Adaptation of unbiased stereological methods to study the effects of feed composition on the morphometrics of the different layers of the digestive tract of juvenile Atlantic cod (*Gadus morhua* L.).

by

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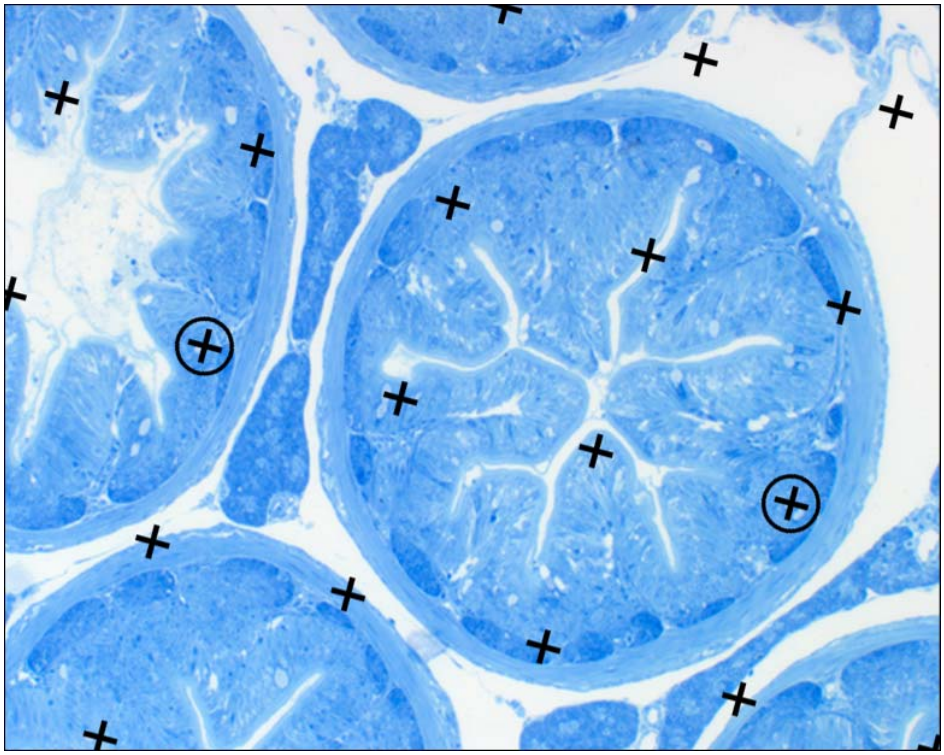

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Front page: Picture of pyloric region with a superimposed point counting grid.

CONTENTS

1 ABSTRACT

Juveniles of Atlantic cod (*Gadus morhua* L.) were fed food, containing different compositions of macro-nutrients (proteins, fat and carbohydrates). The aims of this study were to evaluate the use of unbiased stereology to find out if there were any differences in estimated weight, volume and/or total surface area and to describe a general digestive tract in these juvenile cod.

By employing Technovit 7100 as embedding medium, it was possible to apply a VUR (vertical uniform random) sampling protocol and obtain samples unbiased. Hits from a point-counting grid were used to calculate estimations of volume fraction, and hits from a cycloid counting grid were used for calculating estimations of total surface area of the digestive tract. The estimations showed that there were no significant differences between any of the tested food types in any of the calculated parameters, apart from one, found in the estimated surface ratio of the intestine. This may, however, be due to high variability present in the material. There were no differences between diet groups, regarding the layers of the digestive tracts whatever diet the fish were given, and the description of the layers in the digestive tract, supports previous findings.

2 INTRODUCTION

Coastal cod (*Gadus morhua* L.) is an important food source and economic trade article, and has been so for centuries. The extensive industrial fisheries have reduced the market value of Atlantic cod, and the production of feed for cultivation is expensive. This makes commercial cultivation economically challenging. Today, however, with the diminished cod stocks in the North Atlantic Ocean, and probable future reduction in fishing quotas, the importance of commercial cultivation will increase. Already in the late 19th century, cod was reared through the first months after hatching in closed basins. However, only in 1977, rearing of cod until maturity was accomplished. This was done under laboratory conditions (Hognestad 1984). Atlantic cod has a great biological potential in commercial aquaculture. It has a high fecundity, spawn easily in captivity, larval survival rates are usually acceptable and growth rates can be high even at low temperatures (Howell 1983). Furthermore, rearing of cod does not require any major technical developments, as existing and functional equipment and techniques from cultivation of other marine species can readily be applied (Brown *et al.* 2003).

For cod cultivation to be a competitive industry, the fish must have the right texture, taste and growth. This may only be accomplished through appropriate compositions of the diet, especially with regard to the main nutrients.

Still, many uncertainties remains, regarding the composition of macronutrients, especially the contents of protein and fat in cod feed. High protein contents would most likely make the fish grow fast and also achieve the desired texture in the muscles. Protein, however, is generally expensive. The protein requirement in cod feed seems to be less than 53% (Hamre and Mangor-Jensen (personal communication), Institute of Marine Research, Bergen, Norway). Studies on European sea bass has shown that too much fat in the food reduces the moisture in the fish fillet and increases the lipid content (Lanari *et al.* 1999). High content of fat in cod feed result in increased hepatosomatic index. This is not desirable since liver growth reduces the potential muscle (i.e. marketable) growth (Lie *et al.* 1988).

The present study was part of a larger study, aiming to compose an optimized diet that may enhance the quality and growth of cultivated fish. A total of 20 different diets were composed with a standard diet as basis (Fig. 1a). This diet contained a mean value of nutrients, and was made to determine the tank variation between three-mixture diets (Cornell 2002). The basis diet composition was 65.5% protein, 7.5% carbohydrates and 20% fat. The reminding 7% was the same in all the diets, and was not considered to contribute variation. The basis diet was based on cod fry diet compositions already on the marked.

Studies of cultivated Atlantic fish diet composition have generally been based on analysis of fat accumulation, fluid in liver and muscles, and growth (Jobling *et al.* 1991; Olsen *et al.* 1999; Morais *et al*. 2001).
The part of the study covered by the present study will apply stereological methods to study five different diets with different composition (groups 2, 5, 7, 10 and 18) (Fig. 1b). The weight, volume and surface area of intestinal organs are described. Since all the nutrients necessarily are absorbed in the intestinal tract, the analyses focused on detecting any induced changes in the histological layers of the stomach and the intestine, as well as in the pancreas.

The principles underlying stereology has been known for a long time. Modern and generally unbiased stereological methods have, however, mostly been developed in the last decades (Mayhew and Gundersen 1996). Today these so-called design based methods are preferred when studying three-dimensional (3D) structural quantities because of their efficiency. The methods are quick (compared with 3D reconstruction) and estimation can be done from a few chosen sections and precise because the systematic sampling takes away much, and often all of the systematic error (bias). The stereological tools allow quantitative 3D estimates to be obtained from two-dimensional (2D) slices through any object, like histological sections of a tissue sample. While the direct observations are made in the 2D sections, 3D information is obtained through mathematically proven relationships (Howard and Reed 1998).

The accuracy of stereology and its estimates relies on unbiased sampling protocol at all levels (population, individual, tissue sample, section and observation field). This is ensured by using systematic random sampling throughout the sampling hierarchy (Gundersen

1987)(Fig. 2). The numerical estimates are obtained by counting hits from a probe placed randomly on selected microscopic sections (Fig. 3). And for a 3D structure, the dimension of the probe is 3 minus the dimension of the structure (ie. a point one-dimensional (1D) for area (2D)/volume). The estimation of area fraction is used as a direct estimator volume fraction and consequently total volume (Michel and Cruz-Orive 1988).

Using stereology to estimate volume may seem unnecessary since e.g. volume can easily be estimated by putting an object in a jar filled with fluid, and then weighing the displaced fluid, fluid displacement (Scherle 1970). This is, however, often not possible. Many organs consists of several functional parts e.g. the brain with its different kinds of synaptic junctions and glands, the lung with all its alveolar ducts, the intestinal tract with its different histological layers (Fig. 6) and pancreas which is distributed around the cranial region of the intestine (Michel and Cruz-Orive 1988; Fiala and Harris 2001).

The goals of the present study were as follows:

1. To develop a practical way to embed and orient the samples of the digestive tract from the juvenile Atlantic cod.

2. To employ the stereological tools, to determine eventual differences in weight, volume and area of several parts of the tract, from the five diet groups (2, 5, 7, 10 and 18).

3. To obtain quantitative data of the digestive tract in cod fry.

3 MATERIAL AND METHODS

3.1 Material

Fish history

The experiment was carried out in the period of January-March, 2002, at the Institute of Marine Research, Austevoll Aquaculture Research Station, Storebø, Norway. A broodstock of captive costal cod (*Gadus morhua* L.) spawned in September 2001. Prior to spawning, the broodstock was fed a commercial dry diet (Danafeed, 16 mm). The broodstock was kept in Ø = 4 m tanks. Fertilized eggs were collected from the effluent surface water and incubated in 100 L tanks with a conic bottom (Fig. 7). Water temperature in the incubator was maintained at 6.0 ± 0.2^0 C throughout the egg stage. Dead eggs were removed daily from the bottom of the incubators. After 14 days the eggs hatched, and 4 days later the larvae were transferred to first feeding tanks, with a diameter of 1.5 m. They were now fed enriched rotifers (*Brachionus sp*.). The rotiferculture was enriched with a mixture of 40% baker's yeast (Idun), 40% Rotimac (Aquafauna Bio-Marine) and 20% Algae paste (*Nanochloropsis*, Aquaculture). The diet was changed to *Artemia* neuplii after 35 days. *Artemia* were for a short time enriched with a DHA-rich Selco product (Fatty acid and vitamins, see http://program.forskningsradet.no/havbruk/prosjekter/del_3/115575-artemiakvalitet.html). The larvae were offered commercially dry diet (Aglonorse < 0.6) after 44 days. 1200 cod fry with an average wet weight of 0.26 grams were collected randomly from the tank 55 days after hatching, and distributed among 24 50 L tanks (50 individuals in each tank). From this point, they were fed the different experimental diets (Table 1), which were based on a three component mixture design, systematically varying the protein, fat and carbohydrate proportions (Cornell 2002).

The 24 tanks were arranged in rows, and they received temperature-controlled and aerated water (34.5 ppt salinity, 12 ºC). Water flow was adjusted to ensure that oxygen saturation in the water was kept at a minimum of 95% through the experiment. Total gas saturation was measured 3 times during the experiment. It varied from 98% to 100% saturation. To

get an exact feeding regime, an automatic belt-feeder (Hølland technology, Sandnes, Norway) ensured continuous feeding.

Daily food rations were calculated as the fish grew according to the fish size, and the mortality was also taken into account. Throughout the experiment, food was given in abundance and redundant food was removed daily by siphoning and flushing of the bottom of the tanks. Start rations were 5 grams per tank per day increasing to 15 grams per tank per day at the end of the experiment. Dead fish were removed daily from the tanks, if present.

At the termination of the experiment, after 63 days, 120 fish were randomly selected, five from each tank. The fish were weighed, and the digestive tract was removed and fixed in formalin (Appendix 7.3) in individual containers.

3.2 Histological and stereological procedures

Embedding and sectioning

The gills and liver were removed. Oesophagus was removed together with the gills. Excess fluid was wiped off with a paper tissue, and the digestive tract was weighed on a scale with milligram precision. This weight was employed as an estimate for the reference volume.

Four test samples were rinsed in PBS-buffer (Appendix 7.3) for 3 x 10 min to wash off the neutral buffered formalin. Three samples were embedded either in 1%, 4% and 7% Bacto-agar (DIFCO). The agar was mixed with PBS-buffer (pH = 7.2). The agar solutions were kept on a hot plate at approximately 55 ^{0}C until all bubbles had disappeared. Samples were embedded in 50 ml beakers. One sample was embedded directly in 1% agar. The other two were first dipped in 1% agar before embedding in 4% and 7% agar, respectively. When the agar had solidified, they were sliced with a razor blade.

The fourth test sample was embedded in HistOmer. HistOmer and PBS-buffer (1:1) were mixed for 30 s in a beaker. The sample was then placed in the soft HistOmer. After solidification, it was sliced with a razorblade. Samples were also embedded in Technovit 7100. The intestinal tracts which were to be embedded in Technovit 7100 were first dehydrated through a graded ethanol series (50% 30 min, 70% 2 x 48 h and 96% 4 h).

Prior to embedding, each sample was pre-infiltrated in a 50/50 mixture of 96% ethanol and Technovit 7100 base liquid for 24 hours under continuous rotation. Then they were

infiltrated on a rotator for 72 hours in the base liquid Technovit solution that contained 1 activator (1 g per 100 ml).
Embedding was performed in the same Technovit 7100 solution, with an addition of hardener (1 ml per 15 ml) (Fig. 2a). The cranial end was aligned in the same direction in all samples. The wells were covered with transparent plastic in order to avoid the influence of humid air from reducing the polymerization process. The samples were then left for 48 hours for the Technovit 7100 to polymerize completely.

A Technovit embedded test-sample was sawn with an electric jigsaw (Clas Ohlson, scroll saw). This was not successful because the embedding medium melted as a result of the heat of friction. Manual cutting was thus employed for the remaining part of the material.

The embedded samples were attached to a wooden block with Technovit 3040, 1 part solution and 2-3 parts powder, and held in place in a vice during sawing.

Starting in a random position at the cranial end of the digestive tract, a grid of evenly spaced lines was drawn to aid the cutting. These lines constituted a systematic random sample of the specimen. Depending on the size of the digestive tract, it was sawn into 8-10 equally thick slices (Fig. 2b). The distance between the resulting slices was 1-1.6 mm, depending on the digestive tracts which wearied in size. Sawing was done vertically along the lines with a Japanese handsaw (0.3 mm thick). These saw cuts when they are pulled away from the samples. This enabled cutting of very thin slices. Every slice was then laid flat with the cranial end facing down in the middle of a 360^0 rotator clock. With a set of randomly chosen numbers from 0-360, a line was drawn through origo in the chosen angle on the plate on each slice of the block (Fig. 2c). Each slice was then cut along this random line. The right hand piece was employed in the further experiments.
Excessive plastic was trimmed off, and the slices were then reembedded in Technovit 7100. They were put side by side in the embedding mould, with the sawn side face down, and the cranial end of the sample slices always facing in the same direction (Fig. 2d).
Semi-thin sections (2 μm) for microscopy were cut on a rotary microtome (Leica, model # 2155). Each section (one per sample) had 8-10 strips containing tissue, each representing a VUR (vertical uniform random) section through the digestive tract (Fig. 2e). The sections were stained in toluidine blue (Philpott 1966).

Stereology

Reference points were made with a diamond pen on each end of the 8-10 strips on every section for estimation of tissue fraction (Fig. 2e). Using a dissecting microscope with a calibrated ocular micrometer, hits on the reference room were counted. Twelve counting points were identified on the micrometer. The magnification was adjusted so that the outer points touched the marks on the widest tissue strip on each section. Then using this magnification, the width of each strip was measured, and the hits on the reference room were counted on each strip. The number of hits on the reference room on each strip per section was summed. The same was done for each strip. To find the tissue fraction, the number of hits on the reference room, were divided on the sum of added widths (n = number of strips):

$$est\,A_{tissue,block} = \frac{\sum_{i=1}^{n}(hits\,on\,tissue)}{\sum_{i=1}^{n}(hits\,on\,tissue\,on\,plastic, between\,X\,marks)}$$

Then a point counting grid with 108 points that contain 9 small points per large (encircled) point, was made on a photographic film, and cut to fit one of the oculars, and calibrated in the microscope (Fig. 3b).

The positions of all the X-marks on the cover glass on each side of the strips on a section were found using the coordinate table on the microscope. These coordinates were entered in a spreadsheet, which then calculated random positions of fields on the strips which should be used for counting. 32-45 random fields, depending on tissue fraction for the section, were needed to reach a total of approximately 240 hits on the specimen.

A primary magnification of 25x was used, with a 10x ocular magnification. The position for the random areas was then found by using the coordinate table on the microscope. All hits from the point counting grids on the reference room and different types of tissue were counted (Fig. 3c).

$$\hat{V}_V(Y, ref) = \frac{\sum_{i=1}^{m} P(Y)_i}{\sum_{i=1}^{m} P(ref)_i} \quad \text{, where}$$

$\hat{V}_V$ is estimated volume fraction (were applied to further calculate total volume), Y is tissue, *ref* is reference room, m is fields of view and $P(\,)_i$ is number of hits.

For surface area estimation a cycloid counting grid was made on photographic film (Fig. 4). This contained 16 reference points and 16 cycloid lines. This was also cut to fit one of the oculars. Then the counting grid was calibrated for the 40x objective on the microscope. The spreadsheet was adjusted to achieve a total of approximate 256 hits in 200 random positions using the same random numbers and tissue fractions as before. The same coordinates on the X- marks was used in the spreadsheet to find the random positions which then were used to count crossings by the cycloid counting grid (Fig. 5).

$$\hat{S}_V(Y, ref) = \frac{2 \bullet \sum_{i=1}^{n} I_i}{l/p \bullet \sum_{i=1}^{n} P_i}, \text{ where}$$

$\hat{S}_V$ is estimated surface density, *Y* and *ref* is the same as above, *n* is fields of view, I_i is the number of intersections between the cycloid and the boundary of interest, *l/p* is known length of a cycloid per point (Fig. 4) and P_i is points within the reference space.

3.3 Figures, tables and statistics

Random numbers were generated using the Marsaglia-Multicarry algorithm available in the statistical software R (Ihaka 1996). 2011926679 and 316834081 were used as random seeds, and the software Sigmaplot 8.0 was used for making figures.

4 RESULTS

4.1 Description of the material

At the start of this experiment the Atlantic costal cod juveniles used in this study had a mean individual weight of 5.45 ± 1.46 g, and ranged from 2.78 to 8.89 g. Reference room (digestive tract) mean weight was 0.51 ± 0.14 g, and ranged from 0.20 to 0.79 g. The mortality in each tank (diet group) varied quite a lot (Table 2).

A correlation was observed between the fish weight and the weight of the reference room (Fig. 8). There was wide variation in fish weight between and within the different diet groups. A significant difference on at least one of the slopes from the regression lines between each diet group were observed (ANKOVA (individual weight * diet group), $p = 0.0159$). Diet group number 10 (much carbohydrate and little fat) had a much wider range in estimated fish weight (2.8 - 6.8) than group number 18 (no carbohydrate and much fat) with the smallest range (5.2 - 7.4). A linear regression for the correlation between fish weight and reference room weight explained 83% of the variance between the diet groups ($R^2 = 0.8277$) (Fig. 8).

4.2 Histology and stereology

The agar (1%, 4% and 7%) – and HistOmer embeddings turned out to be unsuitable, since neither remained attached to the digestive tract when cut with a razorblade. Technovit 7100, which infiltrates the tissue completely, enabled sawing of the sample into thin (>1 mm) slices. Sawing was necessary, since Technovit 7100 is too hard to be cut with a razorblade. The embeddings melted when sawn with an electric jigsaw, but slow sawing with a Japanese handsaw made it possible to saw the 1 mm slices.

The fraction of tissue in the sections was estimated to reduce variance due to the inclusion of empty plastic in the reference room. The mean fraction of tissue on a section was 0.55 ± 0.05 and the range was 0.45 - 0.64.

4.3 Grouped estimates of volume fraction, and weight

The estimated weight of the mucosa in the stomach was generally higher than the weight of mucosa in the intestine, with most of the fish with a mucosa weight above 0.04 g (highest weight 0.12 g), compared to the mucosa in the intestine, which was mostly below 0.04 g (highest weight 0.06 g) (Fig. 9). The weight of exocrine pancreas was generally close to 0.04 g (Fig. 9). There was much variance between individuals, but no significant differences were found between the diet groups in any of these organs.

The individual volume fraction of mucosa in the stomach ranged from 0.01 to 0.21. This was generally higher than the estimated mucosa fractions in the intestine, which ranged from 0.01 to 0.10 (Fig. 10). Estimated volume fraction of the exocrine pancreas ranged from 0.03 to 0.16 (Fig. 10).
The estimates were highly variable between the individuals fed the different diets, but no significant differences were detected in estimated volume fraction between the diet groups in any of the organs.

Estimates of volume of mucosa in the stomach ranged from 0.01 to 0.12 mm^3, whereas estimated volume of mucosa in the intestine was 0.06 mm^3 or lower (Fig. 11). Estimated volume of pancreas ranged from 0.01 to 0.10 mm^3, as may be seen from the figures (Fig. 11). Group number 5 and 18 had less variation in fish size than the other groups.

4.4 Grouped surface estimates

Estimated surface area density showed the area/volume ratio for each tissue type on a section. The estimated surface area density of the stomach had a range from 0.0005 to 0.0048 with much variation through the range between the individuals. The estimated surface area density of the intestine had a range from 0.0003 to 0.0019. In general, this was much lower and with less variation of the range than the estimates for the stomach (Fig. 12). There were, however, no significant differences in surface area between the groups for any of the organs.

The estimated surface area of the stomach was slightly larger than that of the intestines (Fig. 13). Estimated individual surface area of stomach ranged from 0.3 to 2.4 mm^2, and 0.2 to 0.9 mm^2 for the intestine (Fig. 13). Diet groups 5 and 18 showed less variation than the intestinal tract area in the other groups. However, no significant differences were found, despite the tendencies.

The estimated individual surface ratio for the stomach ranged from 2.5 to 11.2. The different diet groups had much the same variation in surface ratio (Fig. 14). No significant differences were found between the diet groups.

Estimated individual surface ratio for the intestine was lower and less variable between groups than surface ratios for the stomach (1 - 4.6) (fig. 15). Significant differences were found between at least one of the diet groups (ANOVA (estimated digestive-tract surface ratio (intestine) * diet group) ($p = 0.0028$).

4.5 Description of the digestive tract in juvenile costal cod based on pooled data

The mucosa and muscularis in the stomach had the same mean estimated volume fraction (0.09), and consequently the same estimated mean total weight (0.047 g). The mean estimated CE for the two tissue types were also similar (0.53).

There was a higher estimated volume fraction of mucosa in pyloric ceca than in the stomach, and in pyloric ceca it was three to four times larger than the volume fraction of the muscularis. The estimated total weight of mucosa in pyloric ceca however, was less than mucosa in the stomach (Table 3, which includes Std. dev.). The estimates of CE were the same for mucosa and muscularis in pyloric ceca (0.4).

Mucosa in the intestine had an estimated volume fraction (0.06) about one third of that in the stomach, and the muscularis in the intestine had only one half the estimated volume fraction than the mucosa in intestine (0.3). The same was represented in the estimated total weight in the intestine, but they had a mean estimated CE on 0.4.

The exocrine pancreas had an estimated volume fraction of 0.08 and estimated total weight of 0.039 g. The mean estimated CE was 0.4 (Table 3, which includes Std. dev.).

Estimated area density was the same for the stomach and pyloric ceca (0.002 mm^{-1}). It was the half for the intestine (0.001 mm^{-1}). The same ratio was seen for estimated total area, with 1.0 mm^2 for the stomach and pyloric ceca, and 0.5 mm^2 for the intestine (Table 4, which includes Std. dev.).

5 DISCUSSION

5.1 Feeding experiments

Five diet groups were tested in this present study. Five individuals from each group were available for histological analysis. The five diet groups represented the extreme points in the three-component mixture design. These groups had combinations of macronutrients, which varied systematically in all variables within given limits (see Fig. 1). A combination with much protein, e.g., and less of the other two macro nutrients, would show if this was better than other combinations. This design allows a general overview over a large studied area, but gives a limitation with no control nor replica groups. When all the groups are included, neighbour groups will, however, serve as partial replicates. Since only five groups were used for histology, selected at the extremes of the nutrient composition, no control groups were available.

The number of individuals was also a limitation. It would clearly have been desirable to have histological samples from more than five individuals in each diet group. However, the rearing and experiment with the feed was finished before the stereological study was designed, and five fish in each diet group was all that were available for testing. A further complication was that the individuals had a large size variation (see Fig. 8, and discussion below). The result was very high variability in the estimates, masking possible effects of the feeding regime. The high CE of the stereological estimates also indicates that the sampling procedure was not fully adequate. This is most likely the result of having only ten slices from each sample. Ideally, there should have been approx. ten slices from each of the organ parts being studied. With a total of ten slices, only three to four were available from each part of the digestive tract. This could not easily have been circumvented, as the thinnest possible slices were already being used. The alternative would have been serial sectioning, which would not have required a substantial increase in the workload. Since there were clear correlations with size for several of the estimates (see Fig. 11 and 13), it seemed that much of the variability could have been removed by selecting fish of the same size for the analysis. This should clearly be done for any future stereological analysis of these tissues.

5.2 Methodological considerations

The selection of an appropriate embedding medium was an initial challenge in the present study. The specimens were relatively large, making good infiltration difficult, as well as preventing conventional serial sectioning. The embedding medium selected would have to hold the sample firmly in place when cut into slices, reducing distortion artefacts to a minimum. Paraffin embedding medium was not an option since it will not infiltrate an digestive tract of this size completely, and is not suitable to cut the approx. 1 mm slices needed for the VUR sampling protocol. The VUR protocol was chosen over the IUR (isotropic uniform random) protocol, since it is easier to know how the sample is oriented, and provided that enough slices can be cut from the samples, gives fully randomized sampling. Embedding in agar (1% and 4%) failed because the agar solution was too soft, and did not keep the intestinal tracts in the right position.

In previous experiment brain specimens have been successfully embedded in 7% agar and HistOmer, an agar-like medium (Bjarkam *et al.* 2001). These media worked to some extent for embedding the intestinal tracts as well. They were firm and held the tracts in place during cutting. They did not, however, penetrate all the small cavities of the folded tract, and did not attach to the tissue. As a result, the tissue disintegrated after cutting the slices. Since the VUR protocol requires keeping track of the vertical axis in the sample, this is not acceptable. It would, however, probably have been adequate for IUR sampling.

If the intestinal tract had been stretched out before fixation in formalin, rather than fixing the viscera as a "lump", it is possible that agar or HistOmer might have been useful for processing the tissue. This was, however, not the case, and other solutions for sampling the VUR sections were needed. Technovit 7100 successfully infiltrated all through the samples of all sizes. The metacrylate resin enabled cutting with the Japanese handsaw of approx. 1 mm thick slices. Since the samples were totally infiltrated and hardened, no pieces were lost during sectioning. Technovit 7100 did not allow cutting with a razorblade, and it melted when cut with an electric jigsaw.

If the intestinal tract had been embedded in a stretched state, it would probably have been possible to saw as much as 20 slices instead of 8-10 slices, which would have reduced the variance and enabled better estimates to be obtained from the samples.

In all stereological studies, it is important that all directions and positions in the sample have the same probability of being sampled. With the directional nature of the intestine,

vertical uniform random (VUR) sampling is a good approach (Howard and Reed 1998). In the VUR protocol, a vertical axis is selected (in this case, chosen to be the same as the body axis). The vertical axis must then remain identifiable throughout the sampling hierarchy (Baddeley *et al.* 1986; Gundersen 2002).

The method used to get the sample unbiased implied cutting the slices in an angle through the centre of the slices (Fig. 2c), and since the digestive tract were folded when embedded, there was a lot of tissue-free space were they were cut (Fig. 2e and 6a). This gave a low estimated tissue fraction on each sample. To compensate for this, 32-45 counting fields for estimates of volume, and as much as 200 counting fields were necessary for the estimates of surface area. It is often assumed that one has to count thousands of points, but it is only necessary to sample enough counting fields to eliminate the variation between the fields. Generally one should never count more fields than to get 2-300 hits on the reference room (Gundersen *et al.* 1988a; Gundersen *et al.* 1988b). The variations in the estimates are mainly due to the fact that it was cut too few slices. And therefore it would not improve CE much by increasing the counting fields or points. The counting fields have to be randomly placed on the tissue section. To facilitate calculation of these positions, a spreadsheet was employed. This spreadsheet calculated the position of the fields, taking into account the position of the marks placed at the end of each tissue strip, as well as the tissue fraction, ensuring random, rather than arbitrary field selection.

To investigate this two sets of counting grids was applied, one point-counting grid for estimation of volume (Fig. 3b), and one cycloid grid for estimation of area (Fig. 5). Both the grids consist of two sets of counting patterns. The point-counting grid has one set of a few points with a ring around, which was used for counting hits on the reference room and another set of many points for counting the desired tissue. The cycloid grid has a set of cycloids for counting crossings of the wanted tissue type, and a set of points for counting hits on the reference room (Fig. 4). The difference in number of points used for the reference room and the organ in question must of course be taken into account when calculating the estimates.

5.3 Feed composition experiments

This study focused on the histological layers in the digestive tract of costal Atlantic cod fry. The analysis has searched for changes in estimated weight, volume and surface area of

selected sections of the tract, after feeding juvenile cod diets with varying proportions of macronutrients. Much of the nutrients are absorbed through the digestive tract, and structural changes in these organs in different regions of the tract may indicate histological adaptation to the diet. Induced changes may therefore be valuable to asses the effects of varying feed composition. Intestinal tracts of carnivorous fishes, as the Atlantic cod, are evolutionary adapted to digest nutrient-dense diets containing high levels of protein and low levels of carbohydrates. Throughout evolution, the diet available in the wild has been quite stable, and cod has little ability to adapt to changes in diet composition (Buddington *et al.* 1997). Significant differences were found in weight between the diet groups. These results are in accordance with studies done by (Hamre and Mangor-Jensen, personal communication). Similar results were found in studies done on Atlantic halibut (*Hippoglossus hippoglossus*, L) with the same experimental setup (Hamre *et al.* 2003).

Studies have shown that high levels of carbohydrates and/or high levels of fat cause an increase in the hepatosomatic index. Low proportions of carbohydrates and fat can cause low growth, as can high proportions (over 65%) of protein (Hamre and Mangor-Jensen, personal communication).

Results in this study showed that there was a good correlation between the individual fish weight and the weight of their intestinal tract, which indicate correlation in anatomy in all the fish in this study. Significant differences were found in the intestine surface ratio between the diet groups (Fig. 15). It should be noted, however, that when many variables are tested for differences, there is a chance of generating significantly different ($P < 0.05$) results by chance. This is due to an increased probability of obtaining a false result as the number of comparisons increases. This effect can be reduced by using the Bonferroni method (Cabin 2000; Garcia 2004). However, this method was not used in the present study. Given the high CE in all estimates, the significant differences found in estimated surface ratio in the intestine are probably not real. The estimates of volume, weight and surface area in this study showed no significant differences between the diet groups for any of the tissues being analysed.

The fish used in this study were selected arbitrarily from tanks. Consequently, there was a large variation in individual weight. Since the Atlantic cod are cannibals this may cause some effects on the variance in the tests. Some of the larger fishes may have eaten smaller fish and by doing this they may have eaten food with more fat or proteins than the smaller ones. This may have influenced the intestines, and these fish may have developed differences in the layers even before the experimental feeding started. Selecting fish of

similar size (both length and weight) may have reduced the variance in the estimates. At the time when the histological study started, selecting the size of the fish was, however, no longer an option.

This study show, as expected, that the estimated weight of mucosa in the stomach was higher than in the intestine. The mucosa in the stomach consists of long and packed tubular glands whereas the mucosa in the intestine is less packed and consists of large villi with a large surface area as shown in both the intestinal tract of cod and human (Morrison 1987; Young *et al.* 2000).

Measurements of the pancreas in cod are difficult to perform because this organ is distributed in the mesenteries close to the digestive tract and liver. The present study focused on the exocrine pancreas since the endocrine part of pancreas is found close to the liver and gallbladder which were removed prior to the embedding (Morrison 1987; Kryvi, personal communication, Institute of Biology, UIB, Norway). The exocrine pancreas was found to have an estimated mean weight of 0.04 g for a mean individual fish weight of 6.0 g.

Grouped volume estimates showed that the estimated volume fraction of mucosa in the stomach was generally higher than of mucosa in the intestine. Estimated volume fraction of pancreas had mean value of 0.08.

The same was found in the estimation of volume of mucosa in the stomach and the intestine. Pancreas had a mean volume of 0.04 mm^3.

The estimated surface area density (area/volume) showed that there was a high variance between individuals in the diet groups, but still the surface area density of the stomachs was higher than the estimated surface area density in the intestines. Much the same differences were shown between the estimated total area in the stomach and the intestine. These differences can be explained by the fact that the Atlantic cod is a carnivorous fish, and most of the food it eats is high in fat and proteins. The cod has a big mouth and a relatively large stomach with Y-form, and the intestinal tract is relatively short. These are adaptations for eating large predated animals (Kent and Miller 1997; Kryvi and Totland 1997).

The estimated surface ratio in the stomachs of the individuals also showed a wide variation. Individuals in all groups had values through the whole range of 2.5 to 11.2. The

present study showed that the different feed compositions most likely did not influence the surface ratio in the stomach. It also showed that there was a significant difference between at least one of the diet groups regarding the surface ratio for the intestine. Since ANOVA tests were performed on many parameters at the same time in present study, one test then may be affected by the Bonferroni effect and come out significant, even if it actually is not.

Estimations on pooled data showed that estimated volume fraction and estimated weight of muscularis in the stomach had the highest values of the studied part of the digestive tract. The muscularis in the stomach is relatively thick in order for the food to be crushed and knead. The mucosa has numerous glands secreting gastric juices for breakdown of proteins, and cells producing mucus for protection from self-destruction (Young *et al.* 2000). It had a higher estimated weight than the mucosa of the pylorus, but a lower estimated volume fraction.

Estimated volume fraction and estimated weight of the muscularis of the pylorus was less than in the stomach. This seems reasonable since there is less movement in this part of the digestive tract.

The mucosa in the intestine had a much lower estimated volume fraction than in the stomach and pyloric ceca. This also seems reasonable, since there is not much abrasion by muscular activity in this part of the tract.

Estimations of pancreas showed that it had about the same estimated volume fraction and estimated weight as mucosa and muscularis in the stomach.

Estimates of CE were similar for all volume fractions and surface area estimates in mucosa and the muscularis of the stomach, pylorus, intestine as well as those for pancreas volume fraction and volume. This indicates that the estimations are realistic, but probably, there must have been some systematic errors, as with the number of slices, early in the methods of the unbiased stereology. The number of specimens used in this study should preferably been higher, and the digestive tracts should probably have been cut into a higher number of slices in order to obtain a lower variation. As mentioned, this was not an option with the present material.

The estimated surface area density (area/volume) and estimated total surface area were both similar in the stomach and the pylorus, while the estimated surface area density of the intestine was only half that of the stomach and the pylorus. Little is known about what happens in the pyloric ceca, but this may indicate that juvenile cod either has a larger

surface area in the pyloric ceca, than in the stomach, for secretion of gastric juices and breakdown of nutrients, or it has a large surface area for abortion of nutrients.

5.4 Conclusions

1. A method for embedding and orienting the digestive tract of the Atlantic cod has been described.

2. The method is suitable for a thorough quantitative description of digestive tracts.

3. The methods are efficient, but they are not sufficient to study the effect of different feed composition since the histological layers of juvenile cod gave no significant differences based on different diets. The layers seem not to be influenced by the feed composition offered in the present study.

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7 APPENDIX

7.1 Figures

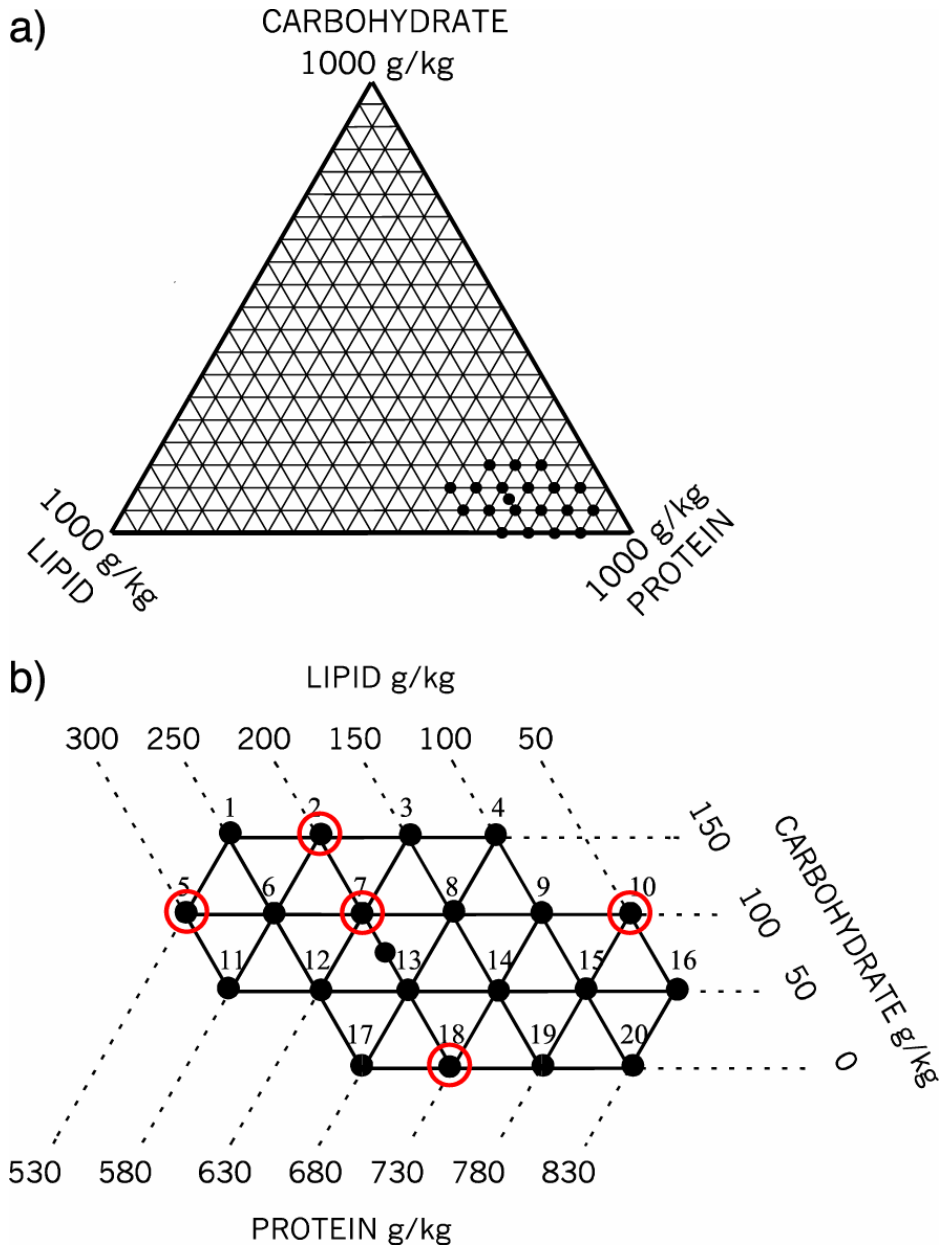

Fig. 1. Diet design. (a) The complete mixture design with the three variables. Diet 2, 5, 7, 10 and 18 (marked with red rings) were used in this thesis. (b) Centre point (basis diet) between diet 7 and 13 at 200 g kg $^{-1}$ lipid, 75 g kg $^{-1}$ carbohydrate and 655 g kg $^{-1}$ protein.

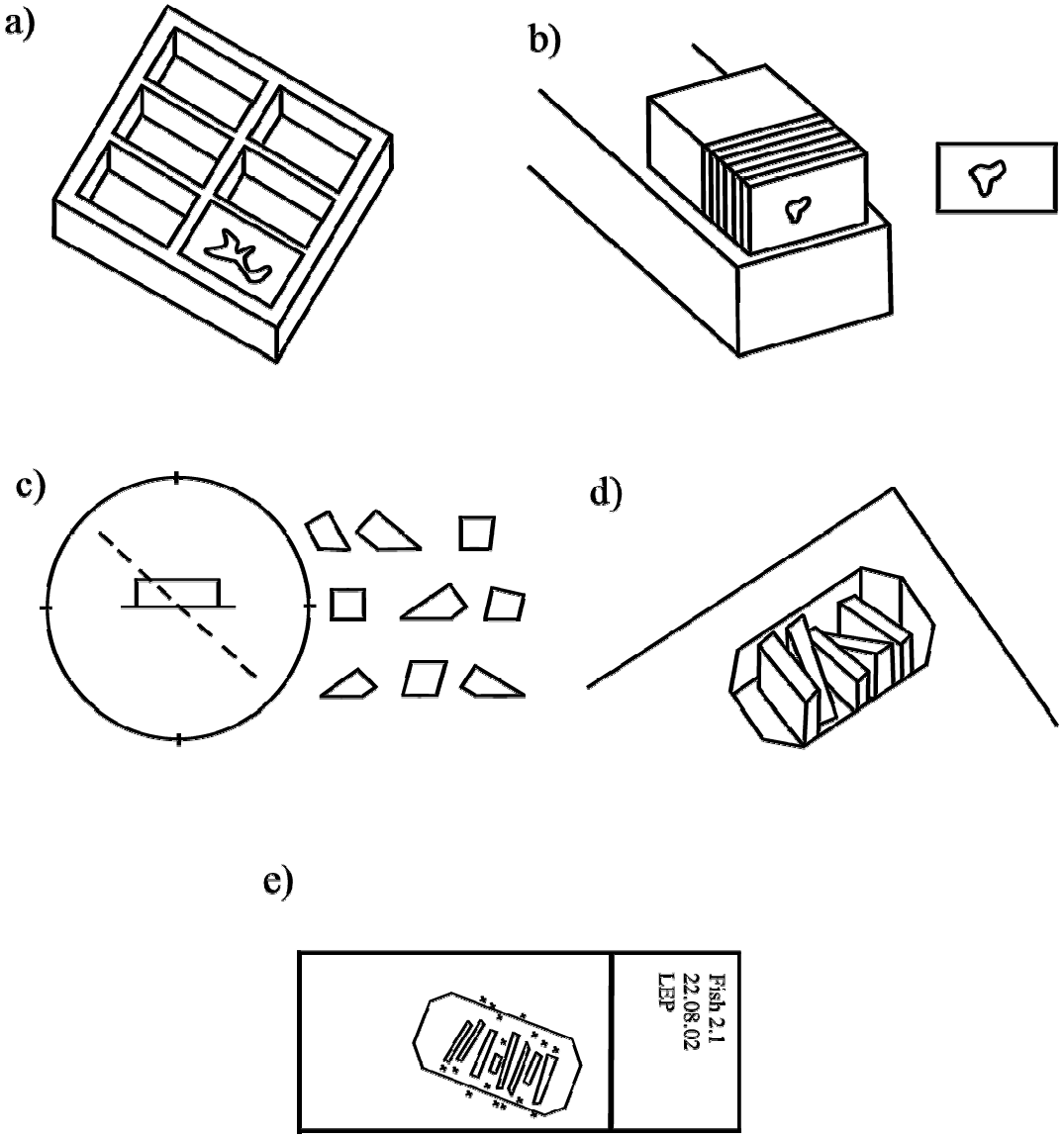

Fig. 2. Systematic random sampling. (a) All the samples (digestive tracts) were embedded in the same direction in Technovit 7100. (b) After hardening the samples were attached to a piece of wood with Technovit 3040. A line was drawn at random outside the tissue, and ten parallel lines were drawn on the block with regular intervals ensuring that the reference room was within these ten slices. (c) After sawing with a Japanese handsaw the ten slices were put on a plate with 360 gratings, always the same side up and sawn at an angle picked at random by angles given by a computer program. (d) One of the two halves from each of the ten slices was then reembedded with the sawn side down. (e) From this block semi-thin sections were made.

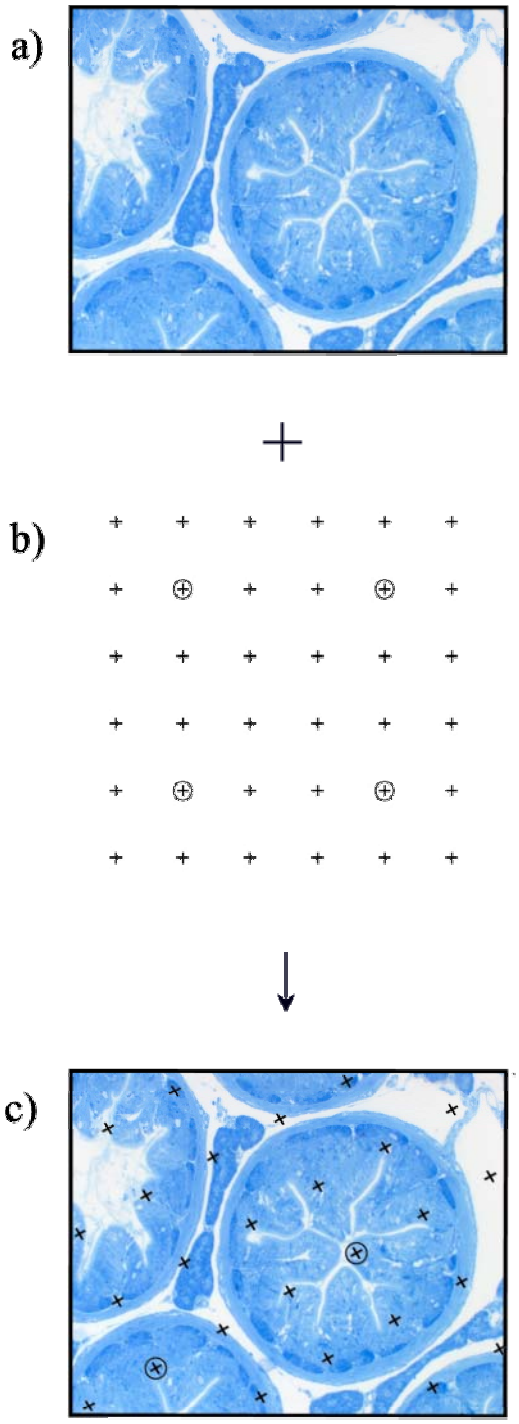

Fig. 3. Placing the counting grid. (a) Picture of a random place on a semi-thin section. (b) Counting grid (here a segment with 2 x 2 large points and 9 small points per large point). (c) Random placement of the counting grid on the picture. This was used for counting.

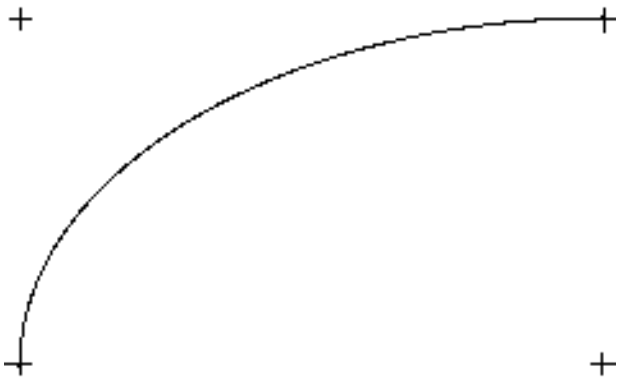

Fig. 4. The cycloid. This was used for estimating surface area. The length of the curve is twice its height (parametric equation of a cycloid: $x = \Theta - \sin\Theta$, $y = 1 - \cos\Theta$, where $0 \leq \Theta < \pi$)(Gundersen et al. 1988b).

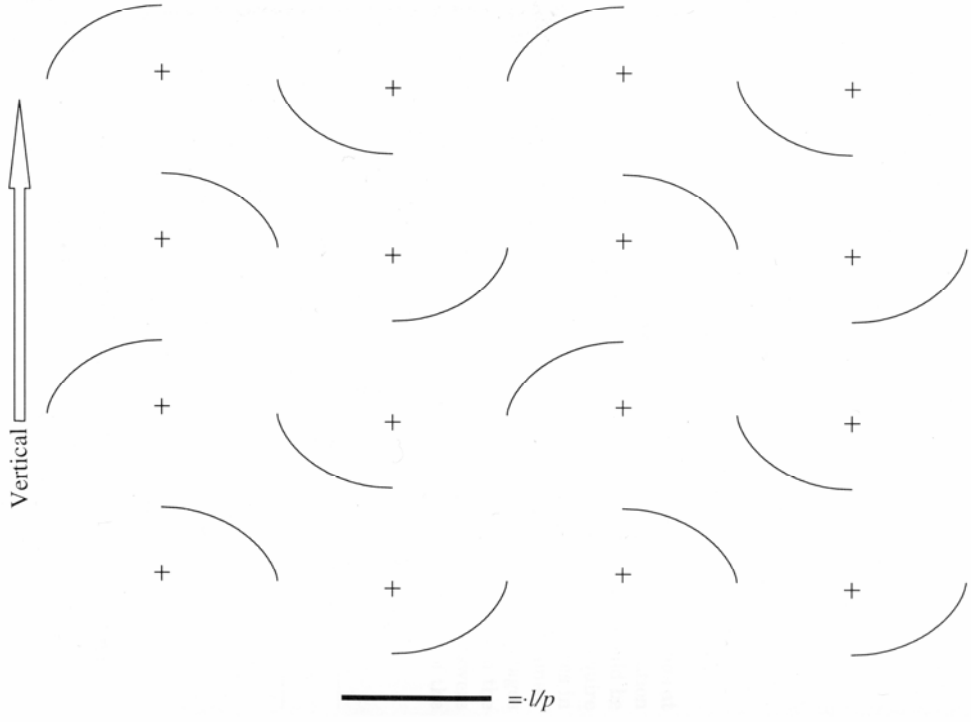

Fig. 5. The cycloid raster in full size (Howard and Reed 1998).

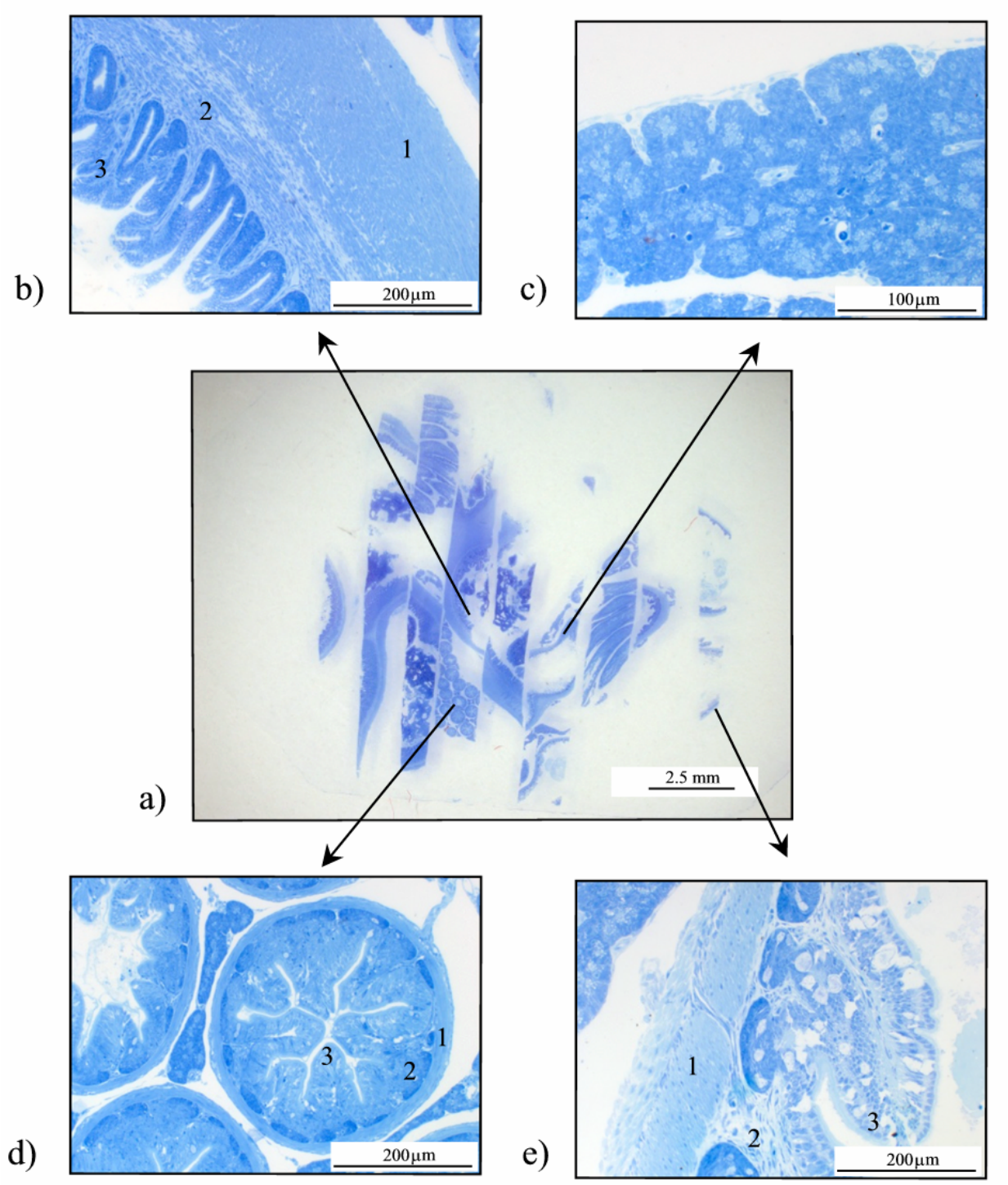

Fig. 6. The studied organs. (a) Overview image from one of the semi-thin sections (5.8x). (b) stomach (20x), (c) pancreas (40x), (d) pylorus area (20x) and (e) intestine (20x). The numbers show: 1. Muscularis, 2. Submucosa and 3.Mucosa.

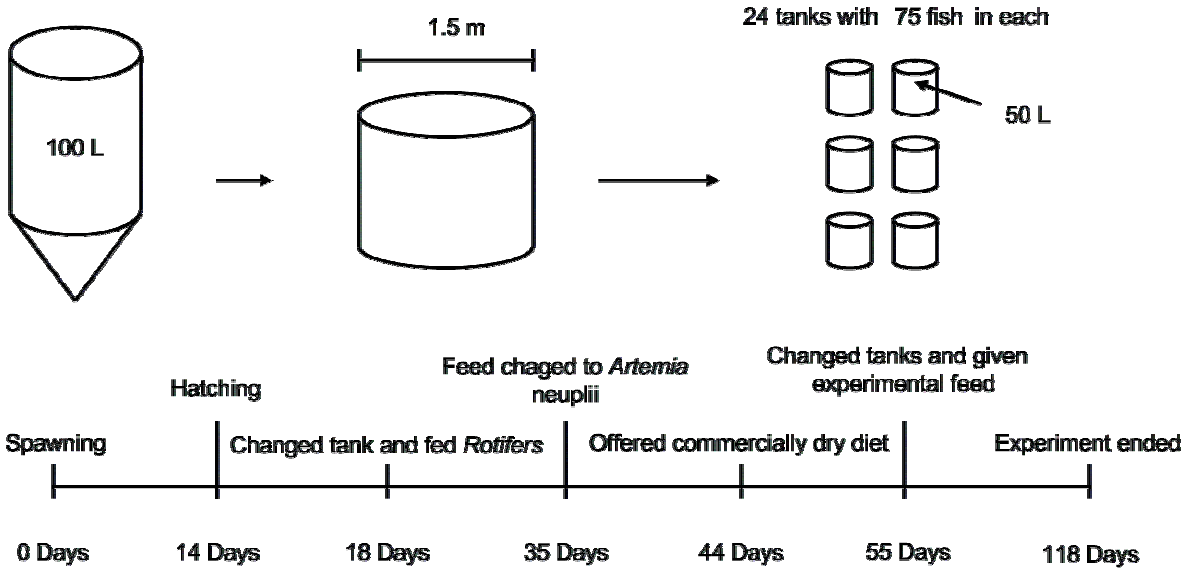

Fig. 7. Fish history. Spawning took place in September 2001. Fertilized eggs were collected and incubated in 100 L tanks with conic bottom. The eggs hatched 14 days later. The fish were transferred to first feeding tanks with a diameter of 1.5 m, and they were fed rotifers (*Brachionus sp.*) for the first time after 18 days. The food was changed to *Artemia* nauplii 35 days after spawning. After 44 days they were fed commercially dry diet. Tanks were changed and the experimental feeding started at the 55th day (75 fish in each of 24 tanks of 50 L). The feeding experiment lasted 63 days, until day 118 post spawning.

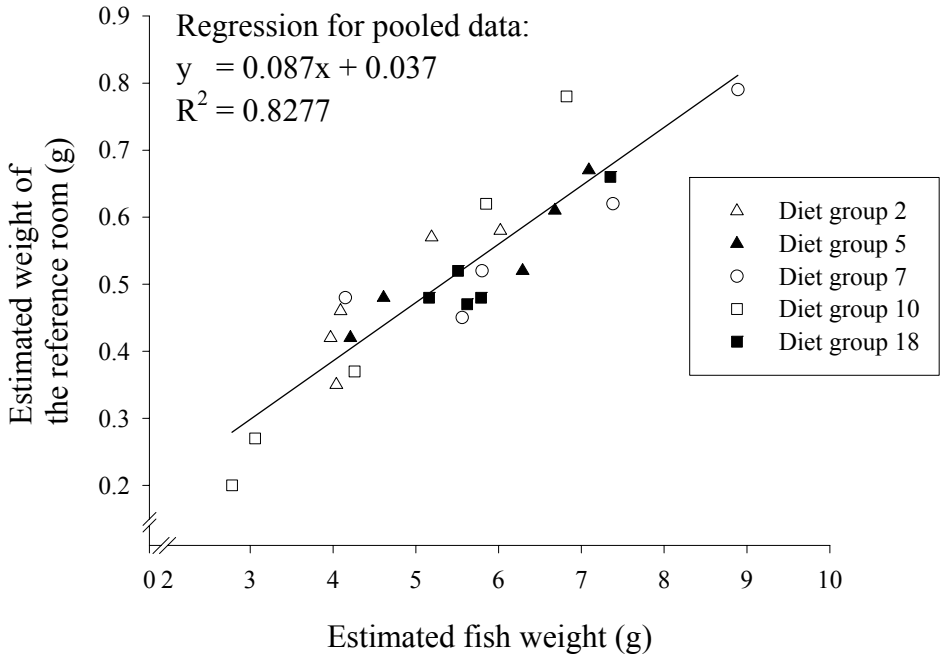

Fig. 8. The relation between estimated individual weight, and the estimated weight of the reference room for all the individuals in this present study. There was considerable variation in fish weight within, as well as between the diet groups.

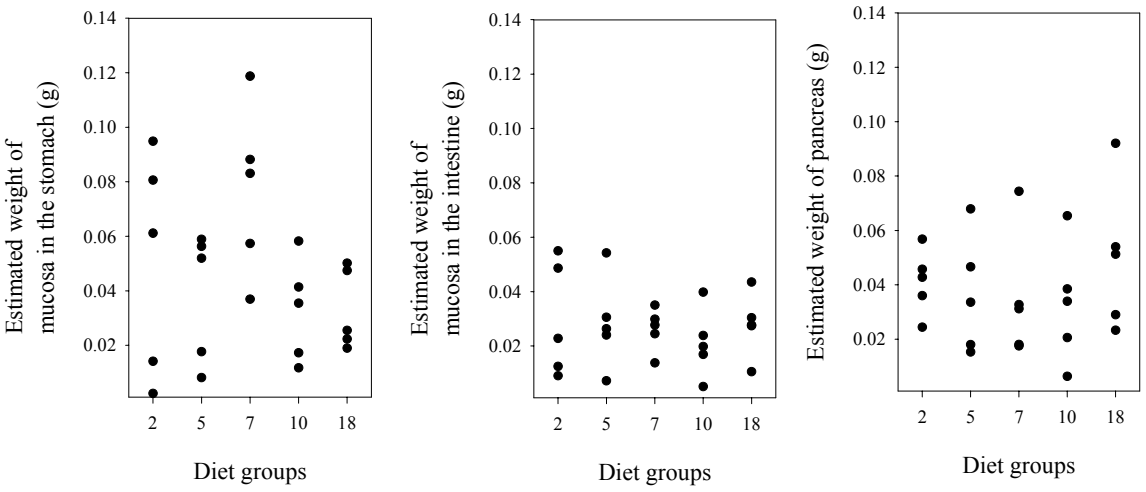

Fig. 9. Estimated weight (g) of mucosa in stomach, mucosa in the digestive tract and pancreas in individuals in the five diet groups.

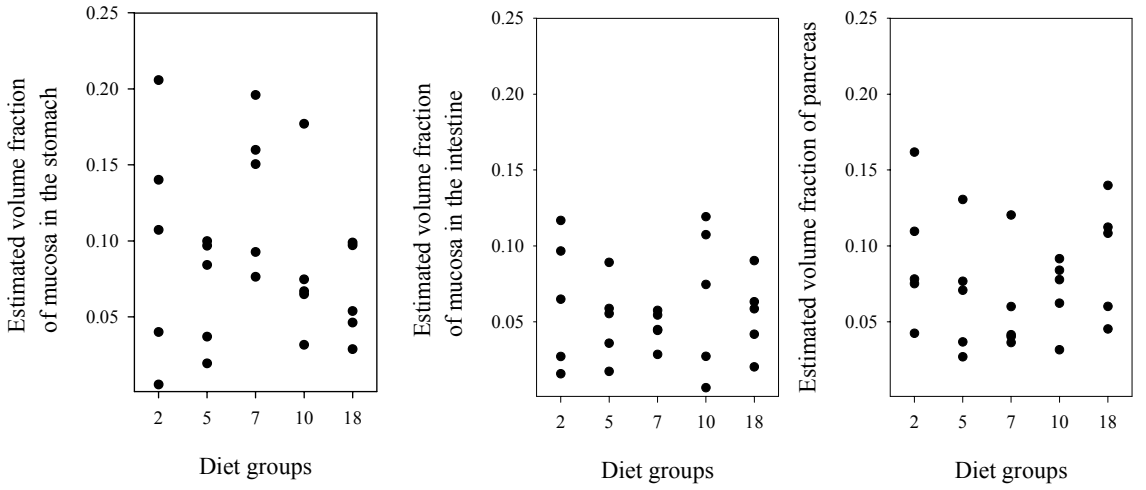

Fig. 10. Estimated **v**olume fraction of mucosa in stomach, mucosa in the digestive tract and of pancreas in the individuals in the five diet groups of the current study.

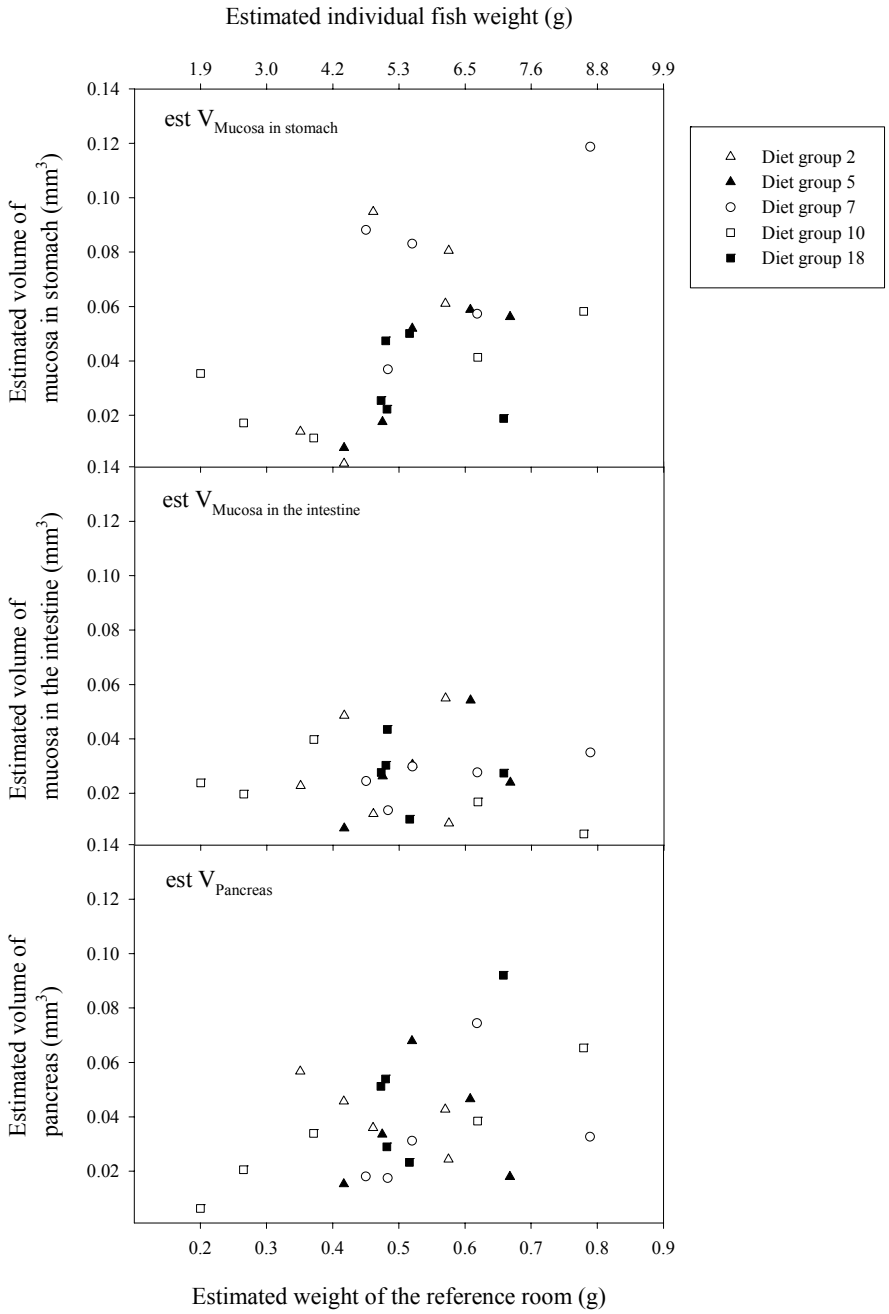

Fig. 11. Estimated volume of mucosa in stomach, mucosa in the digestive tract and in pancreas plotted against the estimated weight of reference room.

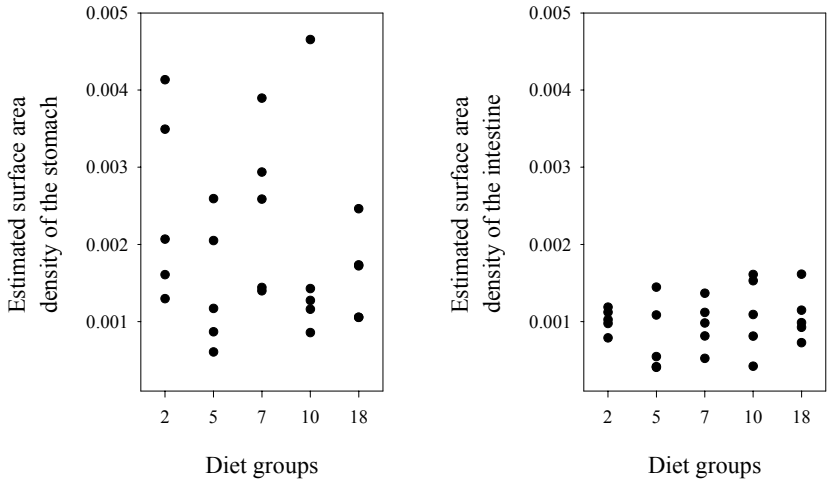

Fig. 12. Estimated surface area density of stomach and intestine. Somewhat surprisingly, the surface area density of the stomach was higher than that of the intestine.

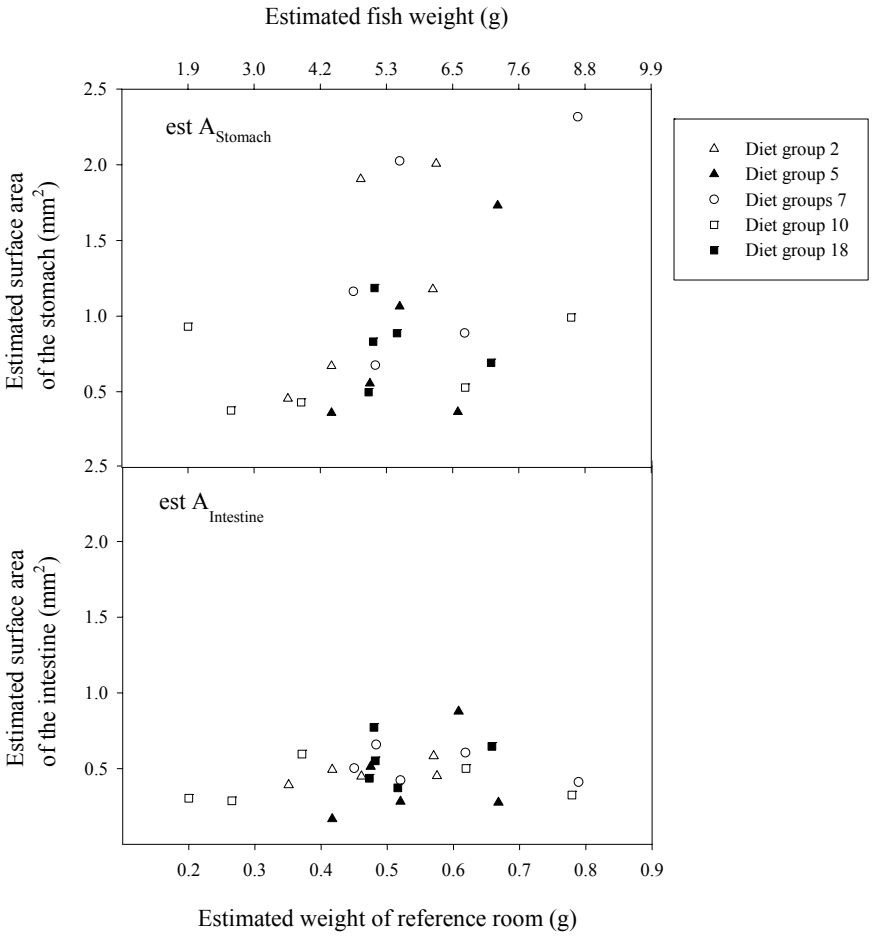

Fig. 13. Estimated surface area of stomach and intestine plotted against the estimated weight of reference room. The estimated fish weight was calculated based on the weight of the reference-room.

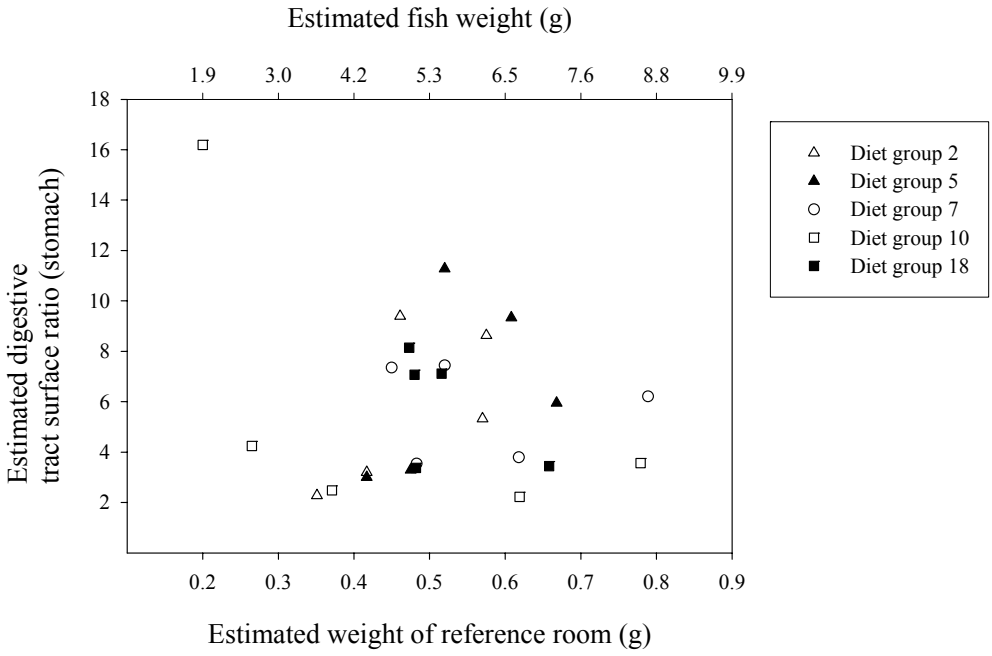

Fig. 14. Estimated digestive tract surface ratio (see text for definition) for the stomach.

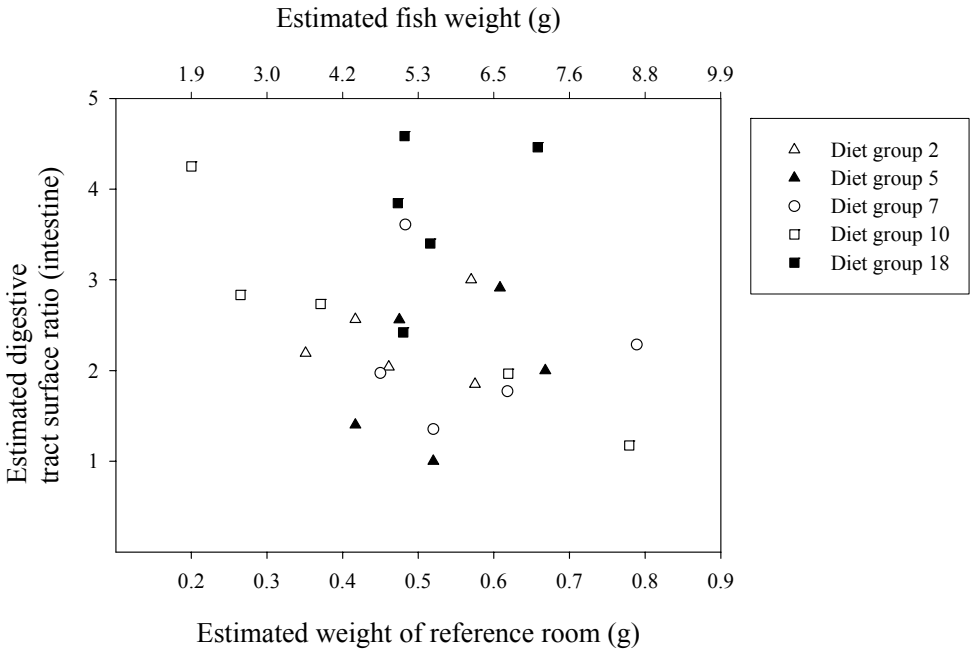

Fig. 15. Estimated digestive tract surface ratio for the intestine.

7.2 Tables

Table 1. Analysed and theoretical proximate composition and energy content of the experimental diets**.**

	Added (g kg^{-1} dry wt.)			Analysed (g kg^{-1} dry wt.)			Energy
Diet no.	Carbohydrate	Lipid	Protein	Carbohydrate[a]	Lipid	Protein	MJ kg^{-1}
1	150	250	530	188	227	530	25
2	150	200	580	166	185	577	24.1
3	150	150	630	176	142	620	23.6
4	150	100	680	179	91	663	22.6
5	100	300	530	135	271	537	26
6	100	250	580	120	240	580	25.6
7	100	200	630	109	197	630	24.9
8	100	150	680	109	150	675	24.1
9	100	100	730	109	98	723	23.1
10	100	50	780	115	42	768	22.1
11	50	300	580	72	284	586	26.6
12	50	250	630	60	243	625	25.7
13	50	200	680	53	202	679	25.3
14	50	150	730	50	150	732	24.4
15	50	100	780	53	98	776	23.4
16	50	50	830	80	48	793	22.3
17	0	250	680	5	253	675	26.4
18	0	200	730	0	202	733	25.7
19	0	150	780	3	150	774	24.6
20	0	100	830	0	92	829	23.6
21-24	75	200	655	86	200	645	24.9

[a] By subtraction: carbohydrate g kg^{-1} = 1000 g kg^{-1} – analysed (fat + protein + ash) g kg^{-1}.

Table 2. Number of individuals in the experiment, and the mortality in each group.

Diet group	2	5	7	10	18
Mortality (%)	15	25	29	20	34
n	67	53	59	55	58

Table 3. Estimated volume fraction and total weight of selected structures of the digestive tract in costal cod fry.

	Est. volume fraction	Std. dev.	Est. total weight (g)	Std. dev.	Est. CE	Std. dev.
Mucosa in stomach	0.091	0.056	0.047	0.030	0.530	0.211
Muscularis in stomach	0.088	0.053	0.048	0.035	0.527	0.140
Mucosa in pylorus	0.135	0.055	0.066	0.029	0.402	0.122
Muscularis in pylorus	0.034	0.013	0.017	0.008	0.398	0.088
Mucosa in gut	0.057	0.032	0.026	0.014	0.557	0.143
Muscularis in gut	0.028	0.018	0.013	0.009	0.614	0.180
Pancreas	0.077	0.036	0.039	0.021	0.398	0.087

Table 4. Estimated surface area density and total surface area of selected structures of the digestive tract in costal cod fry.

	Est. surface area density	Std. dev.	Est. total surface area (mm²)	Std. dev.
Stomach	0.0020	0.0010	0.989	0.528
Pylorus	0.0020	0.0008	1.105	0.476
Gut	0.0010	0.0004	0.476	0.164

7.3 Fixatives, Buffers and Stains for Histological Preparation

Neutral buffered formalin (pH = 7)

8.5 g $Na_2HPO_4 \cdot 2\ H_2O$

4.0 g $NaH_2PO_4 \cdot H_2O$

100 ml Formalin (approx. 37% Formaldehyde)

900 ml H_2O

Phosphate buffered saline (PBS) (0.2 *M*, pH = 7.2)

8.0 g NaCl

0.2 g KCl

14.4 g $Na_2HPO_4 \cdot 2\ H_2O$

2.3 g $NaH_2PO_4 \cdot H_2O$

Dissolved in 1000 ml distilled H_2O

Toluidinblue (1%) (Philpott 1966)

2.0 g borax solved in 100 ml distilled H_2O

1.0 g toluidinblue

The solution was filtered before using.