Microstructural growth patterns in the otoliths of larval and juvenile starry flounder, *Platichthys stellatus*

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Microstructural growth patterns were examined and interpreted in the otoliths of both wild and laboratory-reared starry flounders, *Platichthys stellatus*. Growth increments were not formed with a daily periodicity in laboratory-reared larvae. However, increment counts increased with the degree of sample preparation, suggesting that increments near the resolving limit of light microscopy were not being observed. Increments in wild flounder sagittae were more clearly defined under both light and electron microscopy; in addition, larval and juvenile growth patterns could be easily differentiated. A transition zone between the growth regions corresponded to the size and age at metamorphosis. An increase of increment width with larval age resulted from a curvilinear relationship between otolith diameter and fish size. Larval growth rates of wild fish remained relatively constant at 0.25 mm/day until metamorphosis; juvenile growth rates were substantially higher. Metamorphosis was characterized by a sudden but temporary decline in growth rate.

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La microstructure des anneaux de croissance des otolithes a été étudiée et analysée chez des plies du Pacifique (*Platichthys stellatus*) en nature et des plies élevées en laboratoire. L'addition de calcaire ne se fait pas selon une périodicité journalière chez les larves des poissons de laboratoire. Cependant, l'évaluation des dépôts donne des valeurs plus élevées selon le degré de préparation des échantillons, ce qui indique que les dépôts de taille voisine du pouvoir de résolution du microscope photonique n'ont peut-être pas été observés. L'addition de calcaire dans les sagittae des plies en nature est beaucoup mieux définie, et au microscope électronique, et au microscope photonique; de plus, les anneaux de croissance des larves et des jeunes poissons se distinguent facilement. La zone de transition sise entre les régions de croissance correspond à la taille et à l'âge au moment de la métamorphose. L'augmentation de la largeur du dépôt en fonction de l'âge de la larve correspond à la relation curviligne qui existe entre le diamètre de l'otolithe et la taille du poisson. La croissance larvaire des poissons en nature se fait selon un rythme relativement constant de 0,25 mm/jour jusqu'à la métamorphose; chez les stades plus avancés, les taux de croissance sont légèrement plus élevés. La métamorphose se caractérise par une chute brusque, mais temporaire, du taux de croissance.

[Traduit par le journal]

The early life history of the starry flounder (*Platichthys stellatus*) is poorly known, particularly with respect to the larval and metamorphic stages (Orcutt 1950). Larval rearing experiments have provided basic data on the size and age at metamorphosis (Policansky 1982). However, aside from distributional reports, I am unaware of any studies concerning the larvae in their natural habitat. Information concerning the juveniles is almost as sparse (Orcutt 1950; Campana 1983*a*, 1984*b*).

Otolith microstructure examination may be used to determine both growth rates and life history characters in some fishes (Brothers and McFarland 1981; Methot 1981; Campana 1984*b*; Victor 1983). Through diel variations in calcium and protein deposition, bipartite structures known as daily growth increments often form at the microstructural level of the otolith (Pannella 1971; Brothers *et al.* 1976; Mugiya *et al.* 1981). Given a series of these daily increments and a relationship between otolith size and fish length, an estimate of daily growth rate can then be developed on the basis of increment width. This approach was used in conjunction with other microstructural features to assess some early life history parameters of starry flounders. Larvae reared in the laboratory through metamorphosis were used as controls for otolith interpretation. Calculations of larval growth rates and inferences as to the environmental shifts accompanying metamorphosis were subsequently developed.

Materials and methods

Starry flounders were collected from Bellingham Bay, WA, between 1978 and 1982 as part of another study (Campana 1983*a*). In May and June of 1981, the estuarine nursery area was sampled with a beach seine at biweekly intervals to determine the date of appearance of the newly metamorphosed flounders. First appearance was noted on June 8, 1981 ($N = 4$). The same year class was then sampled ($N = 14$) on September 12, 1981, to verify that the otolith microstructure accurately represented the date of appearance at the estuary. Otoliths for a more complete microstructural examination were removed from fish collected on September 8, 1982 ($N = 46$).

Daily growth increments form in the otoliths of juvenile flounders, both in the laboratory and in the wild (Campana and Neilson 1982). Similar data are not available for the larvae, nor is the otolith growth history before metamorphosis. Therefore, larval starry flounders were reared from the egg stage through metamorphosis in the laboratory. Ripe adults were trawled from Cowichan Bay, Vancouver Island, on March 14, 1981, hand stripped on April 2, and the fertilized eggs were reared in 15-L containers under a 12 h light : 12 h dark light regime. Hatching occurred April 6–8. Water temperature fluctuated around 12°C and was monitored daily. Food was supplied as in Policansky (1982), and the water was changed every 3 days. Larvae were sampled ($N = 23$) for standard lengths and sagittal otoliths at irregular intervals, since the rearing constituted part of another study. Despite low initial numbers, over 70 larvae survived until just prior to metamorphosis; an accident reduced this number to 3, of which 2 survived into the juvenile stage 54 days after hatching.

Otolith preparation

Thin sections of otoliths from wild fish were prepared as in Campana (1984*b*). All preparations ($N = 46$) were characterized by the presence of a central nucleus encircled by two to four peripheral nuclei at radii of 55–110 μm (see Results). Since growth increments distal to the peripheral nuclei were easily resolved microscopically

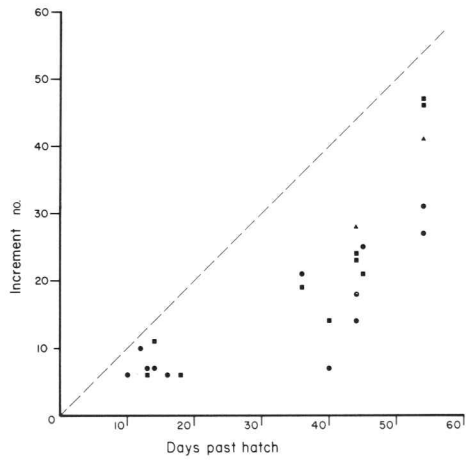

FIG. 1. Otolith increment count as a function of time for laboratory-reared starry flounder larvae. Symbols represent different preparation techniques. ●, unground otolith; ■, ground otolith with video system; ▲, SEM observation.

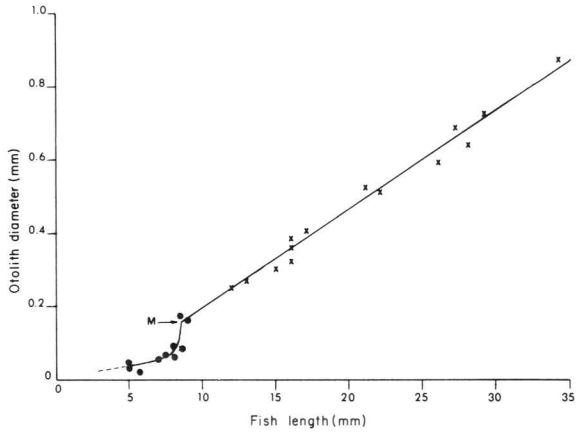

FIG. 2. Otolith diameter as a function of total fish length in larval and early juvenile starry flounders (N = 25) (●, laboratory-reared; ×, wild). Nonlinearity is a characteristic of the larval relationship. M, size at which metamorphosis occurred. Hatching occurred at a length of 2.5 mm.

(>4 μm width), counts of these increments were made at 400× on a Zeiss microscope, and repeated at a later date. Examination of a subsample of 25 otoliths with a scanning electron microscope (SEM) demonstrated an exact correspondence between SEM and light microscope viewed increments in this distal region. Counts derived from left- and right-hand side sagittae did not differ significantly (paired t-test, $p > 0.1$), so the mean was used in later analyses.

As a second stage of preparation and viewing, otolith sections medial to the peripheral nuclei were photographed at 400−1000× and then etched for SEM. Etching was carried out in 2% HCl for 2−4 min or in 0.1 M ethylenediaminetetraacetic acid (EDTA) for 3−5 min. Specimens were coated with Au−Pd and viewed at 20 kV on a Cambridge Stereoscan 180 SEM. Increment widths and counts were made from the SEM photographs. Poorly prepared samples were excluded from all analyses (N = 9 of 46).

Estimates of "growth at age" in wild flounders were developed from measurements of maximum otolith diameter and daily increment width at periodic age intervals (N = 20 per age), in conjunction with the otolith diameter − fish length relationship (N = 25). At a given age, mean otolith diameter was used to predict average fish length at the same age. After having drawn a tangent through the appropriate point on the otolith diameter − fish length curve, the change in fish length corresponding to the observed mean increment width was calculated. For larval otoliths, increment width was doubled to incorporate the symmetrical growth of the otolith on both sides of the nucleus. Otoliths grew asymmetrically in the juvenile flounders. Therefore, the width of a given increment was measured and summed on both sides of the nucleus (along the long axis of the otolith) to more accurately represent the increase in otolith diameter on a given day.

Otoliths obtained from laboratory-reared flounders were prepared for examination in three different ways. After embedding in instant glue, increment counts were made both microscopically (at 1250×) and from light micrographs. Larger otoliths were then polished as per Campana (1984*b*) and viewed under polarized light with a Hitachi CCTV video camera and monitor coupled to a Zeiss microscope. Increment counts were subsequently compared among the viewing techniques. A subsample (N = 3) of the polished otoliths was etched for 1-2 min in 0.1 M EDTA and photographed with the SEM. None of the SEM-viewed otoliths could be polished through the midplane across the entire otolith, owing to a slight curvature of the incremental plane.

Results and discussion

Increment formation in laboratory-reared and wild fish

Growth increments were visible in the sagittae of all laboratory-reared larvae. However, the slope of the regression of increment number against time was significantly less than unity ($p < 0.05$), suggesting that increment formation did not occur on a daily basis (Fig. 1). This suggestion is tempered by the fact that the degree of otolith preparation appeared to have an effect on subsequent increment counts. Significantly fewer growth increments were observed in unground otoliths than in the same otoliths after grinding to the midplane and viewed with a video system (paired t-test, $p < 0.05$) (Fig. 1). This finding was not surprising considering the limited width of some of the increments; growth structures less than 0.5 μm wide were only slightly above the theoretical resolution of light (resolution under green light = 0.28 μm). SEM observations were not constrained by the same resolution limit. However, otolith peripheries remained embedded in glue after the appropriate grinding plane had been reached, thus obscuring the outer increments. As a result, SEM counts probably underestimated the true increment number (Fig. 1).

Increment clarity differed between the laboratory-reared and wild flounders. Increments in the former were characterized by low contrast, irregular width, and poor etch quality when prepared for SEM examination, particularly during the late larval stage. Gaps in the growth record were common, suggesting the presence of increments whose presence could not be reliably confirmed. By contrast, growth patterns in the sagittae of wild fish were clear, regular, and well etched. The differences between the two sets of otoliths were probably related to the presence−absence of diel temperature fluctuations. The latter were weak or absent in the laboratory aquaria, but spanned 2−10°C in the estuarine nursery area (S. E. Campana, unpublished). Since temperature cycles enhance the contrast and clarity of daily growth increments (Brothers 1981; Campana 1984*a*; Neilson and Geen 1984), such environmental effects on increment formation are to be expected. Similar differences in increment clarity between wild and laboratory-reared fish have been noted in other studies (Laroche *et al.* 1982).

Poor resolution reduced the accuracy of the increment counts from laboratory-reared fish. I could neither confirm nor deny the possibility of daily increment formation, although indirect evidence suggests that daily increments were indeed formed. Removal of surface material tended to increase increment counts, particularly in the perinuclear region where increments

TABLE 1. Characteristics of larval starry flounder otoliths reared in the laboratory and in the wild

Rearing location	Mean otolith length at hatch (μm)	SE(N)	Mean no. of larval growth increments	SE(N)	Mean otolith length at metamorphosis (μm)	SE(N)
Laboratory	19–23[a]				95	10(5)
Wild	22.0	0.6(18)	34.9	1.0(18)	100	3.7(28)

[a]Estimated value.

were most narrow. Elimination of irregular refractive effects and a subsequent increase in resolution were the probable causes of this effect. The previously mentioned temperature constancy exacerbated the problem of poor resolution. In addition, many of the first-formed growth structures were so narrow as to be near the theoretical resolution of light. Difficulties in sample preparation prevented the comparison of SEM- and light-derived counts. However, the presence of such narrow increments in the otoliths suggests that increment counts derived from light microscopic examinations may underestimate true values in some species. The problem of inadequate resolution would be compounded by inadequate otolith preparation. "Nondaily" growth increments have been reported in other pelagic larvae (Lough *et al.* 1982; Laroche *et al.* 1982) and have been linked to reduced growth rates (Geffen 1982). Otolith preparation was limited or absent in these same studies. Since increment width may reflect fish growth (Wilson and Larkin 1982), reduced growth may result in increments that are too narrow to be easily resolved, and thus appear falsely as a nondaily growth record.

Microstructural characteristics of otoliths at metamorphosis

Otoliths were not collected from newly hatched flounders. Otolith size at hatch was estimated from the relationship between otolith length and larval length in older larvae (Fig. 2). The estimated value was similar to the observed diameter of the first-formed growth increment in the otoliths of both wild and laboratory-reared flounders (Table 1). Metamorphosis began approximately 45 days after hatch, and was completed in 4–6 days. No evidence of metamorphosis was apparent in the sagittae of postmetamorphic, laboratory-reared fish; neither increment width nor appearance shifted substantially through metamorphosis (Fig. 4), nor were checks formed. However, peripheral nuclei were beginning to form in all sagittae.

The microstructure of wild juvenile flounders was characterized by the presence of a transition zone which may have formed at metamorphosis. The transition zone separated two distinct incremental sequences (inner and outer) which differed in both increment width and appearance (Fig. 3). The most medial of the "outer-zone increments" was arbitrarily defined as the boundary between the two zones; justification for such a division appears later.

Growth increments in the inner zone encircled an amorphous nucleus whose diameter corresponded to that expected of otoliths at hatch (Table 1). Increment width never exceeded 0.6 μm initially, although this value increased gradually with increasing age (Fig. 4). At a radius of 50 ± 20 μm from the nucleus, increment width rose sharply (Fig. 4). As maximal width was reached, increment appearance shifted to one of increased visual contrast and compound structure (Fig. 3). These width and appearance characteristics were used to demarcate the end of the transition zone and the origin of the outer zone. The diameter of this zone corresponded closely to that of otoliths from newly metamorphosed, laboratory-reared flounders (Table 1).

The outer-zone increment sequence originated just medial to the peripheral nuclei ($\bar{x}$ = 5 increments, SE = 1.2), where it assumed a roughly circular shape in the central nucleus (CN) field (Fig. 3). Peripheral nuclei appeared to represent new foci of otolith growth, in which deposition rate could vary substantially with the various possible axes of growth. Individual growth increments could be continuous across the two nuclear fields; however, the width of a given increment was generally 1.6 times greater in the outer (PN) field (Figs. 3 and 4).

Initial formation of outer-zone increments occurred just prior to first availability to the collecting gear. The 1981 year class was first collected June 8, 1981, at which time peripheral nuclei had formed within the past week. Size at capture indicated that metamorphosis had occurred within 1–2 weeks. Otoliths of fish collected September 12, 1981, had 90.5 ± 7.5 outer-zone increments present, corresponding to a starting date of June 13. The two dates are not significantly different ($p > 0.05$), suggesting that outer-zone increments were first formed at or shortly after metamorphosis, and that flounders were first collected several days thereafter.

Examination of the 1982 juvenile growth record was carried out in a similar manner, although the sample size was considerably larger. Initial "juvenile" increments were formed around May 31, 1982 (SE = 1.7 days). As was the case with the 1981 samples, this date is consistent with the expected date of metamorphosis and the flounders' first appearance on the estuarine nursery ground.

Daily increment formation has been validated in wild juvenile flounders (Campana and Neilson 1982). This study reports no direct evidence that increments were formed with a daily periodicity in the wild larvae. However, the increment clarity, width regularity, and absence of gaps in the growth record, as well as the use of the SEM (with its attendant resolving power), strongly suggests that daily increments were indeed being observed. Given these assumptions, my results indicate that the transition zone between the two otolith regions was formed at the time of metamorphosis. The diameter of the outer transition zone was similar to the otolith diameter recorded in metamorphosing, laboratory-reared flounders. Since starry flounders metamorphose at size and not at age (Policansky 1982), such a similarity would be expected. In addition, peripheral nuclei formed shortly after metamorphosis in the experimental flounders, and shortly after the transition zone in the wild fish. Finally, estimated dates of metamorphosis (from the juvenile increment counts) corresponded closely to the date of first appearance at the estuarine nursery area.

The period of metamorphosis was recorded differently in the otoliths of laboratory-reared and wild fish. The latter were characterized by increments of altered appearance, compound

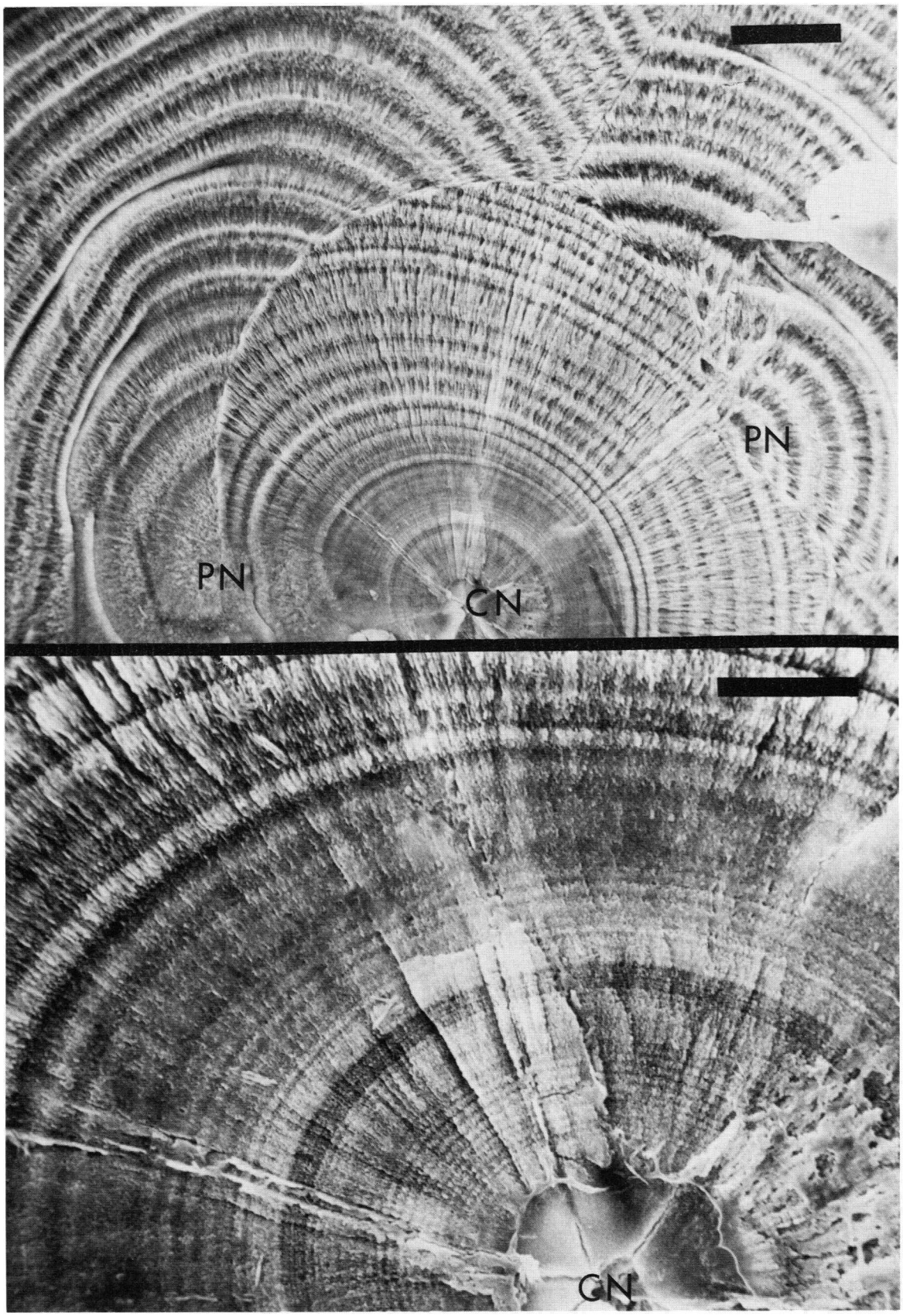

FIG. 3. Scanning electron micrographs of the otolith microstructure of a wild, juvenile starry flounder. Growth fields emanating from the central nucleus (CN) and two peripheral nuclei (PN) are visible (top). The growth increments closest to the central nucleus (inner zone) differ from all others (outer zone) with respect to both width and appearance. Bottom view is a higher magnification of CN in (top) view. Scale bars = 30 μm (top) and 10 μm (bottom).

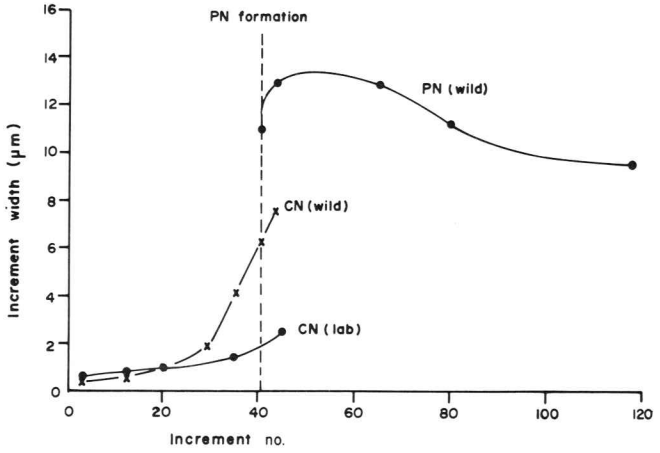

FIG. 4. Otolith increment width as a function of increment number (age) in both wild and laboratory-reared flounders. Growth increments derived from the central nucleus field (CN), but contiguous with increments in the peripheral nucleus field (PN), were substantially wider in the latter. For each wild flounder data point (CN and PN), $N = 20$; for each point in lab curve, N = 2–5.

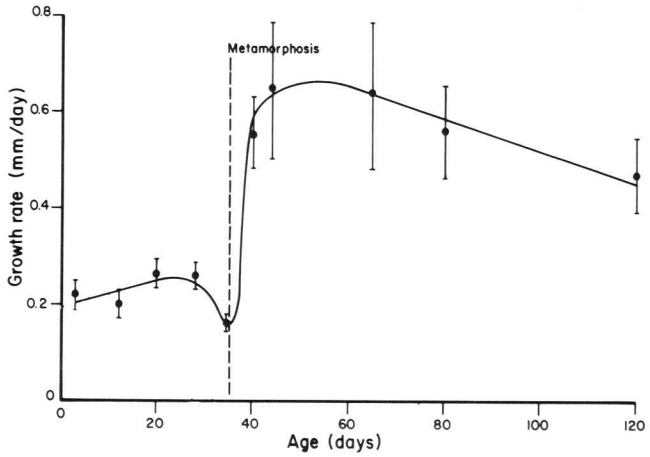

FIG. 5. Age-structured growth rate of wild larval and juvenile flounders, as derived from microstructural examination of juvenile otoliths. Bars indicate the 95% confidence intervals.

structure, and rapidly increasing width. No such features were evident in the sagittae of fish reared in the laboratory. Checks or discontinuities, which often mark periods of stress (Pannella 1980; Campana 1983*b*), were not formed in either set of otoliths. These observations suggest that the process of metamorphosis is, in itself, insufficient to radically change the otolith microstructure. However, concurrent shifts in behaviour and habitat may have such an effect. After the pelagic flounder larvae metamorphose, they settle to the bottom in shallow estuarine areas (Orcutt 1950). Such a shift in habitat is likely accompanied by significant shifts in ambient temperature and available food, variables that are known to influence the appearance of the otolith microstructure (Struhsaker and Uchiyama 1976; Brothers 1981; Neilson and Geen 1982, 1984; Campana 1984*a*, 1984*b*). Therefore, I suggest that the transition zone in the otoliths of wild flounders reflects an ecological (and not an internally derived physiological) shift in the animal's life history.

Early life history

Since the otolith transition zone was formed at metamorphosis, growth increments medial to the transition zone should have been formed during the larval stage. Counts of the larval increments suggest that metamorphosis occurred in approximately 35 days (Table 1). This value probably underestimates the true time to metamorphosis. There was little or no ambiguity in the SEM-derived counts from wild fish. However, the diameter of the most medial increment varied, sometimes exceeding the diameter of the prominent hatch check by several micrometres. It is likely that increments existed (but were obscured) in the intervening distance; therefore, five or six additional increments may have gone uncounted in some otoliths (given a mean increment width of 0.4 μm shortly after hatch). Another possible source of underestimation comes from the age at which increments first formed on the sagittae. The laboratory results did not address this question reliably, but ages of 0–6 days are typical of many fishes (Brothers *et al.* 1976; Laroche *et al.* 1982). Given these two potential sources of bias, an adjusted larval age of 40–45 days is consistent with the laboratory results reported here and elsewhere (Policansky 1982).

Given a relationship between otolith length and fish length, measurements of increment width can be used to estimate the growth rate of young fish at size or age. Otolith growth and fish growth were isometric in juvenile flounders, but allometric in the larvae, resulting in linear and curvilinear relationships, respectively (Fig. 2). The two-stage relationship does not appear to be an artifact of the different origins of the laboratory-reared and wild fish; a single linear relationship fitted both sets of juvenile otolith diameter – fish length data. The allometric larval relationship indicates that increases in increment width with larval age are not necessarily representative of increases in larval growth rate, nor was this observed (Fig. 5). Larval growth rate remained relatively constant at 0.25 mm/day until metamorphosis, at which point there was a sudden decrease. In another study, a temporary cessation of growth was characteristic of metamorphosis in flounders (Policansky 1982). Similarly, Laroche *et al.* (1982) reported a growth rate of 0.35 mm/day in English sole (*Parophrys vetulus*) larvae, with transformation accompanied by a sharp decline in growth rate. In this study, juvenile growth proceeded at a much faster rate, although asymmetric growth of juvenile flounder otoliths (through formation of asymmetrically placed peripheral nuclei) may have resulted in an overestimate of growth rate.

Estimates of larval growth rate in other species are often derived from the analysis of length–frequency modes in sequentially acquired samples. Problems with size-selective mortality (Rosenberg and Haugen 1982) and gear selectivity (Colton *et al.* 1961) often render such estimates suspect. This type of study is not subject to the last-mentioned source of bias, although the calculations of larval growth rate apply only to the survivors. Laroche *et al.* (1982) used daily increments to age sequentially sampled wild larvae, and thus calculate growth rate. The methodology reported here differs, in that daily increment widths of easily collected juvenile fish were used to back calculate the growth rates of the larvae.

Conclusions

Larval fish sampling can be an expensive and bias-ridden procedure, particularly with respect to long-lived pelagic larvae. This study has demonstrated that certain early life history data can be obtained with relative ease through otolith microstructure examination. However, certain aspects of microstructural examination require cautionary notes. First of all, the formation of daily growth increments could be neither

confirmed nor denied on the basis of the laboratory-reared larval flounders. A laboratory growth rate of 0.14 mm/day was 56% of that calculated for wild flounder larvae. Since increment number has been linked to growth rate in other pelagic larvae (Geffen 1982), my results could be explained in a similar fashion. However, an alternate explanation is that of limited resolution of narrow increments at or below the theoretical resolving power of light microscopes. Support for this hypothesis comes from increased increment counts in the larval otoliths after removal of potentially refractory, overlying material. Therefore, I suggest that it is adequate sample preparation and observation, not growth rate, which will define the limit to which microstructural examination can be applied.

Secondly, curvilinear otolith versus fish length relationships appear to be characteristic of many pelagic larvae (Methot 1981; Laroche *et al.* 1982; Lough *et al.* 1982). Given such a relationship, increment width is not directly proportional to larval growth, despite the serial increase in the former. Since the juvenile relationship is often linear, the age at which increment width becomes asymptotic may mark the period of metamorphosis in other species besides the starry flounder.

Although juvenile fish were used for the microstructural observations, there is no reason why adult flounder otoliths could not have been used. Large collections of adult otoliths now exist for many commercially important marine fishes, so the potential source of larval information is extensive. With the examination of sagittal pairs, measurement of larval growth rates from one sagitta of an adult fish and the macrostructural age determination from the matching sagitta is possible. Given the results of a cohort analysis (Pope 1972), correlations between larval growth and year class strength could then be made.

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