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Research Article

In vivo staining with alizarin for ageing studies on chondrichthyan fishes

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Abstract – Age determination for stock assessments and conservation of cartilaginous fishes is mainly obtained by counting the annual growth bands in vertebrae. Recent studies show numerous inconsistencies and the need for systematic validation. We assessed the effectiveness of the fluorochrome alizarin red S, a common skeleton vital marker used as a time stamp for teleost fishes, on chondrichthyan. Twenty-five captive small-spotted catsharks (*Scyliorhinus canicula*) were marked by alizarin red S intraperitoneal injections. The fluorochrome produced a wide fluorescent mark on sectioned vertebral centra of all injected fish. Alizarin red S did not have a deleterious effect on growth during three months monitoring. The marks obtained remained stable in vivo for more than four years after injections and were resistant to fading during the observation under the microscope excitation light. Our results suggest that alizarin red S is an effective tool for long time vital marking of chondrichthyans.

Keywords: Age validation / vertebrae / growth / fluorochrome mark / elasmobranch

1 Introduction

Chondrichthyan fishes are mostly characterised by slow growth rates and long life cycles, making them particularly vulnerable to overexploitation (Goldman et al., 2012). As a consequence, many chondrichthyan species are endangered by human activities (Dulvy et al., 2017) and thus require protection measures. Therefore, gathering consistent and reliable data on their life traits, such as age, is crucial for management and conservation (Matta et al., 2017). Chondrichthyan fishes age and growth curves, especially for elasmobranch fishes (i.e. sharks and skates) are mainly obtained by examining opaque-translucent band pairs deposition in vertebral centra (Cailliet, 2015). This method requires, however, a prior validation of the band pairs counting procedure, to confirm that band pairs are deposited annually, allowing an accurate ageing (Goldman et al., 2012). The predominant method used to validate band pair periodicity in chondrichthyans is based on the examination of the forming band on the distal margin of the sectioned vertebral centra (Cailliet et al., 2006). However, these validations are difficult for slow-growing fishes due to the weak levels of contrasts in the margin of newly formed cartilages (Holden and Vince, 1973; Chin et al., 2013) and according to Campana (2001), they are insufficient and often misused as age validation methods.

The most robust validation methods rely on studying calcified parts of vertebrae from known age specimens, which have grown in natural environment. Obtaining such specimen is possible in particular by studying the increase in radiocarbon (^{14}C) due to nuclear weapons tests sixty years ago (Campana et al., 2002). This method has been successfully used to determine the age of chondrichthyans, however, it is now limited to species with extremely long longevities. An intermediate solution is to confirm the deposition periodicity in calcified parts with a partial known growth. This can be achieved by means of a mark-recapture method along with administration of a chemical marker that binds to growing calcified parts (Holden and Vince, 1973; Natanson et al., 2018). Fluorochrome compounds are the most widely used chemical marker for this purpose (Goldman et al., 2012), mainly because the observation of the fluorescent marks in the calcified parts of fishes is simple and inexpensive (Wickström and Sjöberg, 2014). The three main fluorochrome markers used on fishes are calcein (CAL), various forms of tetracycline (TC) and alizarin red S (ARS).

For the purpose of long term vital marking, the select marker should fulfill several criteria. First, the fluorescent mark needs a sufficient intensity to ensure the marking

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durability (McFarlane and Beamish, 1987), many authors reported failure to detect the fluorochrome marks on shark vertebrae (Smith, 1984; Kusher et al., 1992; Parsons, 1993; McAuley et al., 2006; Chin et al., 2013; Harry et al., 2013). Second, to ensure a valid estimate of age and growth, the marker should have no impact on growth on medium-term (i.e. several month) and long-term (i.e. several years) periods. Inevitably, in the short term (i.e. few weeks), handling and marking cause stress and have an impact on fish growth. This disadvantage is minimized when recapture occurs long after the marking. Third, marks on the processed calcified parts must also have enough intensity not to fade during the first exposure to the microscope excitation light used for fluorochrome activation. Finally, for a given marker these criteria can only be achieved with an optimal dosage. According to McFarlane and Beamish (1987) and Simon and Dörner (2005), the optimal dosage is the one which provides the maximum number of recaptured fish with usable marks.

In Europe, in situ growth studies on teleost larvae commonly use marking techniques by bathing fishes for several hours in a solution of ARS (Wickström and Sjöberg, 2014). The ARS molecule can be a good alternative to TC, whose use is regulated and limited due to its antibiotic action, or to CAL, which is much more expensive and produces less distinct and persistent marks than ARS (Bashey, 2004). Although ARS is commonly used on teleosts, it has not been widely used on cartilaginous fishes so far. About twenty years ago, ARS seems to have been tested among five other fluorochromes on chondrichthyans vertebrae (Officer et al., 1997), but ARS results are not mentioned. More recently, ARS has been used on chondrichthyans (Tovar-Ávila et al., 2008), but only on the fin spines and over a few months period. Therefore, the objective of the present study was to test ARS on catsharks, *Scyliorhinus canicula* (L.), to develop a long term vital marking on chondrichthyan fishes for accurate ageing and growth estimation, that ensures high mark intensity compatible with long-term marking, without significant impact on growth. The present study also proposes, for the first time on fish vertebrae, a method to quantify fluorescent mark intensity, and tests the persistence of fluorescent marks on processed vertebrae, in relation with the fading phenomenon due to the microscope excitation light.

2 Materials and methods

2.1 In vivo experimental design

Three successive in vivo trials were conducted on immature small-spotted catsharks, *Scyliorhinus canicula* (L.) born in captivity at the Concarneau Marine Biological Station (France). This species of small benthic shark (named catshark in this paper) is abundant in French inshore waters. Catshark have relatively slow growth rate with a size of around 60 cm achieved after ten years and late maturity beginning at age eight in the Celtic Sea (Ivory et al., 2004).

On teleost fishes, for vertebral marking with ARS, Yamada (1971) recommended the injection of a dose of 40 to 50 mg kg^{-1}, whereas Meunier and Boivin (1978) reported a dose of 80 mg kg^{-1} to obtain a sure mark on the vertebrae and without effects on growth. Since a higher dosage ensures a better durability of the mark, the 80 mg kg^{-1} was tested first.

In June 2012, five catsharks were given an injection of ARS at a dose of 80 mg kg^{-1} bodyweight, to verify the harmlessness of the recommended dose in teleosts, and the intensity of the fluorescent marks in the short and medium term.

In August 2012, based on the previous results, eight catsharks were injected with a lower dose of 60 mg kg^{-1} ARS, in order to verify the intensity of the marks in the short-term and time required for mark formation.

The third trial, in October 2012 was conducted to detect a possible impact of the ARS injection on growth at the dose of 60 mg kg^{-1}. Twelve catsharks were injected with a solution of ARS while twelve others received the same volume with only physiological saline solution (PSS) to form a control batch. Fish growth was monitored by measurements of the total length at the injection time and then every 30 days during three months. Beyond this period, this trial also allowed to measure fluorescence mark intensity in the medium and long-term, through sacrifices at various time intervals.

Due to ARS acidity, injected solution was composed of ARS powder (Amresco 9436-25) diluted at 0.85% in a solution made of PSS and sodium hydroxide to raise the pH to 6.8. Intraperitoneal injection has been chosen as recommended by Panfili et al. (2002). In contrast with intramuscular injection, it allows the injection of a larger volume and probably a faster assimilation of the marker according to Branstetter (1987).

For individual identification all catsharks were externally tagged through the dorsal fin by small T-Bar Anchor Tags. They were bred in the same tank at a temperature between 10 and 16 °C and for a period ranging from 4 days to 51 months. For mark observation, all ARS marked fish were anaesthetized with eugenol aqueous solution 0.025 ml L^{-1}, and then euthanized by eugenol overdose. Among the control batch, only seven fish were sacrificed to get the natural fluorescence value of unmarked vertebrae.

2.2 Mark observation and intensity measurement

Three post-cranial vertebrae were removed, cleaned and embedded in black epoxy resin (Buehler 20-8502), as done for age reading studies (Panfili et al., 2002, Campana, 2014). For each vertebra studied, a thin longitudinal section of the centrum (Fig. 1) was cut with a diamond saw blade (Struers Discoplan-TS) and the sectioned vertebral centra was progressively polished with a polishing wheel (Struers Labopol-5).

The fluorescent marks were observed with an OLYMPUS BX 51 microscope (excitation filter: 530–550 nm; dichromatic mirror: 570 nm; barrier filter: 590 nm; magnification X100). The luminous intensity of the marks was quantified by the NIS-Element software (NIKON), with a high resolution digital camera (NIKON DSRI-1), along transects crossing the fluorochrome marks (Fig. 1).

Values of the relative intensity of the mark (RMI) were obtained for each recorded photo by adjusting exposure time below the saturation level of the pixel luminosity. It was based on intensity differences between the fluorescent mark and the background as proposed by Lochet et al. (2009) on otoliths, but with a different method of mark measurments and the addition

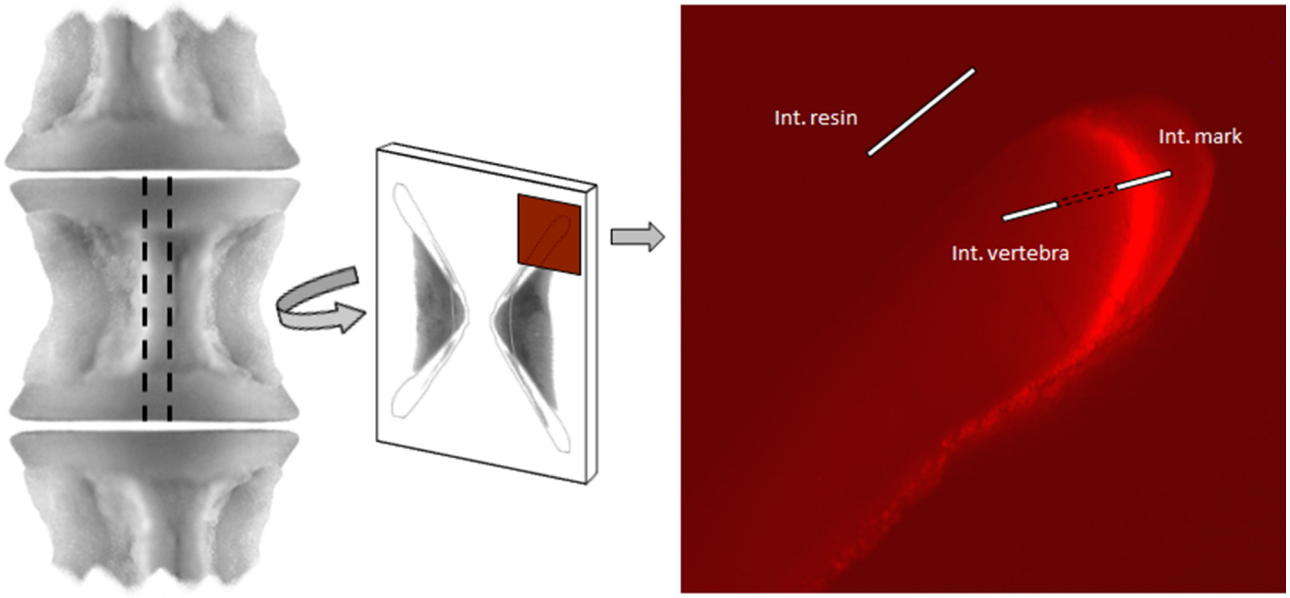

Fig. 1. Diagram of the vertebral centra sectioning plane and ARS mark intensity measurement transect. On the picture the white bar represents intensity measurement transects of the mark, the vertebra and the resin.

of a reference point in the resin (Fig. 1):

RMI = (Int. mark – Int. vertebra) / Int. resin

Int. mark = Intensity value of the first third of the measurement transect, containing the fluorochrome mark;
Int. vertebra = Intensity value of the opposite third of the transect, measuring the natural fluorescence of the vertebra;
Int. resin = Intensity value of the resin transect, measuring the fluorescence intensity of the epoxy resin.

In order to compare the intensity of the marks at different ages and therefore on vertebrae of different sizes, the transect length is based on the width of the sectioned vertebral centra. Similarly, to allow a comparison between fish, an individual value of RMI (iRMI) was calculated by averaging values of relative intensity achieved on the three post-cranial vertebral centra.

When exposed to their excitation wavelength, the fluorescent marks have the capability to produce light, but this reaction leads to mark fading. To prevent this loss of fluorescence intensity, processed samples were stored in the darkness and illumination time was limited to the required time for photo acquisition. Intensity measurement on unmarked vertebral centra of the control batch fish was achieved with the same method, in the part of the vertebral centra corresponding approximately to the injection period.

2.3 Measurement of fading resistance on processed vertebrae

Stability of the 60 mg kg^{-1} ARS marks was tested by measuring the fluorescence decrease during a continuous exposure of marked vertebrae to the fluorochrome excitation wavelength. They were photographed at their earliest exposure, then after 1, 3, 10, 30 and 60 min, which represents a continuous and cumulative time of 1 h and 44 min of illumination. The RMI was calculated as described above. This measurement was performed on a sample of five vertebrae, each from different catsharks marked at the third trial and having all more than 100 days of post-marking growth. This sample includes vertebrae with low, high and medium values of RMI.

Statistical analysis has been performed with R software (http://www.r-project org/) and significance level was set at $p < 0.05$. The data set did not meet the assumption of normality and equality of variances. Thus, non-parametric Wilkoxon-Mann-Whitney tests were used to compare marked and unmarked fishes in terms of growth and mark intensity. In addition, the stability of marks over time was statistically evaluated with a Pearson test.

3 Results

3.1 Mortality and growth monitoring

Injections did not induce mortality during the first and the third trial, but two fish injected with ARS died four days after injection in the second trial. Autopsy showed numerous inflamed zones on the peritoneum and organs, with presence of small crystals of insoluble ARS. Mortality seems linked to undissolved ARS presence at the bottom of the injected solution. Vertebrae of the two dead individuals have not been processed due to this abnormality.

During the three months monitoring of the third trial, individual growth showed no significant difference between fish injected with ARS or PSS (Wilkoxon-Mann-Whitney test, $p = 0.9307$), even between all other time periods considered (Tab. 1).

Table 1. Three months growth monitoring of *Scyliorhinus canicula* and probability of differences between physiological saline solution (PSS) and the 60 mg kg^{-1} alizarin red S (ARS) dosage (total length mean values ± SD. $n = 12$ per protocol, Grey zone: Wilkoxon-Mann-Whitney test results for different considered growth periods).

	at marking		at 1 month		at 2 months		at 3 months	
Treatments	PSS	ARS	PSS	ARS	PSS	ARS	PSS	ARS
Total length	320.9 ± 93.8	321.8 ± 93.3	330.4 ± 96.1	330.8 ± 95.8	338.8 ± 98	340.2 ± 97.9	347.4 ± 100.2	349.3 ± 100.2
Wilkoxon Tests	W = 74, p = 0.9307							
	W = 71.5, p = 1							
	W= 58.5, p = 0.4511							
			W = 82, p = 0.581					
			W = 161, p = 0.1829					
					W = 86, p = 0.4338			

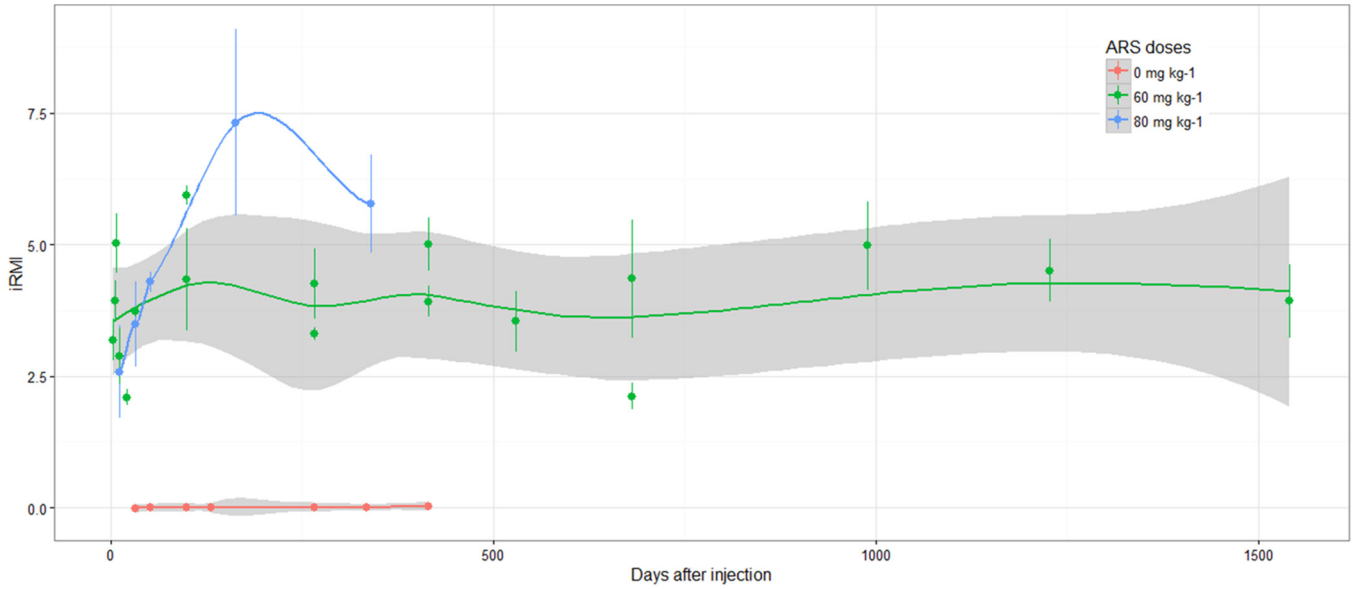

Fig. 2. Individual relative intensity of the fluorescent marks (iRMI) over time. Mean iRMI values and standard deviation of the three vertebrae processed per individual. Continuous lines represent trend curves of the three injection protocols. Grey zone represents the 95% confidence interval for the control batch and the 60 mg kg^{-1} alizarin red S (ARS) dosage.

3.2 Marking rate and mark intensity

Regardless of dosage or time period after injection, all catsharks marked during the various trials showed red bright fluorescent marks on every three vertebra sample processed per individual. The fluorescent marks were visible on the distal margin of the sectioned vertebral centra four days after injection and probably earlier. One month after injection, the fluorescent marks were complete and well delimited on the outer margin of the vertebral centra.

The iRMI of the various trials (Fig. 2) showed values higher than 2.10 (± 0.15) and 2.58 (± 0.88) for the respective dose of 60 and 80 mg kg^{-1}ARS. These lowest mark intensity are, however, greatly sufficient for age validation studies. The highest iRMI value (5.77 ± 1.77) was obtained with an ARS dose of 80 mg kg^{-1}, 164 days after injection and can be considered as a very high intensity mark, more yellowish than red. Marks with RMI value greater than 5 can be seen without fluorescence microscope and appear as a brown line in the sectioned vertebral centra.

Mark intensity was highly variable, independently of ARS dosage, and between individuals as well as between vertebra. In contrast, the control batch iRMI values was close to zero and had a very low standard deviation (0.00 ± 0.01). Over a comparable period of 32 to 415 days post-injection, RMI values differences between control batch and ARS injected batch were highly significant, even with the lowest ARS dosage (Wilkoxon-Mann-Whitney test, $p = 2.772^{-08}$).

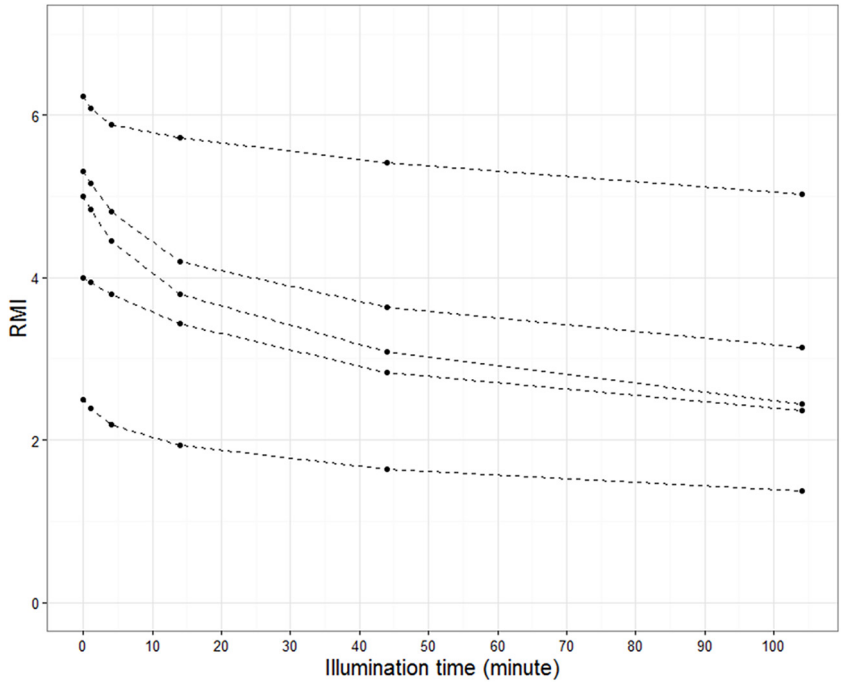

Fig. 3. Decrease of the relative fluorescent mark intensity (RMI) during prolonged illumination time on five vertebrae of *Scyliorhinus canicula* marked with a dose of 60 mg kg^{-1} alizarin red S.

Finally, at 60 mg kg^{-1} ARS dosage, mark intensity remains stable (Fig. 2), with no significant decrease after more than four years of growth post-injection (Pearson test: $t = -0.4149$, df = 10, $p = 0.6869$).

3.3 Fading resistance on processed vertebrae

During a continuous exposure of the sectioned vertebrae to the fluorochrome excitation wavelength (Fig. 3), decrease of mark intensity due to illumination was not constant. Greater reduction were observed during the first fifteen minutes. After 1 h and 44 min of exposure, intensity decreased between 19.3 and 49.9% of the initial RMI value. Similarly, during the first minute which corresponds to the time required to take a photograph, intensity decreased between 1.4 and 4.4%.

4 Discussion

4.1 Mortality and growth

The absence of mortality in the first trial with 80 mg kg^{-1} ARS dosage confirms the results obtained by Meunier and Boivin (1978) on carp *Cyprinus carpio*. The death of two individuals during the second trials with the lower dose of 60 mg kg^{-1} ARS could be linked to the Amreso ARS powder heterogeneity. In order to prevent injection of undissolved ARS particles, it is recommended to pay particular attention to fineness of the available ARS powders and if necessary, to filter the solution before injection.

Growth monitoring over three months did not show deleterious effect of ARS on growth at 60 mg kg^{-1} dosage. Similar results were obtained by Meunier and Boivin (1978) with a 80 mg kg^{-1} ARS dosage, showing however a growth stagnation during the first days. In the present study, the ARS dose of 60 mg kg^{-1} did not induce detectable growth slowdown one month after injection. A general lack of appetite more substantial than those following manipulations for growth monitoring was observed within days following the injection, but it also occurred on the control batch individuals and therefore can be a result of stress caused by injection and handling.

4.2 Marking rate and mark intensity

Clear fluorescent marks of ARS were observed on all three processed vertebra of each injected catshark, while marking rates reported in the literature, are often below 80% with other markers. The intensity of fluorochrome marks and consequently their longevity depend on the injected dose (Casselman, 1983, Lagardère et al., 2000; Baer and Rösch, 2008) and failure in long-term marking are mainly due to a fluorochrome under-dosing solution (Parsons, 1993).

Our results show that the dosage of 80 mg kg^{-1} ARS recommended by Meunier and Boivin (1978) on teleost fishes is largely sufficient for vertebra marking on catshark. However, a lower dose of 60 mg kg^{-1} ARS allow as well to obtain stable marks in vivo and over a period of more than four years, with no marking failures. On chondrichthyan dorsal spines, an ARS mark described as "visible" by the authors was obtained on five individuals several months after the injection, with dosage of 25 mg kg^{-1} (Tovar-Ávila et al., 2008). Given this result, it is likely that the dose of 40 to 50 mg kg^{-1} ARS recommended by Yamada (1971) on teleost fishes should be sufficient.

Several other hypotheses may be advanced as possible cause of failure. According to Blabolil et al. (2018) light exposure of calcified parts in vivo and after extraction may explain some loss of TC fluorochrome marks. A poor uptake of fluorochrome due to weak somatic growth in adult sharks (Harry et al., 2013), or the season of injection due to variations in calcification activity (Smith, 1984) and duration of metabolisation and excretion (Yamada, 1971; Smith, 1984) could also lead to insufficient mark intensity.

Despite the injection of a dose adapted to individual weight, significant variations of mark intensity level were observed. Individual variability has been reported in most articles, regardless of the fluorochrome used. In this study with trials carried out at different seasons and durations, some of the iRMI variability may be due to growth activity related to water temperature (Smith et al., 2013) or seasonal-related changes (Smith, 1984). In the same way, during the short-term marking protocol, mark formation delay could also induce variability between iRMI measurement.

Indeed, iRMI values of incompletely formed marks could be underestimated, if they are measured less than one month post-injection.

Since the end of this study, results of Natanson et al. (2018) showed that in many shark species there is variability in band pair counts between vertebrae along the vertebral column, in relation with vertebrae growth, particularly in girth. Similarly, fluorescent marks are probably wider on the largest vertebrae of the abdominal cavity. Given the RMI measurement, based on a transect whose length was equivalent to the width of the sectioned vertebral centra, RMI value should be stable along the vertebral column. Regardless, in order to compare RMI values, the processed vertebrae must come from the same area along the column.

On teleost fishes, the observation of ARS marked otolith at high magnification shows a continuous and homogeneous fluorescent line (Caraguel et al., 2015). In contrast on the catshark vertebrae, ARS marks appear as a wide and grainy line in a lacunar and irregular structure, regardless of sample polishing. This grainy appearance increase intensity measurements variability and might partly explain variability between vertebrae sample from the same individual. As similarly observed by Yamada (1971), fluorescent marks appear comparatively wider and diffuse in the vertebrae of *Cyprinus carpio*. But this phenomenon could be stronger in chondrichthyans fishes because vertebrae are irregularly mineralized (Dean and Summers, 2006) and poorly crystallized (Clement, 1992).

The fluorochrome mark intensity level obtained is rarely specified in studies using vital markers, and at best it is qualified with a scale of intensity (Tovar-Ávila et al., 2008) dependent on observer. In order to compare marking protocols, Lochet et al. (2009) provide a quantification method of mark intensity for fish otoliths. However, this method requires photo taken under exact same conditions, which was not possible in the present study. With samples obtained over several years, parameters such as camera settings and UV lamp power were hardly reproducible. Moreover, to obtain accurate mark intensity measurements with different fluorescence levels, it is necessary to adapt the aperture or exposure time in order not to saturate the camera sensor. In agreement with Lochet et al. (2009), preparations and images standardization is an important step because mark intensity is influenced by background intensity. Processed vertebrae are surrounded by the inclusion resin and the use of opaque resin avoids fluorescence diffusion from the mark into the background and preserves the contrast. In addition, in order to compare fluorescent marks, the measurement of background fluorescent intensity on opaque resin allows calibrating each photo and thus standardizing the lightning conditions.

4.3 Time required for mark formation

Although the fluorescent mark was already visible on the fourth day after injection, the complete formation takes approximately one month. Beyond that, newly formed cartilages on the outer margin are no longer fluorescent, and the mark can be distinguished from the outer margin of the sectioned vertebral centra. Consequently the values of RMI obtained before one month are therefore not representative of the definitive mark intensity. Similar results were obtained on shark injected with TC. According to Izzo et al. (2007) marks begin to be observable on the sectioned vertebral centra the second day and were clearly apparent on all fish the fifth day after injection. The maximum contrasts were also observed at six months. Likewise, according to Branstetter (1987) the observation of vertebrae of two fish accidentally dead at 36 hours and three days after injection respectively show the absence and the presence of a fluorescent mark. Significantly longer uptake times were reported by Gruber and Stout (1983) with TC, but this could be due to intramuscular injection mode. In sharks, the uptake pattern of ARS seems comparable to TC and allows an almost instantaneous marking with only 2 to 4 days lag time between intraperitoneal injection and fluorescent mark detection.

4.4 Fading resistance on processed vertebrae

Under the microscope, after 1 h and 44 min of illumination with the excitation light, the ARS fluorescence remain at a high level and no mark extinction was observed. Even with the lowest intensity mark, there is no risk of confusion between marked and unmarked vertebrae. Fluorescence decrease is more important during the first five minutes, but does not have the immediate deleterious effect reported by Smith et al. (2003), with the TC fluorochrome. Nevertheless, for a precise mark quantification, photo or intensity measurements must imperatively be performed during the first exposure to the excitation wavelength.

5 Conclusion

Age validation studies are needed to understand the banding pattern on chondrichthyans vertebrae and to acquire reliable growth data on newly studied species. In agreement with Natanson et al. (2014) and Passerotti et al. (2014), validations also allow reassessing growth data of species for which current age reading methodology might tend to underestimate age (Harry, 2018; Natanson et al., 2018) and therefore, having a risk of being incorrectly managed and consequently over-fished

(Cailliet, 2015). Applied to catshark, in vivo staining with ARS represents an effective solution, requiring only conventional equipment for mark observation. Measurement of mark intensity by means of the RMI method showed the stability of ARS fluorochrome during the fish life, and a limited fading during the observation of processed vertebrae. This allows considering a possible reduction of the dose to be injected. In the same way, to confirm these promising results, future studies should be implemented at a wider scale, in wild conditions and conducted on other elasmobranch species.

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