

Marking Zebrafish, *Danio rerio* (Cyprinidae), Using Scale Regeneration

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ABSTRACT Tagging or marking small laboratory-bred fish species is not an easy task. This also holds for the zebrafish, *Danio rerio*, which is widely used throughout the world as a model organism for genetics, developmental biology, etc. We present a simple marking technique based on scale regeneration. A comparative morphological study of various types of zebrafish scales indeed shows that regenerated scales are easily distinguishable from nonregenerated ones. We propose to take advantage of this typical morphology to mark a single or several individuals. This technique, based on a natural biological process, is easy to perform and does not enhance fish mortality in laboratory breeding conditions. It permits assembly of several specimens in a single tank with the possibility of identifying each of them by regenerated-scale coding. Nevertheless, a prerequisite is that the species does not lose and regenerate scales in large numbers in laboratory breeding conditions. To check this, 5,200 scales were removed from a large region of the left flank in 100 zebrafish and the number and position of regenerated scales were statistically analysed. Our results indicate that (1) laboratory-bred zebrafish have only a few regenerated scales (7.48%), (2) the probability of finding a regenerated scale is similar whatever its position in a row (antero-posterior axis), but (3) it differs from one row to another (scales from the back are more frequently lost than those from the pectoral region). This paper presents a procedure to mark small breeding colonies of zebrafish using scale regeneration with the number and position of the scales to be removed with high probability of marking success. *J. Exp. Zool.* 286:297-304, 2000. © 2000 Wiley-Liss, Inc.

In recent years, the zebrafish, *Danio rerio* (Hamilton), has become widely used as a model for vertebrate genetics and developmental biology. Several thousands of mutants have been produced by mutagenesis in numerous laboratories to evaluate gene function (Driever et al., '96; Haffter et al., '96). Most of these mutations are lethal during embryonic stages. To conserve such mutants it is thus necessary to rear heterozygous specimens. However, these specimens cannot be distinguished from wild-type zebrafish phenotype, and the conservation of numerous, often precious, different fish looking identical can appear rapidly problematic in terms of space. Indeed, each stock (often represented by a few individuals) must occupy a single tank. Laboratories must possess large breeding facilities housing several hundred tanks; this is particularly expensive and time-consuming in caretaking. Moreover, breeding problems may occur in zebrafish when populations are so low as to interfere with shoaling behavior.

Small fish such as zebrafish (≤ 30 mm SL) are not aggressive and do not fight; thus, numerous similar-sized specimens can be maintained in a single tank, leading to a drastic reduction of tank

numbers. However, recognizing individual fishes is impossible unless they can be tagged individually. This problem applies also 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 to be useful, notably in fisheries research (e.g., growth rate estimations, ecology (e.g., migration), and population dynamics. Since the end of the nineteenth century, when the first successful mass marking of fish was reported, various techniques have been developed to tag or mark teleost fish. Numerous external or internal tags and marks built in diverse materials (metal, glass, celluloid, plastic, etc.), as well as genetic and chemical marks (Jakobsson, '70; Parker et al., '90), have been proposed. Among the large range of tags used, the most efficient ones appear to be the external tags, which are simple to fix and easy

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to identify; however, they can only be applied to large specimens and are often lost after a few weeks (McFarlane et al., '90). Internal, solid tags have been used as an alternative (Buckley and Blankenship, '90), and more recently small electronic tags (Passive Integrated Transponder, PIT) have been injected into the body cavity as monitoring systems and individual identification (Prentice et al., '90). But these approximate 10-mm long, 2-mm diameter PIT tags can hardly be used for specimens less than 40 mm. Moreover, both external and internal tags can be expensive, and their application time-consuming, when dealing with numerous specimens.

An alternative to mark small specimens can be the chemical markers. For instance, vital staining by chemical administration of fluorochromes (tetracyclines, calcein, xylenol orange, or alizarin red) can be applied to juveniles and even larvae or embryos (Dabrowski and Tsukamoto, '86). These substances have been used to mark either single individuals by injection (see e.g., Meunier, '72, '74; Meunier and Boivin, '74) or several specimens (mass marking) by food incorporation, immersion (Nagiec et al., '88), or osmotic shock (Alcobendas et al., '91). These chemicals are incorporated into the mineralizing tissues (bones, teeth, scales, otoliths) and persist as permanent labels during the entire life span of the fish. This technique has allowed investigations of the mineralized tissues (growth rates, bone remodeling, etc.) and has also been shown useful to validate age estimates (see references in Baglinière et al., '92). This technique can be applied to laboratory-bred teleosts and is not expensive. Nevertheless, there are only a few fluorochromes available, and identification of the labeled specimens requires observation under the fluorescence microscope (Sire and Meunier, '81).

In summary, a wide range of techniques is available nowadays to tag or mark fish, but they are either difficult to apply to small specimens or the marks disappear progressively, as is the case with fin ablation (the fin regenerates and cannot easily be distinguished from a nonregenerated one) and intracutaneous injection of ink or various coloring substances (Herbinger et al., '90). So, if such techniques can be used at all, it is for short-term studies only.

In teleosts, the elasmoid scales, i.e., thin, mineralized dermal plates lying into dermal pockets, regenerate rapidly when they are lost or removed experimentally (Sire and Meunier, '81; Sire and Géraudie, '83, '84; Sire, '86, '87, '88; Sire et al.,

'97a,b). The shape of the regenerated scales is so different from that of normal ones that they can be recognized easily (Sire, '82, '87). Furthermore, when a scale is removed experimentally then put back in its pocket, it remains in place (Sire, '95, unpublished data). Consequently, experimentally regenerated scales could be used to mark small specimens, such as zebrafish, but only if naturally regenerated scales (through loss during fish manipulation [shocks by hand nets], cleaning of tanks, impact with the substrate, etc.) occur relatively infrequently.

With this question in mind, the number of regenerated scales was recorded in a large region of the flank in each of 100 laboratory-bred zebrafish, and, based on this information, we have indicated which area should be selected and how many scales should be removed to obtain the most reliable pattern of regenerating scales for marking zebrafish.

MATERIALS AND METHODS

Biological material

One hundred wild-type zebrafish (*Danio rerio*) (6 months to 1 year old, 20–30 mm standard length, SL, mean 25.91 mm), from diverse populations bred in our laboratory, were reared in 80-litre tanks provided with a sandy bottom and an internal filter at $25 \pm 1^\circ\text{C}$ under 12L:12D light conditions. Fish were fed Tetramin powder (Tetra-Werke, Germany) daily and *Chironomus* sp. larvae twice a week.

Laboratory-bred zebrafish were used instead of pet shop specimens because these had been manipulated several times (approximately every month, and with no particular care) using hand nets, mainly when changing them from tanks for cleaning. These conditions are close to what occurs in other laboratories working with this species.

Anaesthesia

The zebrafish were anaesthetized individually by immersion in a solution of MS 222 (tricaine methane sulfonate, Sandoz, Basel, Switzerland) (0.1 g/litre of tank water). MS 222 was conserved at 4°C as a 1% stock solution in distilled water, and 10 ml were added to 1 litre of tank water for anaesthesia. This solution was replaced every 4 hr. Under these conditions, all specimens recovered rapidly after scale manipulation, and this treatment had no effect on the survival and growth of the experimental fish population. Principles of laboratory care were followed as well as specific national laws where applicable.

Methods

Scale removal

Scales were removed from the left flank, from immediately behind the head to the anterior margin of the anal fin, and from above the pectoral fin to the back (Fig. 1). These scales are large, easy to remove, and thought to interfere less with swimming forces and shocks than those on the caudal peduncle.

Thirteen adjacent scales (I to XIII from anterior to posterior) were removed from 4 adjacent rows (A to D from dorsal to ventral), resulting to a total of 52 scales removed per fish and 5,200 scales for the population studied.

Scales were removed successively (starting from the most posterior ones in each row) using fine forceps under a dissecting microscope; they were transferred onto a glass slide in the same sequence as they had occupied on the flank to reconstruct the original scale pattern. Starting from the most anterior region of the flank, each scale was numbered by reference to its location in a row (for instance, scale number 2 in row C was coded CII), and its state was noted as either normal (nonregenerated) or regenerated.

Fish were allowed to regenerate their scales in a separate tank (addition of antibiotics was not necessary). Scales in the process of regeneration were removed from some specimens 15, 30, and 60 days after scale removal and used as a control for regenerated scale morphology. Normal and regenerated scales were digitalized on a Zeiss photomicroscope equipped with a Sony colour video camera.

Statistical analysis

The number of regenerated scales per individual was tested for correspondence to a binomial distribution (χ^2 test). To differentiate between a po-

tential difference in occurrence of regenerated scales along the antero-posterior or the dorso-ventral axis, two contingency tables were computed with fixed antero-posterior probability (1/13) or fixed dorso-ventral probability (1/4) and tested against the observed distribution of regenerated scales (χ^2 test).

The probability that a regenerated scale in the square [(BII), (CXII)] was bordered by at least another regenerated scale, called hereafter clustering probability, was established. Any side effect within this square was eliminated because each studied scale had 8 neighbours. Then, the positions of the regenerated scales were attributed randomly in 100 hypothetical fish based on the probability of their occurrence in the dorso-ventral and antero-posterior axes previously defined. The numbers of regenerated scales for 100 hypothetical fish were obtained directly from the observed distribution. This procedure was performed 10,000 times. The clustering probability was established for each of the 10,000 simulations. Then, the probability of occurrence of the observed value of clustering probability was obtained directly from the percentage of the simulations showing a higher value than the observed one. This probability could be used directly as the probability that the observed value was obtained by chance.

RESULTS

There was no mortality in the zebrafish population during the 60 days following the manipulation.

Scale morphology

Regenerated and nonregenerated scales were easily recognizable due to their surface ornamentation (Fig. 2). Most of the 5,200 scales (92.52%) removed were normal (nonregenerated), including some lateral-line scales.

Normal scales had roughly the same morphol-

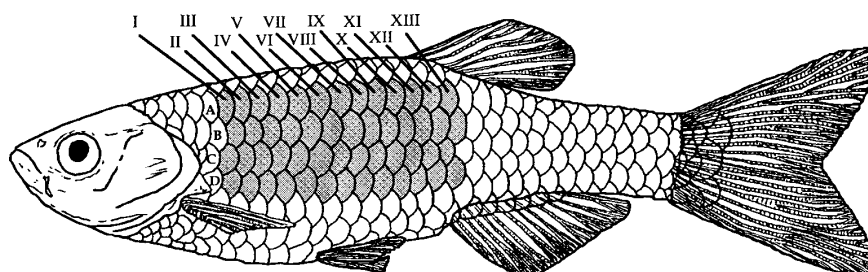


Fig. 1. Left lateral view of a zebrafish showing the region in which scales (in grey) have been removed in rows A to D and columns I to XIII.

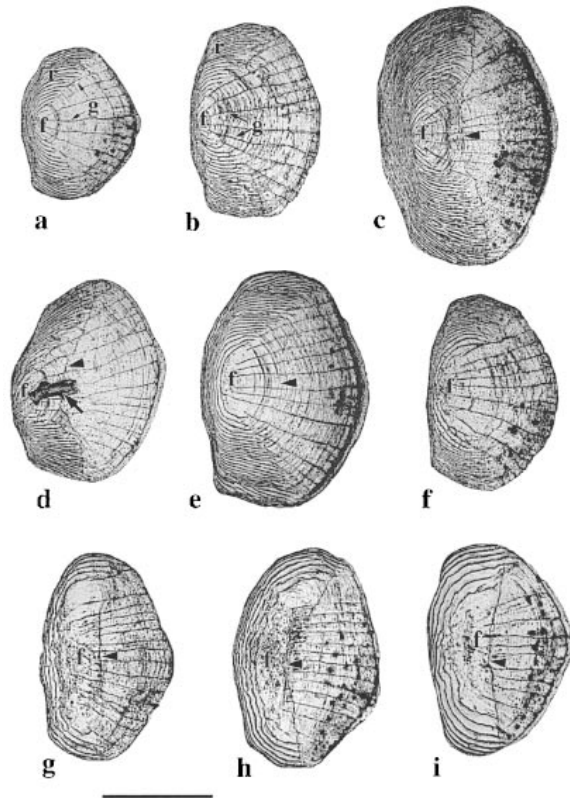


Fig. 2. Scales from the left flank of 20- to 30-mm SL zebrafish. Anterior is to the left, arrowheads indicate the edge of the epidermal cover. a–d: Nonregenerated scales from various regions of the area studied. e, f: Naturally regenerated scales. g–i: Experimentally regenerated scales. a–c: Scales from row A, position VI (AVI) in a 20-, 25-, and 30-mm SL zebrafish. d: Lateral-line scale (CII) showing the typical canal (arrow). e, f: All the regenerated scales fell within these 2 categories; they result from the loss of a scale in juvenile or adult stages, respectively. Note the large regenerated focus and the different organization of the grooves. Compare with a–c. g–i: Zebrafish scales regenerated for 15, 30, and 60 days, respectively. Scale bar = 1.0 mm. f, focus; r, ridges; g, grooves.

ogy whatever the age or size of the individuals (Fig. 2a–c), except for some scales located immediately behind the head, in row C, and which bore a lateral-line canal (Fig. 2d). The scale surface was characterized by a focus in the anterior region, radial grooves (i.e., the radii) from this focus that formed a fan-shaped pattern in the posterior region covered by the epidermis, and ridges disposed around the focus (i.e., the circuli) running parallel to the anterior and lateral regions of the scale. In normal scales the focus was

a small, hardly visible circle that represents the scale initium. The grooves and ridges increased in number during scale growth. In young zebrafish, the flank scales had 4–5 grooves, increasing to 12 or more in adults; the scales hardly grow at their anterior margin, and the new ridges are added at regular intervals mainly on the lateral edges (Sire and Meunier, '81; Sire '86; Sire et al., '97a give further descriptions of scale ornamentation in teleosts). The lateral-line canal is clearly recognizable as a kind of small gutter ornamenting the mid, anterior region of the scales (Fig. 2d).

Both the naturally (Fig. 2e, f) and the experimentally (Fig. 2g–i) regenerated scales were easy to distinguish at low magnification from normal ones because of their large, heterogeneous focus region and the different arrangement of the grooves in this region. The typical morphology of the regenerated focus is the result of the rapid process of scale replacement through activation of a large number of scale-pocket lining cells. These cells reconstruct a lost scale as fast as possible (Sire and Géraudie, '84; Sire et al., '90). However, an individual can lose scales at any time, and this is reflected in the morphological characteristics of the scale (Fig. 2e, f). In a single individual, regenerated scales can thus look different because the surface of their regenerated focus depends on the surface of the scale they replace (Sire, '82). A replacement that has occurred early during scale ontogeny is more difficult to recognize in the adult than a recently regenerated scale because its regenerated focus is small (Fig. 2e, compare with Fig. 2f–i).

The analysis of experimentally regenerated scales (Fig. 2g–i) observed 15, 30, and 60 days after removal led to the following findings: (1) in a single specimen, the focus and the arrangement of grooves and ridges of all the regenerated scales were similar; (2) in all specimens, at a given time, the regenerated scales showed the same morphology; and (3) the morphology of experimentally regenerated scales permitted estimates of their age (cf. Fig. 2g–i and Fig. 2e, f). Most of the naturally regenerated scales in the 100 studied zebrafish were >30 or >60 days old.

Distribution of the regenerated scales

Among the 5,200 scales analysed, 389 (7.48%) were naturally regenerated scales.

The number of regenerated scales per individual differed significantly from the theoretical binomial distribution ($\chi^2 = 30.26$, 5 DF, $P < 0.005$). Signifi-

cantly fewer individuals than expected showed an intermediate number of regenerated scales (Fig. 3).

The distribution of the regenerated scales (Table 1) among the square [(AI), (DXIII)] did not differ statistically from that with fixed antero-posterior probability ($\chi^2 = 58.53$, 47 DF, $P > 0.25$), whereas it did differ from that with a fixed dorso-ventral probability ($\chi^2 = 52.36$, 38 DF, $P < 0.05$). Therefore, the different probabilities of occurrence of regenerated scales in the dorso-ventral axis should be taken into account. Indeed, the frequency of regenerated scales decreased significantly from the dorsal row A (9.5%), to rows B, C, and D (8.9, 6.2, and 5.3%, respectively).

The probability that a regenerated scale in the square [(BII), (CXII)] was bordered by at least one other regenerated scale was 0.0413. Such a value or higher values were observed in 23.53% of the resamplings, indicating that the regenerated scales were not located significantly in clusters on fish (Fig. 4).

The probability of occurrence of a naturally regenerated scale along the dorso-ventral axis is 0.053 for line D, 0.069 for line C, 0.082 for line B, and 0.095 for line A. Therefore, if a scale was removed experimentally from position DIII, the probability that the scale DIII was lost naturally in another specimen in the same tank (thus leading to a misidentification) was 0.053, whereas it would have been 0.095 if scale AIII had been chosen. In the same way, the probability that 2, 3, 4, or more regenerated scales (either adjacent or not) could be lost naturally at a particular site can be

calculated easily by multiplication of probability for each particular position in the lines. The probability (P) decreased to: $2.8 \times 10^{-3} < P < 9 \times 10^{-3}$ for 2 naturally regenerated scales; $1.5 \times 10^{-4} < P < 8.5 \times 10^{-4}$ for 3 regenerated scales; $7 \times 10^{-6} < P < 8 \times 10^{-5}$ for 4 regenerated scales; and so on.

Marking zebrafish using scale removal

Removal of 4 scales was enough to mark zebrafish efficiently with a statistically high probability to recognize the individual later. (A detailed procedure to mark zebrafish is proposed at the end of this article).

Three groups of 10 similar-sized individuals were chosen. A cluster of 4 adjacent scales (CII, DII, CIII, DIII) was removed in group 1 and a different cluster (CVIII, DVIII, CIX, DIX) in group 2, while no scales were removed in group 3. The three groups were placed into a tank, and the 30 individuals were pulled out for decoding the scale regeneration mark after 2 months. We were able to identify each fish to its respective mark group with 100% accuracy.

DISCUSSION

Experimental scale regeneration is a valuable technique to mark small breeding colonies of the zebrafish, *Danio rerio*; this is also another example of the use of scales in fish biology. Indeed, it is known for a long time that scales bring valuable information for age estimates (Ombredane and Baglinière, '92) and for discrimination between individuals of wild and hatchery origin (Ross and Packard, '90).

Scale regeneration in laboratory-bred zebrafish

As a result of studies of elasmoid scales in various teleost families (Sire, '82, '87), it has long been known that the regenerated scales show a typical morphology. For instance, this knowledge has led biologists to reject such scales in aging studies because they provoke a bias in stock composition estimates (Knudsen, '90). The present work confirms that the morphology of the regenerated scales in zebrafish allows them to be distinguished easily from normal scales by the presence of a large "regenerated focus" devoid of ridges and grooves (Sire and Géraudie, '84; Sire et al., '90). This difference is so clear that a regenerated scale can be detected with the naked eye in large specimens, and at low magnification of the dissecting microscope in small specimens, without any particular preparation.

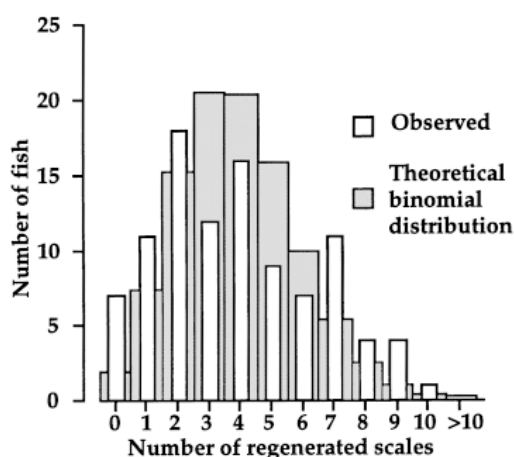


Fig. 3. Number of regenerated scales per fish and adjustment of a binomial distribution.

TABLE 1. Distribution of regenerated scales on the left flank (Fig. 1) of 100 zebrafish¹

Dorso-ventral axis	Antero-posterior axis													Lines
	I	II	III	IV	V	VI	VII	VII	IX	X	XI	XII	XIII	
A	11	11	9	8	11	11	12	13	9	9	3	11	5	123
B	8	10	11	7	14	8	10	9	6	6	8	4	6	107
C	3	6	7	9	18	8	8	5	8	2	8	3	5	90
D	4	4	7	4	2	8	4	9	5	3	5	6	8	69
Columns	26	31	34	28	45	35	34	36	28	20	24	24	24	389

¹Each column represents a total of 400 scales; each row, 1,300 scales.

Scale regeneration is a natural phenomenon that occurs in all species possessing elasmoid scales. In the wild, the number of regenerated scales in an individual depends on the environmental conditions (substrate, intraspecific behaviour, attacks by predators, etc.) and on the manner in which the scales are located in the dermis, either deeply embedded (as generally observed in benthic species) or lying more superficially (as in pelagic species) (Burdak, '86). In benthic species, scales are firmly attached but the species either have territorial behaviour and fight or they have frequent contact with the substrate, while in pelagic species scales can be lost more easily but the species are less in contact with the substrate and have shoal behaviour.

The zebrafish is a pelagic cyprinid that forms shoals; however, its scales are not easily lost due to a considerable overlap by the dermis and the epidermis (Sire et al., '97a,b). It was assumed that the relative protection in the laboratory compared with a natural environment ensured that the zebrafish did not lose numerous scales, and so only a few regenerated scales could be expected in this

population of 100 adults. On average, 7.5% of the scales were regenerated (i.e., 3.9 regenerated scales out of the 52 scales examined per fish). This small number was relatively high considering the relative protection ensured by the breeding conditions. Furthermore, the most dorsal line was more affected than the other lines below, possibly owing to protection by the pectoral fin. This 7.5% scale loss could be explained either by direct shocks by hand nets or by shocks against the substrate (sand, filter) when the fish were frightened. In most laboratories, zebrafish are kept without sand or filters in their tanks. Given the present breeding conditions and the repeated manipulation of zebrafish without any particular care, the percentage of regenerated scales may have been higher than would be expected in less rough conditions. Therefore, the probabilities were probably overestimated compared with those that could be obtained in other laboratories.

The fact that there were significantly less individuals with an intermediate number of regenerated scales than expected indicates that some individuals were more sensitive to scale loss than others, or suffered considerable shocks.

Can this marking technique be applied to other species?

This technique can be applied to all small breeding populations of teleosts having elasmoid scales (e.g., Japanese medaka (*Oryzias latipes*), guppy (*Lebistes reticulata*), and cichlids), provided that they are manipulated carefully and they do not fight when mixed; fights often result in numerous losses of scales. The number of regenerated scales in a laboratory-bred population should be checked before undertaking such a marking experiment. In the wild, the use of this technique is more problematic because wild individuals generally have a high percentage of regenerated scales. Here too should be verified whether these species are suitable for such a marking technique, e.g., that it does not induce mortality.

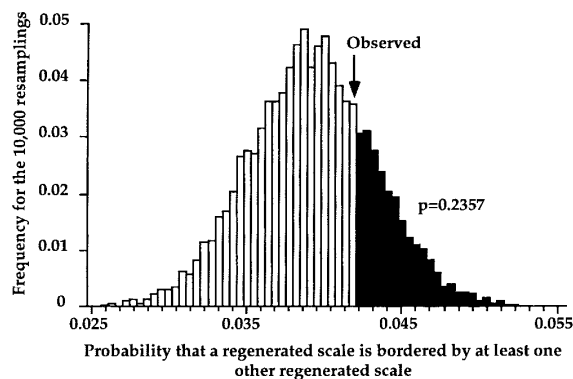


Fig. 4. Distribution of probability that a regenerated scale is bordered by at least one other regenerated scale for 10,000 resamplings. Black bars show the values used to estimate the probability that higher values than the one actually observed (0.0413) are obtained randomly.

Comparison with other marking techniques

In contrast to other marking or tagging techniques applied to different fish species, scale regeneration does not imply any financial investment; the only tools required are a dissection microscope, a pair of forceps, and an anaesthetic. On the contrary, the proposed technique saves both money (less tanks) and time.

The only problem related to this marking technique is the inability to recognize a regenerated scale from a distance. However, the manipulation of the fish is minimal. Anaesthesia is used in most tagging experiments, and the removal of a scale cluster needs only 1–2 min per fish. The mark remains readable throughout the life.

Our technique is based solely on a biological phenomenon, namely, scale regeneration: neither injection of chemicals nor fixation or internal incorporation of a tag is necessary. Scale loss and subsequent replacement occurs naturally and is relatively frequent in fish, so that scale removal and regeneration do not affect the fish (at least in our breeding conditions), in contrast to external or internal tags that could interfere with swimming or chemicals that could perturb physiology.

In conclusion, using experimental scale regeneration as a mark appears to be a valuable technique taking advantage of a natural phenomenon. This technique can save money and time, does not enhance fish mortality in laboratory breeding conditions, and is accessible to everyone working with small colonies of laboratory-bred specimens.

Proposed procedure to mark zebrafish using scale removal

We have demonstrated that removal of a cluster of four scales is enough to mark zebrafish with 100% accuracy. Indeed, when four scales were removed experimentally from the left flank, the probability that a congener had also lost four scales at the same place ranged between 7×10^{-6} and 8×10^{-5} , depending on the row (D to A, respectively). This marking experiment suggested that it was easier to remove a cluster (or maybe successive scales on a line) than four isolated scales because they would be more difficult to recognize. This technique permitted numerous combinations. Once the fish were anaesthetized, it was as easy to remove several scales as only one.

The steps we propose to mark zebrafish are as follows:

1. Prepare precise drawings of the scalation of the lateral flank of the zebrafish (Fig. 1); this

will help in establishing the marking protocol and later to identify the correct row and column to begin assessing scale regeneration. Note the location (line and column) of the cluster of scales planned to be removed in each individual (or in a sample). Use simple combinations and rather spaced scale clusters. For example, if three zebrafish populations have to be mixed in the same tank, remove the cluster (CIII, CIV, DIII, DIV) in sample 1, cluster (CXI, CXII, DXI, DXII) in sample 2, and none in sample 3.

2. Anaesthetize individuals in 0.1 g/litre of MS 222; place the fish on a wet piece of blotting paper under a dissecting microscope; remove the scales as decided in your protocol by seizing the posterior region of each scale with fine forceps, then pull the scale backward to remove it from its pocket; repeat this for each scale chosen to be removed; start with more posterior scales and work forward; this requires only 1–2 min per fish.
3. Place all the operated fish in a separate tank and wait 3 to 5 days (beginning of scale regeneration process) before mixing them with other specimens; the regenerated scales in the marked fish can be removed easily from day 15 on.

To identify marked specimens the following procedure can be applied:

1. Anaesthetize individuals (point 2 above).
2. Start to remove the scales following the expected code of a cluster. Proceed carefully for the first scale; if the first scale is regenerated (large focus), continue until you obtain the expected code; if this scale is not regenerated, meaning that this is not the right fish, put the scale back in its correct place and try either another code or another fish. When a scale is placed immediately back in its pocket, it will remain in place (Sire, unpublished data). When a regenerated scale is removed, it regenerates again so that the code is always the same for each fish. Checking requires only 1–2 min per fish.

Dead specimens can be recognized in the same way. Nevertheless, to avoid tedious research of marked specimens in a tank, mixing numerous specimens with different codes should be avoided, and approximately equal numbers of specimens of each population should be mixed.

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