Evaluation of a European sea bass (Dicentrarchus labrax L.) post-larval tagging method with ultra-small RFID tags

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Abstract

Individual tagging is key to a better understanding of early life stages in fish. Very small RFID transponder microchips (500 x 500 x 100 µm, 82 µg) are now available. The aim of this study was to develop a protocol to tag European sea bass (*Dicentrarchus labrax* L.) larvae from 61 days post-hatching (dph; standard length ~10 mm) to 96 dph (standard length ~28 mm) through intra-coelomic implantation of microchips. The suitability of such a tagging procedure was evaluated, with the purpose of determining the minimal fish age and body size for microchip tagging without adverse effects on survival and growth performance.

We produced an experimental population composed by 50:50 normally pigmented larvae and albino larvae through artificial fertilization. Five tagging trials were performed over 35 days, in fish aged 61, 75, 83, 89 and 96 dph. Each time, 50 normally pigmented fish were tagged, while 50 albino fish were used as controls. Mortality was recorded daily, while biometric measurements were performed at 75, 83, 89, 96, 103 and 110 dph via image analysis.

Microchip tagging was possible in larvae from an age of 75 dph (standard length ~20 mm), with satisfactory performance in terms of survival rate (between 84 to 98% 24 hours after tagging) and growth rate, and without significant differences in comparison with the untagged controls. In contrast, tagging before 75 dph is not to be recommended, as the age group 61 dph was the most affected in terms of survival (only 62% of fish survived 24 hours after tagging) and growth rate, showing significant differences compared to the untagged controls. The overall microchip reading success rate for the age groups throughout the experiment was 51.4%, the overall reading success rate at each biometric measurement was 48.2%, probably due to the change in orientation of the microchip inside the fish body cavity.

The tagging protocol developed was then overall successful, albeit with a moderate reading success. Precocious tagging could allow the collection of new types of data (individual, longitudinal) related to larval development, behavioral studies, physiological and immunological investigations. Future tests could focus on the effects of tagging on baseline locomotion and behavior, as well as the suitability and the efficiency of intramuscular microchip tagging on larger fish.

Keywords: larvae, RFID transponder, individual identification, tagging effects, *Dicentrarchus labrax* L.

1. Introduction

Individual identification and monitoring of an animal within a population through a proper tagging method is increasingly used in aquaculture research. This is especially the case for selective breeding targeting different production traits such as growth, feed efficiency and disease resistance (Das Mahapatra et al. 2001, Lind et al. 2012), but also to track escapees (Uglem et al. 2019). More generally, individual identification is

increasingly used for the investigation of a wide range of life history related features of aquatic species, such as growth and survival rates. It is also used for fisheries research and to study population dynamics, behavioral dynamics, spatial ecology and responses to environmental changes (Pine et al. 2003).

Among the internal tagging methods, Radio Frequency Identification (RFID) electronic tagging using glass Passive Integrated Transponder (PIT) tags or microtags, is widely used due to a series of advantages, such as billions of unique tag numbers, easiness of tagging and reading, high retention rates and limited side effects to the animals carrying the tags. Tagging with standard RFID glass tags (2 × 12 mm, 33 mg or 1.4 × 8 mm, 100 mg) can be performed in fish with a minimum length of 60 mm or a minimum weight of 3 g (Baras et al. 2000; Navarro et al. 2006), while microtags (Nonatec®, size 1 × 6 mm, 10 mg) have been shown to be appropriate in fish with a minimum standard length of 36 mm or a body mass of ~0.84 g (Cousin et al. 2012; Ferrari et al. 2014). A tagging method for even smaller fish, however, is worth developing, as many biological changes occur during very early life stages. Nevertheless, tagging could have drawbacks and affect fish, particularly when the ratio between tag and body weight is high. Moreover, susceptibility to anesthesia and manipulation, tag retention and recovery ability could differ from species to species and in animals of different ages within the same species, so tagging methods, both in terms of tag choice and tagging procedures, need to be tested carefully.

Developing an early tagging method is also interesting from the perspective that the smallest animals that can be tagged may already have body weight considerably higher than hatching body weight. This is particularly true for the European sea bass (*Dicentrarchus labrax* L.), as tagging is possible at around 1 g of mean weight; at this stage, the body weight of the fish has already increased by a factor of nearly 1000 compared to body weight at hatching.

The application of ultra-small tagging technologies at early stages could then provide new insights into different aspects, such as early growth differentiation between sexes in sea bass (Saillant et al. 2001). In this species, indeed, sex dimorphism for growth has already occurred at the age of 105 dph where tagging with microtags is possible (1024 degree days above 10°C, Ferrari et al., 2014). Post-larval tagging can also be useful for selective breeding for production traits (growth) or efficiency traits (feed efficiency, disease resistance) in many species, since recording early individual performance may enable early selection and thus a reduction in the cost of selective breeding. As fish are normally reared together to ensure identical environmental conditions, the identification of individuals is necessary to correlate individual performance with the family structure, which is one of the main aspect of breeding programs, and in turn allows the correct estimation of breeding values, and genetic and genomic parameters (Herbinger et al. 1999).

Despite the availability of tags to track individual organisms, few options are available for tagging small species or early life stages. The miniaturization of technologies has allowed the development of progressively smaller tags, providing the opportunity of the identification and monitoring of very small-bodied organisms, potentially without side effects in terms of survival, growth, behavior and social interactions. Very small RFID transponders characterized by exceptionally small size and weight (500 x 500 x 100 μm, 82 μg) are now available. These microchips have been already tested for biomedical research purposes in laboratory mice (Gruda et al. 2010) and zebrafish (Chen et al. 2013) by subcutaneous injection and for social behavior studies in insects (honeybees, Tenczar et al. 2014; ants, Robinson et al. 2014) by external adhesion, with satisfactory results.

In the present study, we developed a protocol to tag European sea bass larvae from 61 dph (or 372 degree days above 10°C) to 96 dph (or 596 degree days above 10°C) through intra-coelomic implantation of microchips. The suitability and the effects of such tagging procedure were evaluated, with the purpose of determining the most precocious age and the minimal body size for microchip tagging without significant side effects in terms of survival and growth performances.

2. Materials and Methods

2.1. Production and rearing of the experimental fish

All procedures were conducted in accordance with the guidelines for animal experimentation established by the European Union (Directive 2010-63-EU) and the corresponding French legislation. The experiment was approved following evaluation by the Ethical Committee n° 036, under authorization number APAFIS#19713-2019010917222576v3 delivered by the French Ministry of Higher Education, research and Innovation.

The fish used in the experiment were produced in the experimental facilities of IFREMER in Palavas-les-Flots (France). Artificial fertilization was performed as a full factorial mating scheme using the eggs of two albino dams homozygous for recessive albinism (a/a) and the cryopreserved sperm of five sires which were heterozygous (a/+) at the same locus (and thus normally pigmented). This specific mating scheme allowed for the production of normally pigmented (a/+) larvae and albino (a/a) larvae in equal proportions (50:50).

2.2. Microchips, ID reader and software

Microtransponder tags ("p-Chips®") were obtained from PharmaSeq, Inc. (Monmouth Junction, New Jersey). Each microchip measures 500 x 500 x 100 µm (Fig. 1) and carries a specific serial number (ID). When the chip is stimulated by a diode laser (660 nm, 60 mW average power) of an ID reader ("wand"), the photocells embodied in the microchip provide power and synchronization signals for the electronic circuits of the chip. Then, when the on-chip antenna contained by the chip itself is stimulated by the laser light, the chip transmits the ID at 1MHz through a variable magnetic field. Subsequently, the signal is decoded by a field programmable gate array (FPGA), that is part of the wand itself, and eventually through a reader software (www.pharmaseq.com; Jolley-Rogers et al., 2012).

2.3. Implantation protocol

The intra-coelomic implantation was performed using a stereomicroscope. Each sterilized injector (2¼" x 4" sterilization pouch) pre-loaded with the microchip (Fig. 1) was settled on a micromanipulation arm and connected to a piston fixed on a specifically designed and 3D-printed mounting stand. The pressure exerted on the piston caused the subsequent pressure of the injector plunger and the ejection of the microchip from the needle. This mechanism allowed great precision during the tagging operations, avoiding the direct manipulation of the fish and minimizing abrupt movements, which may cause injuries to the larvae.

Fish were prepared for the manipulation in iso-osmotic seawater, to equilibrate internal and external ion concentration (the iso-osmotic salinity is between 10.2 and 11.6‰: Varsamos et al., 2001) and anesthetized with MS-222 (Sigma-Aldrich, 0.07 g/l in iso-osmotic seawater; Chatain and Corraoa, 1992). Each fish was gently placed on a microscope slide covered with dampened absorbent paper and put under the stereomicroscope. The microchip was then injected after the insertion of the needle into the peritoneal cavity of the fish, on the left side (Fig. 1; Supplementary video 1). The whole procedure (preparation in iso-osmotic sea water, anesthesia, tagging) lasted on average 10 minutes for each fish. After tagging, the fish were transferred in a tank of iso-osmotic 0.2 µm filtered and sterilized seawater for recovery (to avoid osmotic stress and prevent infections) and they were allowed to rest for 2 hours before being returned to their rearing tank. The temperature of the water was controlled throughout the entire manipulation in order to avoid temperature shocks, and care was taken to limit the time the fish were kept inside the anesthetic bath and out of the water. Control fish received the same treatment (anesthesia and manipulation out of the water), except for the needle insertion or microchip tagging.

Five tagging trials were performed over 35 days, in fish aged 61, 75, 83, 89 and 96 (days post-hatching) dph. Each time, 50 randomly chosen normally pigmented (a/+) fish from the stock rearing tank were tagged, while 50 randomly chosen albino (a/a) fish from the stock rearing tank were used as controls (total number of tagged fish: 250; total number of untagged controls: 250). After each tagging trial, tagged fish and untagged controls were mixed and transferred to an empty tank, to allow the discrimination of fish tagged on a given day and to easily estimate the mortality per group in case of microchip loss or reading failure. The conditions (temperature and salinity) were strictly identical in all rearing tanks.

2.4. Survival, microchip retention and reading, and growth monitoring

Rearing tanks were monitored daily throughout the experiment to record mortality. Biometric measurements were performed at 75, 83, 89, 96, 103 and 110 dph. During each biometric measurement, the fish were anesthetized as described above (paragraph 2.3) and the microchip ID of each experimental fish was read. As the reading process should be fast and the handling of such small-bodied fish should be minimized, the attempt of microchip reading lasted a maximum of 30 seconds per fish.

The fish (tagged and untagged controls) were then individually placed over a light table (Ultra Slim Light Box, Microlight) to increase the contrast, and a digital picture of each fish was taken using a stand with a digital camera (12.2 megapixel), using a graduated ruler as a reference. Finally, the fish were placed in 0.2 µm filtered and sterilized seawater to recover before being returned to their rearing tank.

Image analysis was performed with the ImageJ software 1.51 (Rasband, 1997-2018), allowing the measure of the standard length of each fish (the caudal fin was not taken into account). The graduated ruler taken on each picture with the fish has permitted to convert all measurements from pixels to mm.

During each biometric measurement (75, 83, 89, 96, 103 and 110 dph), 50 fish from the stock rearing tank were randomly chosen and measured to monitor the survival and the growth of normally pigmented fish and albino fish, and check that (a/a) and (a/+) fish from the same genetic background have similar survival and growth rates (Supplementary material 1).

2.5. Data analysis

The number of survived animals belonging to the tagged fish group and the untagged controls group, both after the implantation of the microchip and at the end of the experiment, were compared using a χ^2 test.

The standard length of the tagged fish and the untagged controls in each tagging group and at each biometric measurement were analyzed for normality using the Shapiro-Wilk test and for homoscedasticity using Bartlett's test. These tests indicated that in general the data did not conform to the assumption of normality or homoscedasticity, even after transformation (log or square root). The Wilcoxon-Mann-Whitney non-parametric test was then used to compare the standard length of the tagged fish and of the untagged controls at each biometric measurement and for each tagging group (one test per tagging group at each biometric measurement).

All the tests were performed in R version 3.5.0, package *stats* (R Core Team, 2018) and the significance threshold was p-value < 0.05.

3. Results

3.1. Survival rate

No significant differences in survival rate were detected between tagged fish and untagged controls across the groups that were tagged on days 75, 83, 89 and 96 post-hatching. However, among the fish tagged on day 61 post-hatching, tagged fish had a lower survival than untagged controls. The increased mortality due to tagging in this group occurred immediately after microchip implantation, within the first 24 hours after tagging (χ^2 = 8.914, p-value = 0.003; Table 1). After that, no fish mortality connected to tagging was registered.

Fish mortality was also registered throughout the experimental trial, but no differences in survival rate were observed between tagged and untagged fish of each group at the end of the experiment (Table 1).

The youngest group subjected to the microchip implantation showed the lowest survival rate (62%), with 31 fish surviving out of 50 fish tagged, whereas the other age groups showed rather higher survival rates, ranging from 82% to 98%, with a minimum of 41 to a maximum of 49 surviving fish out of the total (Table 1).

3.2. Microchip retention and reading

Tag loss was difficult to discriminate from reading failure. The overall microchip reading success rate for the age groups throughout the experiment was 51.4%, the overall reading success rate by biometric measurement (average at each date without taking into account the age at tagging) was 48.2%. The lowest mean value was

observed in tagging age group 61 dph (42.9%), while the highest mean value was observed in tagging age group 89 dph (58.4%). The biometric measurement performed at 103 dph had the highest tag reading success rate (54.1%), whereas the first biometric measurement performed at 75 dph resulted in the lowest tag reading success rate (38.7%; Table 2). However, this latter percentage refers to only tagging age group 61 dph, which was in general the group with the worst performance.

3.3. Growth monitoring

The standard body length of the tagged fish and untagged controls was significantly different in tagging age group 61 dph, starting from the second biometric measurement performed at 83 dph until the end of the experiment. Significant differences in growth were initially detected in tagging age group 83 dph, but in this case, the untagged fish were smaller compared to the tagged ones. However, the body length became homogenous thereafter, and no differences in the body length were found during the fourth and the fifth biometric measurements performed at 103 and 110 dph. For the other groups, no growth differences were observed between tagged and untagged fish (Table 3).

4. Discussion

Our experiment revealed that the microchip intra-coelomic implantation was effective in sea bass larvae from an age of 75 dph (459 degree days above 10°C) or from a standard length of ~20 mm and a body weight of ~0.11 g. On average, fish of 75 dph or more were not affected by the tagging procedure, showing satisfactory performances in terms of survival rat, growth rate and microchip reading success rate.

The group subjected to the earliest microchip implantation (61 dph) was the most affected in terms of survival and growth rate. We can hypothesize that the very small size of the fish at this age (standard length ~10 mm) combined with their developing and thus fragile body may be a reason that explains the higher susceptibility of this group to the procedures of anesthesia, handling and needle insertion. In bigger fish subjected to PIT-tagging, mortality caused by the manipulation and tag insertion was detected up to 10 days after tagging (Dare 2003). In our experiment, we observed mainly a non-recovery immediately after microchip implantation, then a low mortality rate up to 24 hours after tagging. After 24 hours, no fish mortality imputable to the tagging process was registered.

Significant differences in standard length were initially detected between tagged fish and untagged controls in the 83 dph age group, but with tagged fish longer than controls, which was not expected. This could be attributable to a stochastic sampling effect. These differences disappeared after 14 days and were not detected later on.

When we recovered the microchips from the dead fish, we were also able to estimate the retention rate of the tags, which was in general moderately high (76.2%), but the average microchip reading success was lower (~50%). Apart from a certain proportion of reading failure imputable to tag loss, the main explanation could be the change in orientation of the microchip inside the body cavity after tagging. Baras et al. (2000) has already described different orientations of tags and changes of orientation throughout time for PIT-tagged Eurasian perch (*Perca fluvialis*), that could affect the detection of the tag itself. The microchip technology is rather different compared to PIT-tag or microtag technologies, even if they are all RFID transmission protocols. The microchip relies on the laser light stimulation of both the photocells embodied in the chip and the antenna that transmits the ID; both components are situated to one side of the chip, thus a change of orientation of the microchip inside the coelomic cavity of the fish can prevent the laser light to reach the photocells and to stimulate the antenna, making the reading of the chip difficult or even impossible.

The implantation of the microchip in the fish dorsal muscle (as tested on zebrafish; Chen et al. 2013) may avoid or limit chip displacement or orientation change, but this implies the utilization of larger-sized fish (standard length > 30 mm), reducing the comparative advantage of the microchip compared to microtags which can be used at a minimum standard length of 36 mm (Cousin et al. 2012; Ferrari et al. 2014).

Using the microchip technology tested in this study, we showed that it is now possible to individually monitor fish from an extremely early life stage, allowing for the study of many biological, physiological or

behavioral aspects, and the tagging protocol that was developed was overall successful. Anyway, tag implantation should imply minimal or no stress to the fish (Bridger and Booth, 2003) in terms of growth patterns, but also in terms of baseline locomotion and behavior. Further investigations related to the possible effects on swimming behavior due to the procedure and the presence of the tag inside the fish coelomic cavity could be interesting, as Ferrari et al. (2014) found some differences in swimming activity between tagged and untagged controls (105 dph sea bass juveniles). However, they detected such differences only in the period immediately following tag implantation, when tagged fish showed hyperactive behavior compared to the controls. The analysis of the behavioral adaptability to the tagging procedure could be noteworthy, mainly because the fish in our experiment underwent to the tagging process at a younger age and at a smaller size compared to the experiment of Ferrari et al.

In terms of application, microchip tagging is likely to be interesting in all studies targeted at larvae and small-bodied fish, for which other tagging techniques (PIT-tagging, microtagging) are not suitable. Fish at those very early stages are nowadays studied either as groups or with lethal phenotyping. Individual identification could give access to new types of data (individual, longitudinal) that could both improve our understanding of the processes that happen during larval development and the implementation of behavioral studies of larval stages, as well as physiological and developmental investigations. Potential examples include, the individual susceptibility of early stages to different pathologies, the possible recovery from a pathology and the impact on subsequent growth, as well as immunological studies, individual feeding behavior and coping styles of very small fish. Also, for fish treated as groups with "programming" aimed at eliciting epigenetic mechanisms with long term effect (e.g. Balasubramanian et al. 2016), post-treatment individual tagging could enhance the reliability of later phenotyping by enabling common garden rearing of the treated groups, thus better controlling for environmental effects of the tanks. However, the reading success with the implantation methodology we used remained medium-low (36-62% at a given time point), thus operational use of these microchips will require increased sample sizes. Nevertheless, this remains the only method allowing individual identification of fish larvae with a mean weight of 100 mg, while the alternative microtags tested before were operational only for fish of 590 mg of mean weight (Ferrari et al. 2014).

Furthermore, intramuscular microchip tagging could be performed also in larger fish, as an alternative to the common tagging techniques, with the advantage of a very small tag to body weight ratio, but the suitability and the efficiency have to be tested.

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Conflict of interest

The authors declare that they have no conflict of interest.

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326 **Table 1**
327 For each age group, number of survived fish and survival rate (%) of the tagged fish and untagged controls
328 the day of the microchip implantation/first manipulation, and number of survivor fish and survival rate (%)
329 from 24 h post-implantation to the end of the experiment.

Age at tagging	Survival 24h after microchip implantation/first manipulation			
	Tagged		Untagged controls	
	N	Survived	N	Survived
61 dph	50	**31 (62%)**	50	**50 (100%)**
75 dph	50	42 (84%)	50	50 (100%)
83 dph	50	41 (82%)	50	50 (100%)
89 dph	50	49 (98%)	50	50 (100%)
96 dph	50	45 (90%)	50	50 (100%)
Overall	250	208 (83.2%)	250	250 (100%)
Age at tagging	Survival from 24h to the end of the experiment			
	Tagged		Untagged controls	
	N	Survived	N	Survived
61 dph	31	11 (35.5%)	50	24 (48.0%)
75 dph	42	31 (73.8%)	50	44 (88.0%)
83 dph	41	40 (97.6%)	50	48 (96.0%)
89 dph	49	44 (89.8%)	50	48 (96.0%)
96 dph	45	41 (91.1%)	50	49 (98.0%)
Overall	208	167 (80.3%)	250	213 (85.2%)

330 Characters in bold indicate significant differences between tagged and untagged controls (χ^2 test, p-value < 0.05).

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339 **Table 2**
340 Microchip reading success rate (% of total number of fish) at each biometric measurement and for each age
341 group.

Age at tagging	Reading success rate at a given age						Average success rate by group
	75 dph	83 dph	89 dph	96 dph	103 dph	110 dph	
61 dph	38.7	40.0	46.7	46.7	46.7	38.5	42.9
75 dph	-	61.9	58.3	36.1	58.8	43.8	51.8
83 dph	-	-	41.5	46.3	48.8	50.0	46.6
89 dph	-	-	-	61.2	56.3	57.8	58.4
96 dph	-	-	-	-	60.0	54.5	57.3

							51.4
Average success rate by biometric measurement	38.7	51.0	48.8	47.6	54.1	48.9	48.2

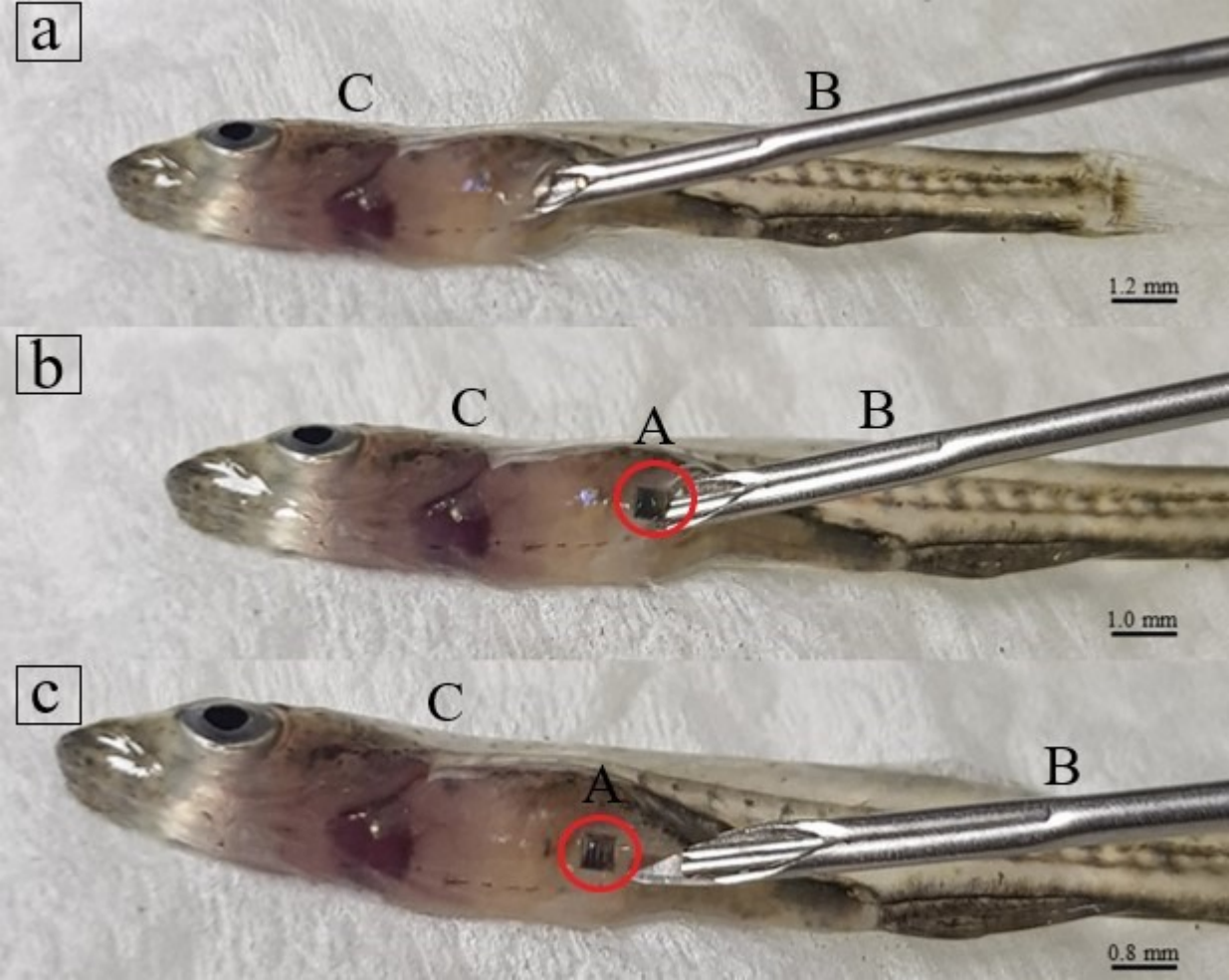
Table 3

Comparison between the standard length (± SD, mm) of the tagged fish and the untagged controls, per each age group and per each biometric measurement.

Age at tagging	Age at measurement					
	75 dph	83 dph	89 dph	96 dph	103 dph	110 dph
			Tagged			
61 dph	19.7 ± 2.3	**20.8 ± 3.1**	**22.2 ± 3.6**	**24.0 ± 4.0**	**26.2 ± 4.5**	**28.7 ± 5.5**
75 dph	-	23.2 ± 2.0	25.1 ± 2.3	27.2 ± 2.5	**30.1 ± 2.8**	33.5 ± 2.5
83 dph	-	-	**26.4 ± 1.8**	**28.6 ± 1.9**	31.3 ± 2.2	34.3 ± 2.4
89 dph	-	-	-	26.4 ± 3.2	28.7 ± 3.4	31.2 ± 3.2
96 dph	-	-	-	-	30.9 ± 2.3	33.3 ± 2.5
			Untagged controls			
61 dph	20.2 ± 2.2	**23.2 ± 1.8**	**25.2 ± 2.2**	**27.6 ± 2.5**	**30.9 ± 2.8**	**33.8 ± 2.9**
75 dph	-	23.2 ± 2.1	25.8 ± 1.8	28.0 ± 2.0	**31.4 ± 23**	34.4 ± 2.3
83 dph	-	-	**25.3 ± 1.8**	**27.6 ± 2.1**	30.8 ± 2.1	33.7 ± 2.4
89 dph	-	-	-	27.3 ± 2.0	29.8 ± 2.1	32.3 ± 2.3
96 dph	-	-	-	-	31.0 ± 3.0	33.4 ± 3.3

Characters in bold indicate significant differences between tagged fish and untagged controls (Wilcoxon-Mann-Whitney non-parametric test, p-value < 0.05).

Fig. 1. Intra-coelomic implantation of the microchip in a 75 dph larva: a) insertion of the injector needle into the peritoneal cavity of the fish; b) ejection of the microchip (A; indicated by the red arrow) from the injector needle; c) withdrawal of the injector needle.


a
C
B
1.2 mm
b
C
A
B
1.0 mm
c
C
A
B
0.8 mm