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Subcutaneous dye injection for marking and identification of individual adult zebrafish (*Danio rerio*) in behavioral studies

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Abstract The zebrafish is increasingly utilized in behavioral brain research, as it offers a useful compromise between system complexity and practical simplicity. However, a potential drawback of this species in behavioral research is that individuals are difficult to distinguish. Here we describe a simple marking procedure, subcutaneous injection of color dyes, that may alleviate this problem. The procedure allowed us to successfully mark zebrafish and distinguish them for a period of more than 30 days, which is sufficiently long for most behavioral paradigms developed for this species. In addition, we also provide data suggesting that the injection-based marking does not significantly alter social interaction, as defined by the frequency of agonistic behaviors within shoals.

Keywords Zebrafish · Individual identification · Individual marking · Social interaction · Aggression

The zebrafish is a small (4-cm long) tropical freshwater fish from South East Asia that has been popular in the aquarium trade for a long time. This popularity is partially due to the fact that the zebrafish is easy to keep and breed. It is a prolific species that can be kept in large groups in small tanks due to its social nature. These same features have made the zebrafish useful in developmental biology and genetics, fields that have utilized the zebrafish for over four decades (Chen & Ekker, 2004; Patton & Zon, 2001). More recently, the zebrafish has started to gain popularity in behavioral brain research (Gerlai, 2010; Sison, Cawker, Buske, & Gerlai, 2006). The use of behavioral analysis coupled with genetics techniques may allow an investigator to discover fundamental characteristics of vertebrate brain function, and perhaps also to model certain human brain disorders in an efficient way (Gerlai, 2010).

Numerous behavioral paradigms have already been developed for zebrafish. However, a simple procedure—the marking and identification of individuals, which is routinely utilized in laboratory rodents—has remained problematic. Zebrafish do not exhibit marked individual differences in body shape, color, or pattern, and thus even for the trained eye they appear uniform. The identification of individuals is crucial in numerous behavioral paradigms, including learning and memory tasks, in which repeated exposure of individuals to the training session and quantification of temporal changes in the performance of each individual may be required. Similarly, the analysis of social interactions—for example, schooling, shoaling, or agonistic responses—would be greatly enhanced if one could identify the interacting subjects individually. Finally, individual differences, which have been well documented in numerous species, including fish (Warren & Callaghan, 1975), could also be investigated. These are but a few areas of research in which the identification of individuals could be important. Although marking or tagging larger-bodied fish have been successfully accomplished (Dunn & Coker, 1951), a paucity of procedures are available that would allow the investigator to mark and distinguish individuals of such small species as the zebrafish. For example, scale-regeneration-based (Sire, Girondot, & Babiar, 2000) or fin-clipping (Saverino & Gerlai, 2008) marking methods have been proposed, but these marks are difficult to see or detect using cameras.

The present study describes a simple marking method that may address the problems above. We utilized subcutaneous injection of dyes to mark the fish. After experimenting with a number of different dyes, we selected a particular set of color dyes originally developed for tissue marking. Below, we describe our methods in detail. In addition, we also provide

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a small data set that investigates whether the injection procedure and the color marking of the fish would alter the social behavior—that is, agonistic interactions among the subjects in a free-swimming shoaling task.

Method

Animals and housing

To enhance our ability to observe the injected dye, we decided to use a color-deficient zebrafish population. A number of color-deficient zebrafish strains are available. We obtained our fish from a local pet store (Big Al's Aquarium Warehouse, Mississauga, Ontario, Canada). These fish, called "gold" in the pet trade, are from a random-bred, genetically heterogeneous, and genetically uncharacterized stock that exhibits reduced expression of the green and blue pigments typical of wild-type zebrafish, hence the appearance of the "gold" phenotype (Fig. 1a). The fish were 6-month-old young adults at the time of the experiments. They were housed in 36-L tanks (27 × 30 × 50 cm, width × height × length) with a density of 30 fish per tank. The water of these tanks was obtained using reverse osmosis (R/O). The R/O-purified water was supplemented with salt (60 mg/L Instant Ocean Sea Salt [Big Al's Pet Store, Mississauga, Ontario, Canada]). This system water was maintained at 26.5 °C using thermostat-controlled aquarium heaters (Tetra Corporation, Melle Germany). Filtration in the holding tank was provided by overhang filters (Marineland Penguin Power Filter, Model 100B [Big Al's Pet Store, Mississauga, Ontario, Canada]) that had a three-stage filtration system (mechanical sponge filter, chemical activated carbon filter, and rotating high-surface area wet–dry bacterial drum). All fish were fed three times a day with a mixture of dry food composed of 50 % Tetra-min flake (Tetra Corp., Melle, Germany) and 50 % spirulina flake (Jemco, USA). In addition, the fish were also fed live nauplii of brine shrimp (*Artemia salina*) once a week. The light cycle was maintained at 12 L:12D, with lights turned on at 7:00 h.

Dye injection procedure

All together, 105 "gold" zebrafish were injected. A subset of these fish were later tested in a simple behavioral task (see below), and the rest of the fish were tested in a separate study (not shown). The individual to be injected was first placed into a 1-L beaker containing a 0.0002 % methylene blue solution in 1 L of system water for 1 min. Methylene blue is bacteriostatic and antifungal, and this preoperative procedure was implemented to minimize the risk of infections. Subsequently, the fish were transferred to another 1-L beaker containing 100-ppm clove oil (Eugenol) in 1 L of system water. Clove oil has been shown to be one of the safest and most effective anesthetic agents for zebrafish (Grush, Noakes, & Moccia, 2004). Upon cessation of locomotory responses, the fish was removed from the beaker and was injected with the dye in the following manner. A 10-μL bevel-tipped Hamilton microsyringe was filled with 4 μL of dye prior to anesthesia. A Styrofoam board was also prepared. A 3-cm-long incision with a transverse profile of a V was cut into the board, and the cut was filled

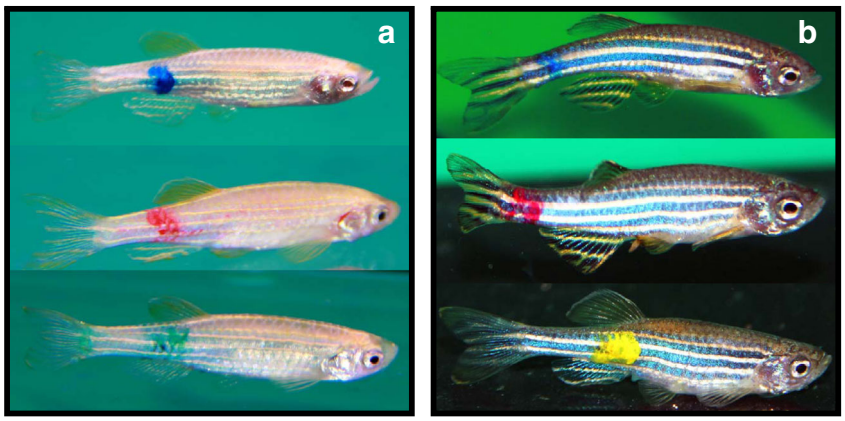

Fig. 1 **a** Representative examples of pigment-deficient zebrafish injected with color dyes (*blue*, *red*, and *green* are shown) can easily be identified and distinguished from each other. But note that the green-dye-injected fish (bottom photograph) may pose a problem if this fish is viewed from a greater distance against the green background, or if the marking is to be detected by a video-tracking system. The pigment-deficient dye-injected fish were further characterized for temporal stability of the marking and behavioral effects of the injection (see the Results section and subsequent figures). **b** In addition, a limited number of wild-type-colored zebrafish were also injected, to see whether the color marking would be visible enough against their darker body coloration. This panel shows three representative examples of these fish, one apiece with *blue*, *red*, and *yellow* dye markings. The photos demonstrate that even these wild-type colored fish can be distinguished on the basis of the color marking. But note that the smaller area covered by the blue dye (top photograph) may represent a problem if this fish were viewed in a larger tank from a greater distance, and that this could be a limitation in the case of video-playback or automated video-tracking methods. All fish were photographed in a thin (30 × 10 × 5 cm, length × height × width) acrylic tank with a black bottom and green background illuminated by a 15-W fluorescent light tube from above

with system water. The anesthetized zebrafish was placed on its side into the V-shaped incision. Then, 4 μL of a particular color dye was injected immediately with the prefilled Hamilton microsyringe, holding the syringe at an angle of 15–20 deg to the horizontal plane. The subcutaneous injections were made near the tail—that is, approximately 5 mm rostral from the caudal peduncle. This location was chosen as it was sufficiently far away from the abdominal region of the fish, and thus represented a less sensitive area. The needle was inserted 2 mm underneath the skin. Dye was slowly pushed in between the skin and muscle with gradual withdrawal of the syringe, to allow the dye to spread throughout the injected area. The injection was performed bilaterally. Immediately after injection, the fish was placed into a 3-L recovery tank containing system water. After a 40-min recovery period, fish were transferred to their 36-L holding tank with a final fish density of 12 per tank.

Five different dye colors were used: red, green, black, yellow, and blue. The dyes were obtained from Sigma-Aldrich (Marking Dyes for Tissue, 5 Color Kit, Cat # MDT 100-1KT). One representative individual injected with the red dye (medium saturation, as compared to the other dyes) was photographed once every week for four weeks—that is, on Days 0, 7, 14, 21, and 28 postinjection—to determine the stability of coloration. The digital photos were saved in JPEG format, and the images were analyzed in "Image pro Plus" software using the "Histogram analysis" program. The size of the image was standardized so that it was made identical to the actual fish size. A 7 × 7 mm rectangle was created surrounding the color-injected area where the intensity of the red color was measured. The color density (saturation score, in arbitrary units) and also the size of the colored area (number of pixels with red color) were calculated by the program. Control fish were chosen randomly from the same pool of fish as the injected fish, but they received no anesthesia or injection procedure. We chose not to use sham injected fish as a control because our pilot data indicated that no injection procedure induced behavioral changes, and we wanted to compare the effects of the entire injection procedure, including handling and anesthesia, relative to completely naïve, nontreated animals.

The advantage of using the pigment-deficient "gold" zebrafish is that against this body color, the dye-injected area stands out. However, numerous zebrafish strains and mutant lines do not exhibit pigment deficiency but show the normal wild-type body coloration, which is substantially darker (blue and yellow horizontal stripes on the side of the fish and an olive brown dorsal area). To explore whether the dye would be observable on this body color, we injected wild-type short-fin zebrafish, a population that we had established from founders obtained from a local pet store 6 years ago (Big Al's Aquarium Services, Mississauga, Ontario, Canada). We injected these fish as described above with the yellow, the blue, or the red dye and took photographs of representative fish 2 days postinjection.

Quantification of behavior

We monitored the injected and control "gold" zebrafish daily for 7 days after the injection and recorded mortality and any observable behavioral abnormality. Subsequently, 32 injected and 32 control fish were selected at random from the pool of fish housed in our vivarium for a simple observation-based behavioral study. For a spectrum of behavioral analyses, we and others routinely use 10–15 fish per treatment condition, a sample size that has been large enough to detect significant effects of a range of treatments, including the effects of acute and chronic alcohol treatment (Gerlai, Chatterjee, Pereira, Sawashima, & Krishnannair, 2009), effects of the presentation of live conspecific stimulus fish (Saverino & Gerlai, 2008) or of images of conspecifics (Saif, Chatterjee, Buske, & Gerlai, 2013), and effects of fear-inducing stimuli, including the zebrafish's natural alarm substance (Speedie & Gerlai, 2008) or the sight of sympatric piscivorous fish (Bass & Gerlai, 2008), to mention but a few treatment conditions. Although in the present study the number of individuals tested was considerably larger than the customary sample size, the unit of statistical analysis was not the individual but the shoal; that is, we had n = 8 shoals (each containing four fish) per treatment condition. The shoal, and not the individual, was regarded as the unit of analysis because the behavior of individuals within a shoal is expected to be dependent on the behavior of other shoal members. Naturally occurring short-time-scale (Miller & Gerlai, 2008), as well as environmental stimulation induced longer-time-scale (Miller & Gerlai, 2007), shoal cohesion changes have been detected using n = 3–8 zebrafish shoals per treatment condition. Significant developmental changes in the shoaling of zebrafish (Buske & Gerlai, 2011a) and alterations in the developmental change of shoaling induced by embryonic ethanol exposure (Buske & Gerlai, 2011b) were also detected using comparable sample sizes (n = 8 shoals). Finally, significant effects of alcohol as well as of nicotine could be demonstrated using n = 8 shoals per treatment condition in zebrafish (Miller, Greene, Dydynski, & Gerlai, 2012). The number of shoals employed in the present study (n = 8 per treatment condition) was comparable to the sample sizes used in previous studies, and thus we expected to have sufficient power to uncover behavioral changes potentially induced by the marking procedure.

We subjected the selected fish to a shoaling test in which four individuals of the same treatment group were allowed to freely swim and interact with each other. The goal of this was to observe whether injected versus control fish exhibited different levels of aggression. For this observation, a group of four fish was removed from their holding tank and was placed in a 30-L testing tank (21 × 34 × 41 cm, width × height × length). The back and sides of the testing tank were covered with corrugated white plastic boards in order to increase contrast and to obscure external and potentially disturbing

stimuli. Three realistic plastic plants were placed at the right side of the tank to create a "desirable zone," which is believed to enhance agonistic responses (territoriality) among zebrafish (Paull et al., 2010). After a 15-min acclimation period, the fish were recorded for 10 min from a frontal view with a Panasonic Everio SDR-S50 HD video camera. Each shoal was tested once. The sequence of testing of the shoals was randomized according to injection treatment. The video-recordings were later replayed, and the numbers of agonistic behavioral events were quantified using the Observer event-recording software (Noldus Info Tech, Wageningen, The Netherlands).

The following behaviors were considered agonistic (for detailed definitions, see Paull et al., 2010). *Chasing* was defined as fast swimming while pursuing another fish. In addition to the aggression component, this behavior may also allow us to judge motor function (ability to swim fast). *Aggressive display* was defined as opening the dorsal anal and caudal fins. This display is not easy to observe in the small zebrafish but usually accompanies two separate motor patterns, one of which was termed *repel* by Paull et al. Repel occurs when the focal fish moves toward an approaching fish, forcing it to make a rapid direction change. The second motor pattern during which aggressive display occurs was defined by Paull et al. as *sparring*. This occurs when two opponents circle each other while undulating their bodies and erecting the fins. Sparring and repelling are pooled under the common terms *aggressive display* or *displaying* in the present study. The last behavior that we recorded and analyzed was *biting*. Biting occurs when the fish opens its mouth and bites into the fin or body of the opponent.

Statistical analysis

The frequency (number of occurrences) of the above-defined agonistic responses was recorded for each shoal member, and the behavior of the shoal as a unit was analyzed (the data of individual fish were pooled for each shoal). This analysis was carried out with SPSS 14 written for the PC. Independent-sample two-tailed t tests were performed to compare the injected versus control shoals. Statistical significance was accepted when the probability (p) of the null hypothesis of "no difference between injected and control fish" was less then 5 %.

Results

Fish recovered from full anesthesia within 3–4 min following immersion into system water, as indicated by reinstated motor activity. Within 1 h of injection, all fish recovered fully and swam actively in their holding tanks. The survival rate was high (96 %), with only four of the total of 105 injected fish dying after the injection procedure. The mortality may have been due to inappropriate injection (damage induced by the needle) and/or to the natural variability in the health status of the subjects.

The injected color markings were clearly visible during behavioral observations through the video recordings, as well as on the photographs of the pigment-deficient "gold" variety of zebrafish (Fig. 1a). A subsequent follow-up study confirmed that in the few wild-type-colored zebrafish that were injected, the dye injection marking was also visible and allowed for easy discrimination of the individuals (Fig. 1b). In the pigment-deficient "gold" zebrafish, the color marking was retained for a minimum of 30 days postinjection, with the longest retention time being observed at 53 days postinjection. Image analysis of a red-color-injected "gold" zebrafish (Fig. 2) also showed the coloration to be present and detectable over the 28-day period analyzed. Although both the size of the red-colored area (Fig. 2a) and the saturation score (Fig. 2b) diminished with time, even after 28 days the values were above background (zero).

The frequencies of agonistic behaviors (Fig. 3) did not appear to be different between the injected and control fish. A series of t tests showed no significant differences in the frequencies of chasing [$t(14) = 0.221$, $p > .80$], displaying [$t(14) = -0.130$, $p > .85$], or biting [$t(14) = 0.00$, $p = 1.00$].

Discussion

Especially in behavioral studies, it is crucial to minimize invasive procedures and to make sure that the subject does not experience pain, discomfort, or fear, which could all interfere with the subsequent performance of the subject. The zebrafish may not appear to be particularly amenable to procedures that involve human handling. Removal of the fish from the water itself is not only stressful for the animal but also exposes the fish to potential pathogens, because the skin and the gills are not adapted to the dry environment. Nevertheless, even larval zebrafish have been successfully utilized in a number of invasive studies that have involved the injection of substances (Bill, Petzold, Clark, Schimmenti, & Ekker, 2009). In the present study, too, with some practice and care, an excellent (very low) mortality rate was attained when using the dye-injection-based marking procedure. Also notably, while being able to visually detect the presence of the injected dye over a prolonged period of time (a month), we saw no signs of infections or skin irritation in the injected subjects. We argue that our injection procedure is safe and effective and will be useful in studies that require identification of individual zebrafish.

Nevertheless, the present study had several limitations. First, we utilized pigment-deficient fish to enhance our ability to detect the color marking. Although both genetically well-defined and genetically heterogeneous aquarium trade

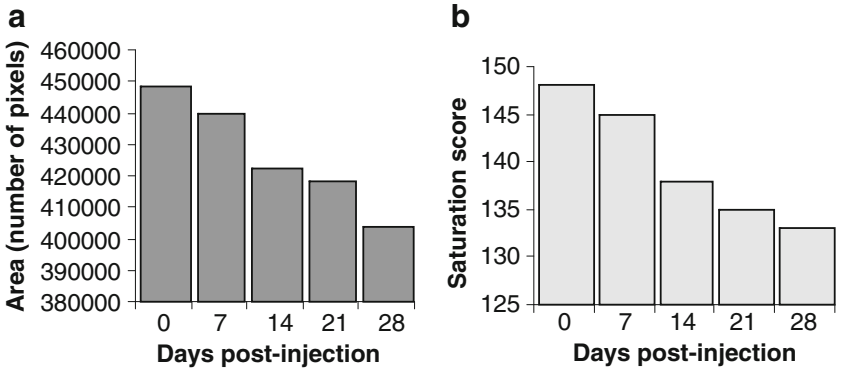

Fig. 2 **a** Temporal change of the size of the colored area for a single representative pigment-deficient zebrafish injected with the red dye, and **b** temporal change in the saturation (strength of coloration) of the red-colored area. Note that both measures suggest fading of the marking with time, but also note that the dye is still observable even at 28 days postinjection

zebrafish strains and populations exist that are pigment deficient, not being able to utilize fish of wild-type coloration might be a significant limitation. Using a limited sample, we attempted to mark fish of wild-type coloration (fish with horizontal stripes). Several genetically well-defined zebrafish strains exhibit this wild-type color and pattern. We have been able to clearly distinguish wild-type fish marked with all of the dyes used (blue, red, and yellow shown), but notably, the green and blue color marks were less obvious, especially when a smaller area was marked, against the naturally bluish-greenish body color of the fish. Thus, we argue that especially with the use of black, yellow, or red dyes, the dye injection-based marking method will be useful not only for pigment-deficient fish but also for regular wild-type fish. Although the markings on the pigment-deficient fish were clearly visible to the human observer using video playback even after a month, an important question concerning the utility of this marking method remains unanswered: whether the dye-injected fish could be distinguished not only by the naked eye but also using commercially available video-tracking systems. This question will be addressed in the future.

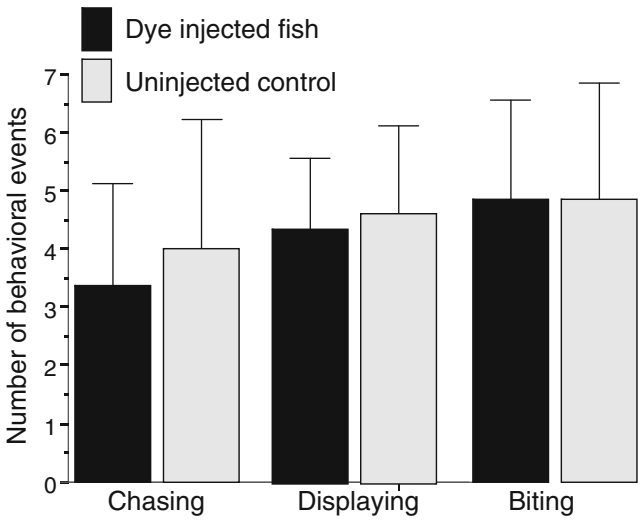

Fig. 3 Injection of color dyes does not significantly alter the frequency of agonistic (aggressive) behaviors observed within four-member shoals. The frequencies of biting, chasing, and displaying are shown. Bars represent the means, and the error bars represent *SEM*s. The sample size was $n = 8$ for each treatment group, which represents the number of four-member shoals tested. Note that no significant difference was found between dye-injected (*black bars*) and uninjected control fish (*light gray bars*) in any of the behavioral measures

Although the questions above require further studies, we argue that the dye-injection-based marking method does have some advantages over other currently existing methods, at least as far as zebrafish research is concerned. We and others have successfully employed marking fish by clipping their fins (e.g., Saverino & Gerlai, 2008, and references therein). Although this method is simple to employ, it suffers from two problems. One, the marks are difficult to see. We could only distinguish two groups of fish, the fin clipped and the nonclipped, but could not differentiate fin-clipped individuals reliably from each other because the fins of zebrafish are small and lightly colored (almost transparent) (Saverino & Gerlai, 2008). Two, clipped fins may interfere with the ability of the fish to swim normally, which may significantly influence several aspects of their observable behavior, including how the fish interact with each other. Other well-established marking methods utilize injectable elastomer implants, attached filaments, or wire tags (Bailey, Irvine, Dalziel, & Nelson, 1998). These methods work well with larger species of fish, for which the size of the implant or tag is small relative to that of the marked fish. However, the 3- to 4-cm-long zebrafish may be too small for the application of these implants or tags; that is, a tag large enough to allow reliable individual identification via video playback may significantly interfere with the motor function of the fish. We suggest that the dye-injection-based marking method is less likely to interfere with the normal behavior of the marked fish, and thus may be a superior method for individual identification.

We base this suggestion on our observation of a lack of gross behavioral abnormalities seen in the dye-injected fish. We observed no motor dysfunction or abnormal postures among the surviving injected fish. The analysis of agonistic behavioral responses within the four-member shoals that we studied also supported this conclusion: The numbers of agonistic behaviors, including chasing, displaying, and biting, did

not differ significantly between injected and control fish. The number of chase episodes, which represents bouts of fast swimming, also suggested that the activity level—that is, the ability of the fish to swim fast—was not grossly altered by the injection procedure and/or the presence of the dye. Therefore, we conclude that the presence of the dye under the skin, the invasive injection procedure, and the anesthesia and human handling that preceded the anesthesia did not interfere with the level of aggression and the ability to exhibit aggressive behaviors in the injected fish, as compared to controls.

Given the focus of this study on the marking method itself, we did not explore several potentially important questions, including whether zebrafish themselves may be able to distinguish each other on the basis of the color mark, and also whether video-tracking systems could distinguish among the color-marked fish in an automated manner. However, because of the simplicity and effective nature of the marking method described above, we hope that now answering such questions will be feasible.

Author note The first two authors contributed equally to the study.

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