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Risk taking behaviour predicts consistent and heritable coping styles in zebrafish



Lianne Koets¹, Tim van der Kwaak¹, Marcel Schaaf¹ and Christian Tudorache^{1*}

Abstract

Background Coping styles are individually coherent sets of behavioural and physiological responses to stress. Coping styles are thought to remain consistent across context and time, and display a certain level of heritability. Here, we examined whether risk taking is a predictor for consistency and heritability of stress coping styles in both larval and adult zebrafish (*Danio rerio*).

Results A group emergence test where fish emerge from a familiar housing compartment into a potentially dangerous novel environment, established the level of risk taking of F0 generation adult zebrafish. The degree of risk taking appeared to be consistent over time and context. Then, the F0 risk taking degree was further correlated with various behavioural parameters related to stress coping of the F1 and F2 generations. In larval fish, these parameters were measured during a light dark challenge which elicits an anxiety like response. In adults, they were measured during a single emergence test and a combined open field and mirror biting test, estimating the degree of risk taking and the level of explorativeness and aggressiveness. The results show that (i) parental risk taking behaviour is a good predictor for a large number of larval and adult behavioural parameters, within and between generations; (ii) a number of these parameters are consistent over ontogenetic (larval and adult) stages within the same generation, and (iii) four of these parameters representing risk taking, aggressiveness, and swimming behaviour, were correlated over multiple generations, establishing heritability of coping styles.

Conclusion We conclude that risk taking behaviour is a strong predictor of coping style within and between generations and behavioural parameters associated with risk taking are consistent over time and heritable over generations.

Keywords Additive genetic variance, Heritability, Parent–offspring regression, Consistency, Stress-coping styles

Background

When faced with internal and external stressors, animals exhibit a range of correlated behavioural and physiological responses that remain consistent over time and across situations [1-3]. These individual patterns of coping with stress, referred to as "stress coping styles", can span a proactive–reactive continuum within a population [2-4]. Proactive individuals are characterised by a high level

Christian Tudorache

¹ Institute of Biology, Leiden University, Leiden, Sylviusweg 72, 2333 BE Leiden, The Netherlands



active attempts to counteract a potential stressor. Reactive individuals, on the other hand, are risk avoidant, show low levels of aggression, and a general tendency to counteract stressors passively [2, 3]. Stress-coping styles have significant fitness consequences, affecting survival and reproductive success [5], and have been studied in a wide variety of vertebrate taxa, including fish (e.g. [6–13]). Previous work in our lab on stress coping in zebrafish demonstrated that risk taking, defined as the willingness to engage in actions with possibly adverse outcome, exploratory behaviour, the curiosity driven tendency to seeking out novel experiences or environments, aggressiveness, the tendency to respond to novel

of risk taking, aggression, and other actions indicating

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^{*}Correspondence:

c.tudorache@biology.leidenuniv.nl

situations or new interindividual encounters forcefully, hostile, or assertive, and biomechanical parameters of swimming behaviour, are correlated and vary consistently among individuals [4, 14, 15] confirming the presence of different behavioural phenotypes within a behavioural syndrome [16]. Furthermore, these behavioural phenotypes were correlated with different types of physiological responses, gene expression and endocrine activity, accounting for a correlation between behaviour and physiology within a coping style [4, 14, 15].

Especially risk taking has been shown to be a strong predictor for various other behavioural and physiological traits within a coping style, such as aggressiveness and explorativeness, the expression of various genes, or energetics and biomechanics [14, 15, 17-19]. In various fish species such as roach (Rutilus rutilus, [20]), carp (Cyprinus carpio, [21]), threespined stickleback (Gasterosteus acculeatus, [22]), or zebrafish (Danio rerio, [4, 14, 15]), risk taking can be evaluated by an emergence test, a wellestablished test with high repeatability. During this test, a group of fish is allowed to emerge from a familiar shelter into a novel and potentially dangerous environment. The emergence rank is considered a measure for the individual tendency of risk taking [4, 14, 15, 17, 18]. The risk taking-avoiding axis, also known as bold-shy continuum [7, 18], is one of the most widely studied continua of behavioural variation. Risk taking animals tend to explore or investigate novel environments or objects more readily than risk avoiding animals, which tend to avoid novelty [23–25]. From the perspective of fitness, risk taking may be adaptive when resources are scarce and predation risk is low, whereas risk avoiding may be more effective when resources are ample and predation risk is high [23]. Next to fish [7], the risk taking continuum has been studied in a wide range of animals including bears [26], lizards [27] and birds [28].

Consistency of coping styles over time and across context [1-3] manifests as the tendency for individuals to display predictable behavioural responses in various situations, and can be evaluated by repeated exposure to similar situations. Consistency exists along a continuum, with some individuals exhibiting more stable behavioural patterns than others [29]. Consistency can predict future performances of individual coping styles within a population [30], since it influences individual fitness by affecting resource acquisition, predator avoidance, and social interactions, and thereby stabilises the response to potential stressors on a population level [31]. Several factors contribute to the observed consistency in coping styles, such as early-life experiences, including maternal care, social interactions, and environmental conditions, such as resource availability, predation risk, and social dynamics. Also, genetic predisposition, a prerequisite for heritability, impacts the expression of consistency across contexts [23].

Coping styles have been shown to be at least partially heritable [2, 32, 33], as they may be influenced by a combination of inherent and environmental factors, such as genotype, development, early life experience and/or social support [3]. Since phenotypic variance is the sum of genotypic and environmental variance [30], individual phenotypic variation of a behavioural parameter within a population affects the fitness of an individual. In order to respond to selection, a significant genetic component must be present which can be estimated by means of heritability [5]. The heritability of a behavioural parameter (h^2) , the variance of breeding values, is often expressed as the proportion of phenotypic variance (V_p) that can be attributed to the additive genetic variance (V_A) , i.e. the deviation from the mean phenotype due to inheritance of a particular allele and this allele's relative effect on the phenotype, with the resulting formula $h^2 = V_A / V_P$. The parameter h² can be estimated by executing a half-sibling test or by performing a parent-offspring regression [30]. Consistency and heritability of behavioural indicators related to coping styles are still not entirely understood. Unravelling the underlying mechanisms, including the genetic and environmental variation, of coping styles is therefore fundamental for understanding the evolution of behavioural and morphological constructs, and is of great value for animal welfare and health.

The objective of this study was to examine the risk taking based association between behavioural indicators of a stress-coping style in both larval (at 5 days post-fertilisation) and adult (>3 month post-fertilisation) zebrafish, and to estimate the consistency and heritability of these behavioural indicators. The individual tendency of risk taking was measured in the parental generation and subsequently correlated with individual scores for aggressiveness and explorativeness, by means of standardised behavioural tests (emergence, mirror image stimulation and open field test, respectively; [4, 14, 15]. Hereafter, a family-crossing emergence rank was performed to produce offspring larvae. At 5 dpf, larvae were subjected to an open field test and a light dark challenge to test for explorativeness and anxiety like response. After raising them to maturity, adults were subjected to the same set of behavioural tests as the previous generation adults. Three generations of fish were tested in this study: the parental fish (F0 adult stage), first generation (F1 larval and adult stage), and second generation (F2 larval stage), and the results of the behavioural tests were correlated with each other in order to (a) establish consistency of the coping style indicators over time and across context, and (b) heritability over various generations. Consistency of the behavioural indicators was evaluated by plotting

the average measurements of the larvae of the first generation against the average measurements of the adult fish of the first generation. Narrow sense heritability of the behavioural indicators was estimated using a parent-offspring regression using data from individuals of the parental and the offspring generation [30]. Here, the average measurements of the offspring generation were plotted against the average measurements of the parental generation fish.

Our results show that (i) parental risk taking behaviour is a good predictor for a large number of larval and adult behavioural parameters, within and between generations; (ii) a number of these parameters are consistent over ontogenetic (larval and adult) stages within the same generation, and (iii) four of these parameters representing risk taking, aggressiveness, and locomotion behaviour, were correlated over multiple generations, establishing heritability between generations. We conclude that risk taking behaviour is a strong predictor of coping style within and between generations and therefore behavioural parameters associated with risk taking are consistent over time and heritable over generations.

Methods

Animals and housing

Zebrafish (Danio rerio) are social teleosts of which clear guidelines exist regarding their welfare conditions. They were bred, maintained and handled according to the guidelines from the Zebrafish Model Organism Database (ZFIN, http://zfin.org) and in compliance with the directives of the Dutch Law on Animal Testing (WOD) and the local animal welfare committee of Leiden University (DEC, number 14058). The wildtype strain was AB/TL, a crossbred strain of AB and Tüpfel Long Fin (TL) strains, originally obtained from the Hubrecht Laboratory (Utrecht, The Netherlands) and maintained in our laboratory for at least ten generations at the time of experimentation. The AB/TL line is considered a segregated hybrid line and contains large genetic variation within as well as between the original AB and TL strains [34–36]. New generations were generated by mating 60-80 fish (ca 1:1 ratio male to female) from the previous generation. The fish were reared in densities of ± 40 individuals (male to female 1:1) per 7.5-L tanks in standardized recirculation systems (Fleuren & Nooijen, Nederweert, The Netherlands); water temperature was maintained at 28±1 °C (n=5), with a conductivity of $518 \pm 12 \ \mu S$ (n=5) and oxygen concentration of 7.9 ± 0.4 mg l⁻¹ (n=5). Light cycles were maintained at 14-h light to 10-h darkness cycle, with light periods from 8:00/7:00 [0 h Zeitgeber Time, (hZT)] to 22:00/21:00 (14 hZT) summer time/winter time, with a linearly decreasing/increasing light intensity between 0 and $320 \pm 21 \ln m^{-2}$ (n = 3) over a period of 15 min. Fish were fed twice daily with dry food (DuplaRinM, Gelsdorf, Germany) and frozen artemia (Dutch Select Food, Aquadistri BV, Klundert, The Netherlands). The fish used in the experiments were between 1 and 2 years old and had a standard length of 32.1 ± 2.3 mm and a body weight of 150.61 ± 17.99 mg (mean \pm SD). There was no correlation between emergence rank and body weight (Spearman rank, N=144, p>0.05).

Adult behavioural testing

A diagram of the sequence of tests is given in Fig. 1A.

Group emergence test

In order to measure the individual tendency of risk taking, an emergence test was performed [15, 17, 18] (Fig. 1B). The experimental setup for this test consisted of a Plexiglas tank $(33 \times 13 \times 13 \text{ cm})$ which was equally divided into two compartments, a slightly darkened holding compartment and an uncovered novel area compartment, by a wall with a hatch $(2 \times 2 \text{ cm})$ at its mid-bottom. This hatch was manually closable by means of a trap door.

Groups of ten fish of mixed sex were netted from the zebrafish facility home tanks into holding tanks $(33 \times 13 \times 13 \text{ cm})$, and brought to the experimental room where they were kept overnight. The next day, fish were carefully poured from the holding tank into the holding compartment of the experimental setup. After an acclimatization period of 10 min, the trap door was opened, giving the fish the opportunity to emerge into the novel area compartment. After each emergence event, the trap door was closed and the emerged fish was netted into holding tanks $(33 \times 13 \times 13 \text{ cm})$, separated by emergence rank 1 to 10. The group emergence test did not last longer than 15 min and was performed between 9:00 and 15:00 a.m. (1 and 9 hZT), with five experimental runs, resulting in 50 ranked fish.

After the experimental runs, the fish were divided into ten ranks of five individuals each, according to their order of emergence. The individual fish were therefore categorized by their coping style, along a proactive–reactive continuum. In the afternoon, the zebrafish were transferred by means of netting and placed individually in holding tanks $(33 \times 13 \times 13 \text{ cm})$. The fish were kept overnight until further experimentation the next day.

Single emergence test

In order to establish consistency over time and context of risk taking tendencies as a trait, fish previously tested in a group emergence test were subsequently tested in a separate experiment for single emergence time, 3 months later. The individual emergence setup consisted of three rows of five Plexiglass emergence tanks, as used for the group emergence tests, with the trap doors connected



Fig. 1 Experimental set up. A The test sequence comprises group and single emergence tests and subsequent open field test and mirror image stimulation for adults of the F0 (parental) generation ("Group emergence test" and "Single emergence test" in "Methods"). After subsequent reproduction per emergence rank ("Method of reproduction" section), F1 were subjected to a light dark challenge as larvae and to an open field test and mirror image stimulation as adults ("Open field test combined with a mirror image stimulation" section). After another round of reproduction per emergence rank, F2 larvae were subjected to a light dark challenge ("Larval behavioural testing: light dark challenge assay" section). B The emergence test assesses the individual tendency of risk taking, by measuring the rank of individuals from a group of fish (group emergence test) or the time of an individual fish (single emergence test) to emerge from a darkened holding compartment into an uncovered and potentially dangerous novel area, compartment. C The open field test combined with a mirror image stimulation was designed to establish a behavioural syndrome of correlated traits. The fish enters from a holding compartment into an open field. After crossing this potentially dangerous novel environment it encounters a mirror image stimulation. The parameters measured were related to locomotion behaviour and aggressiveness.
D During the light dark challenge (LDC) test, larval fish are subjected to a series of alternating dark and light periods, during which they exhibit hyperactivity and hypoactivity, respectively. This behaviour is thought to be an anxiety like response to suddenly changing light conditions

per row so that hatches of the five emergence tanks per row could be opened simultaneously. These 15 emergence tanks were placed on a table surface of 1.0 by 1.0 m, which was covered with a white reflective sheet. A HD video camera (HDC-SD90, Panasonic Inc., Japan. Macro lens: Marumi M-52S050, 52 mm, $0.5 \times$ wide converter) was mounted 1.5 m above the table. Fish were placed individually in the holding compartment and acclimated overnight. At testing time (10:00 p.m., 2 hZT), hatches were opened manually, and emergence was recorded.

Open field test combined with a mirror image stimulation

In order to establish a behavioural syndrome of traits correlated with risk taking, an open field test combined with a mirror image stimulation was conducted (Fig. 1C). The experimental setup for this test consisted of a Plexiglas tank $(33 \times 13 \times 13 \text{ cm})$ which was divided into two compartments, a holding compartment of ca 11 cm long and

an uncovered open field compartment, by a wall with a hatch $(2 \times 2 \text{ cm})$ at its mid-bottom. At the tank wall opposite of the holding compartment a mirror $(13 \times 15 \text{ cm})$ was mounted at an angle of ca. 70°, to ensure that the zebrafish only could see its mirror image when it entered the approach zone, starting at ca 4 cm, or approximately one body length, from the mirror. Fifteen of these set-ups were placed on a table surface underneath a camera as previously described with the single emergence test.

Fish previously tested in the group emergence test were placed individually, by means of carefully pouring, in the holding compartment. After an acclimatization period of 10 min, the trap doors were opened in all 15 tanks simultaneously, enabling the exposure to the open field and mirror, and the video recording was started. The test was performed between 9:00 a.m. and 1:00 p.m. (5 hZT) and lasted not longer than 30 min. Fish that did not emerge within this period were excluded from further analyses. Several parameters were measured from the video footage: swimming velocity (V in cm s⁻¹), angular velocity (Ω ; in °s⁻¹), mobility states (MS), divided into three levels, i.e. highly mobile, mobile and immobile (in %), the time spent in the open field and the mirror approach zone (in s), individual emergence time (in s), and the frequency of aggressive behaviours, i.e. bites to the mirror image and parallel swimming and circling in front of the mirror (AGRf, in s⁻¹ [15]). Individual emergence time and AGRf were determined from the video footage by hand, and the other swimming kinematics were analysed using EthoVision XT 10.1 (Noldus Information Technology b.v., Wageningen, The Netherlands).

Method of reproduction

After the experimental runs, fish were placed in holding tanks $(33 \times 13 \times 13 \text{ cm})$ separated by emergence rank. The holding tanks were subsequently transferred to the zebrafish facility where they were connected to the flow through system. After at least a week of rest, crossings were performed to produce the next generation.

To produce offspring, a family crossing per rank was carried out. Around 5:00 p.m. (9 hZT), the fish were transferred by means of netting into the breeding tanks $(23 \times 13 \times 13 \text{ cm})$, equipped with a sloped mesh bottom to simulate a riverbank and to prevent interaction of the parental fish with their laid eggs. Fertilization occurred by natural spawning the next day at the beginning of the light period. The eggs were collected around 10:00 a.m. (2 hZT) and transferred per rank into two Petri dishes containing egg water (60 µg/ml of "Instant Ocean" sea salts dissolved in demi water), and all Petri dishes were placed in an incubator (HERAtherm Incubator, Thermo Scientific, The Netherlands) at 28.5 °C. Eggs were checked again after 24 h and any unfertilized or dead egg was removed. At 5 dpf, 24 of the hatched larvae were transferred at ca 10:00 a.m. (2 hZT) into 24-well plates (VWR International, LLC Radnor Corporate Center, PA USA) with one larva per well, filled with egg water to 1.5 cm depth, by means of a Pasteur pipette. At 5 dpf a Light Dark Challenge (LDC) assay to measure anxiety like behaviour [37] was performed. This procedure was performed four times for the F1, and 8 times for the F2 (one set had to be eliminated due to mortality), resulting in 96 and 161 tested larvae, respectively.

The remaining larvae were used to raise the following generation. The batches of larvae were reduced to 30 individuals per rank. Hereafter, the fish were raised in densities of 10 individuals per tank $(33 \times 13 \times 13 \text{ cm})$ in standardized recirculation systems (Fleuren and Nooijen, Nederweert, The Netherlands). At maturity age of 3 months, fish were tested in the open field test combined with a mirror image stimulation (N=110).

Larval behavioural testing: light dark challenge assay

In order to estimate degree of anxiety like behaviour, a light dark challenge was performed, where individual housed larvae are after acclimation exposed to a series of alternating challenge phases with ambient illumination turned repeatedly off and on again (Fig. 1D). The larvae are reacting with hyperactivity during the dark and hypoactivity during the light phase, respectively, when compared to baseline activity. The 24 well plate with each well containing a larval zebrafish, was placed in a DanioVisionTM observation chamber (Noldus Inc., Wageningen, Netherlands), equipped with Infrared (IR) illumination from beneath the plate and an IR sensitive camera filming from above at 60 fps in a 1280×960 -pixel resolution. After an initial acclimation period of 10 min in the illuminated chamber, video tracking was initiated for another 10 min before the larvae were subjected to a dark challenge phase of 5 min and another light challenge phase of 10 min. The phase durations are arbitrary but sufficient to give clear data for further analysis. This challenge chain of dark and light phases was repeated, thus resulting in two alternating dark and light phases.

Larval movement tracking and analysis of the resulting behavioural data was conducted using EthovisionTM software (V14; Noldus, Wageningen, Netherlands). Tracking the centre of mass (CoM) of moving individual larvae over time resulted in a number of behavioural parameters: the swimming velocity (V in mm s⁻¹), angular velocity (Ω ; in °s⁻¹), meandering (in °mm⁻¹), acceleration (in mm s⁻²), and mobility state, divided into three levels, i.e. highly mobile (MS_h), mobile (MS_m) and immobile (MS_i, in %).

Statistics

For determining risk taking behaviour as a consistent trait over time and context, we correlated (1-10) with (s) in the F0 generation. Then, in order to test whether the risk taking tendency is associated with explorativeness, we correlated group emergence rank with kinematic swimming parameters from the open field test, i.e. swimming velocity (V in cm s⁻¹), angular velocity (Ω ; in °s⁻¹) and mobility states (MS_h, MS_m, MS_i; in %). Finally, in order to establish a behavioural syndrome between risk taking and aggressiveness, we correlated group emergence rank with the frequency of aggressive behaviours over time (AGRf; in s^{-1}) during a mirror stimulation. Since all data are based on ordinal emergence ranks and a Shapiro-Wilk normality test confirmed not-normally distributed data, the correlations were tested with a Spearman's rank test (N=50, p < 0.05), after performing an outlier test (ROUT, Q = 10%) to clean the data (number of outliers corresponds with the number given between brackets minus the N value).

Similarly, to determine if risk taking tendency of the parents can predict risk taking tendency of the offspring, we correlated the group emergence rank of the F0 generation with the individual emergence time of the F1 generation. We then correlated the emergence rank of the F0 generation with the swimming parameters, i.e. swimming velocity (V in mm s⁻¹), angular velocity (Ω ; in °s⁻¹), meandering (in $^{\circ}mm^{-1}$), acceleration (in mm s⁻²), and mobility states (MS; in %) during a light-dark-challenge (LDC) of the F1 generation, to determine if risk taking tendency of the parents can predict the anxiety like response of the larval offspring. These correlations were performed using a Spearman rank correlation test, after performing an outlier test (ROUT, Q=10%) to clean the data (number of outliers corresponds with the number given between brackets minus the N value).

After that, the LDC swimming parameters of the F1 larval fish were correlated with the swimming parameters during an open field test combined with mirror image stimulation of the F1 adult fish. These correlations were tested with a Spearman's rank test. Bonferroni multiple comparison test was used *post-hoc* to correct for multiple comparisons.

With significant correlations (p < 0.05), a linear regression was conducted ($y=bx+y_{int}$), with r=b. A Bonferroni multiple comparison test was used *post-hoc* to correct for multiple comparisons. This procedure was also applied for the F2 generation.

To determine whether the behavioural indicators of a coping style contained a heritable component, the narrow sense heritability of each behavioural and swimming parameter was estimated by performing a parent–off-spring regression. Per parameter, the individual average measurements of the offspring generation (mid-offspring value; F1 and F2) were plotted against the average measurements of the parent generation (mid-parent value; F0 and F1). Thereupon, a linear regression was carried out with h^2 =b. A Bonferroni multiple comparison test was used *post-hoc* to correct for multiple comparisons.

All analyses were conducted using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Statistical significance was accepted at p < 0.05, and p-values were two tailed throughout. If applicable, the N, ρ , and p-value were indicated. Missing LDC data are due to rank 5 and 10 of the F1 generation and rank 4 of the F2 generation exhibiting low fecundity before the planned experiment.

Results

Adult behaviour: group and single emergence tests

To establish risk taking as a trait consistent over time and context, for predicting individual coping behaviour, adult fish (parental F0) were first tested for group emergence rank and subsequently tested separately for single emergence time, resulting in significant positive correlations (Fig. 2A; Spearman rank test, N=39(50), ρ =0.4393, p=0.0058). These results indicate that risk taking behaviour, as determined in the emergence test, is consistent over time and context.

Subsequently the group emergence ranks of the adult F0 fish were correlated with adult F1 single emergence times. Here too, a significant positive correlation



Fig. 2 Group and single emergence test in parental (F0) and F1 adults. A Single emergence time plotted against group emergence ranks of the F0 parental fish during an emergence test. Data were collected from 5 batches of 10 adult zebrafish (N = 50, individuals not emerging within 10 min were omitted from analysis) obtained during a group emergence test (rank) and subsequent single emergence test (time), with a significant correlation between emergence rank in the group test and time in the single test (Spearman rank test, N = after (before outlier elimination); simple linear regression: y = 86.18x - 135.3, $r^2 = 0.14$). **B** Emergence time obtained during a single emergence test of the F1 adults plotted against emergence rank of F0 parental fish during an (N = 100, individuals not emerging within 10 min were omitted from analysis) in the F0 group emergence test (rank) and F1 single emergence test (time), with a significant correlation between rank and time in the single test (Spearman rank test, N = after (before outlier elimination); simple linear regression: y = 22.73x + 60.69, $r^2 = 0.12$). These results establish risk taking as a consistent and heritable behavioural trait

emerged. (Fig. 2B; Spearman rank test, N = 87(100), $\rho = 0.2841$, p = 0.0077).

Adult behaviour: open field test and mirror image stimulation

Thereafter, the emergence ranks of the adult F0 fish were correlated using a Spearman rank correlation with adult F0 and F1 swimming parameters in an open field test combined with a mirror image stimulation, to investigate possible behavioural correlates within a coping style. The F0 emergence rank was negatively correlated with swimming velocity (V; N=80(100), ρ =-0.2849, p=0.0104, Fig. 3A) and angular velocity (Ω ; N=88(100), ρ =0.2660, p=0.0104 Fig. 3B) and positively correlated with the time to approach the mirror (N=32(50), ρ =0.3804, p=0.0317, Fig. 3C) and frequency of aggressive behaviour (AGRf; N=50, ρ =-0.4459, p=0.0093, Fig. 3D). These results indicate a higher activity and straighter swimming paths during the open field test, and earlier and higher aggressiveness scores, in early than in late emerging fish.

A significant correlation was found between the F0 emergence rank with the F1 behavioural parameters, swimming velocity (V; N=110, ρ =-0.1942, p=0.0421, Fig. 3E) and immobility state (MSi; N=101(110), ρ =0.2044 p=0.0404, Fig. 3F) during the open field test and frequency of aggressive behaviour (AGRf; N=106(110), ρ =-0.2587, p=0.0074, Fig. 3G) during the mirror image stimulation. These results indicated a higher level of aggressiveness and activity in the next generation of early emerging fish than in late emerging fish.

Larval behaviour: light dark challenge (LDC) test

F1 and F2 fish were exposed as 5dpf old larvae to a light dark challenge (LDC) test. The swimming parameters collected during this test were correlated with the emergence rank of the F0 generation. The LDC test results were subsequently divided into baseline, light and dark phases for analysis.

During the baseline phase, the F1 generation of zebrafish larvae showed negative correlations of the F0 emergence rank with swimming velocity (V; N=96, ρ =-0.2052, p=0.0449, Fig. 4A) and acceleration (A; N=96, ρ =-0.1793, p=0.0427, Fig. 4B), and positive corelations with angular velocity (Ω ; N=96, ρ =0.2282, p=0.0315, Fig. 4C) and immobile state (MS_i; N=96, ρ =0.2942, p=0.0036, Fig. 4D). The same profile of significant correlations emerged during the dark phase (V: N=96, ρ =-0.1946, p<0.001, Fig. 4E; A; N=96, ρ =0.2015, p=0.0062, Fig. 4G; MS_i: N=96, ρ =0.2810, p<0.0001, Fig. 4H). No significant correlation was detected of the F0 emergence rank with any of the parameters of the light phases.

The F2 generation larvae showed generally weaker correlations and fewer correlated parameters with the F0 emergence rank, than the F1. Here, a positive correlation was observed of the F0 emergence rank with the angular velocity during the light phase [Ω ; N=195 (161), ρ =0.1178, p=0.0363, Fig. 4I] and the immobile state during the dark phase (MS_i; N=195 (161), ρ =0.1925, p=0.0151, Fig. 4J). Generally, it can be said that we observed a higher general swimming activity



Fig. 3 F0 emergence rank as a predictor for adult behaviour. Behavioural parameters obtained during an open field test combined with mirror image stimulation, in adult fish of the F0 parental generation (**A**–**D**) and the F1 generation (**E**–**G**), plotted over the emergence ranks of the F0 parental fish during a group emergence test: swimming velocity (V; 3A, simple linear regression: Y = -0.10x + 2.17, $r^2 = 0.12$, and 3E, Y = -0.06x + 3.51, $r^2 = 0.04$), angular velocity (Ω ; 3B, Y = 2.414x + 97.16, $r^2 = 0.07$), state of immobility (MS_i; 3F, Y = 0.09x - 0.01, $r^2 = 0.07$), time to approach the mirror (3C, Y = 0.53x + 0.28, $r^2 = 0.021$), and frequency of aggressive behaviour (AGRf, 3D, Y = -2.12x + 43.81, $r^2 = 0.18$ and 3G, Y = -1.30x + 19.61, $r^2 = 0.07$). These parameters yielded a significant correlation with the F0 emergence ranks [Spearman rank test, N = after (before outlier elimination)]



Fig. 4 F0 emergence rank as a predictor for larval behaviour. Behavioural parameters obtained during a Light Dark Challenge (LDC) test, in larval (5dpf) fish of the F1 (**A**–**H**) and F2 (**I**, **J**) generation during the baseline phase (**A**–**D**), the dark phases (pooled; **E**–**H**) and light and dark phases (pooled; **I**, **J**), plotted over the emergence ranks of the F0 parental fish during a group emergence test: swimming velocity (V; 4A, y = -0.08x + 3.65, $r^2 = 0.06$, 3E, y = -0.07x + 4.52, $r^2 = 0.05$), acceleration (4B, y = -0.004x + 0.11, $r^2 = 0.04$, 4F, y = -0.004x + 0.14, $r^2 = 0.02$), angular velocity (Ω ; 3C, y = 1.05x + 69.29, $r^2 = 0.05$, 3G, y = 1.39x + 19.60, $r^2 = 0.04$, 3I, Y = 1.081*X + 57.13, $r^2 = 0.05$), and state of immobility (MS₁₇ 3D, y = 0.13x + 21.25, $r^2 = 0.08$, 3H, y = 0.04y + 9.92, $r^2 = 0.08$, 3 J, Y = 0.02x + 10.01, $r^2 = 0.02$). These parameters yielded a significant correlation with the F0 emergence ranks [Spearman rank test, N = after (before outlier elimination)]

and a straighter and more continuous swimming path, and shorter periods of inactivity in the larval offspring of early emerging fish, than in larvae descending from later emerging ranks.

Consistency of behavioural parameters

To establish whether behavioural indicators of a coping style in the first generation were consistent over age classes, average baseline behavioural parameters of the larvae were plotted against the average behavioural parameters of the adult zebrafish during the open field test of the F1 generation. Here, swimming velocity (V, N=8, ρ =0.7904, p=0.0172, Fig. 5A), angular velocity (Ω , N=8, ρ =0.3333, p=0.0326, Fig. 5B), and immobile state (MS_i; N=8, ρ =0.8382, p=0.0093, Fig. 5C) showed significant correlations between larval and adult values and therefore confirming consistency over developmental stages.

Heritability of behavioural parameters

To establish the heritability of the behavioural indicators of a coping style, parent–offspring regressions were performed on the obtained parameters which were significantly correlated with the emergence rank of the parental (F0) generation.

In adult fish of the F0 and F1 generation, emergence time (ET) during a single emergence test could be correlated significantly (simple linear regression: y=0.49x+94, $r^2=0.4223$, p=0.042, N=10; Fig. 6A), and frequency of aggressive behaviour (AGRf; y=0.41x-0.61, r2=0.67, p=0.0115, N=10, Fig. 6B). Swimming velocity, even though correlating with emergence rank of the F0 generation, did not correlate between the F0 and F1 generation (Fig. 3H).

As in the adults, only a few parameters could be used to establish heritability in larval fish of different generations. A positive correlation was found between the angular velocity (Ω) of F1 and F2 larvae during the dark and the light phase of the LDC test, respectively (y=0.36x+15.1, r²=0.67, p=0.027, N=7, Fig. 6C), and between the immobile states during the dark phases (y=0.42x+5.89, r2=0.63, p=0.0323, N=7, Fig. 6D).

These results show a strong although selective heritability of behavioural parameters of coping styles.



Fig. 5 Consistency of behavioural parameters between larval and adult stage. F1 behavioural parameters obtained during an open field test combined with mirror image stimulation in adult fish are plotted as mean values per emergence ranks of the F0 (group emergence test) over mean values of parameters obtained during the Light Dark Challenge (LDC) test in larval (5dpf) fish of the same generation: swimming velocity (V; 4A, simple linear regression: y=0.68x+0.14, $r^2=0.60$), angular velocity (Ω ; 4B, y=0.63x+7.52, $r^2=0.64$), and state of immobility (MS_i; 4C, y=0.48x+23.00, $r^2=0.70$) yielded a significant correlation (Spearman rank test)



Fig. 6 Heritability of behavioural parameters. F1 plotted over F0 (**A**, **B**) and F2 plotted over F1 behavioural parameters (**C**, **D**), as mean values per emergence ranks of the F0 (group emergence test). **A** Emergence time (ET) obtained during a single emergence test of the F1 adults plotted over the ET of the F0 parental fish. **B** Aggressive behaviour frequency (AGRf) obtained during an open field test combined with mirror image stimulation of the F1 adults plotted over the AGRf of the F0 parental fish. **C** Angular velocity (Ω) of the light phase obtained during a Light Dark Challenge test of the F2 larvae (5dpf) plotted over the Ω of the dark phase of the F1 larvae. **D** Immobility state (MS₁) obtained during the dark phase of a Light Dark Challenge test of the F2 larvae (5dpf) plotted over the MS₁ of the F1 larvae. These parameters yielded a significant correlation (Spearman rank test; h² = heritability)

Discussion

The objective of this study was (i) to examine the risk taking based association between behavioural indicators of a stress-coping style in both larval (at 5 days post-fertilisation) and adult (>3 month post-fertilisation) zebrafish, and (ii) to estimate the consistency and (iii) heritability of these behavioural indicators. First, we established a risk taking hierarchy within a group of adult zebrafish by means of an emergence test [4, 14, 15, 17, 18] over two consecutive generations (F0 and F1). Subsequently, we exposed adults to a mirror image stimulation combined with an open field test. In larval fish of the F1 and

F2 generation we applied a light dark challenge (LDC), to elicit an anxiety-like response [38, 39], and measured locomotion parameters. For adult fish, the results show correlations between risk taking behaviour, locomotion and aggressiveness within the F0 parental generation. Risk taking behaviour of the F0 was also correlated with F1 risk taking, locomotion and aggressive behaviour. Furthermore, risk taking behaviour of the F0 was correlated with locomotion behaviour in larval fish exposed to an LDC. Additionally, three locomotion parameters, i.e. swimming velocity, mobility state and angular velocity, were correlated between larval and adult fish of the F1 generation. Finally, parent-offspring regressions showed a strong correlation between risk taking and aggressive behaviour of adult F0 and the F1 fish, and locomotion parameters of larval F1 and the F2 fish.

Risk taking as a consistent base for trait correlations within a behavioural syndrome

The basis for our study of consistency and heritability of correlated behavioural traits within a behavioural syndrome was the risk taking tendency of the F0 generation during a group emergence test. Risk taking was shown to be consistent over time and context [4, 14, 15, 17, 18]. The group and single emergence tests were previously shown to be separately consistent over time [15, 18]. Earlier we provided evidence for consistency over context, when we tested social zebrafish in a group and in an individual setting [15]. Risk taking was associated with aggressiveness, as previously demonstrated in various species [7, 11, 15, 22], and is considered highly adaptive [16, 31]. Additionally, the risk taking tendency is significantly correlated with a number of locomotor parameters, such as V and Ω [1-3]. Risk taking individuals show a higher aggressiveness, and swim generally faster and straighter and less erratic than risk avoiding individuals. The erratic swimming behaviour of risk avoiding fish on the other hand can be interpreted as an anxiety like state [4, 14, 15, 40-44]. These results confirm risk taking to be an integrative and highly predictive part of a behavioural syndrome [4, 14–16]. Here we confirm that risk taking is a strong and consistent basis for correlations with other behavioural traits within a behavioural syndrome.

Correlations of behavioural traits between developmental stages indicate consistency over time, a necessary condition for the classification of a behavioural syndrome or coping style [3, 4, 11, 14–16, 45]. Here, we correlated larval behavioural parameters collected during an LDC test with those collected in adult individuals during an open field test. Parameters correlated between developmental stages comprised locomotor activity and directionality. However, behavioural traits are often measured repeatedly over a short period of time, maximum a few days [46, 47]. However, in order to proof a consistency over time it would be necessary to repeat experiments over a longer period, possibly in different developmental stages [46, 48]. Here, we show that certain behavioural parameters are indeed not only repeatable over a longer period of time, i.e. ca 3 months, but also over different developmental stages.

F0 emergence rank as a predictor for behaviour in F1 adults and larvae

We found a correlation between the risk taking tendency of the F0 and various behavioural parameters of the subsequent F1 generation. However, this correlation was decreasingly consistent, indicated by generally reduced correlation coefficients of various locomotor parameters (Figs. 3 and 4). Swimming velocity, immobility state and aggressiveness were significantly correlated with the risk taking tendency of the F0 generation. A higher activity and aggressiveness in risk taking fish, and longer freezing bouts in risk avoiding fish when exposed to the open field have been previously described [4, 15, 49–51].

Larval fish of the F1 and F2 generation also showed consistent and correlated traits during the light dark challenge (LDC), within and between generations. Swimming velocity, acceleration, angular velocity and immobile state duration were correlated with the risk taking tendency of the F0 generation during the baseline and dark phase of the LDC. Interestingly, the light phase results were not dependent on the F0 risk taking tendency. Faught and Vijayan [52] have shown that especially the dark responsiveness during the LDC is modulated by the mineralocorticoid receptor (MR). The role of the MR in behavioural phenotypes, including stress coping styles, has been shown previously [53–56].

In the F2 larvae only immobility state duration and angular velocity were correlated with risk taking tendency of the F0 generation, and with a reduced correlation coefficient, indicating a reduced link between risk taking behaviour and other behavioural parameters over consecutive generations. All LDC parameters correlated with the F0 risk taking tendency showed clear differences between the challenge phases, except for Ω , being the only directionality related parameter tested. Rock et al. [37] showed a similar pattern: directionality seems to be linked to the movement quality of activity (e.g. erratic), rather than quantitative parameters such as velocity, and do not show the same clearly distinct pattern in phase difference (Additional files 1 and 2).

All significant correlations were supported by a linear regression $(y=ax+y_{int})$, in order to describe the interdependencies of variables and compare it with previous studies. However, a linear regression only produces straight lines, whereas Spearman rank correlations can include curved lines. A straight line may not be accurately representing the correlation assessed by the Spearman rank test and may result in lower-than-expected r-squared values. However, in order to be able to compare our results with previous studies we chose for this suboptimal solution.

Correlations of behavioural traits between generations indicate heritability of coping style

Our results showed that the mean of individual emergence time of the F0 generation correlated positively with the mean of the emergence time of the F1 generation during the single emergence test (Fig. 4A). Similarly, the means of the aggressiveness score during the mirror image stimulation was correlated between generations (Fig. 4B). This indicates heritability of risk taking behaviour and aggressiveness in adult fish. In larvae, the means of angular velocity and immobility state duration indicate heritability of locomotion directionality and freezing behaviour between the F1 and F2 generation (Fig. 4C, D). For emergence time the additive genetic variance accounted for almost 50% of the total phenotypic variance, for aggressiveness it accounted for 41%, for angular velocity it accounted for 36%, and for immobility state it accounted for 42%. These results indicate not only correlated behavioural parameters within a coping style, as previously shown [4, 15], but also trans-generational heritability of behavioural traits in zebrafish. Interestingly, these results appear when selecting for the ER of the parental F0 generation, indicating the strong link between risk taking tendencies and heritable parameters in up to two generations later. For logistic reasons the individuals used for behavioural testing were different from those used for producing the next generation but originated from the same batch of eggs. However, given the high genetic similarity between siblings from the same batch, we can assume that the conclusion is valid.

Since behavioural traits in zebrafish, like in other organisms, are influenced by both genetic and environmental factors, the extent to which genetic variation contributes to behavioural diversity remains a subject of interest in personality research. Especially quantitative genetic approaches, such as selective breeding [57, 58] shows strong heritability of zebrafish aggressiveness [57] and anxiety-related behaviour within the aggression risk taking syndrome [58]. This is also confirmed by Genome-wide association studies (GWAS) and candidate gene approaches in zebrafish [59].

Zebrafish larvae at 5 dpf are in an active developmental stage of life, and they can already make decisions with significant consequences for their fitness [14]. Therefore, coping styles may emerge from selection pressures on decisions made at an early life stage. The heritability estimates for risk taking (50%) and aggressiveness (41%) are representative in comparison with other vertebrates. In chimpanzees (*Pan troglodytes*), comparable heritability estimates of multiple behavioural indicators showed significant heritability for dominance and well-being with 66% and 40%, respectively [60]. Dingemanse et al. [61] reported significant heritability estimates, ranging from 22 to 37%, for exploratory behaviour in a wild population of great tits (*Parus major*). In another research on great tits, significant heritability estimates, ranging from 19 to 32%, were found for risk taking behaviour [62].

However, heritability estimates cannot be interpreted without taking effects of epigenetic processes and selection into account, since genes can interact through nonadditive genetic variance such as dominance and interactive variation [30, 62]. Additionally, genetic correlations can result from pleiotropic effects or linkage disequilibrium between different behavioural indicators [63]. Furthermore, environmental effects may also stabilise the heritability of a behavioural indicator, since phenotypic plasticity is thought to be a heritable indicator by itself and is stabilised by the environment [62]. In the laboratory however with little or no environmental fluctuations [5], heritability estimates can be lower than in wild populations of zebrafish.

Conclusions

In conclusion, the present study shows that (i) risk taking is the base for associated behavioural indicators within a stress-coping style in both larval (at 5 days post-fertilisation) and adult (>3 months post-fertilisation) zebrafish, and (ii) consistency and (iii) heritability of these behavioural indicators is given, as indicated by correlation within individuals, between generations, and between developmental stages, in both larval and adult zebrafish. Furthermore, these behavioural indicators contain heritable genetic components at least over two generations, and therefore enable populations of mixed coping styles to respond to selection.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12868-025-00944-w.

Additional file 1. Parameters for swimming behaviour during the light dark challenge test in F1 larvae descending from F0 fish of with different ER: A) Swimming velocity (V mm s⁻¹), B) acceleration (A mm⁻²), C) angular velocity (Ω , °s⁻¹) and D) immobile state (%) during the baseline (grey), the dark (black) and the light challenge phases of the test. (letters indicate significant differences, RM Two-way ANOVA with Geisser-Greenhouse corrections, Sidack's post hoc test, p < 0.05, N = 12).

Additional file 2. Parameters for swimming behaviour during the light dark challenge test in F2 larvae descending from F0 fish of with different ER: A) Swimming velocity (V mm s⁻¹), B) acceleration (A mm⁻²), C) angular velocity (Ω , °s⁻¹) and D) immobile state (%) during the baseline (grey), the dark (black) and the light challenge phases of the test. (letters indicate

significant differences, RM Two-way ANOVA with Geisser-Greenhouse corrections, Sidack's post hoc test, p < 0.05, N = 23).

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Author contributions

CT designed the study, conducted the experiments, analysed the results and wrote the paper. LK and TvdK conducted the experiments. MS designed the study and wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated during this study are included in this published article and its additional information files, and all materials generated during this study are available upon request.

Declarations

Ethics approval and consent to participate

Zebrafish were maintained and handled according to the guidelines from the Zebrafish Model Organism Database (ZFIN, http://zfin.org) and incompliance with the directives of the local animal welfare committee of Leiden University (DEC number 14058). Animals: no anaesthesia was used during handling and testing and animals were returned to home aquaria after tests were completed.

Consent for publication

Not applicable.

Clinical trial number

Not applicable.

Competing interests

The authors declare no competing interests.

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