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Tittel: A GUIDE TO THE EXTRACTION AND INTERPRETATION OF OTOLITHS FROM LARVAL AND PELAGIC JUVENILE ARCTO-NORWEGIAN COD *(Gadus morhua)*

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Teknikken forbundet med å plukke ut og tyde mikrostrukturen i otolitter fra larver og postlarver av Norsk-Arktisk torsk er beskrevet i dette arbeidet. Soner blir lagt ned daglig og sonenedleggingen starter ved klekking. Det er imidlertid lett å overse de første 2-3 sonene da disse er smale og utydelige. Det kan også være noen problemer forbundet med tolkningen av dagsonemønsteret i otolitt til postlarver. Årsaken til dette kan være at de vokser svært raskt (dobbelt så raskt som postlarver av torsk fra kysten av Canada), og at de oppholder seg i et miljø med kontinuerlig lys. Ved hjelp av lysmikroskop kan en telle opp til 120 dagsoner. Det anbefales å benytte lapillus framfor sagitta. Det er en lineær sammenheng mellom otolittradius og larvelengde, og det synes også å være en klar sammenheng mellom sonebredde og fødeinntak.

English summary on page 2.

Emneord - norsk:
1. Torskelarver (*Gadus morhua* L.)
2. Otolitt mikrostruktur

Emneord - engelsk:
1. Cod larvae (*Gadus morhua* L.)
2. Otolith microstructure

Seksjonsleder

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Abstract

The techniques for extraction of the lapillar otoliths, and interpretation of otolith microstructure in Arcto-Norwegian cod (*Gadus morhua* L.) is outlined and reviewed. Increment deposition in this species is daily, commencing at hatch, with the possiblity of the first 2-3 daily growth increments being overlooked. Post-larval otoliths of this stock provide some difficulties in interpretation of a daily increment, probably due to rapid growth in the post-larval stage (nearly twice that of western Atlantic cod) and their growth in 24 h daylight. Using the light microscope, increments may be enumerated from 1 to over 120 days post-hatch. Use of the lapillus over the sagitta is recommended. The lapillus radius is linearly related to length, and increment widths seem closely related to feeding rates.

Preface

This is guide is quite specifically written for Arcto-Norwegian cod, using the light microscope, and is not a guide to otolith microstructure in general (see Secor et al. 1991; Stevenson & Campana 1992). It is a brief summary of personal experience, derived from 4 y at the Bedford Institute of Oceanography, Halifax, Canada and a total of 10 months at the Institute of Marine Research, using material from Browns Bank and southwestern Nova Scotia, and from Lofoten and Troms. regions of Norway, as well as known-age material reared at Tromso and Austevoll.

Introduction

Otolith microstructure of larval Arcto-Norwegian cod present some problems in the interpretation of otolith microstructure (Bergstad 1984; Suthers & Sundby 1993), compared to those from western Atlantic cod stocks (Bolz & Lough 1983; 1988; Campana 1989; Suthers et al. 1989). Specifically these problems include lack of clarity and "ring splitting", possibly due to their rapid growth (in excess of 0.5 mm/d), and growth in June and July in 24 h daylight.

This guide sets out some procedures I have found useful in the interpretation of microstructure, to enumerate daily growth increments. A particularly useful reference (Stevenson & Campana 1992) has pictures of cod otoliths, and illustrates some of the problems outlined below.

Growth increments are bipartite concentric rings about the nucleus, or core, and are composed of an accretion zone (broad region of aragonite calcium carbonate) and a discontinuous zone (often narrower region of the protein matrix). To estimate age by the enumeration of growth increments, it must be determined;
a) when growth increments begin to be laid down, and
b) that growth increments are deposited daily.
Unlike herring, it is generally regarded that cod lay down resolvable, daily growth increments (> 0.5 μm) from hatch with the possiblity of the first 2-3 daily growth increments being overlooked (Campana 1989).

Preservation of larvae

Like all other larvae, preservation in 5% formalin destroys all calcium based structures, including otoliths, due to its mild acidic nature. Generally larvae should be preserved in 95% alcohol, but petroleum-based alcohol may be mildly acidic, and alcohol <85% is also mildly acidic. I have successfully recovered otoliths from pelagic juvenile cod after 4 weeks in 5% formalin, that was heavily buffered with 3-4 heaped teaspoons of calcium carbonate per litre. Frozen material is fine. I have successfully retrieved otoliths from cod that have been frozen in liquid nitrogen (Suthers et al. 1992), and/or freeze-dried and oven-dried. Otoliths from larvae that dried in the vial do appear extremely fragile however.

Extraction of otoliths

The three pairs of otoliths lie at the base of the brain case, approximately 0.5 mm behind the eye. Otoliths are birefringent under crossed polarised light. Polarising filters may be purchased for specific microscopes, with one filter fitting into the dissecting microscope stage, and the other attached to the base of the lens, just above the stage. At 250 x (25x objective with 10x eyepieces), the upper filter may be

rotated, crossing the wave field and blackening out all but any birefringent material (revealing calcified portions of the brain case, edges of scales, and sometimes muscle blocks under peculiar conditions). Dried larvae must be hydrated just prior to extraction (else the solution becomes acidic). Using fine forceps, or electronically sharpened tungsten wire probes, the brain case is teased open, and the otoliths frequently tumble out from the vestibular apparatus. Any attached tissue (rarely) must be removed at this stage, or it can completely obscure the ring structure.

In larvae > 20 days post-hatch the sagittae are clearly the largest otoliths, and more globular, with secondary primordia apparent in older specimens. In larvae <15 d, the sagittae is similar in size or smaller than the lapillus, and may be difficult initially to distinguish.
The lapillus is generally smaller, globular like a single squashed ball, with an eccentrically located nucleus (Fig. 1).
The asteriscus in the larvae and post-larvae is similar in size, or slightly smaller than the lapillus, but is clearly thinner and more irregular in shape.

All extraction and manipulation of otoliths should be made within a water drop, as dried otoliths are easily "pinged" into eternity.

Which otolith?

The asteriscus is the smallest of the 3 pairs of otoliths, and is discarded because it is irregular in shape and ring deposition.
The lapillus (Fig. 1) is routinely used for ageing larval and pelagic juvenile cod for the following reasons:
a) it is the largest otolith for the first 5-15 days, and consequently has the most complete, and resolvable growth record,
b) while having an eccentrically located nucleus, growth continues out in a linear fashion from the nucleus without secondary primordia, until at least 150 increments,
c) the radius of the lapillus is linearly related to SL, providing easy back-calculation (Fig. 3),
d) after the first few weeks of life, it is much smaller than the sagitta, and therefore requires less time to prepare a thin, polished section, and,

e) sagittal growth becomes comparatively so rapid that sub-daily rings become harder to distinguish from true, daily rings.
The sagitta does provide a resolvable daily growth record after 1-2 weeks. Its use may lie in microchemistry studies, otolith weight or dimension in relation to age, or to stock discrimination. I routinely store the sagittae on a separate microscope slide for such future uses.

There is no significant difference between the left and right otoliths.

Preparation of the lapillus

Each clean lapillus should be gently transferred to a separate, labelled microscope slide. Transfer takes practice, but water surface tension between fine forceps works well. The dried otolith should be glued to the slide using a very small drop of fresh, non-viscous, cheap, commercial nail polish. I have used Krazy glue in the past, but it may form a white air pocket around the otolith, and it is a much harder glue taking longer to polish.

Gluing is important. Using a probe, draw the nail polish over the dried otolith, and then nudge the otolith (it often seems to dry and stick onto the slide in unusual orientations), and ensure that it settles evenly onto one side (the medial surface may be flatter). Using the probe, draw the remaining nail polish out from the otolith to prevent lumps forming.

It may be better to polish from the lateral side of the otolith rather than the flatter, medial surface but I have not found this distinction to be consistent.

Ring structure can rarely be observed, even in early larvae, without some polishing to the hatch check, as the lapillus is rather globular. Focussing up and down through the three dimensional structure of concentric rings, soon convinces one that the lapillus needs to be polished, at least in part, to a two dimensional structure. Use 3M Imperial lapping film (purchased from Nordbye Engros phone 55 18 30 30, Fax 55 19 21 80, 660 kr for a box of 50 sheets, 6-8 weeks delivery, or 3M Norge, Slipeteknikk, postboks 100, 2031 Skjetten. Telf 638 47 500).

I find 1 µm grit (grey), 3 µm (pink), 6 µm (blue) and 12 µm (yellow) are generally adequate. 30 µm (green) seems to be too harsh and often causes cracking, even when gently polishing.

With a strip of lapping film on one finger, the otolith-slide in the other hand should be gently polished over pink or blue lapping film. Under the 10x or 40 x objective, small scratches on the surface should be visible, and by focussing down, clear growth rings and a very distinctive hatch check should be apparent. When the scratches are almost in the same plane of focus as the hatch check and rings, a light buff with the 1 µm film will provide a smooth surface. You should have almost polished the lapillus down to the widest circumference, or edge, of the otolith. If the lapillus was glued on a slight angle, you may over-grind the outer increments on one side.

The diameter of the lapillus is from 20-300 µm for 5-40 mm larvae.

This polishing technique is generally sufficient to age fish up to 100 d. If ring structures are still not clear, the otolith may need to be unglued with acetone, flipped, and polished from the other side. Krazy glue needs only to be soaked in water for 30 min., allowing the flake of glue and otolith to be easily flipped and the excess glue cut away before re-gluing. Krazy glue is much harder than nail polish, and requires more polishing/grinding.

All increment counts and measurements are usually made under oil (100 x objective). An overall assessment at 40 x is often useful to detect ring splitting, and to determine the longest radius for measuring.

Interpretation of ring structure using OTO software

Accurate ageing must be made by viewing down the microscope. The resolution on the TV monitor is not sufficient for accurate ageing of cod using OTO2.1. During measuring of the growth history using OTO, I routinely check otolith landmarks and numbers of increments down the microscope using the split beam.

Even when using the 10 x and 40 x objective - a thin film of immersion oil over the otolith (not touching the lens) helps fill in strongly contrasting cracks and scratches. After viewing the otolith, it just needs a brisk rub with a tissue. Beware that some immersion oils (Olympus, but Zeiss seems ok) can dissolve or loosen the nail polish. The polished otolith seems clearer and harder to read 6-12 mo. after initially viewing it. It seems possible that the immersion oil can bleach or clear the otolith. If one is confident of the polish, then it can be sealed with a layer of nail polish.

The main feature of the lapillus is the eccentrically located hatch check (Fig. 1), approximately 18-24 µm in diameter.

I have recognised 3 growth zones about the hatch check:
I. Perinuclear zone, just around the hatch check of 3-8 increments (normally about 6/7), often delineated by a check, or 2-3 checks. These increments are very fine, 0.5-1 µm wide, but the brown discontinuous zone is often very faint - especially on the monitor. Considerable re-focussing up and down, and scanning about the whole zone is required to count all increments. This zone probably represents growth during the yolk sac absorption stage.
II. Larval zone, consisting of 10-20 clear increments of about 1 µm in width, beginning to uniformly increase in width to 2-3 µm in width.
III. Post-larval, pelagic juvenile zone. From 20 days to >100 days, these increments can be exceedingly difficult to identify, due to sub-daily rings and ring splitting. Alternatively the dark/white regions can swap contrast. The greatest difficultly may be encountered at 100 x and on the TV monitor. As these increments range from 3 µm to 10 or even 12 µm in width, viewing at 40 x can help to put the various concentric rings into some perspective (Fig. 2).

Subdaily increments are actual physically discrete increments (from feeding bouts or mild temperature shocks), while ring splitting is probably an optical artefact. However both seem to be particularly apparent in fast growing fish (as in pelagic juvenile Arcto-Norwegian cod compared to those from Browns Bank, where increment widths rarely exceeded 5 µm). Ring splitting also becomes apparent if the

otolith has been over-ground. Campana's chapter in Stevenson & Campana (1992) provide some excellent examples, photos, and hints at dealing with this.

Counting growth rings is easier, as one's eye can flicker around the otolith to other clear areas, as looking down the microscope. However measuring at 100 x on the monitor restricts one's field of view, and measurements must continue along the same radius. Measuring at 40 x is possible, but increments <1 µm will not be resolved unless previously identified at 100 x using landmarks.
The following techniques or "rules" I have found useful:

i) Otolith growth is always conservative; increments never suddenly increase and then decrease, or become regularly fat and thin as apparent when ring splitting occurs. In fact pelagic juvenile increments may appear as dark "railway tracks", interspaced by white lines (in many other species such as myctophids, increments are broad light areas that are laid down at night, separated by narrow dark areas).
ii) Put the microscope slightly out of focus to blur ring splitting and the less distinct subdailies.
iii) Examine these broad increments using the 40 x objective - the overall pattern is much easier to discern.
iv) Look for consistent, regular, groups of ring-patterns - at 40 days post-hatch, at least 5-8 rings fit at 100 x across the current monitor in Room 510 at HI. Ring patterns will only be consistent for 3-4 rings.
v) Use the known-age material to help calibrate your interpretation. Blind-labelling, and ignorance of the actual ages is of course useful, and a manual counter prevents mentally converging on a previously identified age.

Growth history and lag effects - application of the data

1. Age frequency and hatch frequency.

The hatch date distribution was derived for the 1988 post-larval survey, and it was found to be approximately 1 week behind the known spawning distribution (Fig. 4, from Suthers & Sundby 1993, their Fig. 6), suggesting differential mortality of the earlier spawned larvae. However, this hatch date frequency was not adjusted to account for the greater cumulative mortality that occurred in the older age classes (Stevenson & Campana 1992, p.89)

2. Lag effects

There is a growing literature on the degree of time lag between feeding events and somatic growth as recorded in the otolith. The only preliminary study of this for cod is found in Suthers and Sundby (1993, their Fig. 11) showing that increment widths reduced within 1-2 days of dry food being provided when natural zooplankton became insufficient (Fig. 5). The growth record therefore seems to be a reliable measure of somatic growth. The effect of relatively smaller otoliths in faster growing fish probably occurs in cod, but has not been demonstrated, and is probably more of an effect in the laboratory when particularly slow growers may not be removed.

3. Backcalculation of size

The lapillus radius is linearly related to standard length (Fig. 3). Campana (1990) provides a robust back-calculation method, using the "biological intercept" which is admirably suited to larval cod using the lapillus. The length at time a, L_a is determined by:

$$L_a = L_c + (O_a - O_c)(L_c - L_i)/(O_c - O_i)$$

where L_c is the length at capture, O_a is otolith radius at time a, O_c is the lapillus radius (from the hatch check) at capture, L_i = 4.0 mm (length at hatch being 3.5-4.5 mm) and O_i = 0 (initial radius, by using radius from the hatch check rather than at the otolith core).

4. Condition, or health in pelagic juvenile cod is a useful technique to assess the effect of oceanographic features or events. Back-calculation of recent otolith growth over the previous seven days is a most practical application ot this technique (e.g. Suthers and Sundby 1993). This is only possible in pelagic juvenile cod which have daily growth increments 1-2 µm in width. Hare and Cowan (1995) provide some useful cautionary information when comparing larvae and pelagic juvenile cod.

Future questions

1) The degree of time lag between the soma and otolith needs to be further explored, so the effect of wind-induced turbulence on growth could be quantified. I believe cod otolith response to be fast, and the lag effect to be very small (1-2 d at the most).
2) Does the influence of daylight length on growth change between stocks?
3) The distinction between larval skrei and krystorsk by otolith morphology needs to be determined.
4) The difference in conditions between the field and aquaculture conditions needs to be carefully considered, before extrapolating lab. results to the field. For example, in aquaculture conditions, the density of prey and cod is very high compared to the ocean. Observations of cannibalism may be a laboratory artifact, and there are others (e.g predation affecting population growth rate). "The life of a larval cod is a very lonely one" (S. Sundby, personal communication) - the same applies to non-schooling pelagic juveniles.
5) Comparison of larval ecology and growth between the eastern and western Atlantic cod (Fig. 6).
6) Otolith microchemistry?
7) Bulk tagging of hatchery-reared cod by use of mild temperature shocks (1-2°C over 6 hours) leaves characteristic sub-daily checks, for subsequent indentification from fishery-caught samples many years later.

Acknowledgments

I particularly thank Svein Sundby, Erlend Moksness and Petter Fossum who have instrumental in allowing my pursuit of larval cod otoliths in Bergen. Per Solemdal, Herman Bjorke, Torstein Pedersen, Terje van der Meeren and many others have assisted in discussions and providing material. I thank Steve Campana for first instructing me in the subtle art of otolith microstructure preparation and interpretation. I acknowledge the Norwegian Research Council for sponsoring my visits to Bergen, and the University of New South Wales for giving me study leave.

List of Figures

Fig. 1 Diagram of cod lapillus showing the location of the measurements, confined to between the two dashed lines: 14-d with, r=radius, h=hatch check, w1-w4 are the 7-d widths (Suthers et al. 1989)

Fig. 2 Examples of ring splitting and the resultant interpretation (triangles). Scale bar is approximately 10 μm. Arrows indicate the problem areas.

Fig. 3. Linear relationship of lapillus radius (μm) on standard length for all cruises off southwestern Nova Scotia (NAFO division 4X). Radius =-16.08 + 6.34 x SL, r^2=0.95 (Suthers et al. 1989). The intercepts between cruises was significantly different (but not slopes). This relationship is also significantly different between Norwegian and Canadian stocks (Suthers and Sundby 1996)

Fig. 4 a) Hatch date distribution derived from otolith microstructure for Tromsoflaket pelagic juvenile cod, and b) the spawning intensity curve at Lofoten spawning grounds during spring 1988 (solid line), and the subsequent hatching curve (dotted line, calculated from the temperature dependent egg development rate, with no mortality component, Suthers & Sundby 1993). The vertical dashed line indicates day of 50% spawning (31 March, mean from 1976-1983 Ellertsen et al. 1987). The otolith derived hatch-date distribution is approximately 1 week later than the hatching curve.

Fig. 5 Response of increment widths to starvation (Suthers & Sundby 1993). Plot of average daily growth increment widths of the otolith (± 1 S.D.) from cod reared in the Tromso mesocosm (n=9). The percentage of gut content containing dry food (data from Olsen et al. 1991) indicates that the natural food was insufficient after 45 days post hatch.

Fig. 6 Relationship of SL on age for Browns Bank-4X (circles) and Tromsoflaket cod (crosses). See Suthers and Sundby for details

SL_{4X} = exp(1.354 + 0.0214*age) (r^2=0.83, n=489)

SL_{AN} = -1.050 + 0.530*age (r^2=0.44, n=159)

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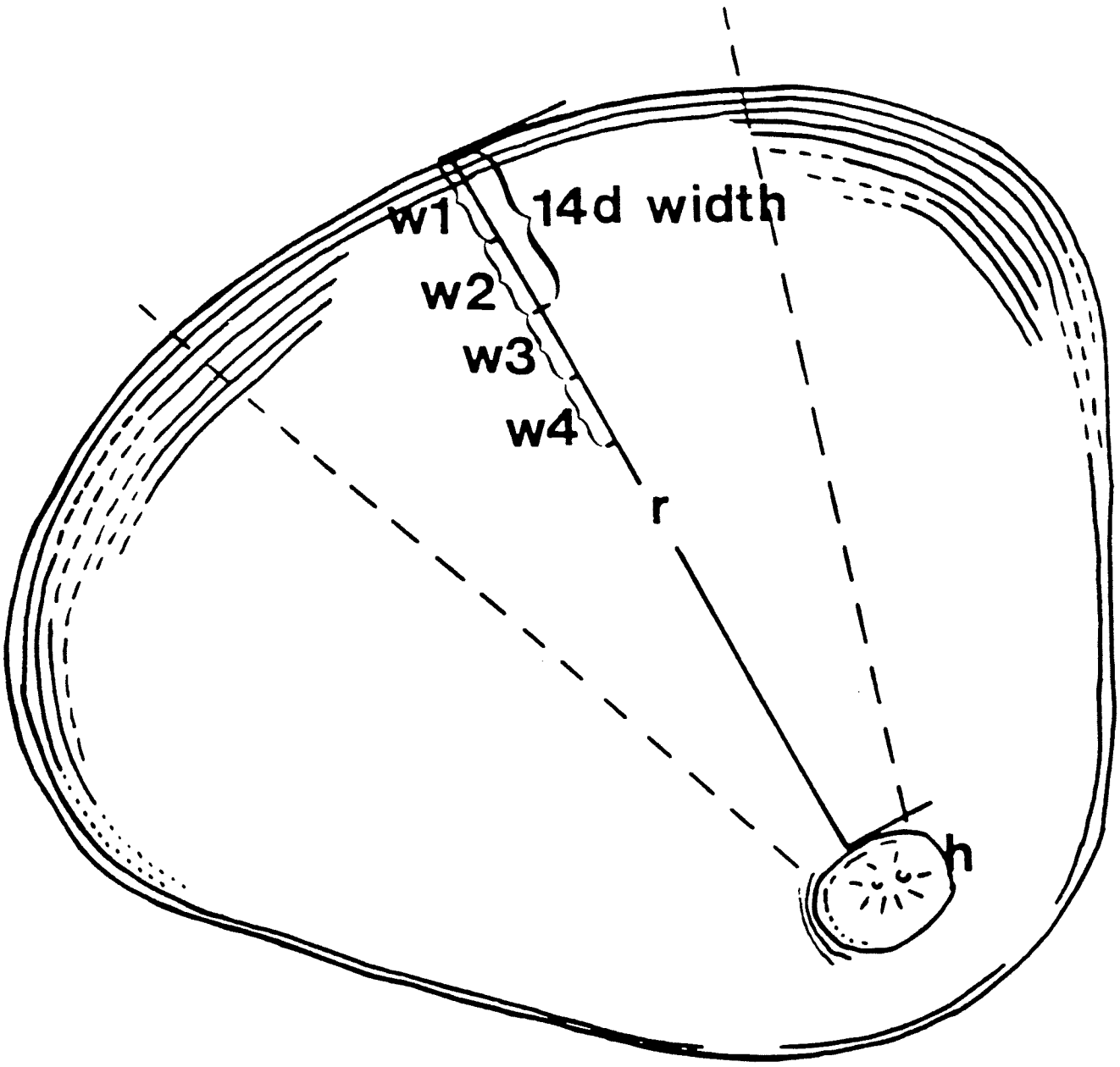


Fig 1

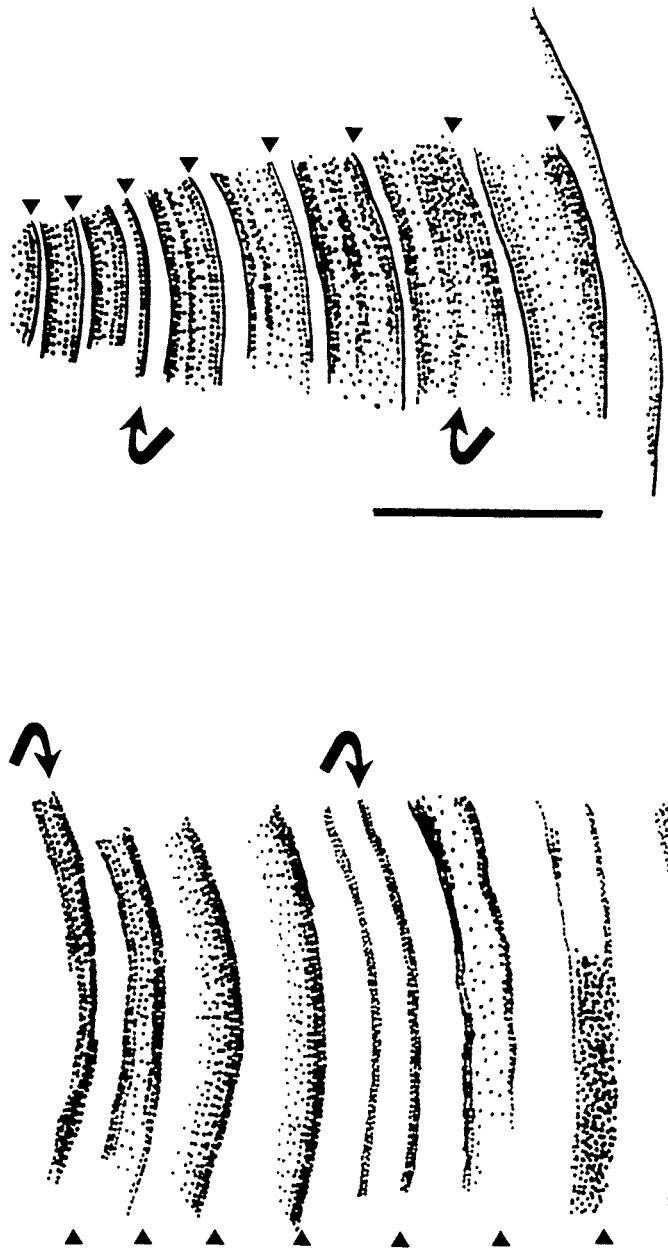
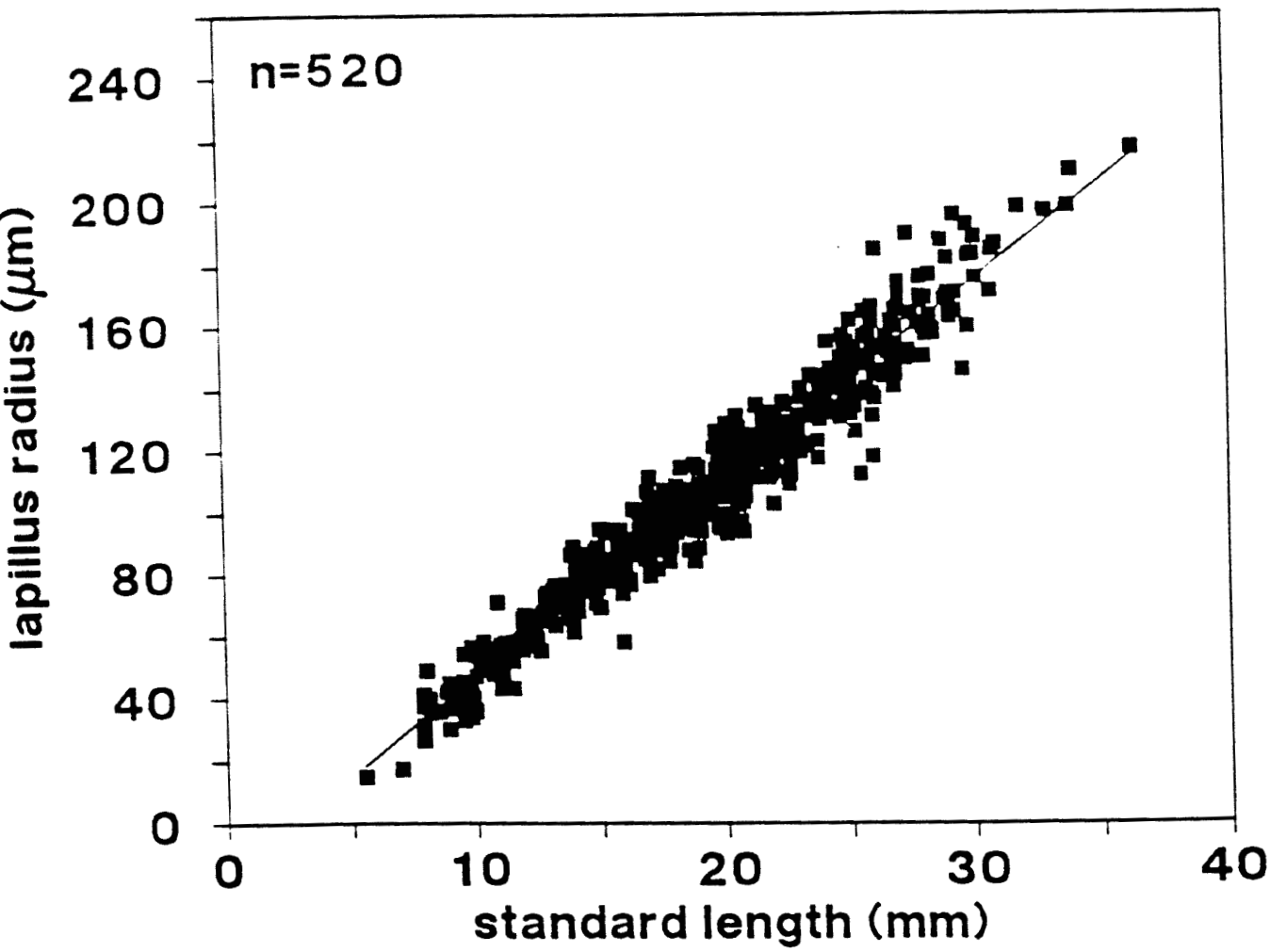


Fig 2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80
40
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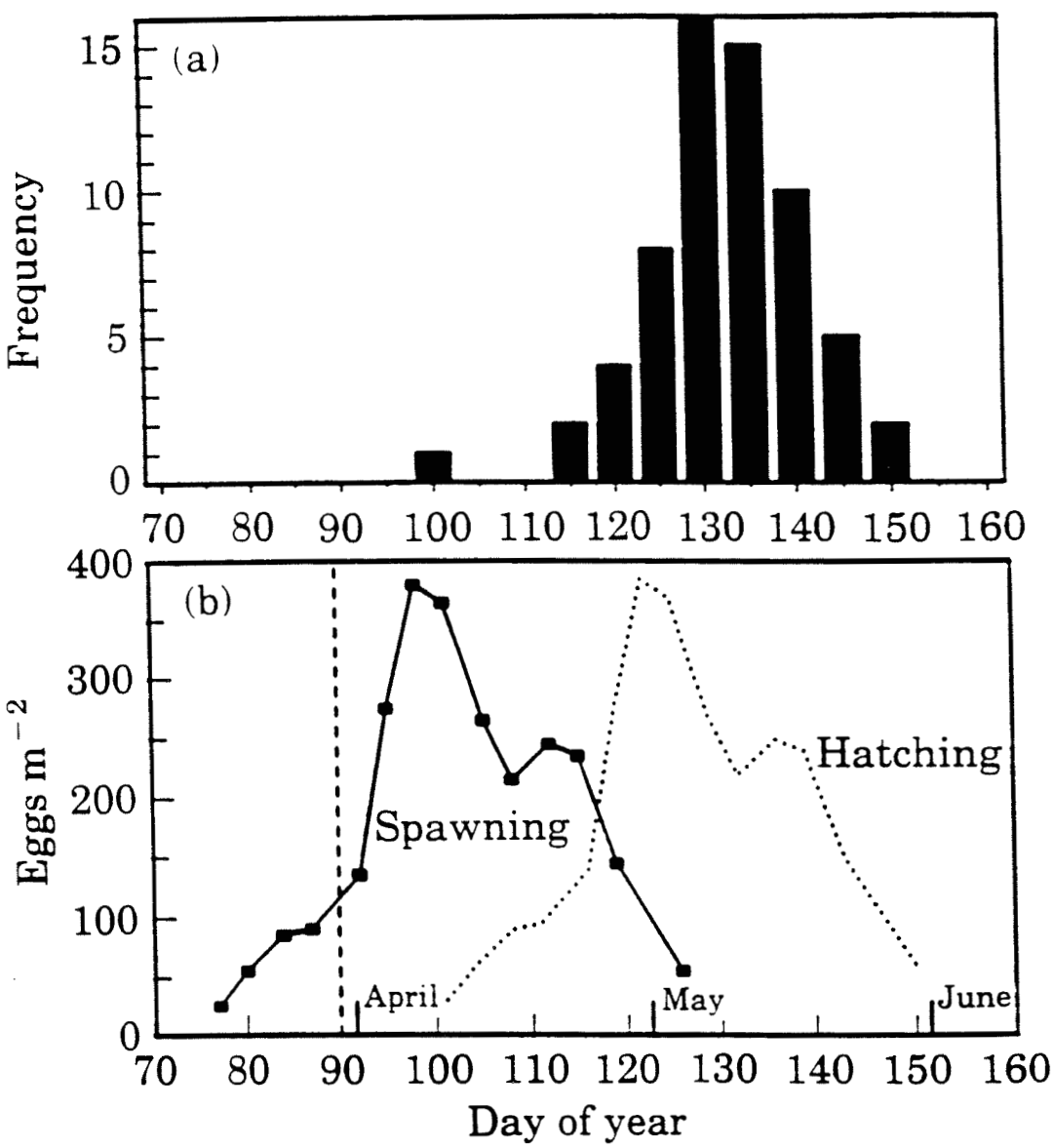
lapillus radius (μm)
0
10
20
30
40
standard length (mm)

Fig 3


(a)
Frequency
15
10
5
0
70 80 90 100 110 120 130 140 150 160
(b)
Eggs m^{-2}
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200
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0
Spawning
Hatching
April
May
June
70 80 90 100 110 120 130 140 150 160
Day of year

Fig. 4

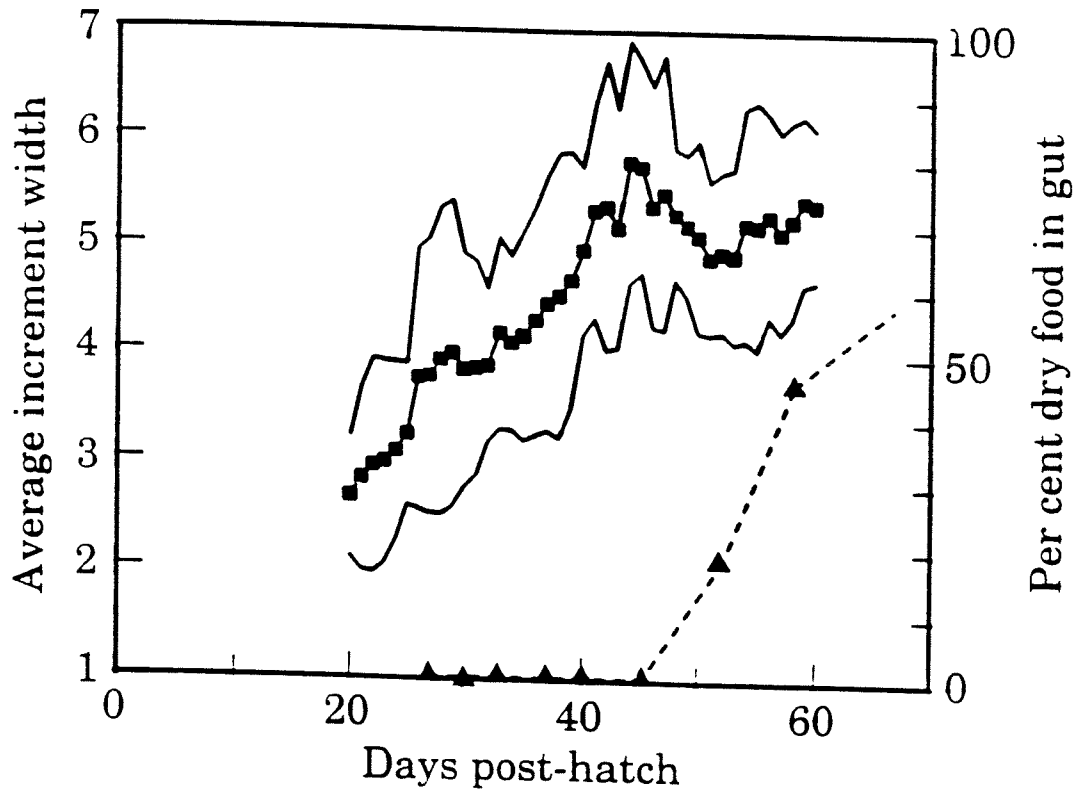
Average increment width
7
6
5
4
3
2
1
0
20
40
60
Days post-hatch
100
50
0
Per cent dry food in gut

Fig 5

dsl14.cg3

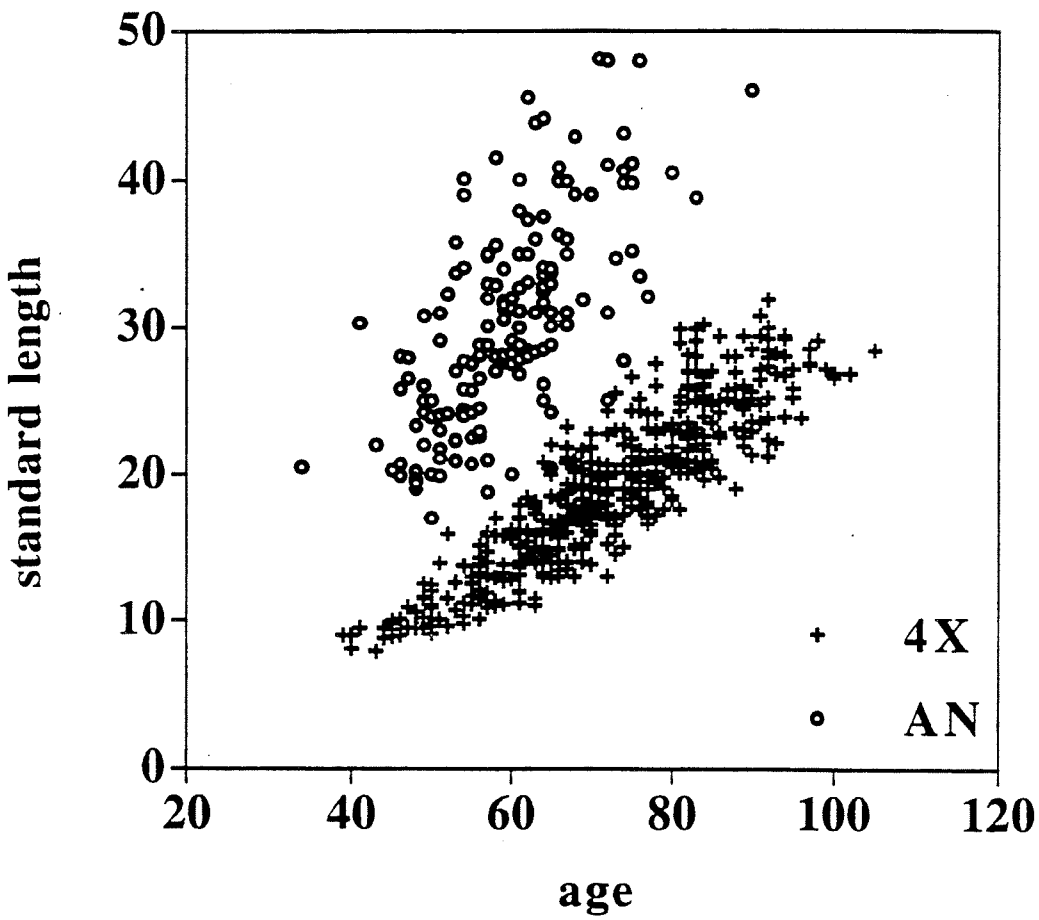

Fig 6