Description of the starving condition in summer flounder, *Paralichthys dentatus*, early life history stages

Gustavo A. Bisbal
Graduate School of Oceanography, University of Rhode Island
Narragansett, RI 02882
Present address: Northwest Power Planning Council
851 S.W. Sixth Avenue, Suite 1100, Portland, OR 97204-1348

David A. Bengtson
Department of Zoology, University of Rhode Island
Kingston, RI 02881

Abstract.—The nutritional status of laboratory-reared summer flounder, *Paralichthys dentatus*, larvae and early juveniles was assessed by morphometric, biochemical, and histological criteria. Conditions of food deprivation were imposed on 6-, 16-, and 33-day-old larvae as well as on 60-day-old juveniles. Samples of ad-libitum-fed or starved individuals were analyzed with regard to standard length, dry weight, eye diameter to head height ratio, pectoral angle, RNA:DNA ratio, total protein content, histological appearance of selected organs, and cell height of the anterior and posterior intestinal mucosae. In general, tolerance to starvation increased with age: 60 h in 6-day-old-larvae, 72 h in 16-day-old larvae, 8 d in 33-day-old-larvae, and 10 d in 60-day-old-juveniles. The results of this study demonstrate that morphological criteria are either not good indicators of nutritional status (eye:head ratio), good only for larvae (pectoral angle), or require extensive calibration (standard length and dry weight). They also show that biochemical criteria are either not good indicators (protein content) or are sensitive to starvation only in juveniles (RNA:DNA ratio). Among the histological criteria, thickness of the posterior intestinal mucosa was the most sensitive and consistent indicator of starvation in summer flounder larvae and early juveniles. The most salient attributes of this histological analysis were sensitivity, objectivity, ease of interpretation, and exemption from shrinkage calibration. These results suggest the use of the histological approach in the face of uncertainties associated with the other methods examined. On the other hand, application of either morphological or histological criteria is appropriate for an aquaculture setting in which age of larvae is known.

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It is currently accepted that starvation and predation are the main agents of marine fish larval mortality (Hunter, 1976; Bailey and Houde, 1989). However, the relative magnitudes of the processes controlling prerecruit mortality are, for the most part, either unknown or controversial (Pepin, 1988/1989; Miller et al., 1991). Furthermore, these forces may at times operate concurrently, adding an additional level of complexity. For instance, although intense food limitation of fish larvae can be lethal per se, it could also be regarded as a sublethal agent that exposes weakened individuals to selective predation by reducing their growth rates (Laurence, 1985; Houde, 1987; Fogarty et al., 1991), reaction capabilities (Hunter, 1972, 1981), or ability to maintain a preferred depth in the water column (Blaxter and Ehrlich, 1974).

Nutritional condition of teleost larvae has been measured and described in a number of ways. The physical deterioration of larvae resulting from experimental conditions of food deprivation has been interpreted by means of morphometric and gravimetric (e.g. Hempel and Blaxter, 1963; Ishibashi, 1974; Ehrlich et al., 1976), biochemical (e.g. Ehrlich, 1974, a and b; Buckley, 1980, 1982, 1984; Fraser et al., 1987; Clemmesen, 1987; Richard et al., 1991), and histological (e.g. Ehrlich et al., 1976; O'Connell, 1976, 1980; Theilacker, 1978; Martin and Malloy, 1980; Watanabe, 1985; Theilacker and Watanabe, 1989) criteria. In some cases, several of these techniques were tested concurrently to determine their relative utility as indicators of starvation (Martin and Wright, 1987; Setzler-Hamilton et al., 1987). Martin and Wright (1987) proposed the combined application of two or three techniques to any given study because of differences in response time of the measure to actual nutritional status.

The summer flounder, *Paralichthys dentatus*, is a temperate paralichthyid flatfish occurring in Atlantic estuaries and continental shelf waters from Nova Scotia to Florida (Rogers and Van Den Avyle, 1983; Able et al., 1990). During 1983–91, the average landings from the commercial and recreational fishery were 11,400 metric tons. Recent surveys revealed that the stock biomass is currently at the lowest average level since the early 1970's which, combined with calcu-

lated present fishing mortality rates, indicates that summer flounder stocks are overexploited (NMFS, 1993). The decline in the natural fishery, together with recent success in culturing other flatfish species, such as the Japanese flounder, *Paralichthys olivaceus* (Sproul and Tominaga, 1992), and the European turbot, *Scophthalmus maximus* (Person-Le Ruyet et al., 1991), stimulated interest in the development of technology for the culture of summer flounder.

Basic information on the ability to distinguish starving from feeding *P. dentatus* larvae and juveniles will be useful for studies of both natural and cultured populations. Studies on the occurrence or frequency of starvation in either field populations or aquaculture operations must be preceded by an experimental study in which specific starvation indicators are validated for fish of known nutritional history. Therefore, the aim of our research was to evaluate and compare alternative criteria for assessing starvation effects at several stages during the early life history of *P. dentatus*. We characterize *P. dentatus* larvae and recently metamorphosed juveniles subjected to conditions of starvation or ad libitum feeding, using biochemical, morphometric, and histological criteria.

Materials and methods

Adult broodstock *P. dentatus* were collected from Narragansett Bay, Rhode Island, and Long Island Sound, Connecticut, and were held in laboratory facilities. They were spawned after artificial induction with repeated carp pituitary injections (2.5 mg/kg) during 8 to 12 consecutive days (Smigielski, 1975). The fertilized eggs were distributed in 38-L glass aquaria covered with opaque black plastic. Each tank was filled with UV-treated filtered (1 μm) Narragansett Bay seawater (adjusted to 34 ±1‰ salinity by brine addition). Antibiotic (200 mg erythromycin activity dissolved in 23 liters of water) was added at one time in each tank, and water changes were performed every 2–3 days to maintain water quality. During the first week, the alga, *Tetraselmis suecica,* was added to the water. No artificial substrate was added to the aquaria. Water temperature was maintained at 19 ±1°C throughout the experiment. Overhead illumination adjusted to a natural photoperiod and aeration were provided.

Hatching began 55 hours after fertilization. During the next 4 days the larval digestive system became morphologically ready to process external food at the time of mouth opening (Bisbal and Bengtson, in press). Since yolk resorption and mouth opening are almost simultaneous, flounder larvae were fed daily on rotifers, *Brachionus plicatilis,* cultured on *T. suecica* (Lubzens, 1987) after day 4. Newly hatched Reference *Artemia* III nauplii (Collins et al., 1991) were offered for the first time 18 days later, and the rotifer supply was progressively reduced. Settlement to the bottom began on day 45 after hatching.

Available literature on the early life stages of fish and previous direct observations on flounder cultures directed our interest toward four developmental stages (Al-Maghazachi and Gibson, 1984; Blaxter, 1988; Youson, 1988). The effects of starvation were evaluated at day 6 (early food ingestion, yolk completely resorbed), day 16 (these larvae have positively ingested and processed food at least once or else they would have died within 10 days after hatching), day 33 (at the beginning of metamorphic eye migration), and day 60 (bottom-dwelling juveniles have metamorphosed) after hatching. At these times, subsamples of the larvae pool were randomly placed in one of two 5-L tanks (25 larvae/L): one (control group) receiving food ad libitum (i.e. *Brachionus* or *Artemia*); the other (starved group) devoid of food. Although the presence of food in the gut was not systematically recorded, the performance of feeding motions and active swimming were visually confirmed on an individual basis. An extra subsample was processed as described below in order to establish the basal levels of the several parameters measured prior to the initiation of the imposed starvation (time 0). Based on previous observations on the progression of starvation at different age intervals and constant visual monitoring of behavioral changes, each group (control and starved) was sampled at least three times, from the beginning of the experimental exposure until the onset of mortality. A larva was considered dead if it did not respond to gentle probing with a glass rod. If that was the case, the larva was captured and placed under the dissecting microscope for a confirmation of its status. At each sampling time the same protocol was followed: 6 to 10 individuals from each group were sampled for histological analysis, 10 for morphometric and dry weight measurements, and 10 for biochemical analyses.

Morphometric measurements consisted of standard length, eye diameter, head height, and the pectoral girdle angle as defined by Ehrlich et al. (1976). Measurements of live larvae were taken under a dissecting microscope equipped with an ocular micrometer accurate to 0.7 μm. Pectoral angles were traced under a camera lucida and measured on a digitizing pad. Each fish was then rinsed in deionized water and placed in a 60°C oven until a constant dry weight was obtained. Weight was measured, on either a Mettler AE 240 balance or a Cahn C–31 electrobalance.

Samples for biochemical analysis were rinsed in deionized water and individually preserved in Eppen-

dorf vials in a –80°C freezer for no more than 45 days until RNA, DNA, and protein determinations were performed. Owing to the extremely small size of the 6-day-old larvae, each determination was performed on samples consisting of two larvae pooled in the same vial. This was the only case where pooling was necessary.

Determinations of RNA and DNA were performed according to the methodology described by Bentle et al. (1981) as modified for individual larvae of small size (Nacci et al., 1992). Total protein determination was assessed by a dye-binding assay (Bradford, 1976) in which bovine serum albumin was the reference standard. Volumes were adjusted to 96-well microtitration plates and, after completion of the colored reaction, absorbances were read at 600 nm in an EL 312 Bio-Tek automated microplate reader.

The fraction of the sample destined for histological examination consisted of 6 to 10 specimens preserved in Dietrich's fixative, embedded in paraffin blocks, and completely sectioned every 4–5 μm on a rotary microtome. Light microscopy analysis was performed after staining with Cason's trichromic (Cason, 1950).

The qualitative histological examination concentrated on the liver, pancreas, musculature, and intestinal mucosae. For quantitative purposes, measurements of the cell height of the anterior and posterior intestinal mucosae were performed as described by Theilacker and Watanabe (1989). These measurements consisted of the distance from the basal membrane to the tip of the brush border and were obtained under a microscope equipped with an ocular micrometer eyepiece accurate to 0.02 μm. In the anterior intestine, the site for this measurement was the ventral row of cells located just cranial of the intestinal valve complex (see Fig. 6, A and B). A similar measurement on the posterior intestine mucosa was performed caudal of the intestinal valve (see Fig. 6, A and B).

Control and starved group parameters at each sampling time were compared by using Student's *t*-tests. The overall level of significance (α) for each data set was fixed at a nominal value of 0.05. The critical *t*-value for k number of tests was adjusted through Bonferroni's correction as α/k (Sacks, 1978). All values were plotted as arithmetic means and standard errors.

Results

Morphometry and biochemistry

Sampling of 6-day-old larvae was conducted at 24, 48, and 60 hours after initiation of starvation (Fig. 1). Mortality in starved larvae began about 60 hours after food deprivation. The mean standard length of starved larvae was lower than their fed counterparts at all sampling times (t_{18}=3.39, P=0.003, at 24 h, Fig. 1A). Mean dry weight (Fig. 1B) and the mean eye to head ratio (Fig. 1C) did not differ significantly (dry weight, t_{18}=2.39, P=0.028, at 24 h; eye/head ratio, t_{18}=1.31, P=0.208, at 60 h). The mean pectoral angle of starved larvae decreased relative to the fed larvae after 24 hours (t_{18}=3.53, P=0.002; Fig. 1D). Mean RNA:DNA ratios of starved larvae were lower than those of fed larvae (t_{18}=2.68, P=0.015, at 60 h; Fig. 1E). After 60 hours, mean RNA:DNA ratios had decreased from an initial value of 3.75 to 2.74 and 2.31 in fed and starved larvae, respectively. Levels of protein remained fairly constant throughout the experimental period (Fig. 1F).

Sampling of 16-day-old larvae was conducted at 24, 48, and 72 hours after initiation of starvation (Fig. 2). Starved 16-day-old larvae began to die after 72 hours. Mean standard length of both groups was not statistically different at any time (t_{18}=2.25, P=0.037, at 72 h; Fig. 2A). However, differences in mean dry weight were significant at 72 hours (t_{18}=3.04, P=0.007; Fig. 2B). A comparison of the mean dry weight at the beginning of the experiment and that for each group after 72 hours indicates that fed larvae incorporated body mass at a daily specific rate of 7.9%/day, whereas starved larvae lost weight at a rate of 10.4%/day. Similarly, the ratio of eye diameter to head height became significantly different only after 72 hours of starvation (t_{18}= 4.41, P<0.001; Fig. 2C). Little difference was observed in the mean pectoral angle between the groups until 48 h (t_{18}=4.59, P<0.001) and 72 hours (t_{18}=8.25, P<0.001; Fig. 2D). For three days, the mean RNA:DNA ratio of fed animals (2.97–2.99) remained near the mean value at time 0 (2.81; Fig. 2E). During the same period, starved fish showed a steady decline in RNA:DNA ratio to a final value of 1.93, although differences were only significant at 72 hours (t_{18}=3.47, P=0.003). Mean protein content initially decreased in both groups but became fairly constant and indistinguishable between groups thereafter (Fig. 2F).

Sampling of 33-day-old larvae was conducted at 24, 72, 120, and 192 hours after initiation of the experiment (Fig. 3). Larvae began to die after approximately 8 days of food deprivation. Starved larvae were significantly shorter than fed ones after 72 hours (t_{18}=3.32, P=0.004; Fig. 3A). Daily specific growth in length of fed larvae progressed at a rate of 2.5%/day but remained almost constant in starved fish. Dry weight of fed larvae also increased significantly relative to starved larvae (Fig. 3B). At the end of the experimental period, the fed larvae had in-

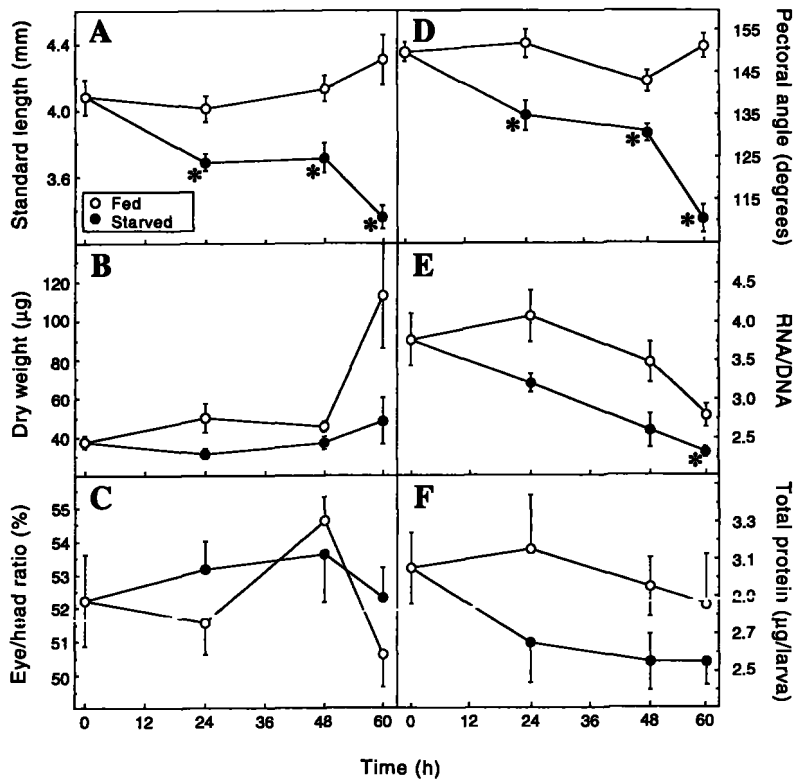

Figure 1

Summer flounder, *Paralichthys dentatus*, 6-day-old larvae. Morphometric, gravimetric, and biochemical changes during ad libitum feeding (○) or starvation (•). (**A**) standard length; (**B**) dry weight; (**C**) eye diameter/head height ratio; (**D**) pectoral angle; (**E**) RNA:DNA ratio; (**F**) total proteins. Symbols represent the arithmetic mean of samples of 9–10 animals ±Standard Error. Asterisks indicate a statistically significant difference between fed and starved groups at a particular sampling time.

creased their dry weight by more than 206% of the initial value, whereas the starved group remained unchanged. This weight difference was significant at 72 hours (t_{18}=4.46, P<0.001), 120 hours (t_{18}=5.54, P<0.001), and 192 hours (t_{18}=4.06, P<0.001). The eye:head ratio of both groups differed at 192 hours (t_{18}=4.28, P<0.001; Fig. 3C). At 72 hours (t_{18}=5.38, P<0.001), 120 hours (t_{18}=7.89, P<0.001), and 192 hours (t_{18}=6.85, P<0.001) the starved group had a lower mean pectoral angle than did the fed group (Fig. 3D). The RNA:DNA ratio showed an initial rise from 2.88 to 3.41 and to 3.26 in fed and starved larvae, respectively (Fig. 3E). After 24 hours, both groups showed a decline, but starved larvae declined to a much greater extent, resulting in significant differences between the two groups at 120 hours (t_{18}=4.85, P<0.001) and 192 hours (t_{18}=5.18, P<0.001). By day 8, starved larvae had ratios 62.4% lower than those of fed larvae. Mean total protein of starving larvae was also significantly lower than that in fed fish, a difference detectable after 192 hours (t_{18}=4.19, P<0.001; Fig. 3F).

Samples of 60-day-old metamorphosed juveniles were taken at 72, 144, and 216 hours (Fig. 4). Mortality in the starved group began after 10 days. While the mean standard length of both groups was different at 216 hours (t_{18}=4.01, P<0.001; Fig. 4A), mean dry weights of the starved and fed groups were significantly different from each other at each sampling time (t_{18}=2.95, P=0.009, at 72 h; Fig. 4B). In 9 days, fed juveniles grew in length at a daily specific rate of 3.1%/day, whereas starved larvae grew at 0.7%/day. During the same time, fed fish gained weight at a rate of 10.1%/day, whereas starved fish lost 1.9% of their body mass every day. The eye diameter to head height ratio in both groups varied in a similar manner (Fig. 4C). A significant difference in the shape of the pectoral angle was only detected at 216 hours (t_{18}=3.15, P=0.006; Fig. 4D). Mean RNA:DNA ratios

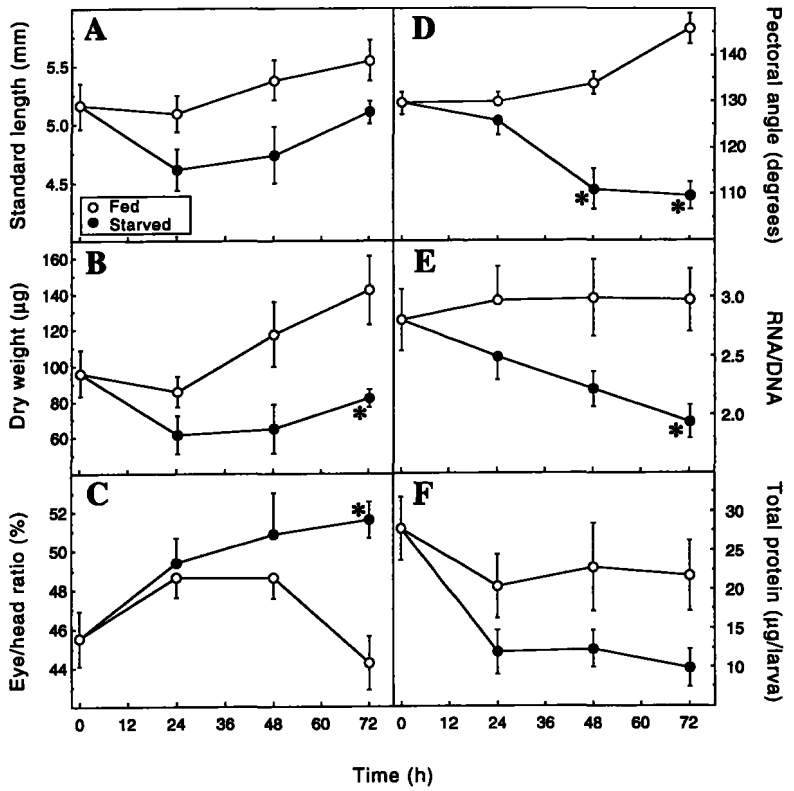

Figure 2

Summer flounder, *Paralichthys dentatus*, 16-day-old larvae. Morphometric, gravimetric, and biochemical changes during ad libitum feeding (○) or starvation (•). (**A**) standard length; (**B**) dry weight; (**C**) eye diameter/head height ratio; (**D**) pectoral angle; (**E**) RNA:DNA ratio; (**F**) total proteins. Symbols represent the arithmetic mean of samples of 9–10 animals ±Standard Error. Asterisks indicate a statistically significant difference between fed and starved groups at a particular sampling time.

of starved juveniles remained consistently lower than those of fed juveniles at all times (t_{17}=3.05, P=0.007, at 72 h; Fig. 4E). During the experimental period, fed fish maintained a mean ratio between 8 and 9. In contrast, the ratio in starved fish dropped from an initial value of 8.49 to a final value of 4.86, a 68% difference from the fed group. Differences in mean total proteins were significant at 72 hours (t_{17}=3.46, P=0.003) and 216 hours (t_{18}=2.71, P=0.014; Fig. 4F).

Histology

The trunk musculature in fed larvae was striated, closely packed, and composed of parallel myofibrils over the lateral surfaces of the notochord (Fig. 5A). However, under starving conditions, the fibrils were not distinguishable and their parallel orientation was disrupted. Further, muscle fibers were widely separated because of shrinkage of the cells (Fig. 5B). In 6 and 16-day-old larvae, degradation of skeletal muscle was evident after 24 hours of starvation. In 33-day-old larvae and 60-day-old juveniles, this effect was detected after 72 and 144 hours of starvation, respectively.

Hepatic tissue of fed larvae appeared continuous and compact, composed of hepatic cells organized in typical liver cords (Fig. 5C). The hepatocytes had a bulky cytoplasm with low staining affinity, several vacuolar inclusions, and round nuclei in their centers. Conversely, liver tissue of starving larvae was fractionated and exhibited loss of the cellular cord arrangement and contained wide intercellular spaces (Fig. 5D). The cytoplasm was severely collapsed and deeply stained (there were no vacuolar spaces) and contained heavily pigmented eccentric nuclei of irregular shape. Liver deterioration was detected after 24, 48, 120, and 144 hours of food deprivation in 6, 16, 33-day-old larvae, and 60-day-old juveniles, respectively.

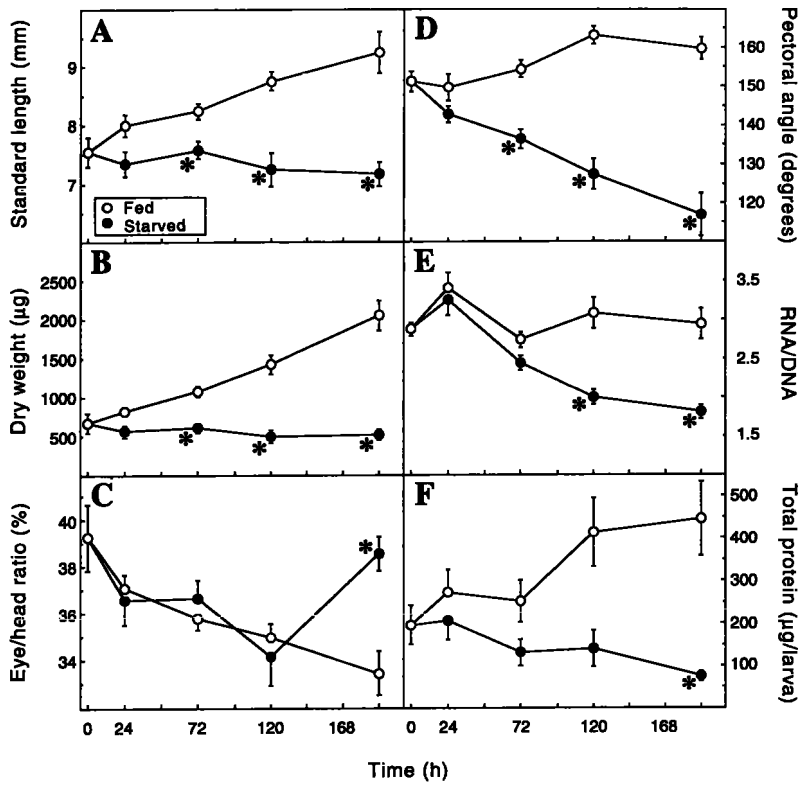

Figure 3

Summer flounder, *Paralichthys dentatus*, 33-day-old larvae. Morphometric, gravimetric, and biochemical changes during *ad libitum* feeding (○) or starvation (•). (**A**) standard length; (**B**) dry weight; (**C**) eye diameter/head height ratio; (**D**) pectoral angle; (**E**) RNA:DNA ratio; (**F**) total proteins. Symbols represent the arithmetic mean of samples of 9–10 animals ±Standard Error. Asterisks indicate a statistically significant difference between fed and starved groups at a particular sampling time.

The acinar arrangement of pancreatic cells was sensitive to starvation. In fed larvae, the typical acinar structure was well defined and symmetrical; cells were arranged around central intercellular lumina (Fig. 5E). Under food deprivation, the acinar structure became increasingly disorganized (Fig. 5F). In 6, 16, and 33-day-old larvae, symptoms of pancreatic degeneration were discernible as early as 24 hours after food deprivation. In 60-day-old juveniles, this effect was detectable after 144 hours of starvation.

The intestinal mucosa of fed larvae was continuous and uninterrupted. A distinct brush border composed of microvilli was evident. The intestinal lumen was wide and the columnar enterocytes were systematically arranged and deeply folded. Cytoplasmic vesicles and vacuoles, suggestive of pinocytosis and intracellular protein digestion, were present in varying numbers and sizes (Fig. 6C). In the starved group, the intestinal mucosa was discontinuous, less compact, and had irregular cells and intercellular spacing. The brush border was not smooth and signs of cell sloughing were evident from the necrotic debris in the lumen. The enterocytes were shrunken and collapsed resulting in a severe reduction of the entire mucosal thickness. The intestinal lumen was comparatively occluded. Cytoplasmic vesicles were not present (Fig. 6D).

The mean cell height of the anterior intestinal mucosa was significantly different between starved and fed groups of all ages. In all cases, these differences were detectable from the first sampling time (t_{18}=2.99, P=0.008, at 24 h in 6-day-old larvae; t_{18}=8.20, P<0.001, at 24 h in 16-day-old larvae; t_{18}=6.06, P<0.001, at 24 h in 33-day-old larvae; and t_{10}=11.0, P<0.001, at 72 h in 60-day-old juveniles; Fig. 7, A, C, E, and G, respectively).

Differences in the cell height of the posterior intestinal mucosa of starved and fed groups were also

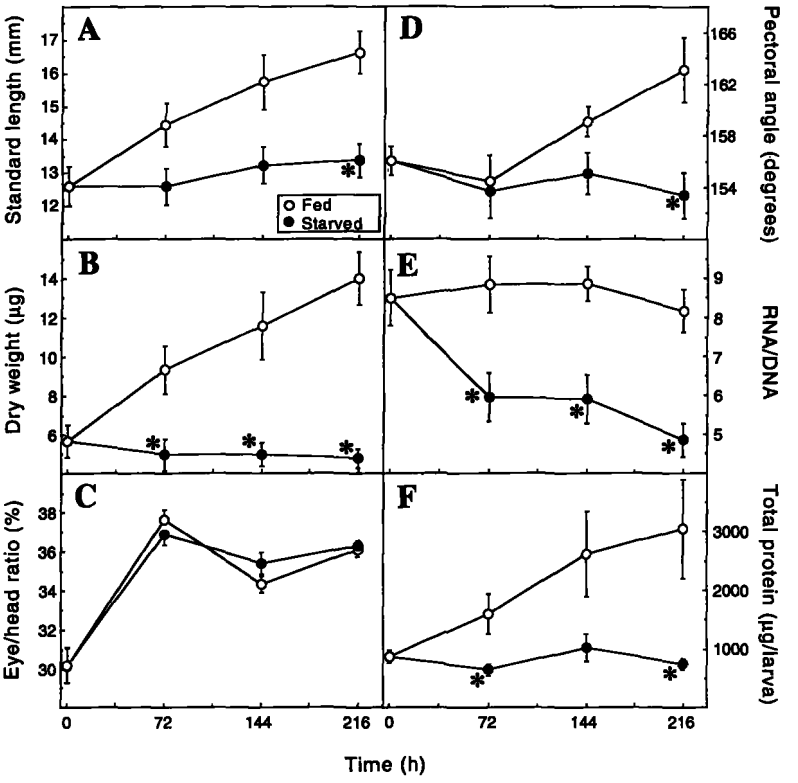

Figure 4

Summer flounder, *Paralichthys dentatus*, 60-day-old juveniles. Morphometric, gravimetric, and biochemical changes during ad libitum feeding (○) or starvation (●). (**A**) standard length; (**B**) dry weight; (**C**) eye diameter/head height ratio; (**D**) pectoral angle; (**E**) RNA:DNA ratio; (**F**) total proteins. Symbols represent the arithmetic mean of samples of 9–10 animals ±Standard Error. Asterisks indicate a statistically significant difference between fed and starved groups at a particular sampling time.

significant from the first sampling time in 16-day-old larvae (t_{18}=6.86, P<0.001, at 24 h), 33-day-old larvae (t_{18}=2.87, P=0.010, at 24 h), and in 60-day-old juveniles (t_{10}=3.05, P=0.012, at 72 h; Fig. 7, D, F, and H, respectively). In the case of 6-day-old larvae, these differences were significant after 48 hours (t_{18}=10.49, P<0.001; Fig. 7B).

Discussion

In summer flounder, the onset of mortality due to starvation occurred later in older ontogenetic stages, similar to observations made by Ivlev (1961) and Wyatt (1972). Response to starvation may depend not only on energy reserves stored in the liver, muscles, and other body tissues but also on more efficient catabolic capabilities attained during ontogenesis (Ehrlich, 1974b). Yin and Blaxter (1987) argued that the relative tolerance to lack of food is the result of reduced energy costs for metamorphosing flounder that increasingly spend more time lying on the bottom.

Morphometric, biochemical, and histological measurements all showed significant differences between starved and fed summer flounder at some point during development. The question then becomes the following: Which individual measurement or combination is the most useful indicator of nutritional status as development proceeds? We define usefulness both in terms of ease and practicality of application. Because of the relatively low resistance to starvation in younger larvae, it is imperative to select an indicator with the sensitivity to respond quickly to changes in nutritional status.

While mean length and dry weight of fed summer flounder showed a steady increase, starving fish shrank or did not grow. Only in 6-day-old larvae did standard length decrease, presumably representing

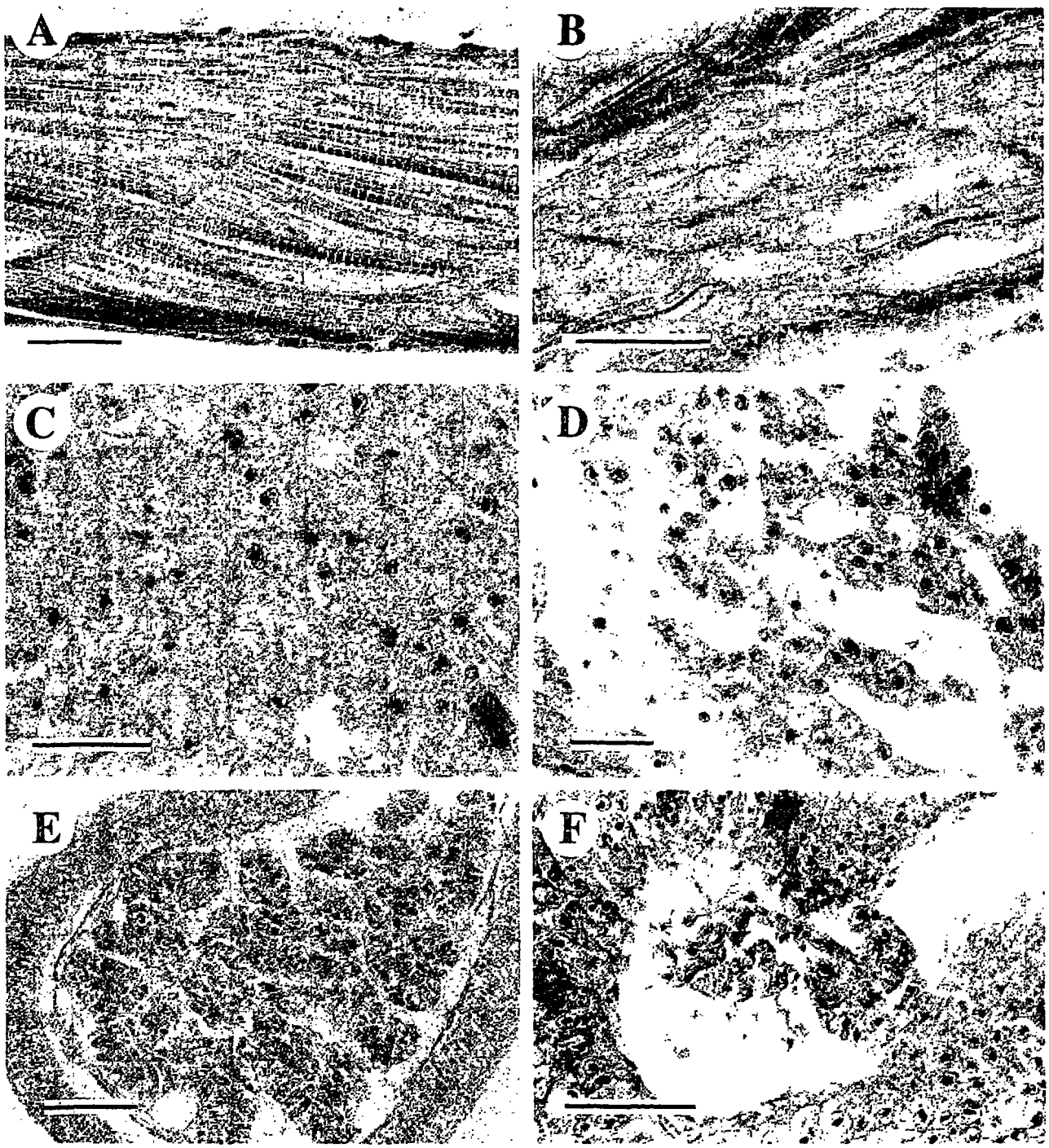

Figure 5

Histological comparisons of ad-libitum-fed and starved summer flounder, *Paralichthys dentatus*, larvae. (**A**) 16 days after hatching (DAH), skeletal musculature, ad-libitum-fed control (bar=20 μm). (**B**) 19 DAH, skeletal musculature, after 72 hours of starvation (bar=35 μm). (**C**) 18 DAH, hepatic tissue, ad-libitum-fed control (bar=25 μm). (**D**) 19 DAH, hepatic tissue, after 72 hours of starvation (bar=20 μm). (**E**) 19 DAH, pancreatic tissue, well-fed control (bar=55 μm). (**F**) 19 DAH, pancreatic tissue, after 72 hours of starvation (bar=30 μm).

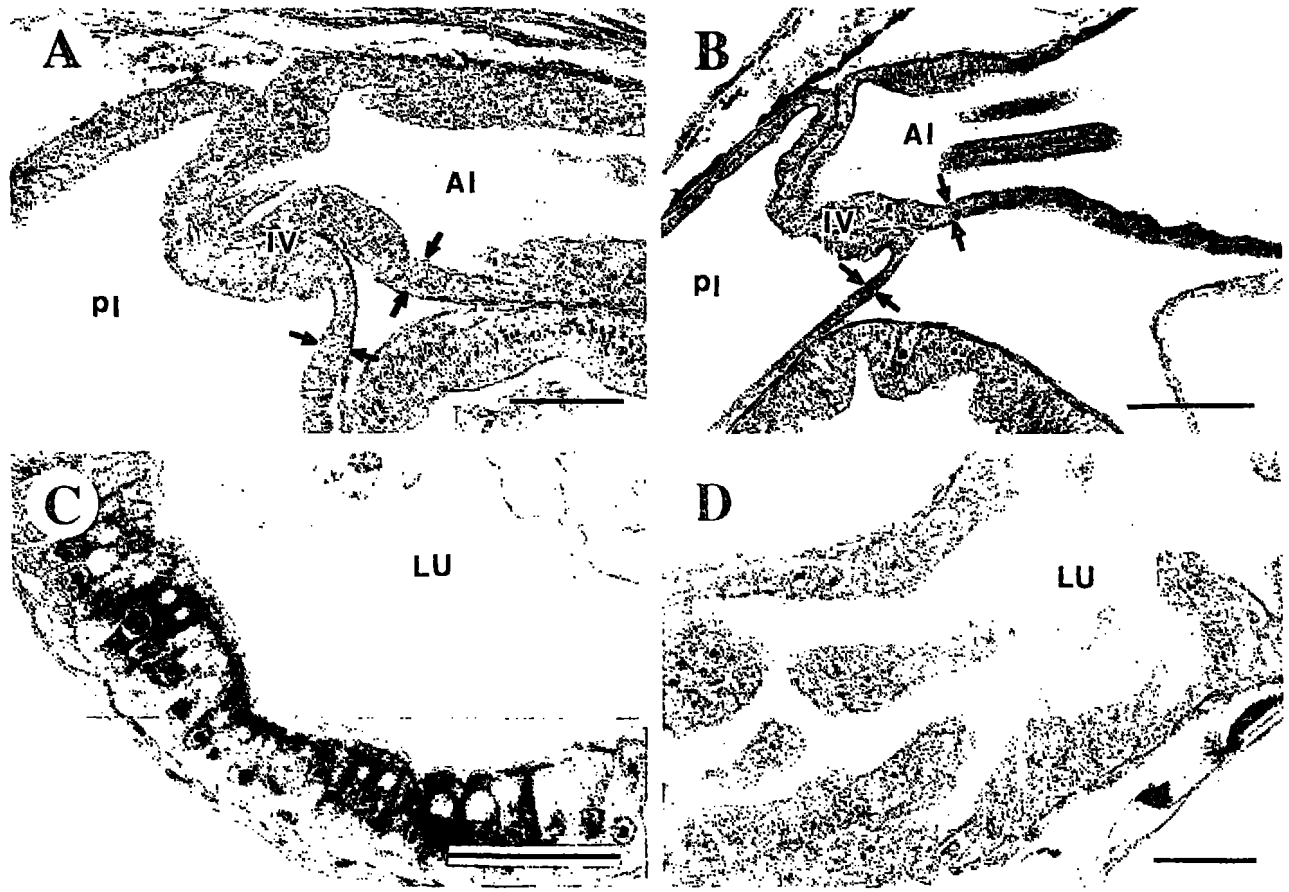

Figure 6

Histological comparisons of well-fed and starved summer flounder, *Paralichthys dentatus*, larvae. (**A**) 19 days after hatching (DAH), intestinal mucosae at the intestinal valve, ad-libitum-fed control (bar=50 μm). (**B**) 19 DAH, intestinal mucosae at the intestinal valve, after 72 hours of starvation (bar=60 μm). The arrows indicate the mucosal height in each intestinal segment. (**C**) 16 DAH, detail of enterocytes showing absorptive inclusions, ad-libitum-fed control (bar=35 μm). (**D**) 19 DAH, detail of enterocytes showing cellular sloughing into the lumen, after 72 hours of starvation (bar=20 μm). Abbreviations: AI=anterior intestine, IV=intestinal valve, LU=lumen, PI=posterior intestine. The arrows indicate the mucosal height in each intestinal segment.

shrinkage of the larvae after yolk absorption. Shrinkage of starved early stage larvae has been reported in herring (Ehrlich et al., 1976) and striped bass (Eldridge et al., 1981). Additionally, large variation in the extent of shrinkage has been reported in preserved larvae as a consequence of capture and fixation (Theilacker, 1980; Hay, 1981). The time of sampling must also be considered to account for changes in dry weight associated with the diurnal rhythms of visual feeders (Arthur, 1976). The dry weight of a larva with a full digestive tract will obviously be greater than that of the same larva with an empty digestive tract. Because extensive calibration between laboratory and field experiments is necessary to compare small larvae at the same developmental stage, length and dry weights are not useful indicators of nutritional status.

The pectoral angle accurately identified the nutritional condition of earlier larval stages. The variability within each group was low and significant differences were established early in the sampling protocol. However, these attributes progressively vanished at later stages. The eye length to head diameter ratio was not a good indicator of the feeding condition at any stage because of large variability within each group. Ehrlich et al. (1976) found the pectoral angle to be a good indicator of starvation in both herring, *Clupea harengus*, and plaice, *Pleuronectes platessa*, but the eye:head ratio was a good indicator in herring only.

Morphological characteristics are relatively simple to measure, inexpensive, and require little time, but the validity of laboratory-derived criteria is uncer-

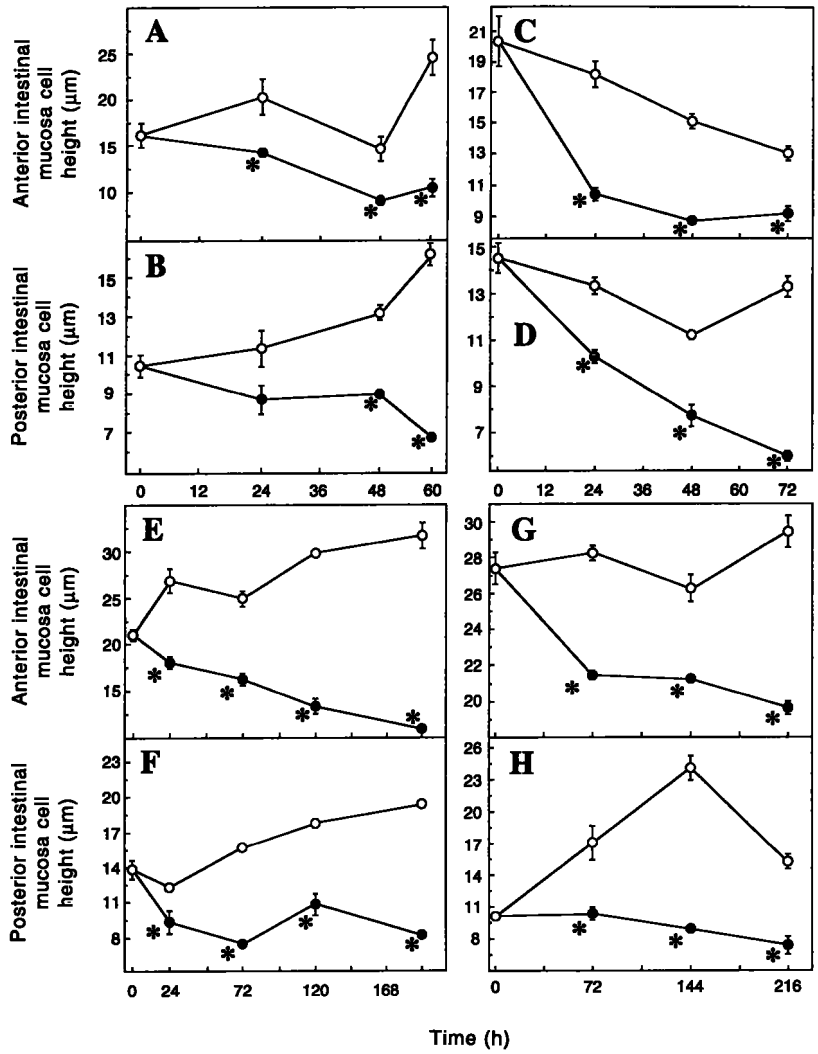

Figure 7

Anterior and posterior intestinal mucosal cell height in summer flounder, *Paralichthys dentatus*, during ad libitum feeding (○) or starvation (•). (**A–B**) 6-day-old larvae; (**C–D**) 16-day-old larvae; (**E–F**) 33-day-old larvae; (**G–H**) 60-day-old juveniles. Symbols represent the arithmetic mean of samples of 9–10 animals ±Standard Error. Asterisks indicate a statistically significant difference between fed and starved fish groups at a particular sampling time.

tain for populations in nature (O'Connell, 1976; Theilacker, 1986; Fraser et al., 1987; Setzler-Hamilton et al., 1987). Confinement in experimental tanks influences growth rates and morphometrics of laboratory-reared larvae (Blaxter, 1975; Arthur, 1976). At present, the applicability of morphometric indices seems more reliable and feasible for reared larvae, where age and historic information are known and feeding can be controlled.

Given the inherent problems of laboratory-to-field calibration and the dynamic changes in body proportions due to allometric growth and progressive ossification of developing larvae, Theilacker (1978) concluded that no single morphological feature can be singled out as a consistent indicator of larval condition. Because some of the variability associated with field-collected larvae is accounted for by differences in age of larvae, interpretation of the data requires the ability to determine age. Ageing of summer flounder from daily growth ring deposition is difficult on field-collected larvae of mixed age (Dery, 1988, Szedlmayer and Able, 1992). Therefore, the use of

length as an estimate of age is a coarse alternative when age data are not available. If this is the case, then the analysis should be restricted to a limited size range (Martin and Wright, 1987).

Among the biochemical criteria, protein data had the largest associated variability. Similar variation in the protein content of winter flounder, *Pleuronectes americanus*, larvae has been obtained by Cetta and Capuzzo (1982). Other studies have shown that protein breakdown is the major source of energy during starvation of herring (Ehrlich, 1974a) and plaice (Ehrlich, 1974b), at least during early larval stages, when lipid reserves are negligible or nonexistent.

The RNA:DNA ratio showed less individual variability and provided a more sensitive index to feeding condition than did protein. The ratio of total RNA to DNA in tissues has been extensively used as an indicator of recent growth rate and changes in feeding levels of various larval fish (Buckley, 1984; Bulow, 1987). In recent years, the relative ease and sensitivity of this analysis have stimulated the development of several procedural variations of the technique. Thus, discretion should be exercised in directly comparing RNA:DNA values obtained with different methods and standards (Caldarone and Buckley, 1991). In addition, it has been demonstrated that temperature can affect the RNA:DNA ratio in fish larvae (Buckley, 1982, 1984; Buckley and Lough, 1987). In the 6-day-old larvae used in our study, the RNA:DNA ratio declined by about 30% over the 60-hour experiment, even in fed larvae. After that decline, which was similar in magnitude to that observed in fed winter flounder larvae 4 days after yolk absorption (Buckley, 1980), the mean RNA:DNA ratio of fed larvae remained within a narrow range (2.7 to 3.1) for the remainder of the larval period. Therefore, it appears that a mean RNA:DNA ratio of less than 2.7 strongly suggests food limitation in flounder. The equilibrium RNA:DNA ratio for *P. dentatus* larvae reared at 14, 16, or 18°C has been reported to be 2.4, 3.1, and 2.6, respectively (Buckley, 1984). Winter flounder and striped bass, *Morone saxatilis*, larvae also appear to establish narrow RNA:DNA equilibrium ranges (Buckley, 1980; Wright and Martin, 1985). After metamorphosis, the RNA:DNA ratio of summer flounder increased to between 8.2 and 8.9, whereas that of starved fish was never above 6. A similar increase in RNA:DNA ratio after metamorphosis has been observed in fed winter flounder (Buckley, 1980).

Although RNA:DNA ratio and pectoral angle were both able to discriminate fed from starved summer flounder, pectoral angle was more sensitive to starvation than was the RNA:DNA ratio in larvae, whereas the opposite was true for juveniles. The quick response of RNA:DNA ratio to food deprivation noted by Buckley (1980), Wright and Martin (1985), and Martin and Wright (1987) was not apparent in summer flounder. An advantage of biochemical methods for field use is that larvae damaged by sampling gear can still be analyzed (Fraser et al., 1987) and distortions due to chemical fixatives are avoided. We conclude, therefore, that RNA:DNA ratios may be useful as indicators of nutritional limitation in summer flounder larvae and juveniles.

Histological analyses indicated that food deprivation of summer flounder larvae and early juveniles had a marked effect on several internal structures. Starvation was readily manifest in the intestine, followed in time by changes in the pancreas, liver, and skeletal musculature, as previously seen in other teleost larvae (Umeda and Ochiai, 1975; Ehrlich et al., 1976; O'Connell, 1976, 1980; Theilacker, 1978, 1986; Cousin et al., 1986; Margulies, 1993). The nutrient shortages that result from food deprivation have an almost immediate manifestation in the intestinal epithelium. In starved summer flounder, lipid and protein inclusions progressively disappeared from the intestinal epithelial cells until they were no longer visible, similar to the previous observations of Ehrlich (1974a), Ciullo (1975), Watanabe (1985), and Govoni et al. (1986). By contrast, Kjørsvik et al. (1991) reported that pinocytic inclusions were visible at all stages of starvation in cod larvae.

Mucosal cell height in summer flounder was extremely sensitive to starvation when applied to the posterior intestine, whereas the height of the anterior intestinal mucosa varied with increasing size or age, or both. The mean height of the posterior intestinal mucosa showed a stable boundary for discrimination of fed and starved individuals (above 10 μm for fed larvae, below for starved) regardless of individual size or age. This criterion therefore provides the best tool to assess starvation in summer flounder during the first 60 days of life. Previous investigators have noted the utility of histological examination of intestinal mucosa, especially cell height, for determination of starvation (Ehrlich et al., 1976; Theilacker, 1978, 1980; Watanabe, 1985; Umeda et al., 1986; Theilacker and Watanabe, 1989; Kjørsvik et al., 1991). The discriminating power of the mucosal cell height criterion incorporates the well known advantages of other traditional histological evaluation procedures. As with the biochemical criteria, specific equipment and some technical proficiency are required to process the samples. One advantage to this criterion is that samples can be preserved on a ship and no subsequent calibration is necessary for shrinkage due to capture or fixation, or for individual size or age.

To summarize, this study has demonstrated that 1) morphological criteria were either not good indicators of nutritional condition (eye:head ratio), good only for larvae (pectoral angle), or require extensive calibration (standard and dry weight); 2) biochemical criteria are either not good indicators (protein content) or are sensitive only in juveniles (RNA:DNA ratio); and 3) the histological criterion of posterior intestinal mucosa cell height is the most sensitive and consistent indicator of starvation in young summer flounder over the stages examined. Although the current study needs to be applied to field-collected larvae, the laboratory data indicate that the additional time and expense of histological sample preparation and analysis is justified in the face of uncertainties associated with the other methods examined. On the other hand, application of either morphological or histological criteria is appropriate for an aquaculture setting in which age of the larvae is known.

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