The Tagging Procedure of Visible Implant Elastomers Influences Zebrafish Individual and Social Behavior

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Abstract

One commonly used method to preserve individual identity in the study of social behavior of zebrafish is through silicone-based visible implant elastomers (VIEs), which represent a safe and durable tagging procedure. While the effects of VIE tagging on welfare and general health have been addressed in detail, whether this procedure influences social behavior remains unclear. In this study, we compared individual and group behaviors exhibited by shoals composed of three individuals: two nontagged and one (focal subject) that was either nontagged (control condition) or sham-, purple-, blue-, or yellow tagged. Traditional behavioral parameters of activity, shoaling, and schooling (speed, polarization, and interindividual distances), along with an information-theoretic measure of social interaction (transfer entropy), were used to study the effect of tagging. Our findings indicate that tagging procedure *per se* significantly increased individual speed of the tagged subjects and of the group. The tagging procedure also altered the level of interaction between individuals, measured by transfer entropy. Conversely, tagging procedure did not influence shoaling and schooling tendencies. These findings suggest that VIE tagging may elicit some level of stress, which may affect some behavioral responses more than others. We recommend use of alternative methods such as multitasking systems when possible.

Keywords: *Danio rerio*, identification, social behavior, tagging, visible implant elastomer

Introduction

WITH THE GROWING USE of zebrafish (*Danio rerio*) in preclinical studies,^{1–4} the experimental procedures in which they are involved have become increasingly complex. Originally presented as the species of choice in developmental biology, zebrafish have now become a central tool in behavioral neuroscience.⁵ In this vein, the richness of their behavioral repertoire is expanding the fields of investigation in which zebrafish are currently used. Such use extends beyond their original use that relied upon the fully sequenced genome⁶ and availability of sophisticated genetic engineering tools.⁷

Social behavior is an important aspect of the zebrafish ethogram.^{5,8,9} Thus, together with the development of new experimental paradigms, it has become necessary to identify individuals swimming in a shoal and maintain such identity throughout the duration of the experiment (be it minutes, hours, or days). In this framework, several tagging procedures have been developed, with the aim of keeping track of

individual fish identities during experiments. These methods range from external tags like spine bands¹⁰ to scale regeneration,¹¹ radio frequency identification technology,¹² injection of colors,¹³ fin clipping,¹⁴ and visible implant elastomers (VIEs).¹⁵ VIEs, based on a series of considerations encompassing durability of the marking, costs, and ethical treatment of experimental subjects,¹⁶ appear to represent the best available option when it comes to tagging laboratory zebrafish. VIE tagging procedure is relatively inexpensive, stable in time, and does not influence zebrafish survival or well-being.^{16–18}

VIE tags are obtained through the injection of a liquid silicone-based material, which is then incorporated in the form of an elastic biocompatible solid.¹⁹ This tagging procedure has been repeatedly reported to beget major advantages in terms of feasibility and durability and, in light of its minimal side effects, is becoming a standard in fish experiments.^{12,20–25}

Notwithstanding its safety, this procedure—developed with the aim of assisting behavioral studies—needs to be systematically assessed to evaluate whether it affects social

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behavior.²⁶ The potential confounds introduced by VIE tagging may take different forms, ranging from simple locomotor effects (the implant may interfere with natural patterns of locomotion)²⁷ to more complex social interactions, where shoal mates may have a differential appraisal of a color-marked individual compared to a naturally colored conspecific.²⁸

Since social behavior of adult zebrafish rests largely upon visual cues,^{29,30} any alteration of visual appearance could substantially bias social behavior. Such alterations may be dictated by different mechanisms and take different forms: for example, a chromatic change diverging from zebrafish reaction norms may induce an “oddity effect” and ultimately result in being repulsive.³¹ In addition, chromatic variations may signal the presence of ectoparasites, thereby eliciting avoidance.^{32,33} Ultimately, specific magnifications in color density may even constitute super-stimuli to which other zebrafish may display increased attraction.³⁴

These concerns constituted the grounding upon which Frommen *et al.* evaluated the preference of individual zebrafish for groups of VIE-tagged conspecifics.²⁸ The authors performed a series of binary choice tests in which one individual was allowed to exhibit a position preference for a shoal of VIE-tagged individuals versus a sham-tagged shoal. Although the authors did not report significant effects of the specific color, they observed that focal subjects preferred VIE-tagged compared to nontagged conspecifics.²⁸ This study thus points at the potential presence of a methodological issue in the use of VIE tagging to study social behavior.

To further investigate this potential limitation, we conducted a systematic experiment that extends the aforementioned study²⁸ along the following research directions. First, we allowed direct interaction between (sham- and color-) VIE-tagged and nontagged conspecifics swimming in the same tank without physical partitions. Second, we broadened the observed ethogram to encompass measures of swimming activity (average speed⁸), shoaling (average nearest neighbor distances³⁵), schooling (polarization—the degree of alignment in the swimming direction of the individuals³⁵), and interactions among fish in the form of anticipatory swimming behavior of some individuals versus others (transfer entropy in pairwise interactions between individuals^{36,37}).

These heuristic improvements were afforded by a dedicated multitarget tracking platform (constituted by an overhead camera, feeding a computer with a custom-made software) developed by our group to simultaneously score the behavior of multiple individuals swimming together, while retaining their identities.³⁸ This platform has already been leveraged in pharmacological studies to elucidate the effect of acute ethanol administration on individual and social behavior.³⁹ Ladu *et al.*³⁹ observed that an individual exposed to ethanol is able to influence the social behavior of four untreated subjects. In addition, it was demonstrated that the presence of a social environment interferes with the effect of alcohol on individual behavior. Just as the ability to preserve individual identities was central in our previous pharmacological work, we expected this methodological tool to support significant knowledge advancements in the study of VIE tagging.

Based on the aforementioned literature, we tested whether tagging procedure (sham-tagged) and different tagging colors (purple, blue, and yellow) altered individual and group behavior in adult zebrafish. To address this goal, we scored groups of three individuals (one focal and two non tagged

conspecifics) swimming together in a shallow water tank. From an overhead camera, we recorded individual trajectories of all the individuals and computed salient measures of individual and social behavior.

Materials and Methods

Ethical note

The experimental procedure was performed in accordance with relevant guidelines and regulations and was approved by the University Animal Welfare Committee (UAWC) of New York University under protocol number 13-1424. Both the housing and the experimental procedure were designed to minimize stress in the tested animals. All experiments were performed at the New York University Tandon School of Engineering in Brooklyn, NY.

Animal care and maintenance

A total of 150 adult zebrafish (*D. rerio*), with a 1:1 male/female ratio and an average body length of ~3 cm (see Data analysis section for details), were used in this study. The fish were purchased from Carolina Biological Supply Co. (Burlington, NC) and housed in 37.5 L (10 gallons) vivarium tanks (Pentair Aquatic Eco-Systems Locations, Cary, NC), with a density of no more than 10 fish per tank. Fish were kept under a 12-h light/12-h dark photoperiod⁴⁰ and fed with commercial flake food (Nutrafin max; Hagen Corp., Mansfield, MA) once a day approximately at 7 PM. Water parameters of the holding tanks were regularly checked, and temperature and pH were maintained at 26°C and 7.2 pH, respectively. Before the beginning of the experiments, fish were acclimatized in the holding facility for a period of 12–15 days.

VIE tagging procedure

The injection kits for VIE tags were purchased from Northwest Marine Technology (NMT), Inc. (Shaw Island, WA). The elastomer was prepared following the instructions for tagging procedure available on the NMT, Inc. website.⁴¹ Briefly, the elastomer is a two-part silicon-based material composed by a colored part and a curing agent; it was mixed in a proportion of 10:1 right before injection since it completely cured within an hour.

Then, ~100 μ L of the mixture was loaded in a syringe (0.3 mL, NMT, Inc.) for the injection. Before the injection, fish were anesthetized with the aim of preventing them from twitching upon the insertion of the needle and avoid wounds other than those caused by the needle. The procedure for anesthesia consisted in placing the fish inside a 1.5 L polypropylene tank, containing a solution of water and tricaine methanesulfonate (MS-222; Western Chemical, Inc., now Syndel USA, Ferndale, WA). The concentration used for the anesthetic solution was 168 mg/L of MS-222.⁴²

Temperature and pH of the solution were kept at the same levels as the water in the holding tanks. Specifically, the temperature was the same as that of the housing tank through a heater, and reductions in pH caused by the addition of MS-222 were counteracted by adding sodium bicarbonate (NaHCO₃; Sigma-Aldrich, St. Louis, MO) as buffer in the anesthesia solution. The effectiveness of the anesthesia was evaluated by observing loss of equilibrium and muscular

tone, decrease of opercular movements, absence of reaction to external stimuli, and loss of all reflex reactivity.

The anesthetized fish was then removed from the solution with a hand-net and placed on a V-shape carved polystyrene surface, such that fish had the dorsal area upwards. The VIE was then injected dorsally close to the head (Fig. 1). The needle was inserted just under the first layer of the skin, paying attention not to perforate the underlying tissues. Once the needle was inserted for ~ 5 mm, the syringe was slowly retracted while the elastomer was injected in the space left by the needle. The injection of the elastomer stopped ~ 2 mm before the full expulsion of the needle from the wound, avoiding overflow of the elastomer and allowing proper wound healing.

At the end of the procedure, the fish was carefully transferred with a hand-net into a different 1.5 L polypropylene recovery tank with system water. Once the fish fully recovered from the anesthesia, it was transferred back to a dedicated new housing tank. Tagged zebrafish were housed in separated tanks and grouped according to the color of their tag.

The postoperative care of the fish consisted of 24–48 h of monitoring. After the tagging procedure, fish were allowed to rest in their holding tank for at least 12 days, to achieve complete healing of the wound and recover from the stress of the operation before experiments.

Experimental setup

The experimental setup consisted of a circular arena (45 cm in diameter and 15 cm in height)—made of aluminum foils (McMaster Corp., Waltham, MA) and covered with white contact paper—positioned in the center of a square tank (120 $\times$ 120 $\times$ 30 cm, length, width, and depth). The water depth was kept at 12 cm to minimize vertical motions of the fish. A webcam (Logitech C920, Newark, CA) and a high-

resolution camera (Flea3 USB 3.0; Point Grey Research, Inc., Richmond, Canada) were mounted on an aluminum frame (McMaster Corp.) 90 cm above the water surface, to record fish behavior during habituation to the apparatus and testing (see Experimental procedure section for details). Four 25-W fluorescent lights (All-Glass Aquarium, Franklin, WI) were installed on the aluminum frame, 150 cm above ground, to ensure uniform lighting of ~ 120 lux on the water surface (Fig. 2). Black curtains surrounded the tank on all four sides to isolate the setup from visual disturbance from the environment.

Experimental procedure

The experiments were performed in August 2017. The study comprised five experimental conditions. We performed a control condition, involving groups of three fish that were not subjected to the tagging procedure. To investigate the effect of the tagging procedure, we performed a “sham-tagged” condition, consisting in the observation of groups of three zebrafish, where one fish underwent the tagging procedure without the injection of any colored tag. Finally, we performed three VIE tag color conditions to investigate the effect of the tag colors. These conditions entailed the observation of groups of three zebrafish, in which one fish was tagged with yellow, blue, or purple elastomer, and the other two fish were not tagged.

The number of trials per condition was determined through a power analysis.⁴³ This analysis was performed on data from our previous research on the effects of acute ethanol administration on zebrafish social behavior.³⁹ Specifically, we used the average speed of four groups of fish, that is, 5.6, 7.8, 4.0, and 3.9 cm/s, associated with the administration of different doses of ethanol. Using a within-group standard deviation of 2.5 cm/s, we computed that a sample size of eight

FIG. 1. VIE tagging. Picture of a purple-tagged (a), blue-tagged (b), and yellow-tagged zebrafish (c), and close-ups of the purple (d), blue (e), and yellow tags (f). VIE, visible implant elastomer. Color images are available online at www.liebertpub.com/zeb

FIG. 2. Experimental setup. Sketch of the experimental setup, consisting of a circular arena positioned in the center of a square tank. Black curtains (not displayed here) were used to isolate the setup.

trials per condition would achieve 85% power in G*Power analysis of variance.⁴³ We increased the sample size by an additional 25% to account for potential experimental loss, thereby achieving a total of 10 trials per condition. During the postoperative care period, one fish tagged with purple elastomer lost its tag, and two other fish, one tagged with the yellow elastomer and one sham-tagged, died following the tagging procedures. As a result, only 9 trials were conducted for sham-tagged, purple, and yellow conditions, while 10 trials were performed for control and blue conditions.

All trials were conducted following the same experimental procedure. For the control condition, three fish were randomly picked from three different holding tanks and hand-netted into the experimental tank. In all the other conditions, two nontagged zebrafish were randomly selected from two different holding tanks and hand-netted into the experimental tank, and then one tagged zebrafish was picked randomly from its housing tank and hand-netted into the same experimental tank. As a result, the tagged zebrafish was always the last one placed in the tank. Although we did not control for the sex of the experimental fish upon testing, we note that since the original male/female ratio was 1:1 and experimental fish were randomly picked, it is tenable to assume that sex of experimental subjects was equally balanced across experimental groups.

Each group was video recorded for 20 min (10 min of habituation and 10 min of testing) at 30 frames-per-second (fps) with the webcam. The last 10 min (testing) were also recorded as images at 30 fps with the high-resolution camera, which was manually started at the end of habituation time. Recording the habituation stage was necessary to preserve the identity of the sham-tagged individual, which could not be discriminated from others. The rationale for limiting high-resolution recording to the testing phase was to reduce the amount of data stored, thereby limiting potential data overflow.

At the end of each trial, fish were hand-netted into different housing tanks. All fish utilized in this study were naive and were tested only once. Ten trials per day were performed, five between 9:30 AM and 12:30 AM and five between 2:30 PM and 6:30 PM.

Data analysis

Images recorded from the high-resolution camera were postprocessed through an automated, in-house developed multitarget tracking software for the measurement of zebrafish locomotion. A thorough description of the tracking software is available in our previous work^{38,39,44}; importantly, the software allows for preserving the identities of the subjects during the entire observation, including those that were not tagged.

To investigate the effects of the tagging procedure and colors of the tags, we recorded locomotory data of each individual in a group. From these data, we scored swimming activity, shoaling tendency, schooling tendency, and leader-follower relationships in the form of anticipatory swimming behavior of some individuals versus others.

Given that body size has been shown to affect shoaling behavior,⁴⁵ we measured the body size of each fish in this study from recorded images utilizing ImageJ 1.54.⁴⁶ Fish belonging to different treatment groups, that is, control, sham, blue, purple, and yellow, regardless of whether tagged or not, were also not significantly different in terms of body size (linear model [lm]: $\chi^2_4 = 9.161$, $p = 0.057$). Measures of the body sizes are reported in Table 1.

For each subject, swimming activity was measured in terms of their average speed during the trial,⁸ estimated from the distance travelled during the trial. For each group, we then

TABLE 1. MEASURES OF THE FISH BODY SIZES

Condition	Treatment	Average size (cm)	SD (cm)
Control	Nontagged	3.44	0.20
	Tagged	3.43	0.35
Sham	Nontagged	3.56	0.36
	Tagged	3.68	0.39
Purple	Nontagged	3.53	0.25
	Tagged	3.43	0.18
Blue	Nontagged	3.44	0.20
	Tagged	3.51	0.27
Yellow	Nontagged	3.30	0.28
	Tagged		

SD, standard deviation.

computed the average speed, as the mean of the average speed of the three subjects (one focal and two nonfocal group members)—for the control group, the focal subject was randomly chosen at the beginning of the testing phase. This granular scoring allowed different analyses: how much a tagged individual modulated the average speed of the entire group; whether and how tagging affected the swimming activity of the focal subject in the group; and whether different colors have different effects on swimming activity of focal and nonfocal subjects.

For each group, shoaling tendency was associated with the cohesion in the group, measured through the average nearest-neighbor distance³⁵ and the average interindividual distance. The former was computed as the mean value of the distances between each fish and its closest conspecific averaged throughout the duration of the testing phase, while the latter was computed as the mean value of the distances between each pair of fish composing the group.

Schooling was instead associated with the degree of alignment of the group members.³⁵ The latter was precisely scored by taking the average in time of the instantaneous polarization,⁴⁷ computed as:

$$P_t = \frac{1}{N} \left\| \sum_{i=1}^N \frac{\mathbf{u}_{i,t}}{\|\mathbf{u}_{i,t}\|} \right\| \quad (1)$$

where t is the generic time step, $N=3$ is the group size, and $\mathbf{u}_{i,t}$ is the velocity vector of the i -th fish at time t . Values of the instantaneous polarization may range from 0 to 1, with 1 being indicated when all the group members swim along the same direction. Notably, for a group of three fish, the chance value of polarization is 0.525, computed by assuming the three orientations of the fish to be independent and uniformly distributed from 0 to 2π .

Finally, mutual interactions in the group, in the form of anticipatory turning response from some individuals, were scored by computing transfer entropy in pairwise interactions between subjects from their turn rates. More specifically, we followed the method demonstrated in our previous work on zebrafish social behavior^{36,37} to score the extent of the interaction between group members, in a completely data-driven perspective. The information-theoretic approach of transfer entropy was introduced by Schreiber nearly two decades ago⁴⁸ to infer causal relationships between coupled systems.

Given two discrete stationary stochastic processes U and V , transfer entropy from V to U is defined as:

$$\text{TE}_{V \rightarrow U} = \sum_{u_{t+1}, u_t, v_t} p(u_{t+1}, u_t, v_t) \log_2 \frac{p(u_{t+1} | u_t, v_t)}{p(u_{t+1} | u_t)}, \quad (2)$$

where u_t and u_{t+1} denote the outcomes of U at time t and $t+1$, respectively; v_t the outcome of V at time t ; p indicates probability, computed from binning the raw time series; and Σ identifies the summation over all possible realizations of u_t , u_{t+1} , and v_t . Transfer entropy quantifies the reduction in the uncertainty predicting the future of U from its present, due to additional knowledge about the present of V . Should U be independent of V , transfer entropy should be zero since V would not encode any relevant information that could be used to better predict the future of U . Otherwise, we should expect a nonzero value, whose magnitude would be indicative of potential causal relationship between the processes.

For each group, we computed six values of transfer entropy, comprising any possible pair in the group. These values were calculated using 18 bins, corresponding to a bin size of 1.667 rad/s (turning rates ranged from -15 to $+15$ rad/s), and a down-sampling rate of 0.2 s, based upon our previous works.^{36,37} The computations were performed using scripts compiled in Matlab R2017a (MathWorks, Natick, MA).

To measure the overall degree of interaction in the group and highlight the emergence of leader–follower relationships, we consolidated the six values of transfer entropies into two numbers: a mean and a standard deviation. The mean should be associated with the overall level of interaction, such that higher value would correspond to groups in which fish tend to interact more strongly with each other. The standard deviation, instead, should be related to heterogeneities in the group, manifested into some fish exerting a larger influence than others.

To further delve into differences between individuals, we followed the approach proposed by our group.³⁶ Specifically, we combined transfer entropy values from pairwise interactions within the tested groups to produce an index of leadership for each member of the group. Such an index summarizes the difference between the propensity of an individual to influence others (i.e., taking a leader role) and the tendency to be influenced by them (i.e., taking a follower role). Therefore, positive values indicate that an individual tends to lead the others, whereas negative values suggest that it tends to follow.

The statistical analysis was performed in two steps. For each parameter, the effect of tagging was evaluated by comparing the sham-tag condition to the control. The effect of color was then evaluated by comparing each color-tag condition to the sham-tag condition. For each of these steps, data were first checked for homoscedasticity using a Levene's test. (Generalized) linear (mixed) modeling was then used to investigate the effect of tagging and color on the behavioral parameters.

For average speed, the average value for each individual fish was used as a response variable in a linear mixed model (lmm), with tagged/nontagged and color condition as categorical explanatory variables and individual body size as continuous explanatory variable and group as a random factor.

For average nearest-neighbor distance, interindividual distances, and polarization, the group average value was used as a response variable, color condition as a categorical explanatory variable, and average body size as continuous explanatory variables. A generalized linear model (glm) with binomial error function was used for the average polarization because the variable is bounded between 0 and 1, and a gamma error function with a log link was used for average nearest neighbor distance to account for high values. A generalized linear mixed model (glmm) was used for the average interindividual distances as dependent variable, with average body size per pair of fish and color condition as continuous explanatory variables, and a gamma error function with a log link.

For transfer entropy, the mean values and the standard deviations were used as response variables and average body size as continuous explanatory variables. The leadership index for each individual fish was used as a response variable in a lmm, with tagged/nontagged and color condition as categorical explanatory variables, body size as continuous explanatory variables, and group as a random factor.

TABLE 2. RESULTS OF LEVENE'S TEST FOR HOMOGENEITY OF VARIANCES

	Control vs. sham	Sham vs. colors
Average speed	$F_{1,17}=0.45, p=0.512$	$F_{3,33}=0.72, p=0.549$
ANND	$F_{1,17}=2.04, p=0.171$	$F_{3,33}=1.34, p=0.279$
Average interindividual distances	$F_{1,17}=2.13, p=0.163$	$F_{3,33}=1.24, p=0.311$
Average polarization	$F_{1,17}=0.10, p=0.760$	$F_{3,33}=2.29, p=0.097$
Mean TE	$F_{1,17} < 0.001, p=0.999$	$F_{3,33}=2.84, p=0.053$
SD TE	$F_{1,17}=0.21, p=0.652$	$F_{3,33}=1.34, p=0.277$

ANND, average nearest neighbor distance; TE, transfer entropy.

Where significant effects were found, a Tukey's honestly significant difference (HSD) criterion *post hoc* test was used for multiple comparisons. All the statistical analyses were performed on R 3.4.4⁴⁹ using "car package 2.1–6"⁵⁰ for the Levene's tests, "lme4 package 1.1–15"⁵¹ for the generalized linear (mixed) models, "lmer package 0.9–35"⁵² for model comparisons, and "multcomp package 1.4–8"⁵³ for *post hoc* analyses. All analyses were conducted with a significance level set at $p < 0.05$.

Results

To investigate whether tagging procedures affected inter-individual differences across all the parameters investigated, we conducted a Levene's test for homoscedasticity of variances. These analyses revealed that within-group variations in speed, average nearest neighbor distance, mean polarization, mean transfer entropy, and standard deviation of transfer entropy did not differ among groups, regardless of tagging procedures (testing sham-tagged vs. nontagged subjects) or specific color adopted (testing sham- vs. blue- vs. purple- vs. yellow tagged), see Table 2 for details.

Measure of activity: speed

We observed that tagging procedures *per se* altered fish speed whereby groups containing one sham-tagged individual showed increased speed compared to groups containing only nontagged individuals (lmm: $\chi^2_1=7.41, p=0.006$) (Fig. 3a). Within the shoals constituting the experimental unit, there was no significant difference in speed between the

focal fish and the nontagged shoal members (lmm: $\chi^2_1=1.81, p=0.178$) (Fig. 3b). This suggests that the tagging-related increment in speed was extended to the other members of the shoal: thus, sham-tagged groups swam faster than nontagged controls. When dissecting the effects of the specific coloration on individual speed, we found no significant effect of any particular color (lmm: $\chi^2_3=6.87, p=0.076$) (Fig. 3b). Conversely, average speed of the color-tagged subjects was indistinguishable from that of sham-tagged subjects. No effect of body size was found on fish speed, neither for the tagging procedures (lmm: $\chi^2_1=0.81, p=0.368$), for different color conditions (lmm: $\chi^2_1=1.03, p=0.310$).

Measure of shoaling: average nearest neighbor distance and average interindividual distances

With respect to shoaling tendency, we observed that the tagging procedures did not induce major variations in average nearest neighbor distance (glm: $\chi^2_1=0.51, p=0.475$) (Fig. 4). Thus, sham-tagged and nontagged subjects showed an indistinguishable pattern of cohesion. Likewise, data analysis revealed that the specific color did not apparently influence shoaling tendency, whereby average nearest neighbor distances did not vary among tagged individuals regardless of the specific color (glm: $\chi^2_3=6.16, p=0.104$) (Fig. 4). The average nearest neighbor distances significantly decreased with larger average body sizes; however, this effect was consistent for both the tagging procedures (glm: $\chi^2_1=5.40, p=0.020$) and for different color conditions (glm: $\chi^2_1=5.59, p=0.018$).

FIG. 3. Speed of the group. Group average speed in control versus sham-tagged conditions (**a**), and average individual speed in sham-tagged versus color-tagged conditions (**b**), separating tagged (*right boxes*) from nontagged (*left boxes*) fish within groups. The *bottom* and *top* of each *box* are the second and third quartiles, the *band* inside the *box* is the median, *whiskers* identify the 1.5-interquartile range of the data, and *dots* represent values beyond the 1.5-interquartile range of the data. *Bar* and *bracket* with *asterisk* indicate a significant difference ($p < 0.05$) in the linear mixed model.

FIG. 4. Average nearest neighbor distances. Average nearest neighbor distance of groups of fish as a function of treatment, control versus sham-tagged conditions (a) and sham-tagged versus color-tagged conditions (b). The bottom and top of each box are the second and third quartiles, the band inside the box is the median, whiskers identify the 1.5-interquartile range of the data, and dots represent values beyond the 1.5-interquartile range of the data.

When looking at interindividual distances between pairs, there was neither significant effects of the tagging procedure (glmm: $\chi^2_1=0.56$, $p=0.452$) nor significant effects of the color conditions (glmm: $\chi^2_3=2.41$, $p=0.492$) (Fig. 5). Similarly, no effect of average body size on average interindividual distances was found for the tagging procedures (glmm: $\chi^2_1=0.89$, $p=0.345$) or different color conditions (glmm: $\chi^2_1=0.06$, $p=0.802$).

Measure of schooling: average polarization

Tagging procedures did not apparently alter the degree of alignment among subjects swimming in small groups. Thus, sham-tagged and nontagged individuals showed an indistinguishable level of polarization (glm: $\chi^2_1 < 0.001$, $p=0.981$) (Fig. 6). In addition, the specific coloration did not affect polarization, whereby tagged zebrafish showed a comparable schooling tendency regardless of the specific color (glm: $\chi^2_3=0.078$, $p=0.994$) (Fig. 6). Fish body size was not affecting the alignment among subjects, neither for the tagging procedures (glm: $\chi^2_1=0.01$, $p=0.930$) nor for different color conditions (glm: $\chi^2_1=0.01$, $p=0.905$).

Measure of sociality: transfer entropy and leadership index

The tagging procedure significantly increased mean transfer entropy, suggesting increased interaction among fish when a tagged individual was part of the group (lm: $F_1=17.88$, $p<0.001$) (Fig. 7a). However, color did not significantly affect such a level of interaction in the groups (lm:

$F_3=0.83$, $p=0.448$) (Fig. 7b). No effect of average body size was found on the mean transfer entropy, neither for the tagging procedures (lm: $F_1=4.35$, $p=0.053$) nor for different color conditions (lm: $F_1=0.06$, $p=0.815$). The standard deviation of transfer entropy was not significantly different between groups with tagged and nontagged fish (lm: $F_1=0.14$, $p=0.715$) neither between different color conditions (lm: $F_3=1.07$, $p=0.373$). No effect of average body size was found, neither for tagging procedures (lm: $F_1=0.89$, $p=0.358$) nor for different color conditions (lm: $F_1=0.39$, $p=0.538$). These results suggest that despite the overall increase in social interactions of fish, tagging did not influence the social role of individual fish. In other words, it is unlikely that the tagged fish interacted with nontagged conspecifics, differently than how nontagged individuals interacted with each other.

The tagging procedure did not significantly affect the leadership index (lmm: $\chi^2_1=0$, $p=1$) (Fig. 8a). There was no significant effect of color condition alone on the index (lmm: $\chi^2_3=0$, $p=1$). However, there was a significant effect of the treatment itself (lmm: $\chi^2_1=4.55$, $p=0.033$) (Fig. 8b) and of the interaction between color condition and tagging procedure (lmm: $\chi^2_3=8.86$, $p=0.031$) (Fig. 8b). A *post hoc* test looking specifically at differences between tagged and nontagged individuals within each color treatment revealed that yellow-tagged individuals were less likely to influence others ($z=-2.98$, $p=0.011$) (Fig. 8b). There was no significant difference within other color treatments (sham: $z=-1.61$, $p=0.368$; purple: $z=-8.67$, $p=0.858$; blue: $z=1.01$, $p=0.774$) (Fig. 8b). No effect of body size was found on the leadership index, neither for

FIG. 5. Average interindividual distances. Average interindividual distances between fish as a function of treatment, control versus sham-tagged conditions (a) and sham-tagged versus color-tagged conditions (b). The bottom and top of each box are the second and third quartiles, the band inside the box is the median, whiskers identify the 1.5-interquartile range of the data, and dots represent values beyond the 1.5-interquartile range of the data.

FIG. 6. Polarization of the group. Average polarization of the groups of fish as a function of treatment, control versus sham-tagged conditions (a), and sham-tagged versus color-tagged conditions (b). The bottom and top of each box are the second and third quartiles, the band inside the box is the median, whiskers identify the 1.5-interquartile range of the data, and dots represent values beyond the 1.5-interquartile range of the data. For reference, the dashed line denotes the chance value for polarization for groups of three individuals (0.525).

the tagging procedures (lmm: $\chi^2_1 = 1.61, p = 0.205$), for different color conditions (lmm: $\chi^2_1 = 0.79, p = 0.375$).

Discussion

Individual tagging represents a crucial need in zebrafish research, since this species exhibits an extremely complex sociality^{5,8,9} and is currently adopted in countless behavioral studies. Studies involving groups of animals demand the ability to identify individual experimental subjects and keep their identity throughout the whole experimentation. One of the most promising tagging procedures in terms of safety, feasibility, and durability is VIE tagging.¹⁶ Notwithstanding the methodological advancements afforded by this procedure, only seldom has the possibility that tagging procedures of VIEs influence experimental validity been systematically addressed.

Based on this grounding, our main aim was to systematically evaluate whether and to which extent tagging procedures may challenge experimental validity. Toward this goal, we compared the ethogram exhibited by nontagged zebrafish with the ethogram exhibited by sham- and color-tagged zebrafish in groups. This experimental design allowed for dissociating the effects of the tagging procedure *per se* (sham tagging) from those dependent on the coloration. Previous studies evaluating the effects of tagging procedures on zebrafish behavior²⁸ addressed, in binary-choice experiments, whether control subjects preferred a compartment containing tagged versus nontagged subjects. It was demonstrated that nontagged individuals were more attracted toward color-

tagged subjects than toward sham tagged. Herein, we complemented and extended these results by performing a fine-grain analysis of individual and group behavior in small shoals of zebrafish constituted by two control subjects (nontagged) and a focal individual. The latter was a nontagged control, sham-, or color-tagged subject.

Our findings indicate that tagging procedures may influence both individual and group behavior, in terms of activity (groups containing tagged individuals swam faster than control groups, containing only nontagged subjects) and social interaction (groups containing tagged individuals displayed a higher degree of interaction than control groups, containing only nontagged subjects). It is important to emphasize that within the tagged experimental units (shoals of three subjects), only one was tagged while the others were not. The fact that the average speed of shoals comprising one tagged subject was higher compared with controls suggests that the tagging-dependent increment in speed affects the entire group and not only the tagged individual. This aspect may depend on the effects of the tagging procedure on the behavior of the treated individual, on the differential appraisal of the treated subject by nontreated shoal mates, or a combination of both.

Regardless of the underlying cause of the higher speed, an increase in the activity of certain individuals might affect other group members.⁵⁴ This has been recently demonstrated in *Poecilia reticulata*, another highly social fish species.⁵⁵ It is known that higher activity may reflect higher fitness or the possession of information regarding potential danger or food sources.⁵⁶ With respect to the influence of tagged individuals

FIG. 7. Mean transfer entropy. Mean transfer entropy in each group of fish in control versus sham-tagged conditions (a) and in sham-tagged versus color-tagged conditions (b). The bottom and top of each box are the first and third quartiles, the band inside the box is the median, whiskers identify the 1.5-interquartile range of the data, and dots represent values beyond the 1.5-interquartile range of the data. Bar with asterisk symbol indicates a significant difference ($p < 0.05$) in the linear model.

FIG. 8. Leadership index. Information-theoretic leadership index as a function of treatment, control versus sham-tagged conditions (**a**), and sham-tagged versus color-tagged conditions (**b**), separating tagged (*right boxes*) from nontagged (*left boxes*) fish within groups. The *bottom* and *top* of each *box* are the second and third quartiles, the *band* inside the *box* is the median, *whiskers* identify the 1.5-interquartile range of the data, and *dots* represent values beyond the 1.5-interquartile range of the data. For reference, the *dashed line* denotes symmetry in the interactions between individuals, whereby neither leadership nor followership emerge in the group. *Bar* and *brackets* with *dollar symbol* indicate statistically significant comparison ($p < 0.05$) in *post hoc* comparisons, with yellow-tagged individuals less likely to influence nontagged individuals.

on the group, our data indicate that while the presence of tagged individuals alters group speed, the behavior of tagged individuals is indistinguishable from that exhibited by nontagged shoal mates. It is tenable that the presence of the elastomer altered individual behavior and that such change was appraised by the other shoal mates which, in turn, influenced each other and the tagged subject.

This global effect is revealed by mean transfer entropy, which indicates an increase in the level of interaction in the group. Thus, the macroscopic change identified in the analysis may reflect the additive effect of individual modifications. This hypothesis, although speculative, would be reconciled with the fact that tagging increases overall speed and social influence without having a remarkable role on individual behavior. The latter is supported by the absence of variations in activity and social influence within each group, such that tagged fish swim as fast as nontagged individuals and the standard deviation of transfer entropy does not fluctuate across conditions. Furthermore, for most color treatments, no significant difference was found in terms of leadership between tagged and nontagged individuals.

Unlike Frommen *et al.*,²⁸ we did not observe differences in behavior between sham-tagged fish and color-tagged fish. However, our findings of increased speed in tagged individuals may also shed light on the higher attractiveness of tagged individuals described by Frommen *et al.*²⁸ It is possible that the finer interaction allowed between tagged- and nontagged fish in our experimental setup lowered the threshold in detecting a difference in preference, which could not be measured in a binary choice setup.²⁸ Specifically, although no color produced a significantly different effect than sham tags across all the metrics we used, we found that yellow-tagged fish were less likely to take leadership in their group, partially contrasting with Frommen's overall conclusions. This small but measurable difference calls for further studies.

Differences in colors could be perceived by the nontagged conspecifics as different types of injuries, parasites, or even necrosis.⁵⁷ Within this context, the nontagged fish in a group might try to swim faster in the attempt to isolate themselves

from the tagged- and sick-looking individual.⁵⁸ Indeed, yellow appears more contrasting on a zebrafish's natural color and is likely more visible also by conspecifics than the other colors that we used (purple, blue), potentially creating an oddity effect,³¹ as shown in previous studies on differences in body coloration.⁵⁹

Similar coloration-dependent influences are also well-documented in zebrafish.²⁹ However, if that were the case, we should expect also an increase of the average nearest neighbor distance and interindividual distance, or a difference between social interactions in the group, neither of which we did observe. In contrast, the color of the tag could trigger interest from the other individuals, as the tagged fish may display a novel color pattern that would increase its attractiveness as mating partner.^{60,61} It is still unclear whether the increases in activity and social interactions within the groups reflect attraction, repulsion, or another type of response between the tagged and the nontagged fish. Future experiments should be performed to clarify these aspects.

Furthermore, it is important to note that the current study has been performed ~ 2 weeks after the tagging procedures. Ferrari *et al.*⁶² observed, in sea bass juveniles, that tagging procedures altered individual behavior in a time-dependent manner: a transient hyperactivity immediately after tagging, a transient hypoactivity 3 weeks after tagging, and then no variation 4 weeks later.⁶² We note that the procedure adopted in this study is identical to that adopted in previous independent experiments^{20,21} and that we precisely followed the guidelines provided by the NMT, Inc. manuals.⁴¹ Thus, while our study represents a standard in this field of research, we nonetheless believe that additional experimental data, repeating the behavioral measurements by testing tagged individuals after different time intervals, may be required to understand the long-term effects of tagging procedures.

One last aspect that warrants consideration relates to the fact that our experimental population was constituted by male and female experimental subjects. Since sex has been shown to remarkably influence zebrafish social behavior,^{63,64} it cannot be excluded that our results may vary if the study were

conducted on an experimental population characterized by a different sex balance. We note, however, that our group recently performed an experiment to explore the influence of sex on leadership in zebrafish in an experimental setting analogous to that used in this study.⁶⁵ Specifically, we studied pairs of zebrafish composed by a female and a male subject freely swimming in a tank. The results detailed in the Supplementary Materials of that work indicate that sex was not a significant factor in zebrafish leadership.⁶⁵

Although VIE is a valuable tool to keep track of individual animals in many situations such as field studies and long-term experiments spanning over weeks,^{66–68} alternative solutions to the tagging procedures should also be considered when available. Among them, multitasking systems allow for the identification of all subjects in groups of limited size during the whole duration of experimental trials,^{38,39,44,69,70} see Delcourt *et al.*⁷¹ for a review. Multitasking systems can afford the tracking of trajectories in three dimensions of multiple fish.^{72–74} However, when increasing the number of experimental subjects, the reliability of these systems tends to decrease due to unresolved occlusions or missing detections that may occur during multiple trajectory crossings.⁷³

Therefore, time has to be invested by the experimenters to manually check the trajectories of each individual. Within this context, VIE tags might facilitate, for example, individual identification following occlusions, but care should be taken when choosing to use VIE in studies on social behavior. With technological advances in hardware (high speed and high resolution cameras, faster processors) and software (computer vision techniques, such as machine learning), we might expect multitarget tracking systems to better handle occlusion resolution, prediction of trajectories involving multiple individuals, rendering superfluous the use of tagging, thereby sparing potential confounds on social behavior.

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Authors' Contributions

T.R., D.N., and M.P. designed the research; T.R. and D.N. performed the experiments; T.R., D.N., and C.S. scored animal behavior; R.J.G.C., S.M., and M.P. performed statistical analyses; C.S. and D.N. provided input on Materials and Methods; T.R., R.J.G.C., S.M., and M.P. compiled a first draft of the article. All the authors reviewed the final draft and offered comments.

Disclosure Statement

The authors declare no competing financial interests.

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