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Development of aggressive phenotypes in zebrafish: interactions of age, experience and social status

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Aggression is important in the life history of most vertebrate species and influences ecological and social relationships by regulating the dynamics of territoriality, hierarchy, predation and resource utilization. The expression of aggressive behaviour develops as an individual ages and is often shaped by social experience. Even though the heritability of traits that yield dominant–subordinate relationships is high, strong environmental influences also shape the expression of these individual behaviours. In this study we present evidence for the first time of a necessary interplay between factors that generate behavioural phenotypic plasticity in zebrafish. Select presumptively genetic and environmental elements interacted developmentally to produce the behavioural phenotypes necessary for dominant–subordinate relationships in dyads. Aggressive and submissive acts increased in focus and intensity and were correlated with social rank during development, but decreased with duration of the paired interaction.

Social aggression influences ecological and conspecific relationships by regulating the dynamics of territoriality, hierarchy and resource utilization. Ontogenetic development of aggressive behavioural phenotypes, which contribute to social aggression in many vertebrate species, is not well understood, but certainly involves genetic, epigenetic, developmental, social and age-related phenomena. In addition, experience helps to mould the final phenotype. Increased aggression among adolescents and young adults is exacerbated by neurochemical and neuroendocrine manipulation (Melloni et al. 1997; Delville et al. 2003; Ricci et al. 2004). Early life stress during vertebrate development alters neurochemical and neuroendocrine function, contributing to cognitive impairment, social dysfunction, and anxious and depressive behaviour (Liu et al. 1997, 2000; Lukkes et al. 2009). While neurochemical changes throughout development certainly influence the expression of aggressive and submissive behaviour, little is known about how dominant–subordinate relationships develop ontogenetically. Multiple factors, including social experience, appear to be involved (Oliveira et al. 1998), as individual expressions of dominant or subordinate behaviour develop with age. Insults and injuries, whether exclusively environmental or internal, comprise observable, emotional and/or valence changes that affect behavioural coping styles (Koolhaas et al. 2007). Repeated physical defeat and conquest are critical variables. How other psychosocial and biological factors determine the ontogenetic progression of overtly aggressive or submissive behaviour is unknown. Here we show that the development of dominant–subordinate relationships in zebrafish is shaped via interactions between age, experience and social status, despite genetic predispositions.

Zebrafish are gregarious animals that show a variety of social behaviours, such as schooling, shoal choice, territoriality, kin recognition and social learning (Krause et al. 1999; Mann et al. 2003; Kato et al. 2004; Spence & Smith 2005; Trapani & Nicolson

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2010; Zala et al. 2012). Zebrafish establish dominant–subordinate relationships, show agonistic behaviour (Larson et al. 2006) and, like salmonid fish, show aggression in schools (Ryer & Olla 1991; Winberg & Nilsson 1993). Although previous studies of aggression in zebrafish have examined adult behavioural phenotypes, surprisingly none have detailed the development of aggressive behaviour (Basquill & Grant 1998; Pyron 2003). This is the first report of the ontogeny of aggression in zebrafish.

To examine the development of behavioural phenotypes over time, we chose a model organism, the zebrafish, *Danio rerio* (Grunwald & Eisen 2002; Spence et al. 2008). The zebrafish model combines an in-depth developmental history (Kimmel et al. 1995; Grunwald 1996) and a fully developed ethogram for complex behaviours, including agonistic interactions and social status establishment (Gerlai 2003; Blaser & Gerlai 2006). In addition, a completed genomic sequence for *D. rerio* offers a unique model species for examining the establishment of candidate genes likely to be expressed in complex behaviours. Zebrafish aggressively interact with their own mirror image and show place preference for nearby conspecific opponents (Gerlai et al. 2000; Gerlai 2003). In addition, naïve *D. rerio* pairs interact to form social rank hierarchies and stable dominant–subordinate relationships (Larson et al. 2006). Furthermore, the neurocircuitry underlying aggression, which controls socially agonistic and rank relationships in mammals and other vertebrates (Ferris et al. 1997; Nelson & Chiavegatto 2001; Summers et al. 2005b), is evolutionarily conserved in *D. rerio* (Larson et al. 2006; Summers & Winberg 2006). Given the ability of this model to produce an accurate representation of the ontogeny of dominant–subordinate relationships, we designed experiments to determine the temporal development of agonistic behavioural phenotypes.

METHODS

Zebrafish

Wild-type zebrafish, *D. rerio*, were obtained from a commercial supplier (Scientific Hatcheries, Huntington Beach, CA, U.S.A.). Fish were originally group housed in a 10-gallon (37.84-litre) tank prior to social interaction in pairs. Water was recirculating and contained 160 mg of Instant Ocean (http://www.instantocean.com) per litre and was maintained at 28 °C on a 14:10 h light:dark (LD) cycle. Fish were fed twice daily with flake food. Fish used in the developmental study were adult males (22 ± 3 mm standard length, SL), at least 12 weeks old, and several ages of juvenile fish reared from breeding in the original cohort: 2 weeks old (5 ± 1 mm SL), 4 weeks old (8 ± 2 mm SL), 6 weeks old (10 ± 2 mm SL), 8 weeks old (12 ± 2 mm SL) and 10 weeks old (15 ± 2 mm SL). Zebrafish do not sexually mature before 12 weeks of age. Juveniles were treated the same as adults with the exception of the 2-week-old larvae. Two-week-old larvae were reared in Hank's solution in 12-well culture plates in an incubator at 28 °C on an LD 14:10 h cycle. They were fed twice daily with paramecia and Hatchfry Encapsulation 0 and I (Argent Laboratories, Redmond, WA, U.S.A.). After 5 days of social interaction, we used mass (g) and standard length of dominant and subordinate fish to calculate a condition factor ($K = \text{mass}(100) \times \text{SL}^3$) typically used by fisheries biologists as an indicator of fish health. We used this measure to test the hypothesis that subordinate individuals experience negative health effects due to their social position. All experiments were conducted in accordance with ASAB/ABS Guidelines and the ethical standards established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), under approved protocols (031021R and 010513R) by the Institutional Animal Care and Use Committee of Northeastern University.

Social Interaction

We allowed pairs of sized-matched *D. rerio* to interact for 5 days and form dominant–subordinate relationships. This was done for six age classes: 2, 4, 6, 8, 10 and 12 weeks of age. All age classes had only generalized social interactions in group housing, except for 2-week-old larvae, which were isolated prior to behavioural experiments. Experiments for fish that were 4–12 weeks old were conducted in glass aquaria (5.5 gallon, 20.8 litre) divided into four compartments by removable PVC walls. Fish were transferred to the experimental tanks after a minimum of 5 days of acclimation. Each fish was measured and size-matched with a counterpart that differed by no more than 1 mm. Animals were held individually for 5 days separated by the PVC walls. To keep the size of the behavioural arena similar for fish of different ages, we varied the volume of water in the aquarium depending on the age of the fish, so that smaller animals had smaller arenas (adult 12-week-old fish: ca. 5.2 litres of water/pair; 10-week-old fish: 4.6 litres; 8-week-old fish: 4.4 litres; 6-week-old fish: 3.8 litres; 4-week-old fish: 2.2 litres). After 5 days of isolation, we removed the walls separating the two fish and allowed the individuals to interact for 5 days ($N = 10$ pairs) to establish a dominant–subordinate relationship. Two-week-old larvae were reared and allowed to interact in 12-well culture plates. The volume of water was approximately 7.5 ml. We videotaped behavioural interactions of each pair on the first and last day of interaction, and thus after each fish had experienced 1 day and 5 days of aggressive interaction with a conspecific. Culture plates containing larvae were placed on a light box and filmed from above with a video camera.

Behavioural Analyses

We scored videotapes of the behavioural trials by first analysing day 5 (when social rank was obvious) and then day 1. The analysis was randomized with respect to the order of the pairs, and the videotape was slowed to half speed to allow for accurate identification of individual fish. Subordinate animals are pale in colour and occupied a relatively stationary position in the corner of the tank. Dominant animals have normal coloration and patrolled the behavioural arena. By analysing day 5 initially, it was possible to retroactively indicate which animal was the future dominant or subordinate individual on day 1. Analysis of day 1 was based on future dominant or future subordinate status. Chases, bites and retreats were recorded.

Statistical Analyses

We analysed aggressive behaviour of zebrafish using three-way (age × experience × status) ANOVA and two-way ANOVA with repeated measures. Effects of age, experience and status were further examined by two-way and one-way ANOVA, followed by Bonferroni's post hoc tests. Paired comparisons were made using Student's *t* test. Age × aggression interactions were analysed relative to social status and experience using linear regression.

RESULTS

Although aggressive behaviours were absent in larval stages, the resulting interactions defined a behavioural phenotype in which aggression increased with age, became dependent upon rank and was shaped by social interaction ($F_{5,122} = 3.62$, $P < 0.01$). Repeated exposure to dominant–submissive interactions did not adversely affect 4- or 6-week-old fish, as evidenced by condition factor data (i.e. Student's t test: 4 weeks: $t_{12} = 0.47$, $P > 0.65$; 6 weeks: $t_{20} = 0.95$, $P > 0.37$). However, older submissive animals (8–12