# INCORPORATION OF $^{14}$C-GLYCINE IN THE SCALES OF WINTER FLOUNDER (PSEUDOPLEURONECTES AMERICANUS, WALBAUM) DOES NOT REFLECT SCALE GROWTH



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by

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## ABSTRACT

The hypothesis that the rate of incorporation of $^{14}C$-labelled glycine into isolated fish scales reflects the instantaneous growth rate of fish (Ottaway and Simkiss 1977a) was tested for scales of winter flounder (Pseudopleuronectes americanus), a marine fish which has a strongly seasonal biology.

A seasonal study of $^{14}C$-glycine incorporation into scales of wild fish was conducted over a 22 month period. A separate study of $^{14}C$-glycine incorporation was carried out on laboratory-held fish, which were serially sampled over 17 months. In addition, scale growth was estimated by examining scales from all fish sampled, and measuring the width of the growing edge. Changes in length and weight of serially sampled fish were monitored.

The seasonal cycles of glycine incorporation into scales did not correlate with cycles of scale growth of wild or serially sampled laboratory fish. The cycles of growth in length and weight observed in the serially sampled fish also did not correlate with the cycle of glycine incorporation rates. Peak $^{14}C$-glycine incorporation rates were observed from late July to September, when annual scale growth was nearly complete in most fish. The peak in $^{14}C$-glycine incorporation occurred about two months after peak rates of increases in length and weight.

Incorporation into anterior scale portions, which included the growing edge of the scale, accounted for only 16 to 34% of whole scale incorporation. The cycle of incorporation of anterior scales paralleled the cycles of incorporation of the epidermis bearing posterior

scale portions and of whole scales, but did not correlate to the cycle of scale growth.

The clear seasonal cycle in $^{14}$C-glycine incorporation was strongly correlated with seasonal changes in epidermal thickness, which have been correlated to changes in gonad condition (Burton and Fletcher 1983). Peak $^{14}$C-glycine incorporation occurred when the epidermis was thinnest, and processes of regeneration were probably highly active.

Analysis of incubated scales showed that an average 36% of $^{14}$C-glycine was not incorporated into any other molecules within the scale, and only about 13% of the $^{14}$C-glycine was associated with collagen molecules. There were seasonal variations in the distribution of $^{14}$C in various scale fractions, with more incorporation into larger molecules in summer- and autumn-incubated scales than in winter-incubated scales. The highest level of incorporation of $^{14}$C-glycine into collagen molecules occurred in September, which coincided with the period of epidermal regeneration.

The seasonal cycle of $^{14}$C-glycine incorporation into scales was also influenced by other factors, though only slightly. Seasonal variations in temperature resulted in a variable $Q_{10}$ of the incorporation rate. Fluctuations in the scale free glycine pool changed the relative concentration of $^{14}$C-glycine within the scale, but did not change the timing of the seasonal cycle of incorporation rates. The effects of inhibitors of protein biosynthesis suggested that microorganisms may colonize the degenerating epidermis and contribute to $^{14}$C-glycine incorporation rates during summer months.

This study indicated that the basic hypothesis of Ottaway and

Simkiss (1977a) was not valid for winter flounder: the rate of $^{14}C$-glycine incorporation was not correlated with the rate of growth of scales or the rate of growth of the fish. Instead, the seasonal cycle of $^{14}C$-glycine incorporation was correlated to the seasonal cycle of degeneration and regeneration in the epidermis. For winter flounder, $^{14}C$-glycine incorporation into scales seemed to be sensitive to generalized processes of protein metabolism, but was not specifically sensitive to collagen synthesis at the growing edge of scales.

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# 1. INTRODUCTION

Fish grow throughout their lifespan, increasing in both length and weight. The rate of growth varies throughout life and may be characterized as consisting of one or more "stanzas" (Ricke. 1979), which are separated by dramatic physiological or environmental changes such as metamorphosis or migration. The rate of growth for each stanza generally defines an S-shaped curve, but there are recurring patterns of growth within the stanza consisting of seasonal and even daily changes in growth rate. Such rhythms of growth reflect the combined influences of numerous varying factors, including hormones, food quality and availability, environmental temperature and day length. An increase in body size represents the sum of modifying influences on growth, over an extended period of time.

The hard tissues of a growing fish, the bones and scales, grow with the fish. Scales are a structure of the integument, and grow in size by depositing new scale material around the perimeter, forming more-or-less concentric circles (circuli), similar to the rings of a tree (Wallin 1957). Regular annual variations in the rate of growth of the scale result in variations in the spacing between circuli, forming annual marks (annuli) (Tesch 1970). Because scale growth is correlated with body growth, the scale represents a record of the growth history of an individual. The annuli, or other recurring checks (such as those due to spawning), provide a method of aging fish, and of estimating past body size and growth rates. Unfortunately, the growth record on a scale provides no resolution of the effects of the various factors which have influenced the rate of growth. Examined in the traditional

ways, a scale can reveal nothing about the rate of growth at the moment of sampling, when factors which influence that rate could be assessed.

Ottaway and Simkiss (1977a) have suggested that the rate of $^{14}C$-glycine incorporation into a growing scale can provide an index of the instantaneous growth rate of the scale because the principal protein involved in scale growth, collagen, contains a high proportion of the amino acid glycine (Stryer 1975). The mechanisms of scale growth have been characterized as occurring in four phases (Fouda 1979):

1. production of a collagen matrix at the growing edge of the scale;
2. calcification of the collagen matrix;
3. growth and thickening of the collagen fibre fibrillary plate, which underlies the calcified layer of the scale; and
4. formation of circuli in the calcified layer.

The instantaneous growth rate of scales is determined by incubating isolated scales in physiological saline containing $^{14}C$-labelled glycine. The level of incorporation of radioactive glycine after a fixed period of time is considered to be an index of the rate of scale collagen synthesis, and thereby, of scale growth. In their initial investigations, Ottaway and Simkiss (1977a) demonstrated that the incorporation of $^{14}C$-glycine by scales was a metabolic event; that incorporation increased linearly with the period of incubation, and had a $Q_{10}$ (the change in metabolic rate with a 10°C increase in temperature), of 1.65. Subsequent researchers have not found a consistent temperature relationship within a species, and have suggested that the rate of incorporation at a given temperature is influenced by

acclimation temperature (Adelman 1980; Smagula and Adelman 1982). Incorporated $^{14}$C-glycine was found in the scale-forming cells at the scale margin (Ottaway and Simkiss 1977a) and seemed to be most actively incorporated by the less numerous cells of the anterior part of the scale, as compared to the posterior scale portion, which included epithelial tissue (Ottaway 1978). The rate of incorporation was significantly inhibited (up to 90%) by inclusion of cycloheximide, an inhibitor of eukaryote protein synthesis, in the incubation medium (Goolish and Adelman 1983). These observations support the hypothesis that an index of scale protein synthesis is being measured by $^{14}$C-glycine incorporation into scales.

Growth of fishes can be influenced by stress due to handling and starvation, which affect growth in length and weight and also scale circuli formation (Bilton 1974). Oxygen deprivation, starvation, or handling and confinement all reduced $^{14}$C-glycine incorporation rates into scales (Ottaway and Simkiss 1977b; Goolish and Adelman 1983). The influence of handling stress was not evident if scales were sampled within one hour of the onset of the stress (Goolish and Adelman 1983), but handling was the only condition, in these experiments, which resulted in subsequent "false check" formation on scales (Ottaway and Simkiss 1977b).

Regular cycles of fish growth have also been correlated to scale $^{14}$C-glycine incorporation rates. Ottaway (1978) reported a circadian rhythm of $^{14}$C-glycine incorporation in scales of roach, Rutilus rutilus, although no diel variations in $^{14}$C-glycine incorporation were found in scales of three other fish species (Goolish and Adelman 1983). A seasonal cycle of $^{14}$C-glycine incorporation was

observed in the scales of juvenile sea bass, Dicentrarchus labrax, with summer rates reaching four times winter levels (Ottaway and Simkiss 1979). Enzymes involved in formation of hard tissues, the alkaline phosphatases, showed a seasonal cycle in the skin of the common goby (Pomatoschistus microps) which reflected the cycle of scale growth (Fouda and Miller 1979).

The instantaneous growth rate of scales indicated by $^{14}$C-glycine incorporation has been correlated to overall growth of fish. There was an inverse relationship between age group and the rate of incorporation of $^{14}$C-glycine in scales of roach and sea bass (Ottaway and Simkiss 1977a; 1979). Three populations of roach with different growth rates, determined from back-calculation on scales, had correspondingly different rates of $^{14}$C-glycine incorporation (Ottaway and Simkiss 1977a). Growth of a juvenile sea bass population over a two-month interval was strongly correlated to the mean glycine incorporation from the two sampling dates (Ottaway and Simkiss 1979). Similar results were obtained with individual bluegills (Lepomis macrochirus) over a two-week interval (Goolish and Adelman 1983).

The results of these investigations indicate that scale protein metabolism is reflected by the rate of $^{14}$C-glycine incorporation, and that the index of instantaneous growth rate obtained correlates well with actual growth over longer intervals. However, the relationship between short-term rates of scale growth and $^{14}$C-glycine incorporation has not been examined, nor has the basic hypothesis that $^{14}$C-glycine is actually incorporated into collagen molecules within the scale. Further, the impact of glycine already present within the scale on $^{14}$C-glycine incorporation has not been considered. This

study addresses these points.

This study focused on seasonal aspects of $^{14}$C-glycine incorporation into the scales of winter flounder (Pseudopleuronectes americanus). All previous work with $^{14}$C-glycine incorporation into scales has used scales from young, or relatively fast-growing, freshwater fish. By using winter flounder, we could test the effectiveness of the method with mature fish of a slow-growing marine species. The winter flounder in Newfoundland coastal waters has a strongly-seasonal biology: virtual dormancy from November to March, when temperatures are low; with increasing activity levels and resumption of feeding in April, continuing through October and November (van Guelpen and Davis 1979). The annual growth increment is small, but occurs in such a protracted period that a clear seasonal cycle of $^{14}$C-glycine incorporation should be revealed.

The hypothesis that the rate of scale growth is accurately reflected by the rate of $^{14}$C-glycine incorporation by scales (Ottaway and Simkiss 1977a) was tested by comparing scale growth rates measured by standard methods to $^{14}$C-glycine incorporation rates. A wild population of winter flounder was sampled, at least monthly, for 22 months to determine if there was a seasonal cycle in $^{14}$C-glycine incorporation rates. Growth of these fish was determined by examining scales for growing edges and measuring its width. The correlation of $^{14}$C-glycine incorporation to actual growth of individuals was tested through serial sampling of two groups of laboratory-held fish, one group fed and the other group starved. The scales of these fish were also examined monthly for growing edges, the width of which were measured.

The possible influence of three varying factors on $^{14}$C-glycine incorporation were investigated: seasonal changes in epidermal thickness of wild fish, as reported by Burton and Fletcher (1983), were monitored; seasonality in the effect of temperature on the rate of incorporation was tested by incubating scales over a range of temperatures throughout the year; and fluctuations in free glycine pools of scales were measured by amino acid analysis of extracts of scales from fish used in the seasonal study.

In addition to these seasonal studies, the incorporation of $^{14}$C-glycine into collagen molecules was investigated by testing the sensitivity of scale extracts from incubated scales to collagenase activity, and examining distribution of $^{14}$C-glycine within the incubated scales. The effects of inhibitors of prokaryote and eukaryote protein synthesis on $^{14}$C-glycine incorporation by scales were also investigated.

# 2. METHODS

## 2.1 GENERAL PROCEDURES

### 2.1.1 Collection of fish

Winter flounder were collected by SCUBA-equipped divers at Chapel's Cove, Conception Bay, Newfoundland, or on three occasions, at nearby Harbour Main. Live fish were transported in a seawater tank truck to the Marine Sciences Research Laboratory (MSRL), Memorial University of Newfoundland, at Logy Bay, and were held in running seawater in 2 000 l tanks, at ambient temperature and daylength. Only healthy fish of 25-40 cm total length (TL) (mean 33 cm), were sampled.

### 2.1.2 Incorporation of $^{14}$C-glycine by scales

Fish were sampled between 1030 h and 1330 h. Because winter flounder scales are small, two samples of 10 scales each were removed with forceps from a 1 $cm^2$ area above the lateral line at the thickest part of the body. The scale samples were placed in flounder saline (Table 1) in loosely-capped, individual 10 ml test tubes, shaken gently to remove excess mucous and held on ice until all samples for incubation had been collected. Incubation was started when the saline and mucous debris was removed and replaced with 0.5 ml of flounder saline containing 0.8 µCi/ml $^{14}$C-glycine (universal label, specific activity 1.17 mCi/mg, from New England Nuclear NEN). The scale samples were incubated for 2 h at 10°C and at ambient seawater temperature, in

Table 1. Flounder Saline

| | |
|---|---|
| KCl | 0.201 g $l^{-1}$ |
| $CaCl_2$ $2H_2O$ | 0.403 g $l^{-1}$ |
| $MgCl_2$ $6H_2O$ | 0.130 g $l^{-1}$ |
| NaCl | 10.227 g $l^{-1}$ |
| | - made up as a 10X concentrated Stock Saline and kept refrigerated |
| Glucose | 0.4 g $l^{-1}$ |
| TES[a] buffer | 0.688 g $l^{-1}$ |
| | - added to fresh saline at time of use<br>- pH adjusted to 7.8 |
| Glycine | 0.150 g $l^{-1}$ |
| | - added to Flounder Saline for post-incubation wash |

Note: [a] TES = tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid (from Sigma Chemical Company)

shaker water baths. The incubation saline was then removed and the scales washed for 1 h with five changes of saline containing 2 mM unlabelled glycine. Glycine was included in the wash saline to reduce the level of free $^{14}$C-glycine in (or on) the scales. Samples remained under incubation conditions during the wash period. After washing, scales were gently blotted on filter paper and dried to constant weight at 55°C, for at least 24 h.

Dried scales were either left whole, or a few scales from each sample were cut with scissors near the line of abscission into two sections: posterior (epidermis-bearing) and anterior (normally embedded in the dermis). Whole and sectioned scales were weighed to the nearest 0.01 mg and put into glass scintillation vials, dampened with 1 or 2 drops of distilled water, tightly capped, then digested in 1 ml Protosol (NEN) for at least 24 h at 55°C. Digested scales were counted in 10 ml Liquifluor (NEN) on a Packard 2425 liquid scintillation counter. Results were corrected for colour quench with a flounder scale extract quench curve. The incorporation of $^{14}$C-glycine, in disintegrations per minute (DPM) per mg of scale, were calculated for whole and cut scales of each fish. Samples of the incubation saline and the pooled post-incubation salines were also counted to enable standardization of incorporation results to a constant incubation concentration of 0.8 µCi/ml. The $^{14}$C-glycine concentration in the incubation saline did not decline during the incubation period, indicating that the availability of $^{14}$C-glycine did not limit the rate of incorporation. Mean values $\pm$ SE of $^{14}$C-glycine incorporation were calculated for whole scales and cut scales for all fish sampled, and by sex, for each incubation temperature. The term "incorporation rate" as used in this

study indicates the uptake of $^{14}$C-glycine per mg of scale for a standard 2 h incubation period.

## 2.2 SEASONAL STUDIES

### 2.2.1 Seasonal incorporation of $^{14}$C-glycine into scales

A total of 513 fish (average total length 32.2 ± 0.1 cm) for seasonal studies were collected on 29 field trips over 21 months. Of these, 279 fish (53% male) were sampled in the field immediately upon capture. When possible (most dates), scale samples were collected from five males and five females, but on 5 dates less than 10 fish were sampled: the number of fish sampled, N, was 9 on 1 July 1980; N = 8 on 8 December 1980 and 14 September 1981; N = 7 on 12 January 1981; and N = 6 on 15 January 1980. After scale samples were taken, the fish were killed and packed in polyethylene bags. The fish and samples were held on ice until return to the MSRL, within 2 h. Incubation proceeded as soon as possible, at ambient seawater and 10°C temperatures.

Live fish returned to the MSRL were held in shallow 250 l tanks with running seawater without food for one week before sampling, to reduce effects of capture stress. A total of 234 "laboratory" fish (55% males) were sampled on 28 dates and scales incubated as for "field" fish. The number of laboratory sampled fish was 10, except on 14 dates: N = 9 on 9 April and 7 October 1980; N = 8 on 5 August, 3 September and 28 October 1980; N = 7 on 16 September and 25 November 1980; N = 6 on 27 July 1981; N = 5 on 27 February, 12 May, 3 December 1980 and 25 August 1981; and N = 4 on 29 January 1980 and 14 July 1981.

Field and laboratory fish were also sampled for free amino acid analysis of scales by scraping a 5 $cm^2$ area above the lateral line at the thickest part of the body. The scraped scales were put into 15 ml test tubes and held on ice, or refrigerated, until scale incubations were underway. The scales were washed with 1% NaCl, drained in an unglazed Buchner funnel with vacuum aspiration, returned to the rinsed test tube, capped and frozen until processed further (see section 2.4).

The sampled field and laboratory fish were measured: total length (0.1 cm), total weight (1.0 g), gonad and gut weight (0.01 g). Gut weight is the weight of the entire gastrointestinal tract. Gut contents were weighed in 1980 and estimated in 1981 by subtracting empty gut weight of laboratory fish (as % body weight) from full gut weight of individual field fish (as % body weight). Estimated body weight (EBW) was calculated as total body weight less gonad and gut contents. The weight of the gonad and full gut were calculated as a proportion of the estimated body weight for all field fish. Condition factor, K, was calculated using the formula:

$$K = (EBW/TL^3) \times 100.$$

Mean whole scale $^{14}C$-glycine incorporation (±SE) was calculated, and Students t-test ($p < 0.05$) was used to compare mean values between dates, between sexes, and between field and laboratory fish (Sokal and Rohlf 1969). The incorporation rates of anterior (ANT) and posterior (POST) scale portions were measured on all sampling dates after 29 July 1980. The proportion of total incorporation in the

anterior scale was calculated using the formula:

$$ANT/(ANT + POST) \times 100$$

Mean values for each sample date were calculated.

Scales of 436 field and laboratory sampled wild fish were examined for the presence of a growing edge. The mean monthly proportion of all scales with growing edges, and mean monthly width of the scale growing edge as a proportion of the scale radius, were calculated. An annulus on the outside edge of the scale was assumed to indicate cessation of growth for the year. For such scales a zero value was used for the width of the growing edge.

### 2.2.2 Epidermal thickness

A separate study (Burton and Fletcher 1983) measured seasonal changes in epidermal thickness in field fish throughout 1980; epidermal samples for 1981 were obtained from field fish which had been sampled between May and September 1981, and frozen after initial sampling. A 1 $cm^2$ skin sample from the lower body surface was taken just above the lateral line, at the thickest part of the thawed fish. The skin sample was fixed, embedded in paraffin, and sectioned using techniques of Burton and Fletcher (1983), and mean lower epidermal thickness ($\pm$SE) calculated for each date. Samples for 1981 were analysed from fish collected 19 May (5 male, 2 female); 15 June (4 male, 3 female); 20 July (4 male, 3 female); 12 August (6 male, 1 female); 14 September (4 male); 20 October (3 male, 1 female).

### 2.2.3 Serially sampled fish

A 17 month program of serial sampling of 36 laboratory-held fish was undertaken to investigate the relationship between rates of fish and scale growth and $^{14}$C-glycine incorporation into scales. Healthy male winter flounder, of 25-30 cm TL, were randomly assigned to one of two treatment groups, fed or starved, at the beginning of the program. The fish were held in two 250 l tanks under ambient conditions, and allowed to acclimate for one month before sampling commenced. Each individual was sampled every two weeks, from May until December 1980, and then monthly to August 1981. Two scales were removed from the 5 cm$^2$ sampling area for incubation, then the fish was lightly anaesthetized in a basin containing 0.5 g MS222 in 4 l of fresh seawater, before measuring weight (0.1 g) and total length (0.1 cm). Once a month, a single scale was removed from the caudal peduncle for examination of the growing edge. The fish was returned to the tank as rapidly as possible. When each fish had been sampled, the scales were incubated at 10°C. Individuals were identified by coded bead tags sewn through the base of the upper pectoral fin.

The physical condition of each fish was noted when samples were taken, and unhealthy fish (usually with degeneration of the caudal fin) were removed. Such fish were replaced during June and July of 1980 by fish acclimated for 2 weeks. On the final sampling date, there were four fish in the fed group, which had all survived from the original fifteen. None of the starved fish survived beyond 10 October 1980.

One group of fish was fed daily with a weighed amount of chopped capelin (Mallotus villotus). Uneaten food was collected and weighed

the next day to estimate the rate of feeding. The fish were not fed for 24 h before sampling. This group was fed from April through November 1980, by which time food consumption had ceased; food was provided again in April 1981, which is when wild fish were observed to have resumed feeding. The starved group was not fed throughout the experiment.

The rate of food consumption for each sampling interval was calculated by dividing the weight of food consumed by the mean weight of the fish at the end of the interval. The per cent rates of change in length and weight of individual fed and starved fish were calculated as the difference between consecutive measurements, divided by the earlier measurement, times 100. The mean rates of growth were computed for each interval of sampling. The mean rate of $^{14}C$-glycine incorporation into scales was calculated from individual values, as described in section 2.1. Differences between the mean values were analysed by t-tests.

Scales were read at 17 different dates for the fed fish, and eight dates for the starved fish. Mean values of the proportion of scales with growing edges and the width of the growing edge as a per cent of total scale radius, were calculated for each group, for each date. When an annulus was present on the outside edge of a scale, a value of zero was included in the mean width of the growing edge calculation.

### 2.2.4 Seasonal temperature effects

On eight occasions over the 16 month period, the influence of temperature on $^{14}C$-glycine incorporation into scales was

investigated by incubating scales over a naturally occurring range of temperatures (Fig. 1). Fish used for these experiments were held in the laboratory for periods of two weeks to two months, under ambient conditions of light and temperature, and fed chopped capelin. On each date, one to three fish were sampled, with two to eight samples of 10 scales being removed from a 5 $cm^2$ area, for incubation at each of four or five temperatures (Table 2). Scales from different individuals were not mixed. Several shaking water baths, and a multipurpose rotator (Scientific Instruments Inc., model 150V) with sample test tubes held in a horizontal position, were used to provide incubation temperatures of: 0°C or 1°C, 5°C, 10°C, 15°C, and ambient seawater temperature in the laboratory.

At each date, the mean $^{14}C$-glycine incorporation rate was calculated for all replicates at each incubation temperature. The relationship of incorporation rate to temperature was linear over the temperatures tested, and linear regressions of incorporation rate against incubation temperature were calculated from mean values using the equation:

$$y = ax + b,$$

where a = slope and b = intercept. The coefficients of determination, $r^2$, were also computed. The incorporation/temperature regressions were examined by analysis of covariance (ANCOVA) to test the influence of ambient temperature on mean rate of $^{14}C$-glycine incorporation.

The increase in incorporation rate with a 10°C increase in temperature ($Q_{10}$), was calculated from linear regression values for each date according to the equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/T_2-T_1}$$ Prosser 1973

Figure 1. Seasonal cycles in ambient temperature (°C), winter flounder condition (K), and gonad and full gut weights as % of estimated body weight (EBW), for field sampled wild fish, 1980 and 1981. °N, number of fish sampled = 10, except on 5-dates noted in text. Data are combined from male and female flounders.

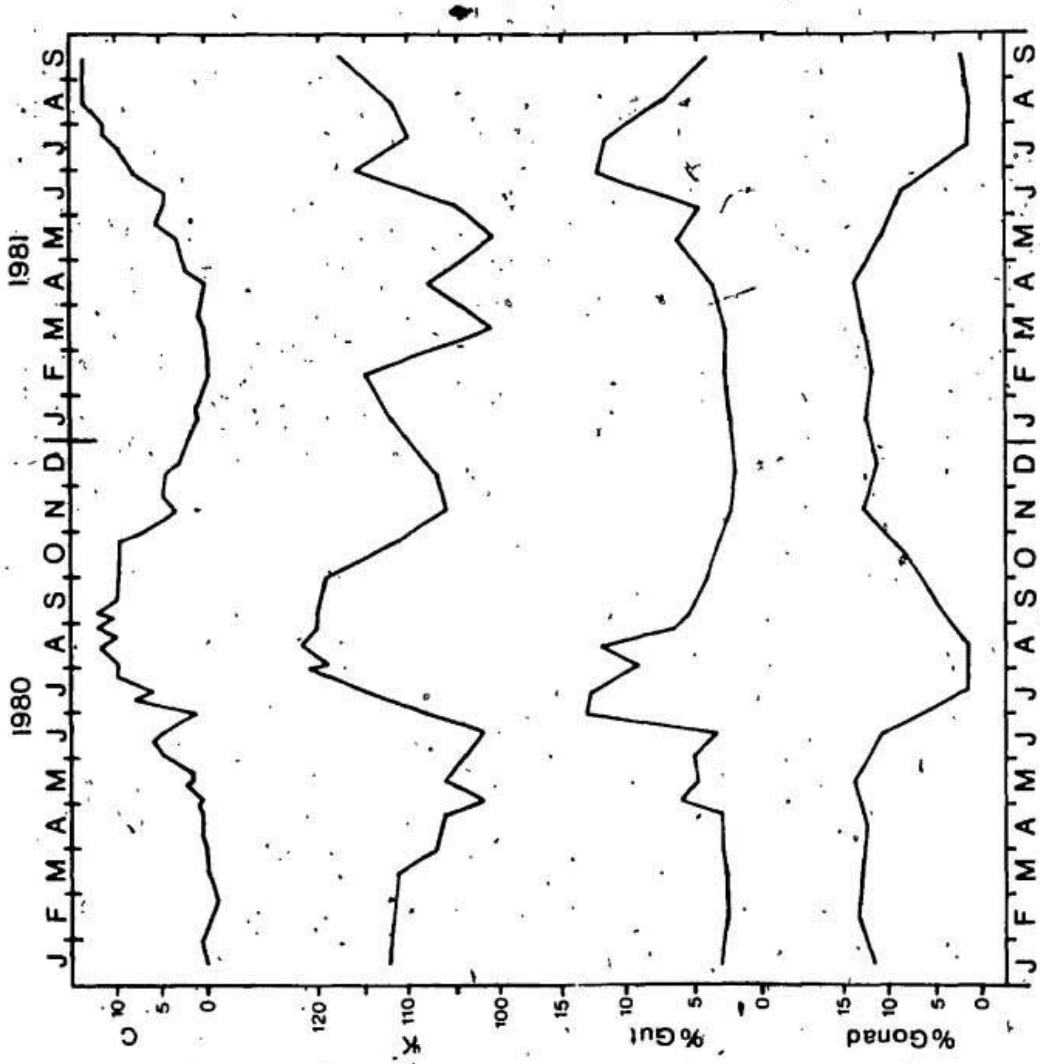

1980
1981
J F M A M J J A S O N D J F M A M J J A S
C
K
% Gut
% Gonad
J F M A M J J A S O N D J F M A M J J A S
Months

Table 2. Temperature Curve Regressions and $Q_{10}$

| Date | Ambient T°C | N[a] | n[b] | Linear Regression | | | $Q_{10}$ |
|---|---|---|---|---|---|---|---|
| | | | | $r^2$ | slope | intercept | (0-10°C) |
| 19-05-80 | 1.5 | 5 | 5 | 0.55 | 92.7 | 1273.4 | 1.75 |
| 04-06-80 | 5.0 | 3 | 3 | 0.87 | 94.2 | 1056.0 | 1.62 |
| 26-06-80 | 7.5 | 2 | 2-4 | 0.84 | 153.3 | 1264.5 | 1.86 |
| 31-08-80 | 10.0 | 2 | 8 | 0.93 | 161.7 | 822.7 | 2.37 |
| 06-11-80 | 7.0 | 1 | 4 | 0.92 | 172.7 | 1341.4 | 2.17 |
| 10-12-80 | 4.5 | 1 | 6 | 0.96 | 118.4 | 1742.7 | 1.52 |
| 05-02-81 | 0.0 | 1 | 4 | 0.95 | 156.2 | 1570.5 | 1.94 |
| 31-05-81 | 6.0 | 1 | 8 | 0.85 | 60.1 | 560.7 | 2.56 |

Notes:
[a] N is the number of fish sampled
[b] n is the number of replicate scale samples from each fish incubated at each temperature
[c] Linear regressions were calculated from the mean of all replicates at each temperature, using the formula $y = ax + b$, where y = incorporation rate, x = temperature, a = slope and b = intercept.

where $R_1$ = incorporation rate at $T_1$, the lower temperature, and

$R_2$ = incorporation rate at $T_2$, the higher temperature.

2.2.5 Scale glycine pool

Scale samples of 176 fish were selected from 29 field sampling dates for glycine pool analysis. Four samples from male and female fish of similar size were processed, except on 9 dates: N = 10 on 1 April and 15 May 1980; N = 9 on 12 March and 5 May 1980; N = 6 on 15 January 1980; N = 3 on 12 January 1981; and N = 2 on 22 April, 1 July, and 12 August 1980.

For each scale sample, the frozen scales were lyophilized for 24 h, weighed to the nearest mg, milled to a powder in a Prolabo ball mill and reweighed. The powder was suspended in 10 ml of distilled water (acidified to pH 2.5 with HCl) in the original test tube, tightly capped and extracted by inverting for 48 h at 4°C on a multipurpose test tube rotator. The scale extract was centrifuged (4 000 x g) for 10 minutes, and the supernatant removed. The scale pellet was resuspended in 2 ml acidified distilled water, recentrifuged, and the pellet wash combined with the scale extract.

The protein in the extract was precipitated in 80% EtOH and discarded, the deproteinated extract was reduced by evaporation to approximately 5 ml and passed through a 2 ml bed of Amberlite anion exchange resin (BDH IR-120 ($H^+$)) packed in an 11 ml polypropylene mini-column (BIORAD Econocolumn) at a rate of 0.2 ml/min. The resin was washed with three 10 ml volumes of 50% EtOH, and samples eluted with 50% $NH_3$ in EtOH.

The protein free eluate, containing only amino acids and small molecules, was reduced to 1 ml on a Rotovapour flash evaporator (Buchi, Switzerland). The reduced extract, with rinsings of the evaporation flask, was transferred to a 7 ml capped plastic test tube and dried under nitrogen on a N-Evap (Organomation Ass'n, Worcester, Massachussetts) at 30°C. The dry extract was made up to 0.5 ml with redistilled water, deproteinated once more with 0.125 ml 5% sulfasalicylic acid in lithium citrate buffer (pH 2.2, 0.15 N) and made up to 1 ml with the lithium citrate buffer. The extract was centrifuged and the level of free amino acids analysed using a Beckman model 121 amino acid analyser, as described by Squires et al. (1976). The amount of glycine in each sample was quantified as nanomoles (nM) per gram of scale powder extracted, and mean values calculated for each sample date.

The influence of the scale glycine pool on $^{14}$C-glycine incorporation rates was assessed by expressing the incorporation rate in terms of the specific activity of the scale glycine pool, in the presence of $^{14}$C-glycine in the incubation medium. The specific activity of the scale glycine pool was calculated based on the assumption that equilibrium is established between $^{14}$C-glycine in the incubation medium and free glycine within scale cells during incubation. Although this assumption was not tested specifically it was supported by the fact that $^{14}$C-glycine incorporation was linear over incubation periods of 0.5 h to 3 h (for example see Figure 14).

$$\text{Scale glycine pool specific activity} = \frac{\text{specific activity of incubation medium (0.8 } \mu\text{Ci/ml)}}{\text{scale glycine pool}} \quad (\text{nM/mg})$$

$$= \frac{1.76 \times 10^6 \text{ DPM mg}^{-1}}{\text{pM} \times 10^3 \text{ Gly mg}^{-1}}$$

$$= \frac{\text{DPM} \times 10^3}{\text{pM Gly}}$$

The rate of incorporation of glycine from the active glycine pool is:

$$\frac{\text{Rate of } ^{14}\text{C-glycine incorporation}}{\text{Pool activity}} = \frac{\text{DPM} \times 10^3 \text{ mg}^{-1} \text{ scale}}{\text{DPM} \times 10^3 \text{ pM}^{-1} \text{ Gly}}$$

$$= \text{pM Gly mg}^{-1} \text{ scale}$$

The rate of $^{14}$C-glycine incorporation into scales of individual fish was converted to glycine incorporation using the mean scale glycine pool for the sampling date; and the mean glycine incorporation rate $\pm$ SE was calculated for each date. Between year glycine incorporation rates at date were compared using Students t-test.

## 2.3 EFFECTS OF INHIBITORS OF PROTEIN BIOSYNTHESIS

The effects of three inhibitors of protein biosynthesis on $^{14}$C-glycine incorporation by scales were examined in four experiments, from July to October 1980. The inhibitors used were: cyclo-

heximide (actidione) at a concentration of 5 g $ml^{-1}$, an inhibitor of protein chain initiation and elongation in eukaryote cells; puromycin dihydrochloride, 30 g $ml^{-1}$, an inhibitor of ribosomal protein synthesis in prokaryote cells; and chloramphenicol, 10 g $ml^{-1}$, which inhibits peptide bond formation of most bacteria. The inhibitors were obtained from Mannheim-Boehringer. Scales were collected from laboratory-held fish and preincubated for 2 h, incubated with $^{14}C$-glycine for 2 h and then washed at 10°C. On 31 July, incubation periods of 0.5 to 3.0 h were used to test the linearity of incorporation and the time course of inhibition. Inhibitors were included in the preincubation, incubation and wash media of inhibitor-treated samples. Two scale samples were incubated under each treatment, including controls, and the scales of one sample from each treatment were cut into anterior and posterior sections. Details of the incubations are presented in Table 3. Samples treated with chloramphenicol were protected from light with an aluminum foil sleeve, as chloramphenicol is light sensitive.

Results were expressed as proportions of control values. For the 31 July experiment, regressions of incorporation on time were calculated from mean results for each incubation period. The incorporation results of controls and cycloheximide inhibited incubations were analysed by one-way analysis of variance to determine if there were significant differences in incorporation between the dates. Data from all replicates preincubated and incubated for 2 h, including the cut scales, were combined to provide a mean value for each date and the means were compared by Students t-tests.

Table 3. Incubations with inhibitors of protein biosynthesis

| Date | Inhibitor | Incubation | Incorporation Rate (as % of Controls) Whole | Post | Ant |
|---|---|---|---|---|---|
| 23 July | Puromycin | 2.0 h | 46.9 | 33.9 | 73.9 |
| | Puromycin and cycloheximide | 2.0 h | 47.0 | 25.5 | 67.4 |
| | Cycloheximide | 2.0 h | 46.9 | 42.1 | 132.4 |
| 31 July | Cycloheximide | 0.5 h | 94.3 | 88.9 | 145.7 |
| | | 1.0 h | 224.9 | 319.1 | 414.7 |
| | | 1.5 h | 130.7 | 161.1 | 218.9 |
| | | 2.0 h | 73.1 | 90.8 | 117.4 |
| | | 2.5 h | 69.3 | 51.6 | 128.8 |
| | | 3.0 h | 69.4 | 81.2 | 67.5 |
| 15 August | Cycloheximide | 2.0 h | 52.6 | 46.9 | 98.1 |
| 17 October | Cycloheximide | 2.0 h | 71.5 | 59.2 | 145.9 |
| | Cycloheximide and chloramphenicol | 2.0 h | 70.5 | 70.9 | 133.0 |
| | Choramphenicol | 2.0 h | 100.2 | 106.3 | 126.1 |

## 2.4 MOLECULAR INCORPORATION OF $^{14}$C-GLYCINE

How much of $^{14}$C-glycine incorporation into scales is actually associated with collagen, or smaller molecules, and how much remains as free glycine? To approach these questions, large numbers of scales were incubated on three dates (11 September 1980, 28 January 1981 and 9 July 1981). Twenty samples, of 10 scales each, were collected from the 5 cm$^2$ sampling area of fish which had been in the laboratory for one to two weeks. All of the samples were incubated at 10°C with 1.6 µCi/ml of $^{14}$C-glycine in saline, except 11 September 1980 when 0.8 µCi/ml was used. The average incorporation at each date was determined from two to four representative samples composed of one scale from each incubation vial. Some of these scales were cut into anterior and posterior sections, and all were weighed and digested individually in Protosol. The remaining scales for each date were combined and cut into anterior and posterior portions, weighed and frozen until all samples could be processed together (2 November 1981). The scale samples from each date were then lyophilized and reweighed before being taken through four main steps of analysis:

1. extraction of soluble components of scale;
2. gel filtration chromatography of the scale extract;
3. analysis of extract components soluble in 15% TCA by collagenase digestion and dialysis; and
4. collagenase digest of 15% TCA insoluble material.

At each step, the $^{14}$C content was determined by counting a small sample. Every effort was made to account for all of the $^{14}$C at each step, and to reduce the proportion that was lost. For example,

Kim Wipe tissues used to wipe the sampling micropipettes were also counted. The procedures outlined below are presented as a flow chart in Figure 2.

For each step of the procedure the recovered counts of $^{14}C$ in each fraction was expressed as a proportion of total DPM recovered for the entire scale sample (Table 4), and as proportion of DPM recovered at each step. The amount of radioactivity that was solubilized or reduced in molecular weight by collagenase digestion ("collagen"-associated $^{14}C$) was calculated as a proportion of total counts in the sample.

### 2.4.1 Extraction of scales

The procedure for lyophilizing and extracting scale samples is described in section 2.2.5. Scale pellets were washed with acidified distilled water, and the wash was added to the extract after the protein content of the extract had been determined. The scale pellets (scale fraction 1) were frozen until the extracts were processed, at which time they were thawed and a weighed portion of each digested with collagenase (see section 2.4.4).

### 2.4.2 Gel filtration chromatography

Each of the scale extracts (scale fraction 2) was applied to a column of Sephadex G-25 (Pharmacia), and eluted with a 0.05 M glycine buffer (pH 7.4). Samples of 0.5 ml from each 2.5 ml fraction were dissolved in Aquasol scintillation fluid (NEN), counted, and the

Figure 2. Flowchart of molecular incorporation procedures used to determine the percent of total radioactivity associated with various scale fractions. Numbers refer to scale fractions in Table 4.

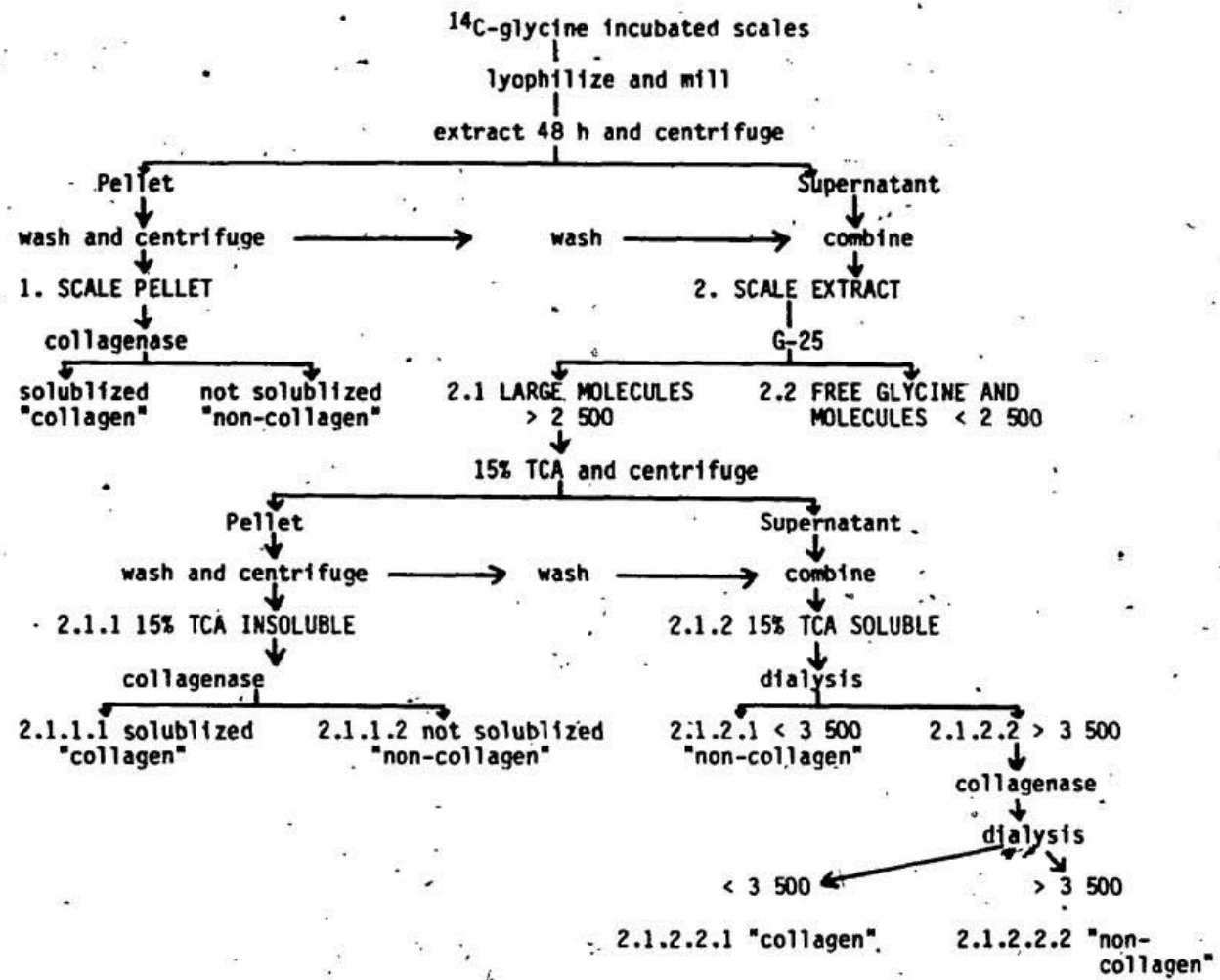

$^{14}$C-glycine incubated scales
lyophilize and mill
extract 48 h and centrifuge
Pellet
Supernatant
wash and centrifuge
wash
combine
1. SCALE PELLET
2. SCALE EXTRACT
collagenase
G-25
solublized "collagen"
not solublized "non-collagen"
2.1 LARGE MOLECULES > 2 500
2.2 FREE GLYCINE AND MOLECULES < 2 500
15% TCA and centrifuge
Pellet
Supernatant
wash and centrifuge
wash
combine
2.1.1 15% TCA INSOLUBLE
2.1.2 15% TCA SOLUBLE
collagenase
dialysis
2.1.1.1 solublized "collagen"
2.1.1.2 not solublized "non-collagen"
2.1.2.1 < 3 500 "non-collagen"
2.1.2.2 > 3 500
collagenase
dialysis
< 3 500
> 3 500
2.1.2.2.1 "collagen"
2.1.2.2.2 "non-collagen"

Table 4. Molecular Incorporation: Percent of Total Recovered Radioactivity Associated with Various Scale Fractions

| Scale Fraction[b] | 11 Sept 1980 Ant | 11 Sept 1980 Post | 28 Jan 1981 Ant | 28 Jan 1981 Post | 9 July 1981 Ant | 9 July 1981 Post |
|---|---|---|---|---|---|---|
| 1. Scale Pellet | | | | | | |
| "collagen" | 9.5 | 25.2 | 8.0 | 4.1 | 5.4 | 6.9 |
| "non-collagen" | 15.7 | 56.1 | 6.3 | 16.3 | 20.8 | 24.8 |
| 2. Scale Extract | | | | | | |
| 2.1 large molecules[c] | | | | | | |
| 2.1.1 15% TCA insoluble | | | | | | |
| 2.1.1.1 "collagen" | 3.2 | 0.3 | 0.3 | 1.7 | 3.3 | 0.2 |
| 2.1.1.2 "non-collagen" | 1.0 | 0.2 | 0.2 | 0.7 | 1.5 | 0.1 |
| 2.1.2 15% TCA soluble | | | | | | |
| 2.1.2.1 (non-collagen) MW < 3 500 | 28.8 | 2.7 | 10.3 | 8.6 | 10.3 | 12.0 |
| 2.1.2.2 MW > 3 500 | | | | | | |
| 2.1.2.2.1 "collagen" | 1.8 | 0.2 | 2.0 | 3.3 | 0.9 | 0.8 |
| 2.1.2.2.2 "non-collagen" | 1.7 | 2.2 | 11.1 | 5.9 | 14.4 | 10.8 |
| 2.2 free glycine and small molecules[d] ("non-collagen") | 30.8<br>1.8 | 10.7<br>0.5 | 52.8<br>2.6 | 48.6<br>3.6 | 36.0<br>1.2 | 36.4<br>2.0 |
| 3. Lost | 6.3 | 1.9 | 6.3 | 7.4 | 6.2 | 6.0 |
| Total % collagen[a] | 14.5 | 25.7 | 10.3 | 9.1 | 9.6 | 7.9 |
| Total DPM recovered | 74 160 | 96 150 | 54 580 | 122 250 | 78 170 | 262 290 |

Notes: [a] Total DPM recovered - scale pellet DPM plus total recovered DPM from gel filtration chromatography.

[b] Scale Fraction - number refers to the scale fraction identified in Figure 2.

[c] Large molecules - gel filtration chromatography fractions 10-19.

[d] Free glycine and small molecules - gel filtration chromatography fractions 20-35.

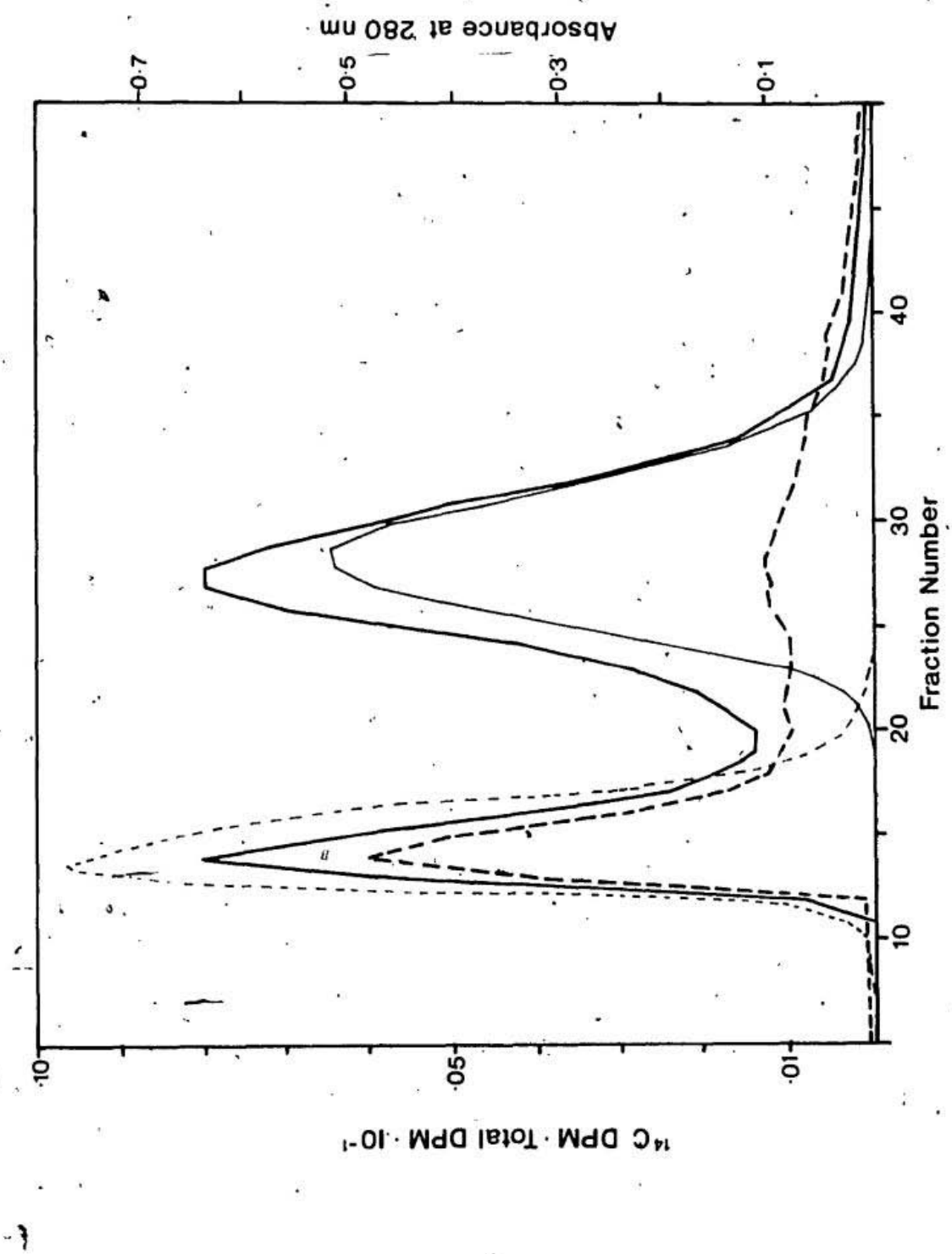
fraction DPM expressed as a proportion of total DPM recovered from the column. Protein concentrations were determined by measuring absorbance of each fraction at 280 nm. Standard curves for the G-25 column were obtained by applying a 5 ml sample of acidified distilled water containing 60 mg of BSA (beef serum albumen) and 8 µl of $^{14}$C-glycine. The BSA standard indicates the location of the void volume (molecules larger than 2 500 MW); while radioactive decay locates the $^{14}$C-containing fractions. The absorbance and radioactive profiles of the standards and of the July 1981 scale extract are shown in Figure 3.

The free glycine and small molecular weight (<2 500) fractions (scale fraction 2.2) were discarded. The larger molecular weight (void volume) fractions (scale fraction 2.1) were pooled, lyophilized and reconstituted with 2 ml distilled water and a sample counted. The pooled void volume fractions were then deproteinated by addition of 0.86 ml of 50% TCA (trichloracetic acid), giving a final concentration of 15% TCA, refrigerated for 1 h, and centrifuged for 10 minutes at 4 000 x g.

### 2.4.3 15% TCA insolubles

The 15% TCA precipitated proteins (scale fraction 2.1.1) were washed with 0.5 ml each of absolute EtOH and ether, and the washes reduced to dryness under $N_2$ gas on a Rotovapour. The alcohol wash containing TCA was neutralized by adding 0.25 ml 6% NaOH, and then all washes were dissolved in Protosol, counted and the counts included in values for TCA solubles. The dry, washed protein pellets were digested with collagenase (see section 2.4.4). After the 24 h digestion period,

Figure 3. Gel filtration chromatography of scale extract of scales incubated July 1980, and of beef serum albumen (BSA) and $^{14}C$-glycine standards. The $^{14}C$ content of each fraction is expressed as a percent of total DPM recovered from the column (——July 1980 scale sample; — $^{14}C$ standard). Protein content of each fraction is indicated by absorbance at 280 nM (– – July 1980 scale sample; - - BSA standard).


Absorbance at 280 nm
0·7
0·5
0·3
0·1
Fraction Number
10
20
30
40
$^{14}C$ DPM · Total DPM · $10^{-1}$
·10
·05
·01

all protein was in solution. The collagenase solublized proteins were separated from proteins not affected by collagenase by reprecipitating the samples in 15% TCA. The resulting TCA soluble supernatants ("collagen" (scale fraction 2.1.1.1) and precipitated proteins ("non-collagen) (scale fraction 2.1.1.2) were dissolved in Protosol and counted.

### 2.4.4 Collagenase digestion of scale proteins

The reaction mixtures of Yoshida and Noda (1965) were used to hydrolyse scale proteins with collagenase Type 1A (Sigma), 9.4 units/ml distilled water. Samples were incubated in a shaking water bath for 24 h at 37°C. The collagenase digest solution plus wash of any non-digested material was dissolved in Protosol and counted against a blank consisting of the reaction media without scale protein. Scale material which was not solublized by collagenase ("non-collagen") was digested in Protosol at 55°C and counted.

### 2.4.5 15% TCA solubles

The 15% TCA soluble portions (scale fraction 2.1.2) of the large molecular weight fractions were sampled for radioactive content and then dialysed against a membrane with a 3 5000 MW pore size (Fisher Scientific Co.) in 2 l of 10 mM ammonium bicarbonate buffer for 24 h at 4°C. The buffer was changed after 1.5 h and after 17 h of dialysis, then discarded. Radioactive counts associated with molecules of less than 3 500 MW were discarded with the dialysis buffer and were

considered to be associated with "non-collagen" molecules (scale fraction 2.1.2.1). The dialysed proteins of more than 3 500 MW (scale fraction 2.1.2.2) were washed out of the dialysis bags with redistilled water, lyophilized, and reconstituted to 2 ml with redistilled water. A sample was taken for counting. The samples were then relyophilized, and when dry, digested by collagenase. After collagenase digestion, the samples were redialysed and counts which were dialysed were considered to be associated with "collagen" molecules (scale fraction 2.1.2.2.1). The non-dialysed proteins (scale fraction 2.1.2.2.2) were dissolved in Protosol and counted. Counts associated with the dialysis bag were included in the non-dialysed total.

# 3. RESULTS

## 3.1 SEASONAL STUDIES

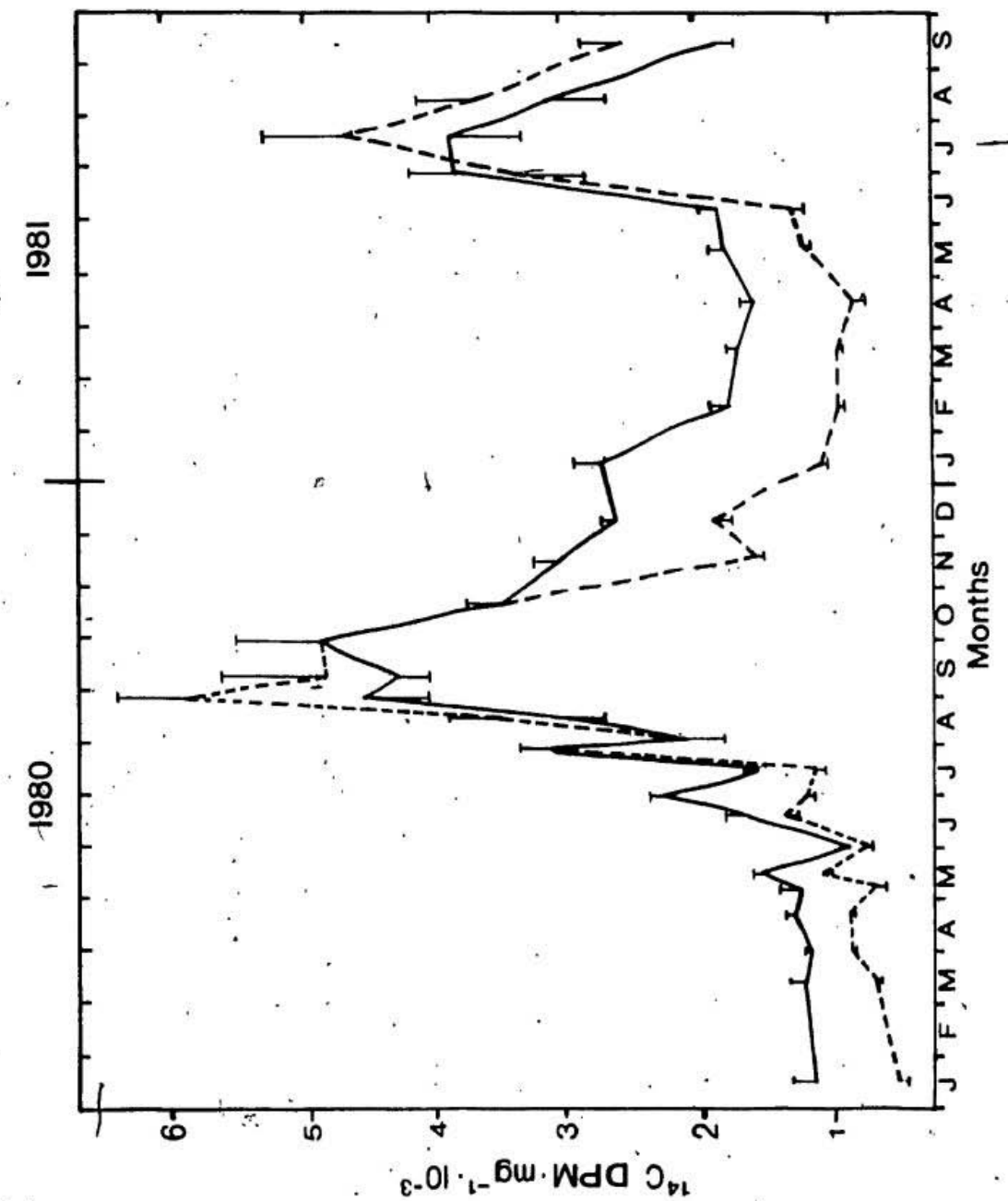
### 3.1.1 Seasonal biology

The pronounced seasonal biology of winter flounder was reflected in the changes in condition (K) and full gut weight (feeding) during 1980 and 1981 (Figure 1). The periods of rapid change in condition correspond to completion of spawning in June and cessation of feeding in the autumn. Condition did not differ between sexes on 27 of 29 sampling dates, and therefore the values from both sexes were combined. Except for March, mean monthly condition was the same for both years, which indicated that there was a regular seasonal cycle in growth. Regular seasonal cycles in gut weight were apparent in both years. The changes in gonad weight show the seasonal cycle of spawning and gonad ripening.

### 3.1.2 Seasonal incorporation of $^{14}$C-glycine into scales

There was a seasonal cycle in $^{14}$C-glycine incorporation by scales at ambient temperature and at 10°C (Figure 4). In field fish, the incorporation values averaged 1 578 $\pm$ 42 DPM mg$^{-1}$ at 10°C from January through April ("winter"), increasing significantly from mid-June to peak levels, during mid-July to mid-August, of about 2.5 times mean winter incorporation (4 289 $\pm$ 300 in 1980; 3 575 $\pm$ 275 in 1981). The timing of the peak incorporation rate varied slightly between the

Figure 4. Seasonal rates of $^{14}C$-glycine incorporation by field-sampled scales of wild fish, incubated at 10°C (—) and at ambient seawater temperature (---), 1980 and 1981. Mean ± SE $^{14}C$ DPM $mg^{-1}$ scale x $10^{-3}$. The rate of $^{14}C$-glycine incorporation was measured after a 2 h incubation period. N, number of fish sampled = 10, except on 5 dates noted in the text.


1980
1981
J F M A M J J A S O N D J F M A M J J A S
Months
$^{14}C$ DPM·mg$^{-1}$·10$^{-3}$
1
2
3
4
5
6

years, but peak incorporation rates were not significantly different. There were no differences between male and female fish in 28 out of 29 sampling dates, or between winter field and laboratory fish glycine incorporation rates. However, laboratory fish incorporation rates were significantly higher from March to early July in 1980 ($P < 0.05$), and significantly lower than field fish incorporation from July to September 1980 ($P < 0.02$) and in July 1981 ($P < 0.02$) (Figure 5).

Anterior and posterior scale portions from field fish showed the same seasonal cycle in rate of incorporation as whole scales (Figure 6). The anterior scale accounted for 16 to 31% of the summed cut-scale incorporation of $^{14}$C-glycine, with peak values averaging 26.32 $\pm$ 0.77%, and winter values averaging 19.81 $\pm$ 0.42% ($P < 0.001$). The rate of seasonal change in glycine incorporation rate was not different between anterior and posterior scale portions, which suggested that both portions respond in a similar fashion to factors which influence the rate of incorporation of $^{14}$C-glycine.

### 3.1.3 Growth of scales

Peak rates in $^{14}$C-glycine incorporation by scales of field sampled fish did not coincide with scale growth in field and laboratory sampled fish, except during July and August 1981 (Figure 7). Growing edges were present on scales of more than 50% of fish sampled from April to July, with a peak in May when over 90% of scales had growing edges (Figure 7). Increases in the percent growing edge (width of the growing edge as a proportion of total scale radius) occurred most rapidly from March to May, with an average maximum width of the growing

Figure 5. Seasonal rates of $^{14}C$-glycine incorporation by scales of laboratory-sampled wild fish one week after capture, incubated at 10°C (—) and at ambient seawater temperature (---), 1980 and 1981. Mean ± SE $^{14}C$ DPM $mg^{-1}$ scale x $10^{-3}$. The rate of $^{14}C$-glycine incorporation was measured after a 2 h incubation period. N, number of fish sampled = 10, except on 14 dates noted in the text.

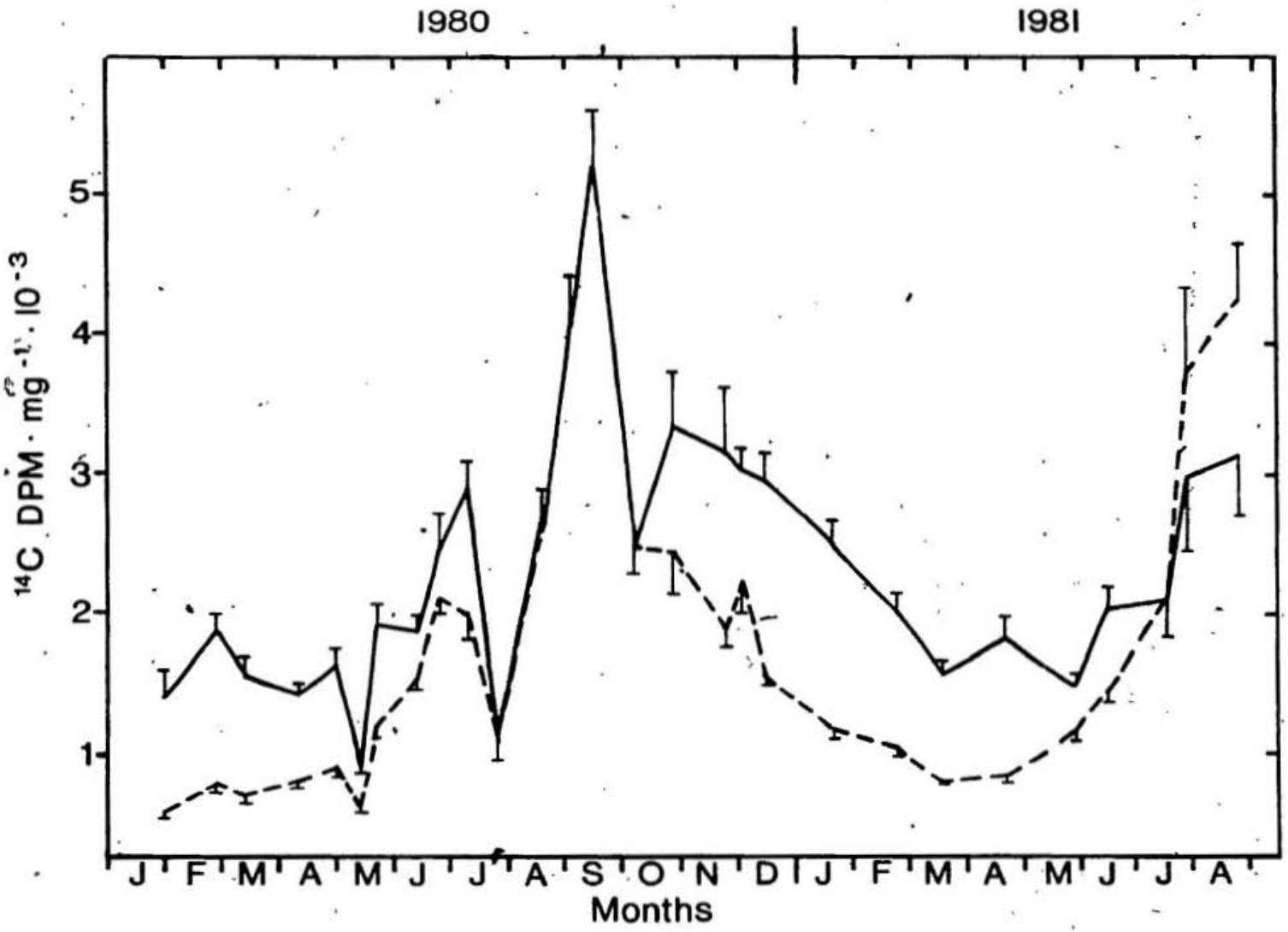

1980
1981
$^{14}C$ DPM · mg$^{-1}$ · $10^{-3}$
5
4
3
2
1
J F M A M J J A S O N D J F M A M J J A
Months

Figure 6. Seasonal rates of $^{14}C$-glycine incorporation in field-sampled scale portions, 1980 and 1981. The rate of $^{14}C$-glycine incorporation was measured after a 2 h incubation period.

a. Whole scales and anterior (ANT) and posterior (POST) scale portions, incubated at 10°C. Mean ± SE of $^{14}C$ DPM $mg^{-1}$ scale x $10^{-3}$.

b. Seasonal anterior scale incorporation, as a proportion of summed anterior plus posterior scale incorporation at 10°C. Mean ± SE of ANT/(ANT + POST) x 100. N, number of fish sampled = 10, except on 5 dates noted in the text.

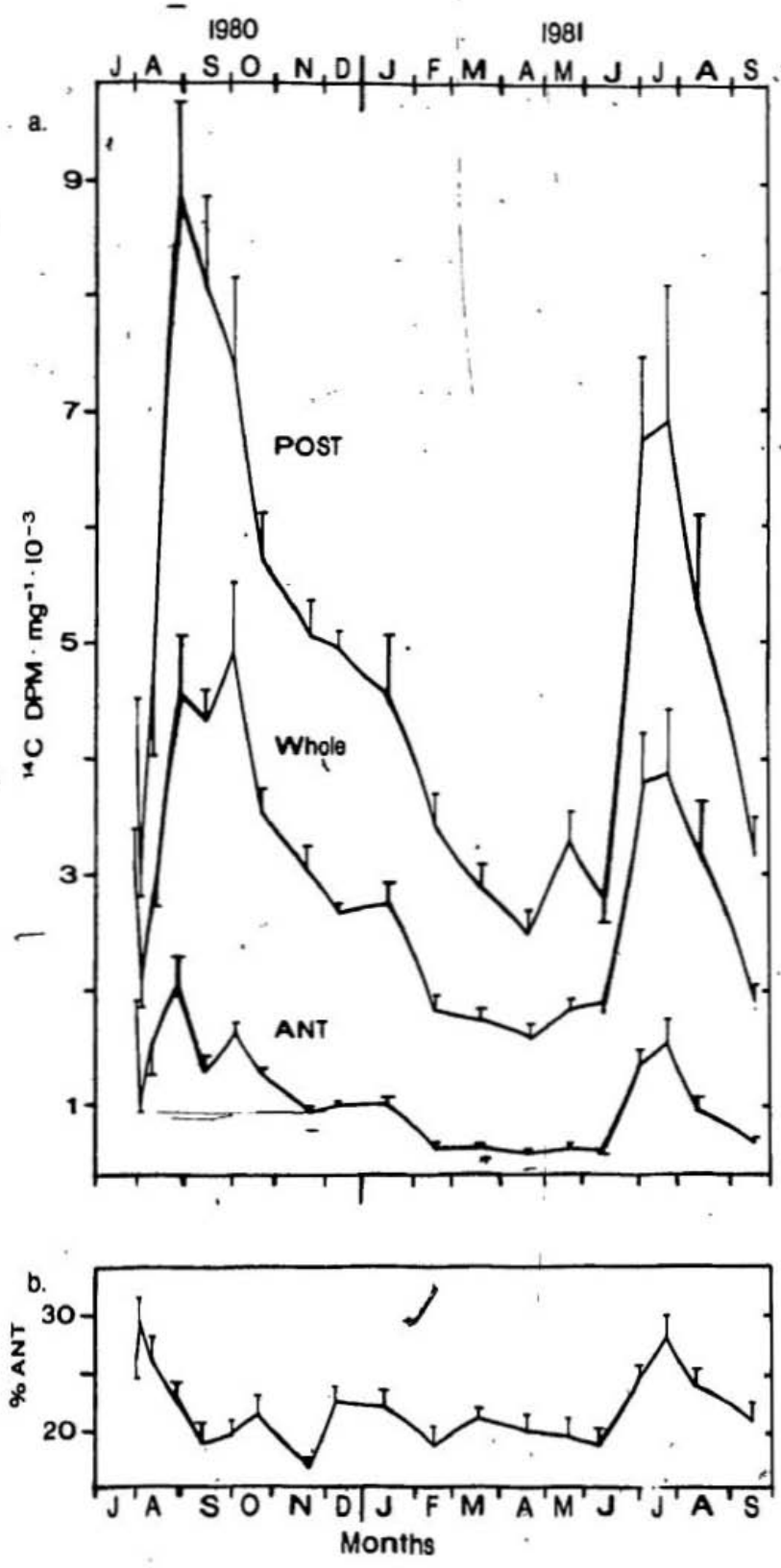

1980
1981
J A S O N D J F M A M J J A S
a.
9
7
5
3
1
POST
Whole
ANT
$^{14}C$ DPM · $mg^{-1}$ · $10^{-3}$
b.
30
20
% ANT
J A S O N D J F M A M J J A S
Months

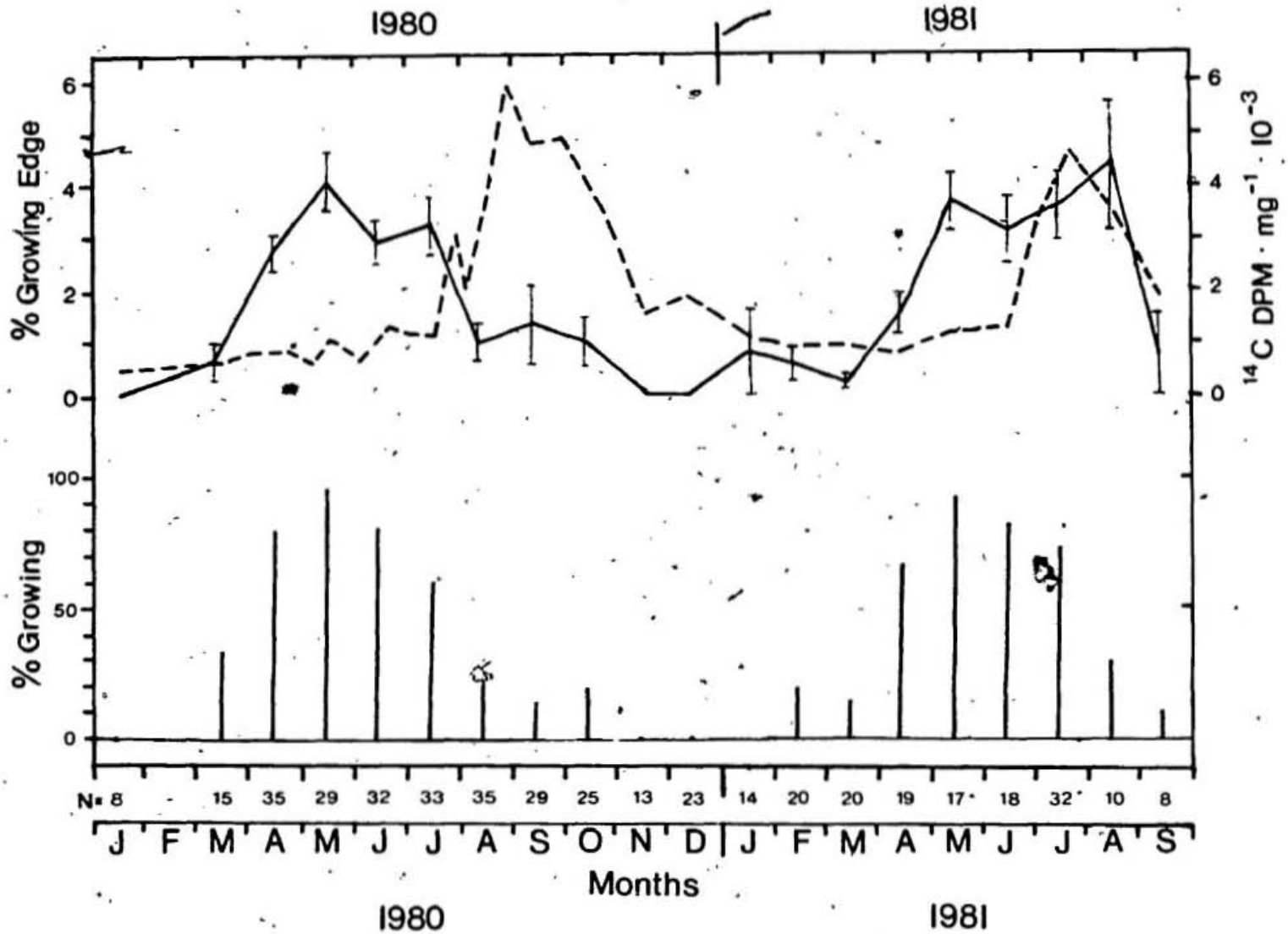
Figure 7. Cycles of scale growth in seasonally sampled wild fish, 1980 and 1981.

Percent growing edge of field and laboratory-sampled wild fish (—) (monthly Means ± SE) and $^{14}$C-glycine incorporation rates (after 2 h incubation period) of field sampled fish (---) (sampling date Means).

Percent growing edge is the width of the scale growing edge as a proportion of total scale radius, and is equal to 0 when an annulus is present at the edge of the scale.

Percent growing is the number of scales with growing edges as a proportion of all scales sampled for each month.

N = the total number of fish sampled in the field and laboratory for each month.


1980
1981
% Growing Edge
$^{14}C$ DPM · mg$^{-1}$ · $10^{-3}$
% Growing
N= 8 - 15 35 29 32 33 35 29 25 13 23 14 20 20 19 17 18 32 10 8
J F M A M J J A S O N D J F M A M J J A S
Months
1980
1981

edge of 4.11 ± 0.55% in May 1980, and 4.37 ± 1.21% in August 1981. During the period of peak scale growth the rate of $^{14}$C-glycine incorporation increased only slightly, and peak levels of incorporation of field fish scales (Figure 7) and laboratory fish scales (Figure 5) generally occurred when the proportion of scales that were still growing was decreasing. There was no relationship between scale radius and scale $^{14}$C-glycine incorporation rates at any time of the year.

### 3.1.4 Serially sampled fish

The seasonal rates of food consumption, increments of body growth and scale $^{14}$C-glycine incorporation rates for the serially sampled fed fish are presented in Figure 8. All of the fed fish gained weight between June and October, with mean peak rate of increase of 7.5% over 2 weeks in July 1980. Eight of the eighteen fed fish grew in length, with the greatest mean increase of 0.44% occurring during June 1980, and an overall mean increase in length of 2.78% for the season. There was no growth in length from October until May 1981. Incorporation of $^{14}$C-glycine reached peak levels through September 1980, and dropped over 4-fold to winter incorporation levels from January to May.

There were no significant differences between years (April to August) in water temperatures or rates of body growth; however food consumption was greater in 1981. $^{14}$C-glycine incorporation rates were not different between years except in mid-July ($P < 0.001$). The mean rates of growth and incorporation of the eight fish which grew in length did not differ significantly from the mean rates of all fed fish combined ($P > 0.05$).

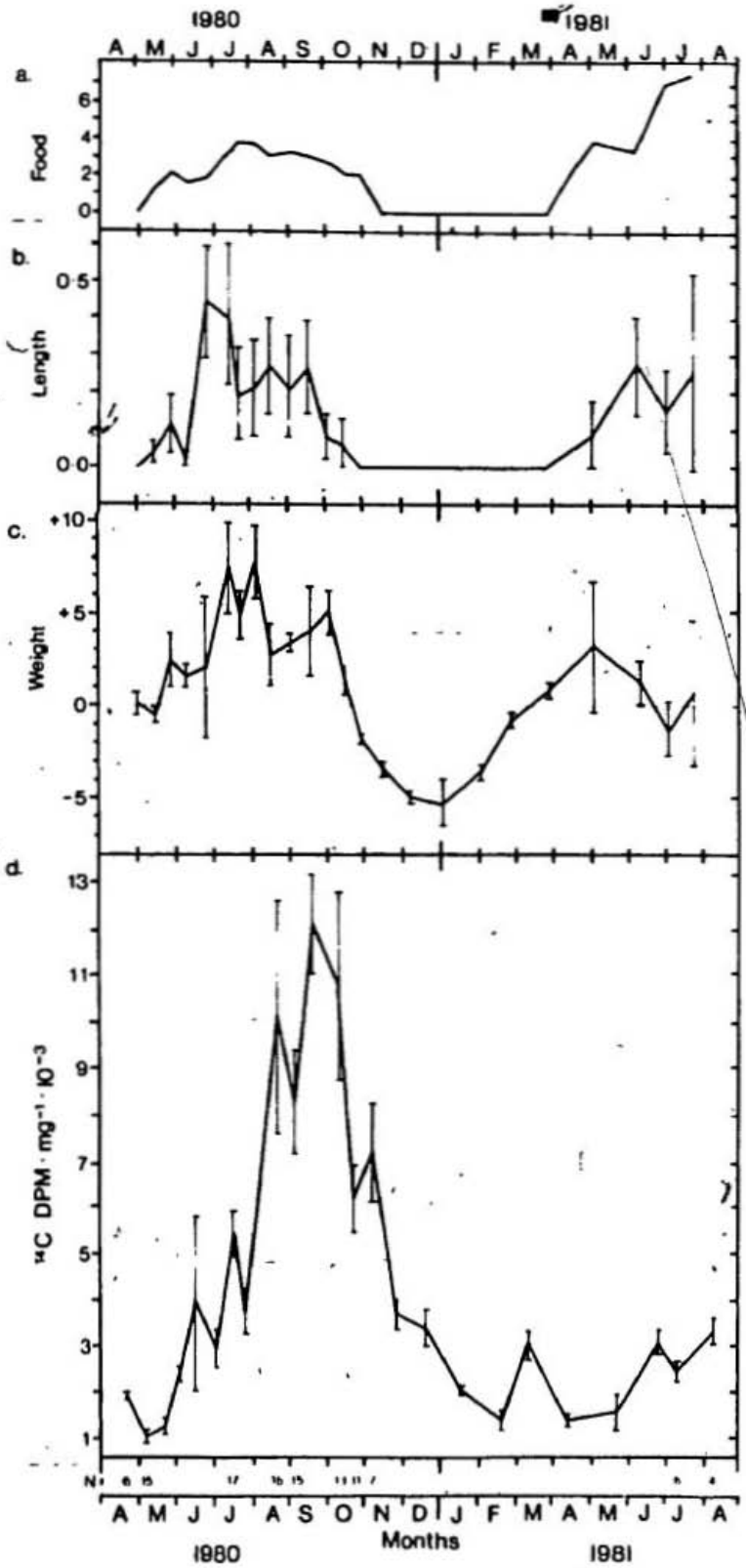
Figure 8. Fed serially sampled fish changes in length and weight and $^{14}C$-glycine incorporation in scales. Mean values $\pm$ SE. N = number of fish sampled. Where not stated N sampled is the same as for the previous date.

a. Food consumption (total food eaten as a proportion of mean fish weights for each sampling interval).

b. Mean per cent change in length for each sampling interval.

c. Mean per cent change in weight for each sampling interval.

d. $^{14}C$-glycine incorporation rate at 10°C $^{14}C$ DPM $mg^{-1}$ scale x $10^{-3}$.


1980
1981
A M J J A S O N D J F M A M J J A
a.
Food
6
4
2
0
b.
Length
0.5
0.0
c.
Weight
+10
+5
0
-5
d.
$^{14}C$ DPM · mg$^{-1}$ · $10^{-3}$
13
11
9
7
5
3
1
A M J J A S O N D J F M A M J J A
1980
Months
1981

The rates of weight change and glycine incorporation into the scales of the starved serially sampled fish are presented in Figure 9. All fish lost weight during the sampling. There were significant differences in change in weight between the fed and starved fish from April through October 1980. The rate of incorporation of $^{14}C$-glycine by the starved fish scales was significantly less than that of fed fish scales, but changes in the rate of incorporation occurred at about the same time. There were no significant differences between the incorporation rates of the two groups until mid-July ($P < 0.001$), when the fed group showed a rapid increase. Significant differences between the two groups also occurred throughout September, when fed-fish scale incorporation of glycine was at peak levels. In starved fish, the mean peak of glycine incorporation was 5 139 $\pm$ 404 DPM $mg^{-1}$ compared to fed fish mean peak levels of 8 587 $\pm$ 607 DPM $mg^{-1}$; a more than 1.5-fold difference in magnitude.

The serially sampled fed fish showed a seasonal cycle in the proportion of scales with growing edges and in the width of the growing edge (Figure 10a). Growing edges were observed from April to December 1980 and after March 1981. The peak proportion of scales with growing edges was 100% at the end of July 1980, and in June and July 1981. Maximum width of the growing edge occurred in August 1980 (averaging 4.00 $\pm$ 0.53% of total scale radius) and in July 1981 (3.20 $\pm$ 0.58%). Peak rates of $^{14}C$-glycine incorporation in August and September 1980 (Figure 8) occurred after the period of increasing width of the growing edge of the scales, and when an increasing number of scales had formed annuli (Figure 10a). Scale growth did coincide with growth in length of the serially sampled fed fish (Figure 8).

Figure 9. Starved serially sampled fish changes in weight and $^{14}C$-glycine incorporation in scales. Mean values ± SE. N = number of fish sampled. Where not stated, the number of fish sampled is the same as for the previous date.

a. Mean per cent change in weight for each sampling interval.

b. $^{14}C$-glycine incorporation rate at 10°C $^{14}C$ DPM $mg^{-1}$ scale x $10^{-3}$. The rate of $^{14}C$-glycine incorporation was measured after a 2 h incubation period.

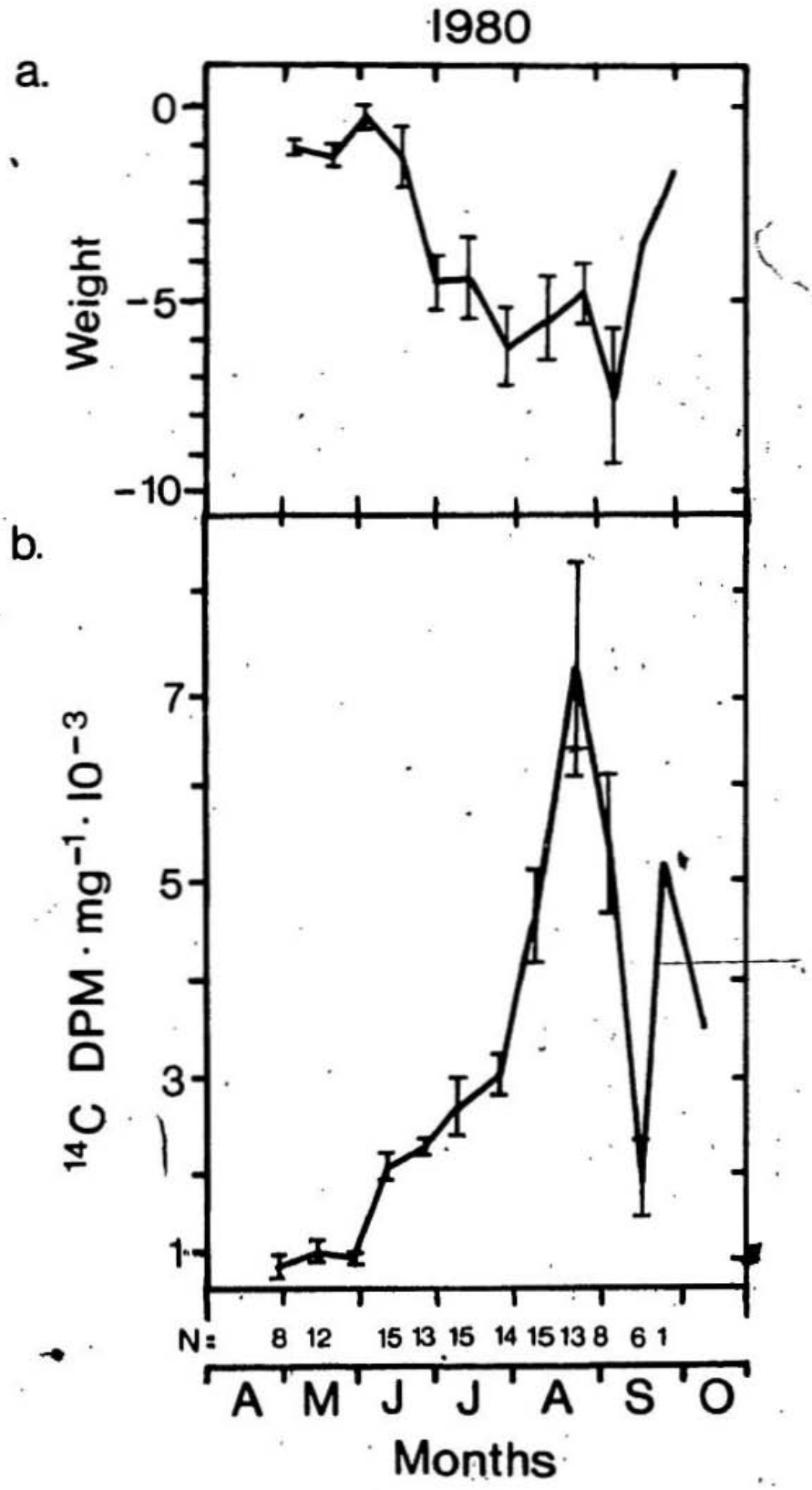
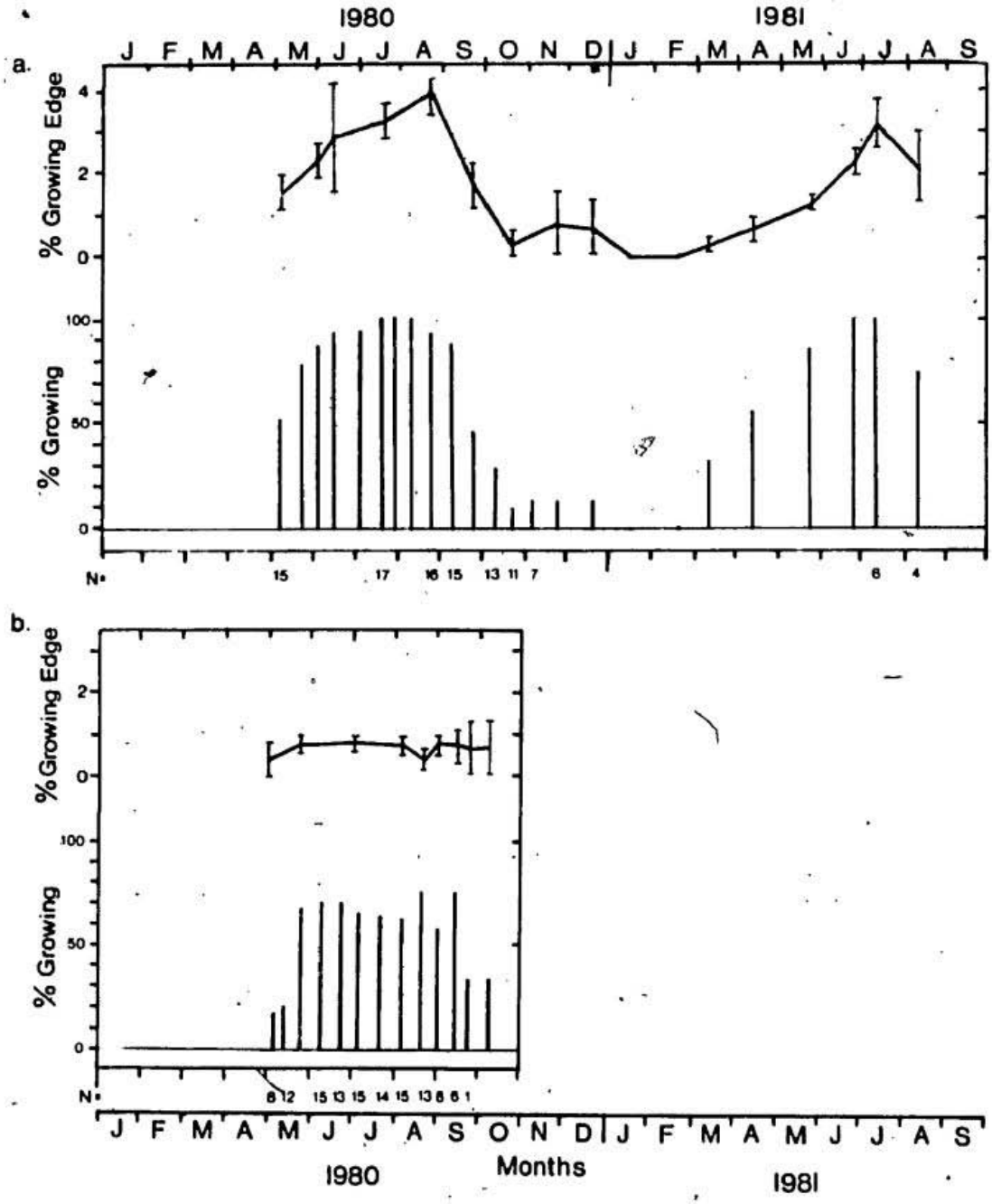

1980
a.
Weight
0
-5
-10
b.
$^{14}C$ DPM · $mg^{-1}$ · $10^{-3}$
7
5
3
1
N= 8 12 15 13 15 14 15 13 8 6 1
A M J J A S O
Months

Figure 10. Cycles of scale growth in serially-sampled fish in 1980 and 1981. Per cent growing edge is the width of the scale growing edge as a proportion of total scale radius, and is equal to 0 when an annulus is present at the edge of the scale. Mean ± SE for each sampling date. Per cent growing is the number of scales with growing edges as a proportion of all scales sampled for each sampling date. N = the number of scales sampled for each date. Where not stated, N is the same as for the previous date.

a. Fed serially-sampled fish.

b. Starved serially-sampled fish.


1980
1981
J F M A M J J A S O N D J F M A M J J A S
a.
% Growing Edge
4
2
0
100
% Growing
50
0
N•
15 17 16 15 13 11 7 6 4
b.
% Growing Edge
2
0
100
% Growing
50
0
N•
8 12 15 13 15 14 15 13 8 6 1
J F M A M J J A S O N D J F M A M J J A S
1980
Months
1981

Among the starved fish, growing edges were observed from April to September, when the last sample was collected (Figure 10b). The proportion of scales with growing edges peaked in late August through September at 75% (disregarding the final sample which consisted of only one fish), but the mean width of the growing edge did not increase significantly from May through September. Because no samples were obtained after September, the occurrence of a seasonal cycle of growth in scales of starved fish was not determined.

There was no correlation between the rate of $^{14}$C-glycine incorporation into scales of serially sampled starved fish and changes in the width of the growing edge of the scales.

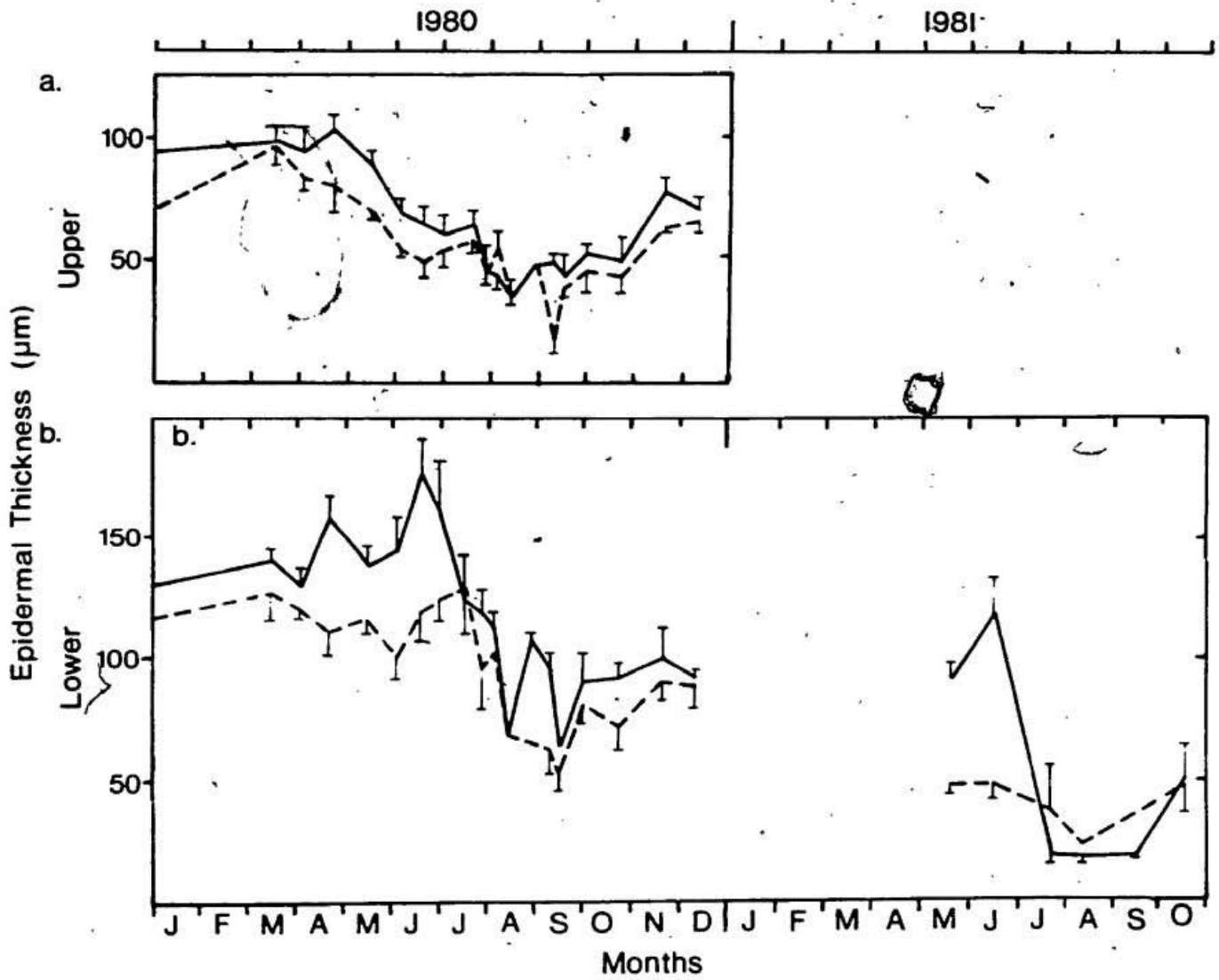
### 3.1.5 Epidermal thickness

The seasonal cycle in upper and lower epidermal thickness for 1980 (from Burton and Fletcher 1983) and lower epidermis for part of 1981 is shown in Figure 11. Although the previously frozen 1981 samples are about 25% thinner than the 1980 samples, the seasonal cycle was the same. Epidermal thickness of both sexes declined from peak thickness in June to a minimum by August and September. Burton and Fletcher (1983) report similar changes in the upper epidermis, with changes of similar magnitude occurring at the same time as the changes observed in the lower epidermis. There was a strong temporal relationship between these decreases in epidermal thickness and increases in $^{14}$C-glycine incorporation into scales.

Figure 11. Seasonal changes in thickness of the lower and upper epidermis of males (—) and females (---), 1980 and 1981. Mean values ± SE. The number of fish sampled in 1981 is noted in the text (1980 data from Burton and Fletcher 1983).

a. Upper epidermal thickness, 1980.

b. Lower epidermal thickness, 1980 and 1981.


1980
1981
a.
b.
Upper
Lower
100
50
150
100
50
Epidermal Thickness (µm)
J F M A M J J A S O N D J F M A M J J A S O
Months

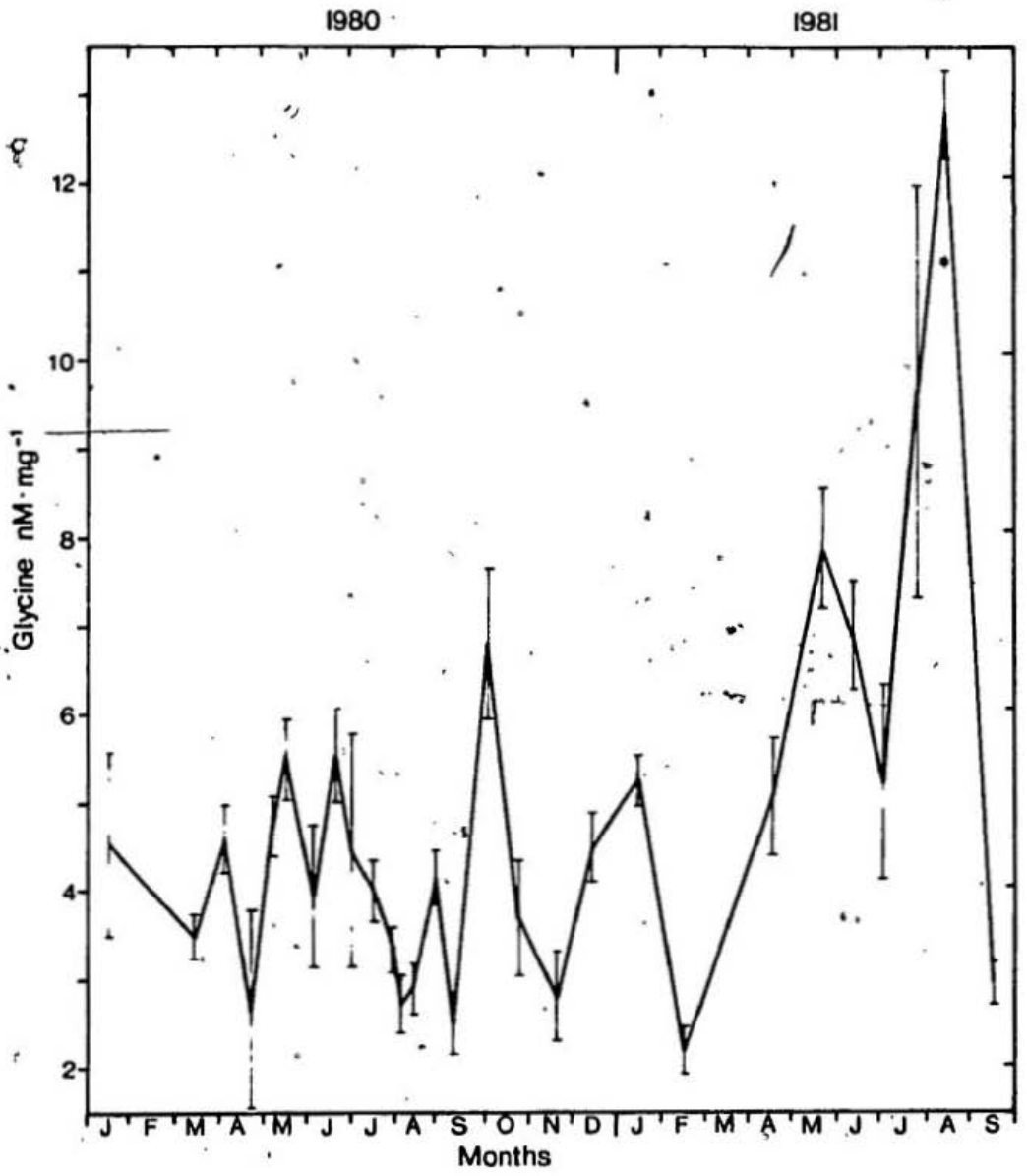
### 3.1.6 Seasonal temperature effects

The seasonal cycle of seawater temperature at Logy Bay ranged from -1.1°C minimum to 14.4°C maximum (Figure 1). Incubation of scales at 10°C throughout the year reduced extreme values of $^{14}$C-glycine incorporation observed at ambient temperatures, but did not eliminate the seasonal cycle (Figures 4 and 5). However, temperature did not have a consistent effect on the incorporation rate thrbughout the year. For each date that incorporation-at-temperature was examined, good linear regressions were obtained (Table 2), but there was significant variation between dates in the mean incorporation rate at any given temperature (ANOVA $P < 0.01$), resulting in significant variations between slopes and intercepts of the regression curves ($P < 0.005$) (Table 2). The source of this variation did not appear to be attributable to season or growth. The ambient water temperature did not significantly influence the rate of glycine incorporation at temperatures of 0 to 15°C. Differences between individuals were the most likely source of variation. The $Q_{10}$'s calculated from incubations at 0 and 10°C, ranged from 1.52 to 2.56 (Table 2).

### 3.1.7 Scale glycine pool

The amount of free glycine in scale extracts from field fish was highly variable, and differed between the two years (Figure 12). The differences in peak glycine pool levels between the two years could not be attributed to differences in the rate of feeding or seawater temperatures (Figure 1). The seasonal cycle of glycine incorporation,

Figure 12. Seasonal variations in scale free glycine pools in 1980 and 1981, nM glycine per mg of scale. Mean values ± SE. N, number of fish sampled = 4, except on 9 dates noted in the text.


1980
1981
Glycine nM·mg⁻¹
12
10
8
6
4
2
J F M A M J J A S O N D J F M A M J J A S
Months

calculated by adjusting $^{14}$C-glycine incorporation to the specific activity of the scale glycine pool, was similar to the $^{14}$C-glycine incorporation cycle (Figure 13), although there was a greater magnitude of change between summer and winter values (11 times compared to about 4 times).

## 3.2 EFFECTS OF INHIBITORS OF PROTEIN BIOSYNTHESIS

The three inhibitors of protein biosynthesis employed in this study all reduced the rate of $^{14}$C-glycine incorporation into scales. Cycloheximide produced significantly decreased rates of incorporation on 3 of the 4 dates it was used. However, there appeared to be a seasonal factor in its effect, as the degree of inhibition produced was significantly different between dates, with the highest degree of inhibition of incorporation rates occurring in July and an insignificant inhibition of incorporation in October (Figure 14a).

When the period of incubation was varied, the rate of $^{14}$C-glycine incorporation by scales treated with cycloheximide was at or above control rates for the first 1.5 h of incubation, but there was significant inhibition of incorporation after 2 h of incubation with the inhibitor (Figure 14b). The incorporation rate of control scale samples increased linearly, nearly doubling each hour.

Of the inhibitors of prokaryote protein biosynthesis, puromycin, used in July, produced the most inhibition of incorporation, while chloramphenicol, used in October, produced only slight inhibition (Table 3). The degree of inhibition of incorporation produced by puromycin was the same as that produced by cycloheximide on the same

Figure 13. Seasonal glycine incorporation (pM glycine $mg^{-1}$ scale) (——) and $^{14}C$-glycine incorporation ($^{14}C$ DPM $mg^{-1}$ scale x $10^{-3}$) (---) in field-sampled wild fish scales incubated 2 h at 10°C, 1980 and 1981. Mean values ± SE. N, number of fish sampled = 10, except on 5 dates noted in the text.

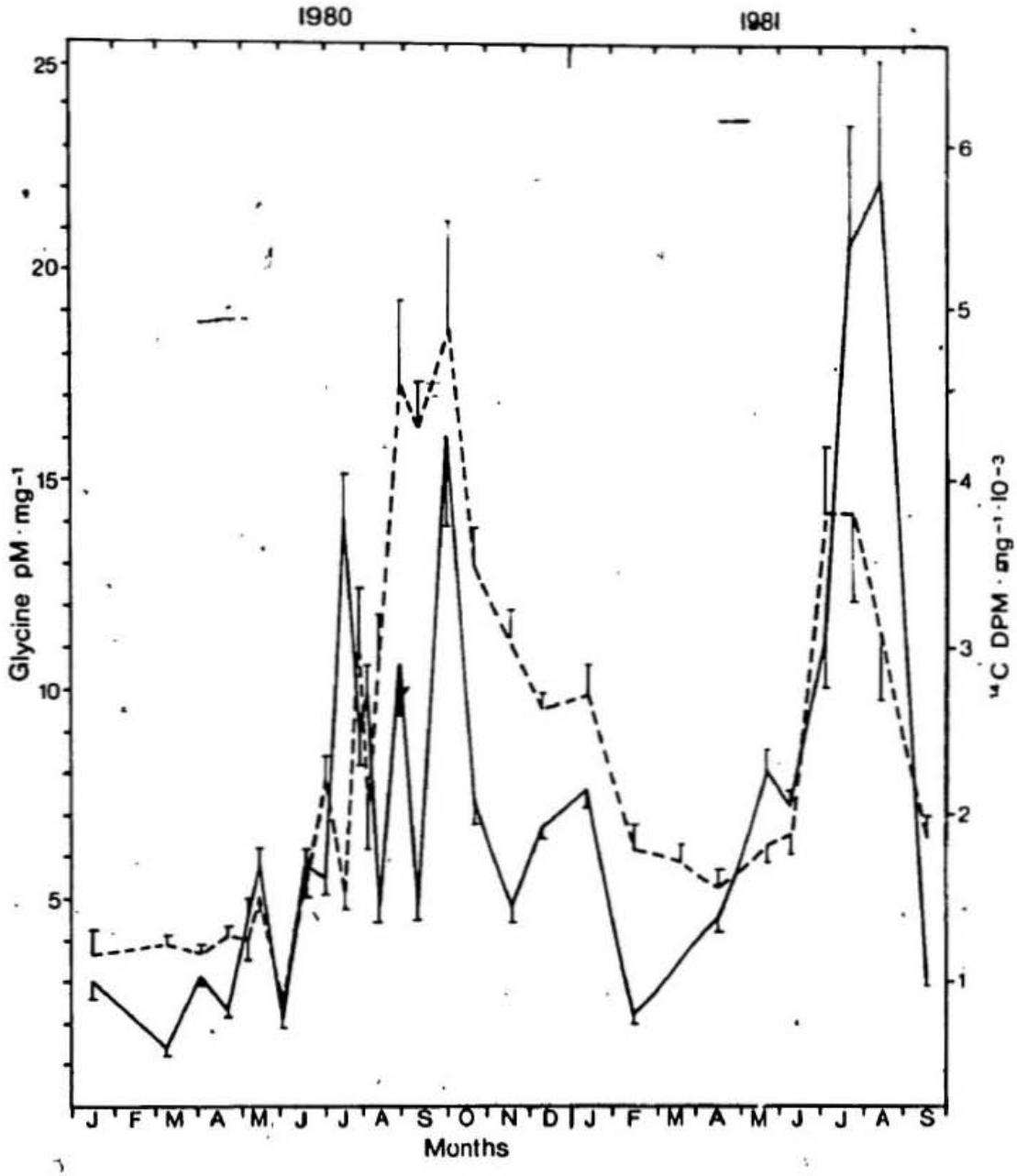
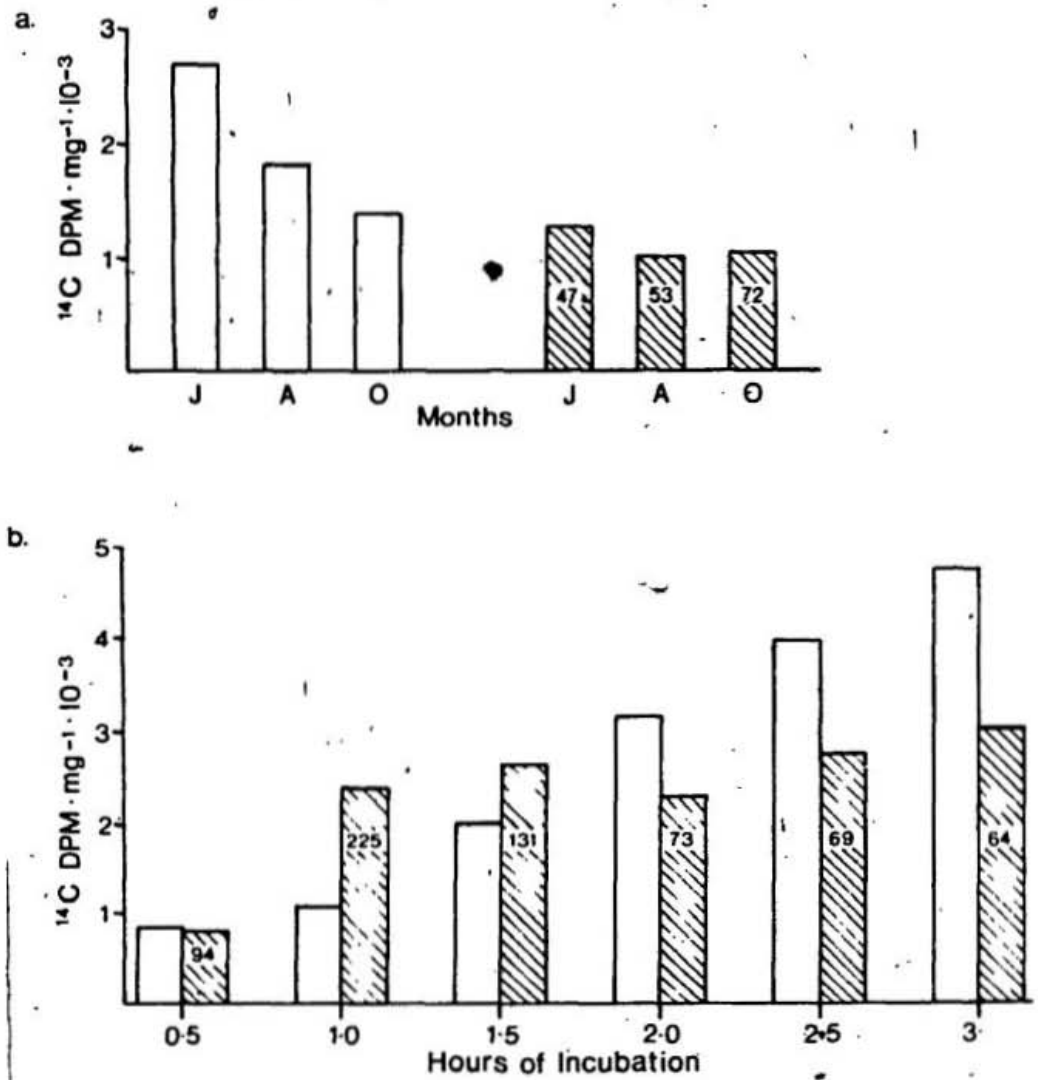

1980
1981
Glycine pM · mg⁻¹
¹⁴C DPM · mg⁻¹ · 10⁻³
Months
J F M A M J J A S O N D J F M A M J J A S

Figure 14. Effects of a protein synthesis inhibitor on $^{14}C$-glycine incorporation rate after a 2 h incubation period.

a. Seasonal variation in cycloheximide inhibition of $^{14}C$-glycine incorporation rate in July, August and October 1980 samples.

b. Changes in incorporation rate with cycloheximide over time.

Control □ ; and cycloheximide treated ▨ , with per cent of control incorporation shown inside the bar.


a.
$^{14}C$ DPM · mg$^{-1}$ · 10$^{-3}$
3
2
1
47
53
72
J
A
O
Months
J
A
O
b.
$^{14}C$ DPM · mg$^{-1}$ · 10$^{-3}$
5
4
3
2
1
94
225
131
73
69
64
0·5
1·0
1·5
2·0
2·5
3·
Hours of Incubation

date. When the two inhibitors were combined, incorporation was not inhibited more than when they were used alone. Chloramphenicol combined with cycloheximide also resulted in no increase in inhibition, compared to cycloheximide used alone.

Inhibition of incorporation occurred primarily in the posterior scale portion (Table 3), while anterior scale incorporation appears in most cases to have been enhanced by inclusion of the inhibitors, compared to controls. This result suggests that there may be technical problems when inhibitor molecules, designed for cell-free systems, are employed in in vitro systems. Decreases in whole scale incorporation rates in the presence of inhibitors were, in almost every case, due to reduced posterior scale incorporation.

## 3.3 MOLECULAR INCORPORATION OF $^{14}$C-GLYCINE

The distribution of $^{14}$C-glycine within scale components was studied on three dates. The scale fraction numbers refer to numbers used in Table 4.

### 3.3.1 Percent of radioactivity within scale fractions

The DPM recovered in each of the fractions can be proportioned into "collagen" or "non-collagen" molecules. In the scale pellets (scale fraction 1), a mean of 31.1 $\pm$ 5.7% of recovered radioactivity was associated with "collagen" molecules. The January anterior scale sample had more "collagen", 55.6%, than the other samples. After gel filtration chromatography, an average 55.9 $\pm$ 2.5% of the recovered

counts were in the glycine fraction and 3.6 ± 1.1% was associated with small molecule fractions (scale fraction 2.2).

The large molecule fractions (scale fraction 2.1) averaged 43.3 ± 3.5% of recovered counts, most of which (84.6 ± 1.2%), were soluble in 15% TCA (scale fraction 2.1.2). Within the TCA soluble fraction, an average 5.6 ± 2.0% of the counts were associated with "collagen" molecules (scale fraction 2.1.2.2.1). Posterior scales incubated in January contained more collagen soluble in TCA (15.2%) than other scale samples. In the TCA insoluble fraction (scale fraction 2.1.1), 7.25 ± 1.41% of the recovered radioactivity was associated with collagen molecules (scale fraction 2.1.1.1). The January anterior scale sample had less $^{14}C$ associated with "collagen" in the TCA insoluble fraction (1.4%) than other samples.

### 3.3.2 Incorporation into collagen

The mean proportion of total $^{14}C$-glycine that was incorporated by "collagen" molecules was 12.5 ± 2.7%. This value should be considered an estimate because small peptides < 3 500 MW, which may include collagen precursor molecules, were not tested for collagenase sensitivity. The distribution of "collagen" measured in these experiments appears to vary seasonally, with more $^{14}C$ associated with "collagen" molecules in the September posterior scale sample than at other dates.

# 4. DISCUSSION

The seasonal cycle of $^{14}$C-glycine incorporation by scales of winter flounder reflected the seasonal biology of this fish. Investigations of the nature of the cycle of incorporation leads to five general conclusions:

1. The seasonal cycle of $^{14}$C-glycine incorporation did not coincide with the cycle of scale growth; nor did the size of the incubated scale influence the rate of incorporation.
2. The seasonal cycles of $^{14}$C-glycine incorporation and scale growth were the same for fed, laboratory maintained fish as they were for wild fish. The cycle of $^{14}$C-glycine incorporation of the fed fish did not coincide with the cycles of growth in length and weight.
3. Anterior scales, which contain the scale-forming cells, had a pattern of $^{14}$C incorporation which paralleled $^{14}$C incorporation into posterior scales. This corroborated the observation that the cycle of $^{14}$C-glycine incorporation was not related to the cycle of scale growth.
4. There was a relationship between peak rates of $^{14}$C-glycine incorporation and seasonal degeneration of the epidermis. The increase in the rate of $^{14}$C incorporation, which occurred when the epidermis was thinnest, may reflect a metabolic response to the physiological stress of epidermal degeneration. The increased rate of $^{14}$C incorporation may also be due to the presence of microorganisms in the degenerating epidermis. This was suggested by the effects of protein synthesis inhibitors.

5. Most "incorporated" $^{14}$C-glycine was not associated with any molecule within the scale; and of the actually incorporated $^{14}$C, only a small amount was associated with collagen molecules.

There was a pronounced seasonal cycle in $^{14}$C-glycine incorporation by winter flounder scales, but it was not coincident with the seasonal cycles in condition or scale growth. Increases in condition factor, following spawning in May (Figure 1), indicated that fish were growing in weight at a faster rate than increases in length. A peak in the number of fish with growing scales was observed in May of both years, and the peak rate of scale growth (based on the change in width of the scale growing edge), occurred between March and May (Figure 7). Incorporation of $^{14}$C-glycine by scales, however, remained at winter levels between March and May. Peak rates of incorporation in July through September 1980 occurred after more than 50% of scales had stopped growing. In 1981, peak $^{14}$C-glycine incorporation coincided with the end of the period of scale growth, when the number of scales with growing edges was declining.

The size of the incubated scale which is related to fish length (Tesch 1970) did not influence the rate of incorporation. This indicated that there was no relationship between $^{14}$C-glycine incorporation and length of fish over the size range sampled (25-40 cm). Such a relationship may have occurred, even though $^{14}$C incorporation rates did not correlate with seasonal scale growth, if smaller fish had an overall faster rate of growth than larger fish. Ottaway and Simkiss (1979) reported an inverse relationship between scale radius and incorporation rate which varied seasonally, and suggest that this was due to

the faster rate of growth of younger fish, which have smaller scales. Smagula and Adelman (1982) note that the growing edge is a greater proportion of small scales, which would increase scale-size errors due to relative growth rates, and recommend that incorporation be expressed in terms of scale area instead of weight. The relatively small scales and slow growth rate of winter flounder may cause scale-size errors of this type to be minimal. The seasonal patterns of scale growth and incorporation of $^{14}$C-glycine were the same both for wild fish in the seasonal study, and in the serially sampled fed fish (Figures 7, 8 and 10a). Some studies have found that repeated sampling of laboratoryheld fish creates stress effects, such as loss of weight (Bilton 1974), but in this study, the general patterns of growth and incorporation in the fed fish group were not different from wild fish. The fed fish showed peak rates of growth in length between mid-June to mid-July, and greatest changes in weight during July (Figure 8). Scale growth was maximum between May and August (Figure 10a) at the same time that growth in length occurred. Thus, the laboratory fish were growing during the same period as wild fish. And, as in the wild fish, peak rates of $^{14}$C-glycine incorporation occurred after most growth in length, weight and in scales was completed.

There was also a seasonal cycle of $^{14}$C-glycine incorporation into the scales of starved fish (Figure 9). Although the starved fish showed no growth in length or weight, or any scale growth (Figure 10b), the seasonal cycle in $^{14}$C-glycine incorporation was similar to that observed in the serially sampled fed fish (Figure 8) and in wild fish (Figures 4 and 5). These observations suggest that $^{14}$C-glycine uptake may indicate metabolic rhythms other than body growth. Ottaway

and Simkiss (1979) suggests that glycine incorporation may reflect the general protein metabolism of the fish, because stresses such as starvation influence the rate of $^{14}C$ incorporation without producing changes in the rate of scale growth, such as formation of "checks". The seasonal increase in incorporation rates observed in scales of starved fish in this study supports this view, and may be reflecting the effects of seasonal temperatures on protein turnover rates.

If whole scale incorporation does not reflect growth, does the rate of glycine incorporation into the anterior scale, which contains most of the scale-forming cells provide a closer correlation? The results of this study suggest it does not. Anterior and posterior portions of incubated scales showed the same seasonal pattern of $^{14}C$-glycine incorporation as did whole scales (Figure 6). A seasonal peak in anterior scale incorporation occurred in early August 1980 and late July 1981. This observation does not support the assumption of Ottaway and Simkiss (1977a) that the proportion of incorporation in each scale portion is constant. In general, however, the seasonal cycle of glycine incorporation and the rate of change in incorporation of the anterior scale paralleled that of the posterior scale. As such, the anterior scale metabolism measured by $^{14}C$-glycine incorporation appeared to reflect the metabolism of the scale as a whole.

Because the scale is a structure of the skin, patterns of $^{14}C$-glycine incorporation into scales were compared to changes in the epidermis. The period of minimum epidermal thickness corresponded to the period of peak scale $^{14}C$-glycine incorporation rates. Decreases in epidermal thickness occurred after spawning and the upper

epidermis of winter flounder had only 1-4 cell layers at minimum thickness during the summer, compared to 8-9 in the winter (Burton and Fletcher 1983). Thinning of the epidermis was apparent in early May, just before the rate of glycine incorporation began to increase from winter levels (Figure 11).

The dramatic seasonal changes in epidermal thickness are the result of post-spawning degeneration and erosion, followed by regeneration in October to December, and are correlated with changes in gonad condition (Burton and Fletcher 1983). These authors discussed the influences of testosterone and thyroid hormones on epidermal thickness reported by other researchers, and noted that seasonal variations of both types of hormones in the plasma of winter flounder (Campbell et al. 1976; Eales and Fletcher 1982), corresponded to changes in the epidermis. Seasonal behaviour of the fish may also influence epidermal condition since minimum thickness is observed during summer months, when Newfoundland winter flounder are in warm inshore shallow water (van Guelpen and Davis 1976). These fish typically bury themselves in the substrate when disturbed or resting, and their high activity levels may result in increased dermal abrasion, particularly if a hormone effect that reduced epidermal thickness was also involved.

It is beyond the scope of this study to determine the reason for the inverse relationship between incorporation of $^{14}$C-glycine by scales and epidermal thickness. However, elements of this study touch upon two possibilities: First, the seasonal stress of epidermal degeneration may have stimulated the overall metabolic rate of the skin, in particular the processes of skin regeneration and growth. $^{14}$C-glycine incorporation into scales of the serially sampled fed

fish was significantly higher than that of field fish (Figures 4 and 8). The repeated sampling of scales within a relatively small area may have stimulated the metabolism of the skin in the area, as lost scales were regenerated. Fouda and Miller (1979) report increased activity of the enzyme alkaline phosphatase, which is involved in calcification of hard tissues, in regenerating scales of the common goby (Pomatoschistus microps); and in the skin during the spawning season (when about 60% of the fishes' scales are lost due to behavioral activities). Thus a seasonal stress to the skin was accompanied by a seasonal increase in metabolism involving at least one enzyme. It would be interesting to examine the physiological response of the skin of winter flounder to the seasonal loss of so much epidermal tissue, and to determine if enzymes or hormones are influencing the rate of $^{14}$C-glycine incorporation by scales.

A second hypothesis to explain the inverse correlation of peak $^{14}$C-glycine incorporation with epidermal thickness is that there may be a population of micro-organisms in the degenerating epidermis, which metabolised the $^{14}$C-glycine during the incubation period (Cone and Wiles 1984). There was a substantial reduction in the rate of incorporation of $^{14}$C-glycine in scales incubated with puromycin at the end of July (Table 3), suggesting that there was a high level of protein synthesis in non-scale tissue while the epidermis was in poor condition. By mid October another inhibitor of prokaryote protein synthesis, chloramphenicol, produced no significant reduction in incorporation rate, which suggested that the level of prokaryote $^{14}$C-glycine incorporation was reduced when the epidermis regenerated. Similarly, cycloheximide produced a high level of inhibition in late

July, but had no significant effect in mid-October (Figure 14a), when the epidermis had regenerated.

The observation that all inhibition of protein synthesis occurred only in the posterior scale portion (Table 3) may support the hypothesis that some of the seasonal $^{14}$C-glycine incorporation can be attributed to non-scale tissue incorporation. However, a higher concentration of inhibitor may be required to influence anterior scale incorporation than is required to influence the posterior scale incorporation. Goolish and Adelman (1983) achieved a much higher degree of inhibition of whole scale incorporation when cycloheximide was employed at 5-100 times the concentration used in this study.

The maximum inhibition of $^{14}$C incorporation produced by both types of protein synthesis inhibitor, in July, was less than 50% and a combination of inhibitors produced no increase in the level of inhibition (Table 3). The molecular incorporation results indicate that almost 40% of "incorporated" $^{14}$C-glycine remained in the "free glycine" fraction (Table 4), and therefore this proportion could not be affected by either the prokaryote or eukaryote inhibitors of protein biosynthesis.

The degeneration of winter flounder epidermis in late summer appeared to be responsible for the peak rates of $^{14}$C-glycine incorporation that occurred at the same time. Such a dramatic event may overshadow a relationship between growth and incorporation in the scale. The results of the molecular uptake experiments showed that the $^{14}$C-glycine was absorbed by the scale, and some was incorporated into collagen molecules (Table 4). Anterior scale portions averaged 11% of total $^{14}$C incorporated into "collagen" molecules. This

value may represent collagen synthesized at the growing edge of the scale, but probably includes synthesis of collagen fibres in the underlying fibrillary plate, which increases in thickness as the scale grows (Fouda 1979). The highest proportion of $^{14}C$ associated with collagen molecules, in the September posterior scale samples, coincided with regeneration of the epidermis. Thus, even $^{14}C$ incorporation into scale collagen molecules in whole scales is not necessarily a reflection of the instantaneous growth rate of the scale.

Most incorporated $^{14}C$ was not associated with any molecules within the scale: an average 36% was in the "free glycine" fractions, and 2% in molecules of less than 2 500 MW. There was evidence of seasonal variation in the distribution of $^{14}C$-glycine in the scales. Scales sampled in January had comparatively more incorporation as "free glycine and small molecules", while in July and September there was more incorporation into larger molecular weight molecules (Table 4). This suggests a more active protein synthesis occurs in both anterior and posterior scales during summer months, which may reflect growth or temperature effects. More sampling, and identification of collagen precursor molecules, may clarify the relationship between scale growth and $^{14}C$-glycine incorporation.

The metabolic processes of incorporation and protein synthesis were influenced by temperature, but this influence was not consistent throughout the year. Adelman (1980) and Smagula and Adelman (1982) reported temperature effects which were correlated to acclimation temperature of the fish, and suggested that the metabolic processes involved may be temperature-compensating: warm-acclimated fish scales incubated at low temperatures showed a large decrease in incorporation;

while cold-acclimated scales showed less change. Kent and Prosser (1980) found temperature compensation in protein synthesis of liver cells from green sunfish (Lepomis cyanellus). In this study, $^{14}C$ incorporation at a fixed temperature did not covary with ambient temperature. An examination of the $Q_{10}$ results, however, suggests that higher $Q_{10}$ values were obtained for warm-acclimated fish, and lower values for cold-acclimated fish (Table 2). This would be consistent with the results of Adelman (1980) and Smagula and Adelman (1982). Temperature compensation in scale protein synthesis could mean that the incubations at 10°C in this study were not comparable throughout the year, as summer $^{14}C$ incorporation values would be suppressed and winter values elevated. Further, the standard incubation temperature of 10°C was probably below the temperature required for optimal incorporation rates, as incorporation was consistently greater at 15°C, the highest temperature tested in the temperature-incorporation experiments. Whether incorporation at ambient temperature or at optimal temperature provides the best indicator of scale metabolism, and possibly growth, will require further investigation. However, it is apparent that the temperature adjustment based on a single $Q_{10}$ employed by Ottaway and Simkiss (1977a; 1979) is inappropriate to standardize results.

Finally, variations in the scale glycine pool also influenced the rate of incorporation of $^{14}C$-glycine, by changing the effective concentration of $^{14}C$-glycine within the scale. The seasonal cycle in glycine incorporation rates had an overall greater magnitude of change than the cycle of $^{14}C$-glycine incorporation (Figure 13). The general pattern of lower winter scale glycine pools and higher

summer pools (Figure 12) is similar to the cycle of glycine in winter flounder plasma (Squires et al. 1979) although the scale pool values are lower and more variable. Because changes in the scale glycine pool affect the apparent rate of $^{14}$C-glycine incorporation, an investigation of factors which influence changes in the scale pool, such as hormones which mediate glycine transport and use in the cell, may be valuable in understanding how $^{14}$C-glycine incorporation is related to scale metabolism and the metabolic status of the fish.

The rate of $^{14}$C-glycine incorporation into scales of winter flounder did not reflect the rate of growth. There was a clear seasonal cycle of $^{14}$C-glycine incorporation into scales, but late summer peak incorporation occurred several months after the period of rapid scale growth in the spring and increases in body length in early summer. Instead of reflecting scale growth, the cycle of $^{14}$C-glycine incorporation was coincident with a seasonal cycle of epidermal degeneration and regeneration. The peaks in $^{14}$C incorporation when the epidermis was thinnest appeared to reflect increased metabolic rates in the epidermis in general, and perhaps the metabolism of $^{14}$C-glycine by a population of microorganisms colonizing the degenerating epithelium.

Although $^{14}$C-glycine was incorporated by scales, very little was incorporated into collagen molecules. In both anterior and posterior scale portions, an average 38% of incorporated $^{14}$C remained in the form of "free glycine", or small molecules and incorporation into "collagen" molecules averaged only 13%. The highest levels of $^{14}$C incorporation into "collagen" were coincident with the period of rapid regeneration in the epidermis in the autumn, rather

than the period of rapid scale growth in the spring.

Incorporation of $^{14}C$-glycine into scales appears to be sensitive to more generalized influences than the specific processes of scale collagen synthesis involved in scale growth. In the case of winter flounder, factors influencing overall epidermal metabolism appeared to control the seasonal cycle in $^{14}C$ incorporation by scales. In other species, factors influencing epidermal metabolism, and also general protein metabolism of the fish, may affect the rate of $^{14}C$-glycine incorporation into scales. Because this method is sensitive to metabolic processes other than scale collagen synthesis, $^{14}C$-glycine incorporation into scales should not be considered an index of instantaneous growth rate unless the influence of other anabolic systems can be determined.

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