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Age validation studies on the centra of *Raja clavata* using tetracycline

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The results of an experiment in which 348 *Raja clavata* were tagged and injected with tetracycline in November 1968 are described. This drug is deposited in calcified tissues and fluoresces in ultra-violet light, and can therefore be used as a time marker in calcified skeletal structures. There were 86 recaptures from the experiment by mid-October 1971, but only 16 fish were actually returned. Examinations of these showed that tetracycline was deposited in all the calcified skeletal structures present at the time of injection. It was not found in the secondary sexual alar and malar spines of a male ray which was immature at tagging and mature at recapture. The deposition of tetracycline in relation to the opaque and hyaline zones occurring on the centra of the vertebrae is described in detail. Six of these rays had just started the formation of a hyaline zone at the time of injection, and six were still forming an opaque zone; one ray was in a transitional stage. These observations, combined with those on the appearance of the opaque zone at the periphery of the centrum, show that formation of the opaque zone probably occurs in the latter half of the year but usually does not become visible, when the centra are observed by reflected light, until the early months of the subsequent year. By relating the opaque and hyaline zones to the deposition of tetracycline it is proved that one of each type of zone is laid down annually. The difficulties in interpreting the edge structure in relation to the use of the centra for age determination are discussed.

Introduction

Rings have been described on the centra of the vertebrae of *Raja fusca* by Ishiyama (1951), of *R. eglanteria* by Daiber (1960) and of *R. erinacea* by Richards, Merriman and Calhoun (1963). They have also been observed on the centra of porbeagle sharks, *Lamna nasus*, by Aasen (1963) and on those of basking sharks, *Cetorhinus maximus*, by Parker and Stott (1965). None of these authors was able to prove that the rings which they observed were annual and, in fact, Parker and Stott concluded from a comparison of the number of rings on the centra at a given length with length-for-age derived independently that two rings of the same appearance were laid down annually. Rings also appear on the centra of rays occurring in British waters, and in order to determine whether these are annual a method was used based on the injection of tetracycline to introduce a mark of known date into the skeletal structures. This technique was described by Kobayashi et al. (1964) who worked on goldfish, and it has also been used successfully by Jones and Bedford (1968) working with North Sea cod. Tetracycline is deposited in vertebrates at sites where calcification is taking place, and it fluoresces in ultra-violet light. In the teleost species which have been studied to date it is incorporated into skeletal structures in the growing zone which is being formed at the time of injection, thus giving a region which is visible under ultra-violet light, when these structures are subsequently examined, and which is of known date. The timing of occurrences of any natural variations, such as growth zones, can then be deduced by relating their positions to that of the fluorescent zone.

Methods

During November 1968, 348 *Raja clavata* caught in the southern North Sea, off the east coast of England between latitudes 51°55′N and 52°02′N and longitudes 1°29′E and 1°33′E, were tagged with Petersen discs and injected with tetracycline which was made up by dissolving 500 mg of tetracycline hydrochloride

in 19 ml of isotonic elasmobranch saline (Bialaszewiez, 1933, quoted by Pantin, 1948). The first 134 rays tagged were injected at a dose rate of 100 mg tetracycline hydrochloride per kg of body weight, and the remaining 214 rays at half this rate. In order that the solution would enter the body cavity only, each fish was laid with its ventral surface uppermost and held by the tail at an angle which caused the viscera to move away from the posterior region of the body cavity. The tetracycline was injected into this region, just anterior to, and to the left side of the cloacal opening. This technique was not suitable for large mature females which contained egg capsules, because the eggs occupied the space otherwise formed when the viscera moved forwards, but only three rays which possibly c ontained eggs were injected.

Results

General deposition of tetracycline

The first ray returned, in March 1969, had heavy depositions of tetracycline in all its skeletal structures; all the placoid scales fluoresced, the bases of the "buckler spines" (placoid scales with swollen bases) being particularly bright. The quantity of tetracycline in the teeth was so large that it was visible as a greenish hue in daylight. Both the region of tagging and the liver fluoresced brightly. All injected rays subsequently returned had deposits of tetracycline essentially similar to that of the first recapture, although the intensity of fluorescence varied slightly from ray to ray. This did not appear to be related to the dose rate; at least one of the rays injected at the full dose rate fluoresced much less brightly than others injected at the half dose rate and recaptured later. Unfortunately, only the first ray was returned ungutted, so it was not possible to determine for how long tetracycline was stored in the liver. One of the most interesting recaptures was of a male ray which was immature when injected and mature on recapture; all the placoid scales and spines fluoresced except those which are secondary sexual characteristics, the alar and malar spines.

Deposition in the centra

The centra form the main portion of the vertebral column, less its neural and haemal arches and ribs. Each centrum consists essentially of two, obtuse, hollow cones of partially calcified cartilage with their apices opposed (Figure 1). Viewed under reflected light the inside of each cone presents a series of concentric rings of alternating degrees of brightness, surrounding a hole marking the position of the primitive notochord: the brighter of the two types of rings are the opaque zones and the less bright the hyaline zones. At the periphery of each cone there is a narrow band of connective tissue which has a higher reflectivity than the opaque zones.

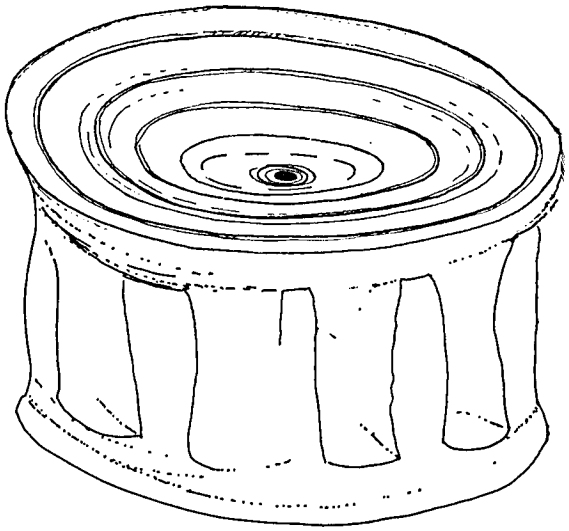

Figure 1. Centrum of *Raja clavata*.

Sixteen rays had been recaptured by Mid-October 1971, details of which are given in Table 1, in date order of recapture. The whole face of each centrum of the first two rays recaptured fluoresced brightly although there was a brighter ring at the periphery. The faces of the centra of the third recaptured ray were similar except that there was a narrow hyaline zone distal to the very bright fluorescent ring. The appearance of the faces of the centra of the remaining 13 rays is shown diagrammatically in Figure 2. There are three main points of interest:

1. for each ray, except three (EZ 3540, EZ 3664 and EZ 3872), there is a ring which fluoresces very intensely, distal to which there is no fluorescent material at all;
2. within this distal, very intensely fluorescing ring there is great variation in the amount of absorbed tetracycline, ranging from none in rays EZ 3913 and EZ 3523 to deposits in all the zones in rays EZ 3684, EZ 3806, EZ 3740 and EZ 3718;
3. the position of the distal very bright ring does not always coincide with the same type of zone; in five instances it coincided with a hyaline zone, in six with an opaque zone, and in one case with the transition from an opaque to a hyaline zone (Table 1 and Figure 2).

Table 1. Details of recaptured *Raja clavata* and appearance of the edge of the centrum. H = hyaline; O = opaque

Tag number	Date of recapture	Sex	Total length (cm) at release	Total length (cm) at recapture	Dose rate (mg/kg)	Edge of centrum at injection	Edge of centrum at recapture
EZ 3738	21 Mar 1969	♀	69	70	50	?	?
EZ 3911	13 May 1969	♂	51	52	50	?	?
EZ 3915	27 Jul 1969	♂	40	43	50	?	H
EZ 3920	30 Sep 1969	♂	50	55	50	O	H
EZ 3540	6 Oct 1969	♀	37	45	100	O	H
EZ 3545	15 Oct 1969	♀	67	71	100	O	H
EZ 3777	5 Mar 1970	♂	56	63	50	H	H
EZ 3684	20 Apr 1970	♂	43	57	50	O/H	H
EZ 3913	19 Aug 1970	♀	44	53	50	H	H
EZ 3523	17 Sep 1970	♀	36	57	100	H	H
EZ 3664	12 Oct 1970	♂	66	74	50	H	H
EZ 3806	9 Mar 1971	♀	54	75	50	O	H
EZ 3872	23 Mar 1971	♂	55	63	50	H	H
EZ 3770	20 Jul 1971	♂	61	73	50	O	O
EZ 3740	5 Sep 1971	♂	46	66	50	H	H
EZ 3718	Oct 1971	♂	55	79	50	O	H

Conclusions

The time of formation of the opaque and hyaline zones

Except for two rays (EZ 3913 and EZ 3523) there was not a single, sharply defined fluorescent ring on the centra as observed by Kobayashi et al. (1964) on those of goldfish, but it is assumed that the brightest distal fluorescent ring corresponds to the time of injection and that tissues peripheral to this were laid down subsequently. The widespread distribution of tetracycline throughout the skeletal structure and its absence from the secondary sexual characteristics of the one male which matured between injection and recapture, as described earlier, also indicate that the line of cessation of the tetracycline deposits corresponds approximately with the time of injection. It is approximate because the tetracycline is deposited

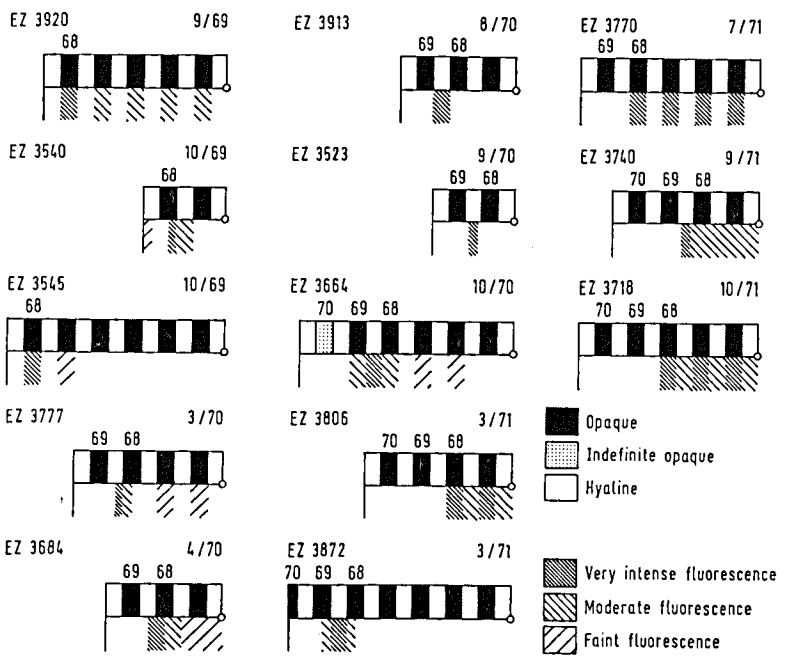

Figure 2. Diagrammatic presentation of the centra of recaptured *Raja clavata* showing relationship between the opaque and hyaline zones and the deposition of tetracycline.

over a period of weeks; Kobayashi et al. (1964) could not distinguish separate bands of tetracycline from injections at monthly intervals but could at two-monthly intervals.

As five rays had an opaque zone coincident with the distal brightest fluorescent ring, six with a hyaline zone and one with a transitional zone from opaque to hyaline (Table 1), it is clear that November marks the average time of changeover from the deposition of opaque to hyaline zones. However, this transition is spread over a period of months. Rays EZ 3913 and EZ 3523 had completed the formation of their opaque zones at the time of injection and the latter had definitely started the formation of a hyaline zone. In comparison an opaque zone was still being formed on the centra of rays EZ 3920, EZ 3545, EZ 3806, EZ 3770 and EZ 3718 and, allowing for a minimum of a month during which tetracycline was being deposited, these zones were not completed until mid-December at earliest. Thus, although it is not possible to state from the results of this experiment over what period the formation of the opaque zone extends, the most probable period is July to December. There is also some evidence that its formation is complete earlier in smaller rays than larger ones; only one of five rays smaller than 49 cm at tagging had the distal, brightest, fluorescent ring coincident with an opaque zone, compared with four of the seven rays 50 cm and longer (Table 1).

Time of appearance of the opaque and hyaline zones

The time of formation of the opaque zone is not necessarily the time at which it becomes visible at the periphery of the centrum; only one ray (EZ 3872), recaptured in March, had an opaque edge. Unfortunately, observations on the type of zone present at the periphery are made difficult by the presence of connective tissue; this has a higher intensity of reflection which appears to obscure the opaque zone until the two become separated by a hyaline zone. Thus, there is probably a time lag of up to six months between the time of formation of the opaque zone and its appearance at, or near, the periphery of the centrum. This implies that the appearance of a hyaline edge is normal and that the distal opaque zone will always be that which was laid down in the year previous to the year of capture. Thus, between July and December the opaque zone being formed will be masked by reflections from the connective tissue, and the distal opaque visible will be that of the previous year; between January and June the distal opaque zone will be that of the previous year, separated from the connective tissue by a hyaline zone. This will be the usual situation; however, there will always be doubtful instances in the later months of the year, comprising those fish which have laid down an opaque zone early so that it is visible by November–December.

Validation of annual growth zones

Taking November as the month in which 50% of the rays have an opaque edge to their centra and 50% a hyaline edge, as shown by the positioning of the distal, brightest, fluorescent ring, the 1968 opaque zone would be either that which is wholly or partially coincident with this ring or that which is immediately adjacent centrally to the hyaline zone coincident with this ring. These opaque zones are labelled "68" in Figure 2, and all subsequent zones serially numbered. Using the criteria detailed above, if the opaque zones are formed annually, the final opaque should correspond either with the year of recapture or the preceding year, depending upon the month of recapture. These conditions are fulfilled with one exception. All the rays caught in March and April (EZ 3777, EZ 3684, EZ 3806 and EZ 3872) had the opaque zone of the previous year fully formed except for ray EZ 3872, the oldest of the four, in which it was still forming. Rays caught in August and September (EZ 3920, EZ 3913, EZ 3523 and EZ 3740) showed the same appearance; the opaque zone of the year of capture, supposing it to be forming, was not visible. In October, three of the four rays recaptured still showed no sign of the opaque zone of the year of capture at the peripheries of their centra (EZ 3540, EZ 3545 and EZ 3718) but one (EZ 3664) had forming an indistinct opaque zone, which was assumed to be that of the year of recapture. All the rays exhibit the formation of one opaque and one hyaline zone a year except for ray EZ 3770, the distal opaque zone of which should have been formed in 1970, not 1969. Therefore, with one exception, the recaptured rays whose centra are described in Figure 2 had laid down one opaque and one hyaline zone a year since they had been injected with tetracycline.

Discussion

At the dose rates used, tetracycline was absorbed by all the skeletal tissues of the injected rays and this, combined with the apparent greater permeability of cartilage compared with bone, resulted in the deposition of tetracycline in all the calcified tissues present at the time of injection. A dose rate of 25 mg per kg of body weight (half that used on the majority of

rays), or even less, would probably be sufficient to give a sharply-defined ring.

In general there was no sharply defined zone marking the time of injection as found in teleosts. Although the tetracycline was deposited at high concentration in the hyaline zones being formed at the time of injection it was taken up most markedly by the opaque zones of those which had been laid down previously, indicating that part of the difference between opaque and hyaline zones in the centra of rays is due to greater calcification of the former.

There was also some deposition of tetracycline subsequent to the distal, brightest, fluorescent ring in rays EZ 3540, EZ 3664 and EZ 3872, indicating that the tetracycline is secreted slowly; the very bright fluorescence of the liver of the first recaptured ray suggests that it is stored in this organ. It may be this factor which accounts for the anomalous zone formation of ray EZ 3770, suggesting that the deposition of tetracycline coincident with the opaque zone labelled "68" resulted from the utilization of tetracycline stored in the liver, and that this opaque zone was actually formed in 1969, which would make it consistent with the hypothesis of annual ring formation.

The main problem in using the centra for age determination stems from the difficulties of both presentation and the highly reflective connective tissue at the periphery of the centrum, making interpretation of the edge difficult, and this will present particular problems in the period December to March, particularly in the older fish in which the appearance of the opaque zone is delayed; for example, compare rays EZ 3777 and EZ 3806 with ray EZ 3872. Unfortunately, owing to the seasonal nature of the fishery into which these rays were tagged there were no recaptures from this experiment in these winter months. However, interpretation of the edge of any structure used for age determination is a common difficulty and does not invalidate the conclusions drawn from this experiment.

Summary

1. An experiment is described in which tetracycline was injected into tagged rays. Tetracycline is a drug which is deposited in calcified tissues and fluoresces under ultra-violet light.
2. Using the tetracycline deposited in the centra of the vertebrae as a time marker it was shown that the period of formation of the opaque zones, which appear on the centra of the vertebrae, is protracted but that in November, when the experiment was conducted, half the recaptured rays had just completed formation of an opaque zone.
3. It was concluded that one opaque and one hyaline zone are laid down each year.
4. It is concluded that a dose rate of 25 mg of tetracycline per kg of body weight, or less would be sufficient for elasmobranchs.

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