed acutely in drugs before locomotion and aggression was measured. A single fish was placed into a small basket (17 x 17 x 22 mm) made out of wire mesh. The baskets were then placed into a tank containing 100 ml of drug solution (Fig. 1). Drugs were applied for thirty minutes. This time period was chosen to maximise the number of drugs that could be analysed in a single experimental session. Following treatment the baskets containing the juveniles were washed in tanks containing drug-free water. These washes ensured that the drug did not contaminate the recording apparatus. Fish were then placed into the cubicle as described above. Control untreated fish received a mock treatment (i.e. immersion in aquarium water in a basket) and underwent the same handling procedure including the washes.

The following drugs were used in this study: lithium carbonate (Tocris cat. number #4740) used at a final concentration of 0.05, 0.5 and 5 mM (Nery et al., 2014); and ethanol (Fisher Scientific cat. number #10342652) used at a final concentration of 0.05%, 0.15% and 0.25% v/v (Fontana et al., 2016).

2.2.5. Data analysis

The aggression software automatically exports tracking parameters from all three cameras into a single Excel file. Statistical analysis of behavioural data was performed in GraphPad Prism 7 and IBM SPSS Statistics 24. The data set was assessed for normality using a Shapiro-Wilk test. Homogeneity of variances was assessed using the Levene's test. A Mann-Whitney *U*-test was used to compare two groups of fish. When comparing more than two groups, a one-way ANOVA followed by a Dunnett or Games-Howell post hoc was used. A Kruskal-Wallis test followed by Dunn post hoc was used for non-parametric data. To examine possible seasonal changes in aggression and locomotion, data recorded between February and December 2016 were analysed by fitting a linear regression model. The correlation between aggression and locomotion, between automatic and manual quantification of aggression, and between the distance swum measured by an Ethovision video tracking system (Noldus) and the novel ViewPoint software was calculated using the Pearson's correlation coefficient.

3. Results

3.1. Automatic detection of juvenile aggression and locomotion

In this study we have developed a protocol to automatically quantify zebrafish aggression and locomotion. Preliminary data from our laboratory suggested that 28 dpf fish could interact aggressively with their own mirror image (data not shown). We therefore designed and built a setup to record the behaviour of 12 1-month old fish in parallel (Fig. 1). We also developed software to automatically detect aggression. We validated the software by comparing its output (in aggression units (AU) and locomotion units (LU)) to the distance swum measured with an Ethovision videotracking system (Noldus) and to manual quantification of aggression in the same films. We found a strong significant correlation between the number of AU and the time spent in aggression (Fig. 2a; Pearson $r = 0.918$; $p < 0.0001$). Comparison of LU to the distance swum measured using Ethovision also revealed a significant positive correlation (Fig. 2b; Pearson $r = 0.774$; $p < 0.0001$). In

contrast to this, the correlation between locomotion and aggression was weaker (Fig. 2c; Pearson $r = 0.3188$; $p < 0.0001$). This suggests that the two behaviours are related, with locomotion representing an important component of aggressive behaviour.

3.2. The ontogeny of aggression

We identified the earliest age at which zebrafish would exhibit aggression against the mirror by recorded the behaviour of 21 dpf juvenile zebrafish. We chose this stage because the lateral pallium of the telencephalon matures at this time (Dirian et al., 2014) and zebrafish are capable of operant conditioning from 21 days onwards (Valente et al., 2012). At 21 dpf the majority of fish did not show significant mirror-induced aggression (median 0 AU) (Fig. 2d). However, a small number of animals, perhaps representing the larger more mature fish, did show agonistic interaction. By 28 dpf there was a significant increase in aggression directed against the mirror (Fig. 2d) with a median group value of 50.55 AU.

In order to compare data collected using juveniles to our previous studies of adult zebrafish (Norton, 2012) we compared the aggression levels of three groups of fish at 21 dpf, 28 dpf and 3 months. We had previously calibrated our software to quantify juvenile aggression. When we placed adult fish into the cubicle we found that they were not tracked properly (judged by comparing automatic to manual quantification; data not shown). We therefore compared manual quantification of aggression of 21 and 28 dpf fish recorded in the cubicle to 3 month old fish measured in our adult mirror test setup (Norton et al., 2011). In agreement with the output of the novel software, manual analysis revealed a significant increase in aggression between 21 and 28 dpf (Fig. 2e; $p < 0.01$). However, there was no increase in aggression between 28 dpf and 3 months (Fig. 2e; $p > 0.05$). This suggests that data generated using juveniles can be used to predict the behaviour of adult fish.

3.3. Validation of screening setup and software

Automatic detection of aggression is triggered when juvenile fish enter a predefined zone within one body-length of the mirror. Proximity to the end of the tank suggested that our software could be detecting thigmotaxis (a preference for the bottom or side of an arena (Maximino et al., 2011)) rather than actual aggression. We investigated this possibility by measuring aggression levels in the dark. Switching off the lights in the cubicle triggered a significant reduction of aggression compared to the normal ambient lighting conditions (Fig. 3a ON vs OFF $p < 0.0001$). Fish recorded in the dark showed almost no aggression (median of 0 AU in dark compared to 125.4 AU in light) suggesting that this behaviour is visually based. Locomotion also decreased when the lights were turned off, although the difference was less marked with fish swimming a median of 631.8 LU in the dark compared to 2246 LU in the light (Fig. 3b ON vs OFF $p < 0.0001$). Together, these results suggest that the software is specifically detecting visual interaction with the mirror.

3.4. Alterations to aggression over time

Although the setup to measure juvenile behaviour could be used to screen for novel drugs or mutant lines that alter aggression or locomotion it would take several months to carry out a screen. We therefore examined changes to aggression and locomotion over time. We first quantified both behaviours at three time points during the day – morning (10 am), afternoon (2 pm) and evening (8 pm). Juvenile zebrafish showed stable levels of aggression (Fig. 3c) and locomotion (Fig. 3d) at these time points suggesting that there is no circadian fluctuations in these behaviours. We next investigated whether animals that have been reared in the laboratory exhibit circannual changes in behaviour by examining aggression and locomotion levels over an 11-month period (Fig. 3e,f). Although there were clear differences in the mean levels of both behaviours each month, the influence of time could not explain these changes (Fig. 3e,f aggression, linear model $R^2 = 0.0023$; and locomotion, linear model $R^2 = 0.0057$). Taken together, these results suggest that we can compare the results of experiments conducted at different times of the year making long-term screens feasible.

3.5. Automatic quantification can detect changes in aggression in mutant lines

We used the aggressive *fibroblast growth factor receptor 1a* (*fgfr1a*) mutant line (Norton et al., 2011) to test whether our behaviour setup was sensitive enough to detect mutant behavioural phenotypes. We compared 1 month-old *fgfr1a* mutants and their wild-type siblings and found that juvenile mutants showed heightened aggression (Fig. 4a $p < 0.001$) in line with our previous research (Norton et al., 2011). Juvenile *fgfr1a* mutants were also hyperactive in the recording chamber (Fig. 4b $p < 0.001$) in contrast to adult *fgfr1a* that do not exhibit increased locomotion (Norton et al., 2011).

3.6. Alterations to aggression caused by drug treatment

In a final experiment, we demonstrated the suitability of our setup to screen for drugs that can alter aggression. We applied drugs acutely by immersion for thirty minutes before measuring aggression and locomotion. We tested two drugs that alter aggression in other species including humans: lithium carbonate (Cipriani et al., 2013) and ethanol (e.g. (Miczek et al., 2015). In agreement with published studies, lithium carbonate treatment caused a significant reduction in aggression (Fig. 5a; $p < 0.05$) and locomotion (Fig. 5b; $p < 0.05$) at the highest concentration tested, 5 μ M. Acute treatment with ethanol increased aggression (Fig. 5c; $p < 0.05$) at the highest concentration used (0.25%) in keeping with a previous study that used this drug (Fontana et al., 2016). The total distance swum was not altered upon ethanol application (Fig. 5d; $p > 0.05$) suggesting that our protocol to automatically record aggression is suitable for a drug screen.

4. Discussion

In this study we have established a novel setup to measure aggression and locomotion in juvenile zebrafish. We have also validated software that permits automatic quantification of these

behaviours. Using this platform we have shown that the majority of fish display robust aggression from 28 days onwards. There was no significant difference in the amount of aggression shown by one-month old juveniles and adult fish suggesting that data can be compared between these two life stages. We have also examined circadian and circannual changes to aggression finding that interaction with the mirror is stable over time. Our setup is sensitive enough to detect changes in aggression caused by drug treatment or mutation of genes linked to this behaviour. The ability to record twelve animals in parallel suggests that we can use this approach to identify novel drugs in a medium throughput manner.

4.1. Measuring juvenile zebrafish aggression

In this study we chose to measure behaviour in one month-old zebrafish rather than mature three month-old adults. The small size of juvenile fish allowed us to speed up data acquisition by filming three animals with a single camera. In zebrafish sexual maturity only occurs at three to four months (Liew and Orban, 2014) meaning that juveniles may not produce all the hormones that influence aggression. However, development of the pallium (the teleostean equivalent of the mammalian hippocampus) is complete between three and six weeks (Dirian et al., 2014) and zebrafish are able to perform operant learning at this age (Valente et al., 2012). This suggests that the neural circuits needed to drive complex behaviour are already functional. We measured mirror-induced aggression from three weeks onwards. At this time point interaction with the mirror was highly variable and only a subset of animals showed agonistic display. However by four weeks we were able to record a robust interaction with the mirror. Our findings agree with previous research showing that aggression and dominance appear between 4 and 6 weeks in zebrafish (Ricci et al., 2013). Furthermore, a substantial variability in learning before week four has been described by Valente and colleagues suggesting that individual differences in maturity might influence behaviour (Valente et al., 2012).

Although we have not compared the separate postures that combine to make agonistic display in both juveniles and adults (tail thrashing, biting the mirror), we have demonstrated that similar genes (Fig. 4) and drugs (Fig. 5) control aggression at both these life stages. Together, these observations suggest that one month-old juvenile zebrafish are a valid model to study aggression, with the potential to provide information about the control of behaviour by the immature brain.

4.2. Automatic quantification of aggression and wild-type aggression parameters

Manual quantification of aggression films is time consuming making it unsuitable for high-throughput analyses. We found a strong correlation between automatic- and manual quantification of aggression by two independent observers (Fig. 2a). The time spent in aggressive display can be calculated from the automatic data using the formula: $\text{time} = 0.1978 \times (\text{AU} - 3.720)$. Therefore, each second corresponds to 9.7 AU giving a ratio of about 1:10. Although this definition is not exact (for example it will vary according to individual differences in manual quantification) it does allow data collected using this program to be compared to other studies. The medium throughput nature of our aggression setup permitted us to investigate several aggression parameters. We demonstrated that mirror-induced aggression is a visually-based behaviour (Fig. 3a,b) that does not alter depending upon the time of day (Fig. 3c,d) or month of the year (Fig. 3e,f). The finding that aggression does not show circadian alterations agrees with a previous study that compared zebrafish agonistic behaviour between 9 - 11 am and 2 - 4 pm (Ariyomo and Watt, 2015). This suggests that aggression can be compared in the morning and afternoon, thereby increasing the number of animals that can be measured in one day. The mean values of aggression were also very stable throughout the year, ranging from 254 AU (highest) to 135.2 AU (lowest). Such a stable read-out of aggression over time is useful because it permits comparison of results from experiments carried out over long periods of time.

4.3. Screening for drugs to reduce aggression

Although aggression is a significant burden to society there are very few drugs that specifically target this behaviour. It is more costly and difficult to develop novel drugs for central nervous system disorders than other diseases (Rihel and Schier, 2012; Pangalos et al., 2007) . The signalling pathways that control CNS disorders are complex. Side-effects including dizziness and seizures can be severe, and there is a lack of biomarkers to show target activation once drugs cross the blood-brain barrier (Pangalos et al., 2007). Many novel drugs are derivatives of existing compounds meaning that few novel pathways or targets are being identified.

The zebrafish is an ideal model for behavioural pharmacology since drugs can be applied by immersion and entire neural circuits can be imaged in the intact brain. Recent studies have shown that zebrafish can be used to discover drugs that alter sleep (MacRae and Peterson, 2015; Rihel and Schier, 2012). In a separate approach, drugs that protect fish from the convulsant pentylenetetrazole (PTZ) have been identified as a first step towards developing novel treatments for epilepsy (Baxendale et al., 2012). In this study we have demonstrated the suitability of our setup to identify drugs that reduce aggression. We treated juvenile zebrafish with lithium carbonate, a mood stabiliser that is used to treat suicidality in humans (Cipriani et al., 2013). At the highest concentration tested, lithium significantly reduced both aggression and locomotion with a larger effect on aggression levels (Fig. 5a,b). We also immersed zebrafish in ethanol and measured behaviour. (Fig. 5c,d). Acute treatment with 0.25% ethanol increased aggression levels without altering locomotion in line with previous research (Fontana et al., 2016).

There are several types of screen that could be performed using our novel platform. An unbiased screen would allow libraries of novel compounds to be tested whereas a biased screen could be used to repurpose known drugs (Rihel and Schier, 2012). Using behaviour readout as a readout is also ideal for testing drugs that bind to multiple receptors (Pangalos et al., 2007). Considering the complex neural circuits that control aggression, such a polypharmacological approach may be particularly useful for drugs targeting this behaviour. Since undesirable side effects such as toxicity

or reduced locomotion can be recorded in the same assay drugs can even be counter-screened in parallel. If the molecular basis of an aggression phenotype is known then a target-orientated screen could be performed (List and Barzman, 2011). For example, a drug screen could be carried out in a mutant line that shows altered aggression (Fig. 4a,b (Norton et al., 2011)), uncovering signalling pathways that are epistatic to the known effect (Rihel and Schier, 2012) and paving the way for personalised medicine. Finally, preventative treatments could be identified by pre-treating developing zebrafish with compounds before screening aggression at one month. A reduction of aggression at adult stages might indicate a protective influence of drug treatment. In summary, our juvenile aggression setup is flexible and could provide the opportunity to improve treatment of aggression in several ways. Taken together, these results suggest that zebrafish are an excellent species to screen for novel drugs that can alter this behaviour.

6. Figure legends

Figure 1. Photos of aggression setup. a) Setup to measure the behaviour of 12 juvenile zebrafish in parallel. **b)** Juvenile zebrafish are placed into small wire mesh baskets and immersed in drug solution or untreated aquarium water before behaviour is measured. **c)** Locomotion and aggression is measured in 12 identical plastic tanks. Three sides of the tank are covered with an opaque white material and the fourth side is left clear with a mirror placed outside.

Figure 2. Automatic quantification of juvenile aggression. a) Strong positive correlation between manual and automatic quantification of aggression at 28 dpf (Pearson $r = 0.9184$; $p < 0.0001$; $n = 31$). **b)** Comparison of automatic quantification of locomotion with Ethovision and Viewpoint software. Strong positive correlation in the results obtained using both software (Pearson $r = 0.774$; $p < 0.0001$; $n = 31$). **c)** Correlation between aggression and locomotion. Weak positive correlation between aggression and locomotion values obtained by automatic quantification (Pearson $r =$

0.3188; $p < 0.0001$). A total of 756 28 dpf wild-type fish were measured. **d,e)** Onset of aggression can be detected by 28 dpf. **d)** Automatic quantification detects the onset of aggression at 28 dpf (Mann-Whitney test: $U = 391$; $p = 0.0027$; $n = 36$ each). **e)** Aggression is barely detectable in 21 dpf juveniles (median = 0 s) whereas it can be manually recorded at 28 dpf (median = 7.51 s) with similar levels as in adult fish (median = 23.51 s). Kruskal-Wallis H test: $X^2 (2) = 16.11$; $p = 0.0003$; post hoc Dunn's test (21 dpf vs 28 dpf; $p = 0.0086$; 21 dpf vs 3 months; $p = 0.0007$; 28 dpf vs 3 months; $p = 0.9229$); $n = 36$ (21 dpf); $n = 31$ (28 dpf); $n = 20$ (3 months). The three groups of fish were independent and naive for the mirror test. AU: aggression units. dpf: days post-fertilisation. ** $p < 0.01$; *** $p < 0.001$.

Figure 3. Characterization of juvenile aggression. **a)** Aggression of 28 dpf wild-type fish is reduced when the lights are switched off (OFF) compared to normal illumination (ON) (Mann-Whitney test: $U = 211.5$; $p < 0.0001$; $n = 35$ ON; $n = 32$ OFF). **b)** Locomotion is also reduced in the dark (Mann-Whitney test; $U = 91$; $p < 0.0001$). **c,d)** Aggression and locomotion do not change during the day. **c)** Aggression levels of three different groups of fish measured at different time points (One way ANOVA: $F (2, 62) = 0.010$; $p = 0.9898$). **d)** Locomotion levels of the same groups of fish at the same time points (One way ANOVA: $F (2, 62) = 2.277$; $p = 0.111$; $n = 21$ at 10 am; $n = 22$ at 2 pm; $n = 22$ at 8 pm). **e,f)** Aggression and locomotion levels are stable throughout the year. **(e)** The variability in aggression levels in wild-type fish throughout the year (One way ANOVA (Welch); $F (10, 757) = 2.656$; $p = 0.0034$) is not explained by a linear model ($R^2 = 0.002$). **(f)** The variability in locomotion observed throughout the year (One way ANOVA (Welch); $F (10, 752) = 15.292$; $p < 0.0001$) is not explained by a linear model ($R^2 = 0.0057$). A total number of 756 fish were used. dpf: days post-fertilisation. **** $p < 0.0001$.

Figure 4. Automatic quantification of a mutant phenotype. Automatic quantification can detect increased levels of **(a)** aggression (Mann-Whitney test; $U = 210$; $p = 0.0005$; $n = 30$ WT; $n = 29$ *fgfr1a*

mutants) and **(b)** locomotion (Mann-Whitney test: $U = 210$; $p = 0.0005$) in 28 dpf *fgfr1a* mutants compared to wild-type. dpf: days post-fertilisation. *** $p < 0.001$.

Figure 5. Lithium carbonate and ethanol affect aggression and locomotion in 28 dpf fish. a) Acute treatment with 5 μ M lithium carbonate reduces aggression ($p = 0.0305$) (Kruskal-Wallis H test: $X^2(3) = 12.21$, $p = 0.0067$; post hoc Dunn's test; $n = 10$ each) and **(b)** locomotion ($p = 0.0187$) (one-way ANOVA: $F(3, 36) = 2.813$; $p = 0.053$; post hoc Dunnett's test). **c)** Treatment with 0.25% ethanol increases aggression ($p = 0.03$) (one-way ANOVA (Welch); $F(3, 43) = 3.735$; $p = 0.018$; post hoc Games-Howell test; $n = 11$ control; $n = 12$ treated fish for each concentration) with **(b)** no effects on locomotion (one-way ANOVA; $F(3, 43) = 1.152$; $p = 0.3389$). dpf: days post-fertilisation. * $p < 0.05$.

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