

METHODS & TECHNIQUES

Electronic individual identification of zebrafish using radio frequency identification (RFID) microtags

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SUMMARY

Although individual electronic tagging using passive integrated acoustic (PIT) tags is established, it is mainly for fish >60 mm in length and is unsuitable for fish of <30 mm, like zebrafish. We used radio frequency identification (RFID) microtags (1 mm in diameter and 6 mm in length, with a mass of ~10 mg) to individually identify juvenile zebrafish (length 16–42 mm, mass 138–776 mg) for the first time, and studied the effects of intracoelomic implantation on fish survival and microtag loss, growth, spawning and exploratory behaviour. After 5.5 months, both high survival (82%) and low microtag loss (11%) were achieved. The smallest surviving fish weighed 178 mg, and success in microtag reading was 73% for the size class 350–450 mg (26 mm). Greater success was achieved when fish were larger at the time of tagging but no negative effects on growth were observed for any size class and some tagged fish spawned. No significant differences in behavioural responses could be detected between tagged fish and untagged controls after 2 months. Overall, the results suggest that the tagging method is highly suitable for fish as small as zebrafish juveniles. We think this method will provide significant advances for researchers of the ever-growing fish model community and more generally for all small-fish users. Tagging is essential when one needs to identify fish (e.g. particular genotypes with no external cue), to run longitudinal monitoring of individual biological traits (e.g. growth) or to repeat assays with the same individual at discrete points in time (e.g. behaviour studies). Such a method will find applications in physiology, genetics, behaviour and (eco)toxicology fields.

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INTRODUCTION

In recent years, the zebrafish, *Danio rerio*, has become a widely used model for vertebrate genetics, developmental biology and toxicology studies (Spitsbergen and Kent, 2003). Several thousand mutants and transgenic lines have been produced and laboratories commonly rear several of these lines simultaneously. In most cases, these fish cannot be distinguished by eye, meaning that fish from different lines must be kept in different tanks. From a practical viewpoint, this requires laboratories to manage large rearing facilities housing several hundred tanks, which is both costly and time consuming in terms of husbandry (Sire et al., 2000). Moreover, rearing problems may occur in zebrafish if populations are small because this interferes with shoaling behaviour, especially as shoaling in this species has been shown to increase significantly with age (Buske and Gerlai, 2011). Furthermore, because small fish such as zebrafish (≤ 30 mm standard length, SL) are not aggressive and do not fight when kept in groups, it would be possible to keep numerous similar-sized specimens in a single tank, leading to a drastic reduction in tank numbers. However, recognizing individual fish is impossible unless they can be tagged individually. This problem also applies to other small species (e.g. medaka, guppy) and to juveniles of larger species. The ability to recognize individuals within a large population of similar-sized congeners has long been regarded as useful, notably for fisheries research (e.g. for growth rate estimations), ecology (e.g. in migration) and population

dynamics studies. This is also the case in experiments when one needs to identify specific lines that have no external phenotypes (e.g. mutant or transgenic lines) or to monitor the same individuals over a long time period (e.g. multiple challenges in behavioural analysis, or monitoring of growth or spawning).

Since the end of the nineteenth century, when the first successful mass marking of fish was reported, various techniques have been developed to mark teleosts. These methods now include several ways of tagging relatively large individuals with different types of external or internal tags, and marks made of a diversity of materials, as well as genetic and chemical markers (Jakobsson, 1970; Parker et al., 1990). For small animals, however, few efficient solutions are available and the situation is even more complicated for fish because of their living medium and skin/scales, which preclude the use of external tagging methods such as conventional painting. Overall, although a wide range of techniques is now available to tag or mark fish (Bégout et al., 2012), they are either difficult to apply to small specimens or the marks are liable to disappear progressively, as is the case with fin ablation. One alternative method with a high marking success rate is scale regeneration following precise removal of a number of scales in a known position (Sire et al., 2000), but this method requires careful observation of the scale pattern under a dissecting microscope and long periods of fish handling, which are hardly compatible with studies that require frequent identification. Subcutaneous ink or acrylic paint injection

has also been successful for long-lasting readability in aquatic animals (Herbinger et al., 1990), as have visible implant (VI) elastomer or alphanumeric tags (NMT, WA, USA), which have been reported to be successfully used with small aquatic animals [e.g. shrimp (Brown et al., 2003; Dinh et al., 2012; Imbert et al., 2007; Jensen et al., 2008; Pillai et al., 2009)]. In our experience, however, none of these tagging methods were successful with zebrafish: paint tags faded away and VI tags were lost after 2–3 weeks.

Internal, solid tags have been used as an alternative to external tagging for some time (Buckley and Blankenship, 1990), and more recently small electronic tags (passive integrated transponder, PIT, using acoustic frequency identification) have been injected into the body cavity in several fish species as monitoring systems and for individual identification (Baras et al., 2000; Prentice et al., 1990). The smallest PIT tags are approximately 12 mm in length, 2 mm in diameter and 95 mg in mass, making them unsuitable for use on specimens of less than 60 mm (Baras et al., 2000), but new products are also appearing such as the ‘tiny’ version of the PIT tag (8 mm long, 1.4 mm diameter but still 34 mg). A very recent product, which has been used to document behaviour in insects [ants (Moreau et al., 2011) and bees (Decourtye et al., 2011)] is the radio frequency identification (RFID) microtag (1 mm in diameter and 6 mm in length, with a mass of ~10 mg). These microtags are suitable for zebrafish size but have not, to our knowledge, ever been used on fish. In the present study we tested RFID glass microtags to individually identify juvenile zebrafish and evaluated the effects of intracoelomic implantation on both routine endpoints, such as survival, microtag retention and growth, and sublethal effects, such as those on spawning and exploratory swimming behaviour. Our study is the first use of these microtags for this application and advances research on the surgical implantation of electronic microtags in fish, as recommended in the literature (Cooke et al., 2011).

MATERIALS AND METHODS

Fish and microtag insertion

A batch of 6 week old juvenile zebrafish, *Danio rerio* (F. Hamilton 1822), was bought from a fish supplier (Elevage de La Grande Rivière, Calluire, France) and acclimated to our facilities for 2 months before experimentation. RFID glass microtags (Nonatec RFID, Lutronic International, Rodange, Luxembourg, www.nonatec.net) are a new generation of tags that operate at a high frequency (13.56 MHz), allowing the identification of very small individuals using a laboratory bench RFID reader (designed to read Nonatec microtags; Lutronic) on fish under anaesthesia. Nonatec microtags are 1 mm in diameter and 6 mm in length, with a mass of ~10 mg (Fig. 1). For tag insertion, fish were put under mild anaesthesia in an ethyl-*p*-aminobenzoate (Benzocaine E1501-100G, Sigma, St Louis, MO, USA) solution made by mixing 50 µl from a 10% stock solution of Benzocaine, dissolved in 100% ethanol, per 100 ml of system water. To minimize handling duration, anaesthesia was performed on batches of 10 fish. The glass microtags were cleaned and stored in 70% ethanol prior to use and optimal aseptic conditions were maintained by cleaning the bench and all instruments with 70% ethanol.

About 30 s after anaesthesia induction, fish were measured for mass (to the nearest mg) and length (to the nearest mm) and then placed sideways on the bench. Intracoelomic implantation was then performed by piercing a hole in the abdominal cavity using a 22 gauge needle, taking care only to pierce the body wall muscle and not to insert the needle too far into the cavity so as to avoid damaging any organs. A microtag was then transferred from 70%



Fig. 1. Picture of a zebrafish and a Nonatec radio frequency identification (RFID) glass microtag; diameter 1 mm, length 6 mm, mass 10 mg.

ethanol to sterile physiological serum (9 g l⁻¹ NaCl) for rinsing, picked up with Dumont no. 3 forceps and inserted into the abdominal cavity through the hole made earlier, then pushed until it was fully inside (see supplementary material Movie 1). The whole procedure was routinely performed within 30 s and care was taken to limit the time the fish was out of the water to improve experimental success. Fish were then transferred to a tank of clean water for recovery from anaesthesia and handling, and allowed 10–15 min to rest before being returned to their rearing tanks (201 glass tanks in a zebra rack). Fish death and microtag loss were monitored by daily tank inspection.

Two tagging experiments were conducted: in the first (experiment 1, 71 days duration), 98 fish were tagged and 34 were used as controls (initial mean ± s.d. mass 361 ± 117 mg, within the range 139–712 mg). Tagged fish were put into three tanks in groups with similar mean mass and coefficient of variation of mass; control fish were placed in one tank. For the second experiment (experiment 2, 167 days duration), 140 fish were tagged and 343 were used as controls (initial mean mass of 420 ± 125 mg, within the range 138–776 mg). Tagged fish were divided between six tanks and control fish between 14 tanks. We used a comparative approach between the two treatments (control untagged *versus* tagged fish) to compare the variables detailed below.

Feeding regime and growth monitoring

Fish were fed on dried feed (TetraMin PRO Tropical Crisps), milled for better quantification of the ration, which was set at 1.5% of wet body mass. Every fortnight, biometric measurements were taken according to a standard protocol: fish were fasted the preceding day, anaesthetized as described above, measured for mass (to the nearest mg) and length (to the nearest mm) and put to recover in fresh clean water before being returned to their respective tanks. During the biometry measurements, microtags were read using the RFID reader connected to a computer, and corresponding mass and length were recorded on a spreadsheet. Occasional microtag failure or loss was noted.

Specific growth rate (SGR) was calculated as the mass increase between biometry measurements divided by the time interval between them (days) and expressed as a percentage. For tagged fish, SGR was calculated at the individual level whereas for untagged fish it was calculated at the group level using mean mass:

$$\text{SGR} = 100 \times [\ln(\text{fish mass at date 2}) - \ln(\text{fish mass at date 1})] / (\text{date 2} - \text{date 1}). \quad (1)$$

Spawning

To evaluate spawning ability, couples were set up in spawning boxes every 2 weeks over 2 months, starting 1 month after tagging. Eggs were collected in the morning and fish tag identities recorded. To evaluate reproductive characteristics, eggs were counted and sorted; unfertilized eggs were counted and removed, and the remaining eggs left to develop.

Behaviour

We used swimming behaviour as an integrated indicator to evaluate the possible influence of tagging on fish. Control tagged and untagged fish were subjected to an exploratory assay in which fish were put in the start arm of a T-maze, slightly adapted from a previous study (Ninkovic and Bally-Cuif, 2006), in order to monitor swimming characteristics (distance travelled and temporal space use) in either: (i) a shallow area, with a water depth of 5 cm, composed of the start arm (46 cm long), the arm leading to the deep area ('correct arm') and the 'wrong arm', in the opposite direction to the deep area (total length of these two arms was 66 cm), or (ii) a deep area, with a water depth of 10 cm, which was 23 cm wide and 23 cm long, had marbles and plastic grass and was considered to be a favourable zone (Ninkovic and Bally-Cuif, 2006). Fish were placed individually in a 1 l tank in the experiment room the day before the recording. For each trial, a single fish was removed from its tank and placed in the start area of the longest arm of the T-maze; exploration activity was immediately recorded for 3 min. All runs were accomplished in 1 day (10:00 h–19:00 h) and 20 fish were screened per treatment (control untagged *versus* tagged fish in sequence). This experiment was conducted 2 months after tagging.

Videos were recorded with an analog camera (Panasonic CCTV WV-CL920A) linked to a PC with an acquisition card. Track extraction and analysis were then performed with Ethovision XT software (Noldus, Wageningen, The Netherlands); images were acquired at 25 frames s⁻¹ and extracted data nested every 30 s for saving.

The variables chosen to evaluate behavioural performance were as follows. (1) Time spent (in s) by a fish in the different zones of the T-maze. (2) Velocity (in cms⁻¹) – the distance moved by the centre point of the individual fish per unit time between two consecutive X–Y coordinates acquired. Mean and s.e.m. were calculated for each fish and each zone. Data reported here are of velocity in the shallow zone.

Statistical tests

Data were statistically tested using Statistica 9.0 (Statsoft, Tulsa, OK, USA). For each variable, a Shapiro–Wilk test was performed to check the normality, and a Bartlett test to check the homoscedasticity. As normality and homoscedasticity rules were not respected, Mann–Whitney *U*-test or Kruskal–Wallis test was used (Zar, 1984), followed by multiple comparison rank tests. For survival- and growth-related variables (mass and SGR) we used Kruskal–Wallis tests to analyse sex and treatments effects. To refine our analyses, we made a class analysis of survival, body mass and SGR in relation to mass at tagging and sex for live tagged fish kept until 167 days post-tagging. To this end, we made 100 mg interval classes and used a Kruskal–Wallis test followed by Dunn's *post hoc* tests to compare body mass gain and SGR within sex and between classes. For each swimming variables (time spent in each zone, velocity in the shallow zone), Mann–Whitney *U*-tests were performed to compare results between the two treatments (control untagged *versus* tagged fish). All statistical analyses were carried out at a 95% level of significance.

RESULTS

Evaluation of tagging procedure success

Two sets of fish originating from the same initial batch were tagged in two successive experiments (experiment 1: 98 tagged fish studied for 71 days; experiment 2: 140 tagged fish studied for 167 days). Experiment 1 was a pilot experiment to evaluate the suitability of the microtag size and insertion procedure. During the entire duration of the experiments, fish death and/or microtag loss were monitored daily (Table 1). Survival was expressed as the number of tagged fish remaining over time and tagging success as the percentage of microtag retention in relation to sex and fish mass at tagging (Fig. 2, Table 2).

In the first experiment, some fish death and microtag loss occurred, mainly during the first 3 weeks following microtag insertion. After 2 months there had been a total of 15 fish deaths (15%) and 28 microtags lost or not successfully read (28%). The smallest surviving fish was 178 mg at tagging. Over the 71 days, survival rate of tagged fish (85%) was higher than that of control fish (58%).

For the second experiment, performed later on, we used an improved method in order to shorten the insertion procedure as much as possible and thus limit the period that the fish were out of water

Table 1. Fish number and growth for untagged control and tagged fish, according to sex

	Female		Male	
	Untagged	Tagged	Untagged	Tagged
Initial no.	216	91	127	49
Survival [no. live fish (% initial fish)]				
B5	175 (81.0%)	87 (95.6%)	114 (89.8%)	44 (89.8%)
B8	157 (72.7%)	76 (83.5%)	98 (77.2%)	39 (79.6%)
Body mass (mg)				
B1	521.1±10.6	533.7±13.7	406.9±8.1	423.0±11.3
B5	714.1±15.6	734.8±18.7	512.8±10.2	485.0±14.0
B8	914.8±20.1	958.3±25.8	564.2±14.6	581.6±16.2
Specific growth rate				
SGR1	0.50	0.51 (0.41±0.03)	0.37	0.22 (0.22±0.04)
SGR2	0.44	0.47 (0.49±0.04)	0.17	0.32 (0.26±0.04)

Survival is indicated as the number of live fish and as a percentage (in parentheses) of the initial number of fish in each column on post-tagging biometry measurement days B5 and B8.

Body mass (means ± s.e.m.) indicates data obtained on the post-tagging biometry measurement days B1, B5 and B8.

Specific growth rate (SGR) was obtained from mean mass for both untagged controls and tagged fish between biometry measurement days B1 and B5 (SGR1) and B5 and B8 (SGR2). In addition, for tagged fish, individual SGR was calculated and is given in parentheses (means ± s.e.m.).

There was no significant difference between control untagged and tagged fish.

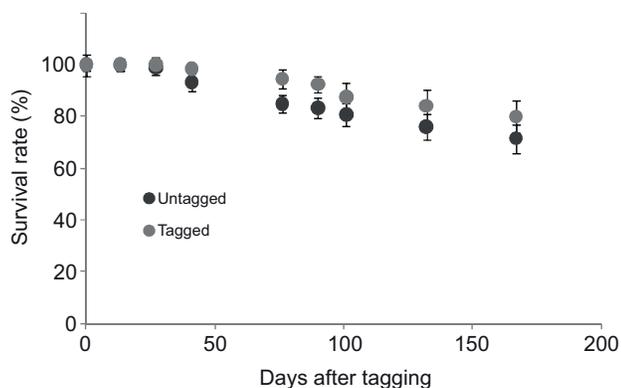


Fig. 2. Monitoring of survival rate of untagged control and tagged fish over time (means ± s.e.m.). No significant difference was observed.

and the stress caused. The tagging procedure took around 30 s (see Materials and methods). In this experiment, 140 fish were tagged. The improved methodology and use of slightly heavier fish meant that fish death was limited to 25 (17.9%) and the number of microtags lost/unreadable to 15 (10.7%) after 167 days (5.5 months) (Fig. 2). The survival rate of tagged fish (82%) was higher than that of control fish (74%) and no significant effect of sex was observed (survival of tagged fish was 83.5% for females and 79.6% for males versus 72.6% and 77.1% for control untagged females and males, respectively; Table 1).

The analysis of survival in relation to mass at tagging and sex showed that there was a trend for a decrease in mortality with increasing initial mass and that this was independent of sex (Table 2). A survival of 82% was obtained for fish from the 350–450 mg mass class.

Growth rate

Mass and length measurements were taken at fortnightly intervals in order to monitor the possible negative effects of tagging on fish growth (Fig. 3). No significant difference in growth was observed between treatments (control untagged versus tagged fish, Table 1). Females

were heavier than males in both treatments and this difference increased over time as their gonads matured. This male/female difference was particularly clear for SGR, which was higher for females than for males. For control untagged fish, SGR could only be calculated based on mean growth. The same calculation was performed for tagged fish, but at the individual level (Table 1). Values obtained could not be compared directly using a statistical test, but it appeared that SGR of individual tagged fish was very probably no different from the mean SGR of the untagged controls.

The actual SGR calculated from individual mass is also indicated for tagged fish and some differences can be noticed with the value calculated based on mean mass (Table 1).

In order to further characterize growth of fish depending on their initial mass, we took the opportunity to monitor fish individually over time, which allowed us to calculate body growth and SGR for each sex and for each fish in the same mass class at tagging as defined in Table 2 and Materials and methods. For each sex, mean mass followed the initial grouping but differences between classes diminished gradually. In addition, variation in mass within any one class increased with time, as illustrated by the increase in s.e.m. SGR appeared inversely correlated to initial mass but this relationship was only significant for SGR1 of males, due to the very large variation of the other calculated SGR values.

Spawning characteristics

Although fish were handled frequently, spawns were obtained over the course of this experiment. Starting 1 month after tagging, in fortnightly trials made over 2 months, we obtained 11 spawns involving 5 different males and 4 different females for experiment 1 and 20 spawns involving 10 different males and 11 different females for experiment 2. A similar low number of spawns was obtained with untagged control fish. In all cases, fertilization rate and larval survival were similar to usual levels.

Swimming behaviour

Behaviour is now recognized as an integrative indicator of both organism stress response and physiological alterations (Champagne et al., 2010; Steenbergen et al., 2011). Two months after tagging, we compared the swimming activity of tagged fish with that of

Table 2. Tagged fish number and growth, according to sex and initial mass

Mass class (mg)	Female						Male		
	0–250	251–350	351–450	451–550	551–650	651–850	251–350	351–450	451–550
Initial no.	3	13	29	21	18	7	15	27	7
Tagging success [no. fish (% initial)]									
B5	2 (66.7%)	9 (69.2%)	27 (93.1%)	21 (100%)	18 (100%)	6 (85.7%)	15 (100%)	24 (88.9%)	7 (100%)
B8	2 (66.7%)	7 (53.8%)	24 (82.8%)	19 (90.5%)	14 (77.8%)	6 (85.7%)	10 (66.7%)	17 (63%)	7 (100%)
Body mass (mg)									
B1	269.3±20.3 ^a	393.3±21.9 ^a	482.8±9.0 ^{a,b}	554.8±12.5 ^{b,c}	645.1±13.4 ^c	770.1±42.1 ^c	352.0±9.8 ^a	438.5±5.6 ^b	547.2±14.1 ^b
B5	412.3±77.6 ^{a,b}	533.8±34.9 ^a	632.3±23.3 ^{a,b}	752±31.5 ^{b,c}	799.1±30.7 ^c	1004±42.9 ^c	417.1±23.1 ^a	510.4±16.8 ^b	595.2±25.6 ^b
B8	706.0±110.0 ^{a,b}	711.3±65.4 ^b	876.8±58.9 ^{a,b}	979.4±51.6 ^{a,b}	1014±50.5 ^a	1146±75.6 ^a	545.6±21.7 ^a	596.7±24.6 ^a	646.2±19.8 ^a
Specific growth rate									
SGR1	0.70±0.30 ^a	0.47±0.12 ^a	0.44±0.05 ^a	0.49±0.04 ^a	0.29±0.04 ^a	0.31±0.08 ^a	0.35±0.02 ^a	0.23±0.04 ^{a,b}	0.13±0.03 ^b
SGR2	0.96±0.29 ^a	0.52±0.16 ^a	0.60±0.04 ^a	0.41±0.08 ^a	0.42±0.06 ^a	0.32±0.07 ^a	0.3±0.09 ^a	0.27±0.05 ^a	0.15±0.06 ^a

Tagged fish were grouped by sex and mass classes at tagging (100 mg classes, except the largest class for female, which ranges from 651 to 850 mg). Tagging success (the percentage of microtag retention) is given as the number of fish for which the microtag was read and as a percentage relative to the initial number of fish in each class (in parentheses; differences arise from either dead fish or a failure to read microtags).

Body mass (mean ± s.e.m.) was measured on post-tagging biometry measurement days B1, B5 and B8.

Specific growth rate (SGR, mean ± s.e.m.) is given between biometry measurement days B1 and B5 (SGR1) and B5 and B8 (SGR2).

Different superscript letters indicate significant differences between classes, by sex, according to a Kruskal–Wallis test followed by Dunn's *post hoc* test; $P < 0.05$.

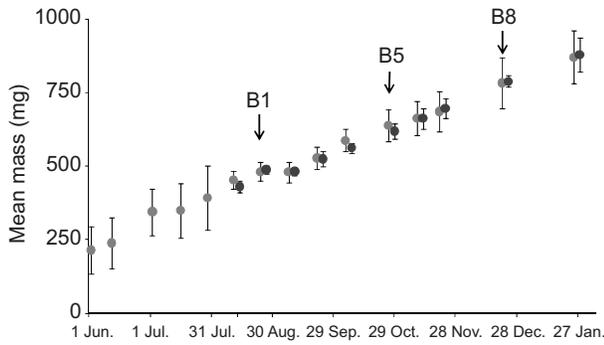


Fig. 3. Growth monitoring of control untagged (grey symbols) and tagged fish (black symbols) over 6 months. Fish growth was monitored for the 2 months before tagging in order to detect possible deviations of growth rate after tagging. Fish mass (means \pm s.e.m.) is shown for every biometry measurement point and no significant differences between control untagged and tagged fish are apparent. Biometry measurement points B1, B5 and B8 (13, 76 and 132 days after tagging, respectively; see Tables 1 and 2) are indicated by arrows.

untagged control fish using an exploratory challenge in a T-maze. This challenge is usually used to measure anxiety and exploration (Ninkovic and Bally-Cuif, 2006) as well as swimming characteristics themselves. No significant modifications were observed for the variables measured (Fig. 4): fish from the two treatments used the different zones of the T-maze equally and swimming velocities in the shallow area were similar.

DISCUSSION

Method choice

For short-term use, e.g. for repetition of a challenge with individual fish within a week, external tags such as paints can be used, even though this might become complicated if a large number of fish are used. External tagging can also potentially pose problems if the challenge involves social interaction, which may be biased because of mark visibility itself. For long-term experiments, a long-lasting tagging method is required, which precludes painting techniques. Internal electronic tagging is the ideal choice in such cases but, as mentioned above, this has so far been limited to fish above 60 mm in length and 1.67 g in mass (Baras et al., 2000).

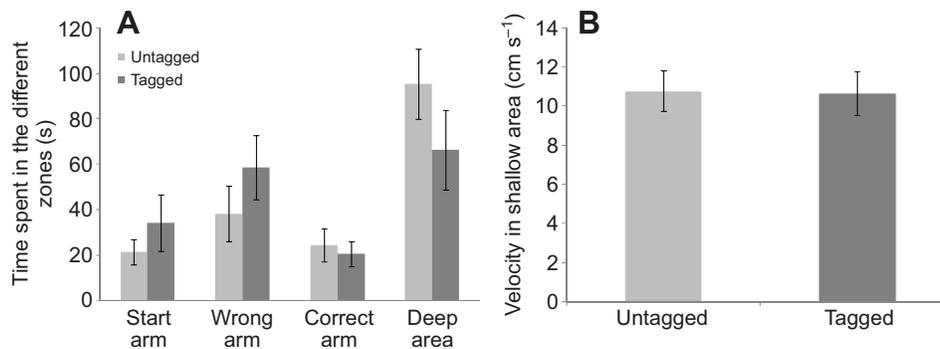


Fig. 4. Swimming activity observed in a T-maze challenge. (A) Fish were introduced in the start arm and had to swim through the correct arm to reach the deep area, which is supposedly the preferred area (Ninkovic and Bally-Cuif, 2006). The other three areas are straight and shallow. Time spent in the deep area was longer than that spent in the other three areas. No significant difference could be observed between control untagged and tagged fish in any of the zones (means \pm s.e.m.; Mann-Whitney $U_{\text{Start arm}}=153$, $P>0.77$; Mann-Whitney $U_{\text{Wrong arm}}=124$, $P=0.23$; Mann-Whitney $U_{\text{Correct arm}}=159$, $P=0.94$; Mann-Whitney $U_{\text{Deep area}}=119$, $P=0.17$). (B) Swimming velocity in the shallow areas was not different between control untagged and tagged fish (means \pm s.e.m.; Mann-Whitney $U=157$, $P=0.87$).

Over the course of projects at our laboratory for which long-lasting tagging was required, we have tried several tagging techniques. Our experience has been that scale painting and under-skin paint (acrylic) or elastomer injections are not successful – colour faded away or injected elastomers were rejected within 2 weeks of tagging. The same problems occurred with VI alphanumeric tags, even when the tags were cut to make them smaller. As we had practical experience of implanting internal transponder tags in larger fish than zebrafish, we searched for RFID microtags because they make it possible to work simultaneously with a large number of code combinations and provide a long-lasting form of tagging. Nonatec microtags were available embedded in resin or in glass. Although the resin microtags offered the advantage of being lighter than the glass ones, they had sharp edges and were rapidly lost following intracoelomic implantation. We therefore focused our experiments on Nonatec glass microtags.

Tagging success and effects

Surgery was adequate for tagging zebrafish, as the mortality of tagged fish was actually lower than that of control untagged fish and was within the range of usual mortality reported for long-term experiments on zebrafish. The overall tagging success with the improved method after 5.5 months of tagging was above 82% for fish survival, among which 11% of fish lost their microtag or had a microtag that became unreadable. In the tagging literature, experiments often follow the recommended ratio of tag mass to fish body mass in air [‘the 2% rule’ (Winter, 1983)]. In our experiment, the lightest surviving tagged fish had an initial mass of 178 mg in experiment 1 and 190 mg in experiment 2, giving microtag to fish body mass ratios of 5.6% and 5.3%, respectively, which are well above the 2% rule. However, this rule has been successfully challenged by several authors who demonstrated that high tag to fish body mass ratios can be used without causing substantial biases in the long term (Baras et al., 2000; Brown et al., 1999; Jepsen et al., 2005). Our findings also agree with this principle, as they show no alteration in equilibrium or swimming behaviour that might be related to the positioning of the microtag close to the centre of gravity of the fish and to the compensatory capabilities of zebrafish as a physostomous cyprinid (Finney et al., 2006). Furthermore, feed intake was not affected and during the first couple of months following tagging, the smallest fish showed catch-up growth (high SGR), although their microtag ratio was still high and hence all initial mass classes exceeded 500 mg after 2 months.

There was, however, a clear increase in success with heavier initial mass at tagging, with a success probability over 73% for fish in the 350–450 mg class range.

None of the variables tested in the longer experiment showed deviation between treatments, control untagged *versus* tagged fish. We were able to obtain only a small number of spawns for tagged and untagged fish, which was probably due to the combination of two factors: frequent handling under anaesthesia at fortnightly intervals and restrictively low food levels. Analysis of the swimming activity in a T-maze indicated that swimming ability as measured 2 months after tagging was not modified by tagging and that tagging did not induce modification of behavioural traits that could have been interpreted as enforced anxiety (Champagne et al., 2010).

Possible development of the techniques

We have demonstrated that fish as small as 200 mg can survive after the insertion of a 10 mg glass microtag. This represents a great decrease in the lower mass limit for fish to be tagged compared with other tags available on the market and therefore creates the possibility of tagging very young individuals of larger fish species in order to conduct long-term life history monitoring, as well as providing a means of tagging small model species such as zebrafish. However, because of the short reading distance of the RFID reader (1 cm) and its functioning mode, some occasional microtag reading failures were noted that could not be distinguished from microtag loss unless the microtag was read at the following session. Also, if frequent monitoring is required as fish grow (e.g. for biometry measurements, behavioural challenges) and when working with juveniles of large fish, an additional tagging should be done later on, using regular PIT tags (e.g. when fish are above 5–10 g depending on the species), so as to avoid losing the signal.

An automated identification portal at specific checkpoints would make an excellent research tool for behavioural challenge monitoring in fish, like the equipment already developed for insects (Decourtye et al., 2011; Moreau et al., 2011), but this would require the RFID reader to be waterproof, and improvements in the detection range and speed, which are currently weak points because they require a close positioning of the fish for reading.

In conclusion, we have demonstrated that long-term tagging of small fish such as zebrafish is now possible using RFID glass microtags. The tagging procedure is simple and fast. None of the tested variables indicated an adverse effect of the tagging procedure or of the microtag itself in the long term.

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