Further studies on Vitamins and the Reproductive Cycle of Ovaries in Cod (*Gadus morrhua*)

By

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INTRODUCTION

The relation between the contents of the B-vitamins pantothenic acid, riboflavin, niacin, vitamin B_{12} and biotin, and the reproductive cycle of the ovaries in cod (*Gadus morrhua*) has been reported in previous studies (BRÆKKAN, 1955, 1958 a). For pantothenic acid was found a unique relation, the values being very high in ovaries from juvenile fish and mature fish in the first stages of regeneration. Calculated on the basis of fresh weight, riboflavin, niacin and biotin did not show any variation of importance, while vitamin B_{12} showed a slight, but definite decrease during the maturation or regeneration. The values for this vitamin were not numerous enough to allow a final conclusion to be drawn.

In a preliminary communication (BRÆKKAN and BOGE, 1960) results were reported from an investigation of the relation between vitamin B_6 and the reproductive cycle of the ovaries in cod. The present paper gives a full account of this study. Further are reported additional results for vitamin B_{12} and biotin. In the previous studies the vitamin B_{12} content seemed to vary with the stages of maturation and regeneration, and it was found of interest to verify this finding and if possible reach a more final conclusion on this point. Biotin was not found to show variation of importance in the previous investigation. It has been included to assure a control of the comparative aspect of the studies.

The previous studies reported extensive data on the contents of moisture, protein and ash in the ovaries in relation to the reproductive cycle. In the present study only moisture has been determined, and the variation of the total dry matter in relation to the ovarian development has been summarized.

METHODS

Samples. The ovaries were collected from live cod brough directly from the fishing ground to the fish market in well boats. The fish was weighed and the ovaries were taken out, recorded and brought to the laboratory. Here they were weighed and examined on maturity. The

classification was carried out according to the method described by
SIVERTSEN (1935, 1939). The size of the ovaries as expressed by percent-
age of the body-weight was used as the main criterion for the deter-
mination of the stage of maturation or regeneration. The samples were
homogenized and either analysed at once or frozen and stored at below
— 15° C until further studies could be carried out.

Vitamin B_6 was determined microbiologically with *Saccharomyces
carlsbergensis* as test-organism. The method employed is mainly as
described by ATKIN et al. (1943). An experimental volume of 5 ml per
test tube was applied, as this volume had been found to give the most
reproducible results. Usually 3 g sample were weighed out. To this were
added 5.5 ml 2N H_2SO_4 and 195 ml water to give a final concentration
of 0.055 N H_2SO_4. Smaller samples were extracted with proportionally
less volumes of the acid. The mixture was autoclaved at 120° C for 4 hrs
and cooled. pH was adjusted to 5.5 with NaOH, and the extract diluted
to an exact volume before filtering. Aliquotes of the filtrate were diluted
with water to a final concentration of about 0.002 μg vitamin B_6 per ml.
After inoculation the tubes were incubated for 18 hrs in an air-incubator
at 30° C with constant shaking. The growth was measured turbidimetri-
cally in a Beckmann Modell B Spectrophotometer at 660 μm against
an inoculated blank.

Vitamin B_{12} was determined microbiologically with *Lactobacillus
leichmannii* as test-organism, according to the method of THOMPSON et al.
(1950). The vitamin was extracted by autoclaving with 50 ml M/15
sodium acetate buffer of pH 4.5 + 5 ml 1 % KCN per g sample, and
autoclaving for 15 min. at 120° C. The incubation was carried out for
20 to 22 hrs at 37° C in a water bath. The response was measured turbi-
dimetrically.

Biotin was determined microbiologically with *Lactobacillus plan-
tarum* as test organism. The extraction was carried out by autoclaving
1 g of the sample with 25 ml 3N H_2SO_4 for 3 hrs at 120° C. The digest
was neutralized to pH 4.5, diluted to volume and filtered. Aliquots
were neutralized to pH 6.8 and diluted to suitable concentrations. The
incubation was carried out at 30° C for 20 hrs in a water bath. The
response was measured turbidimetrically.

Moisture was determined by drying in a oven at 105° C for 20 hrs.

RESULTS

88 ovaries from live cod were investigated. When possible, all analyses
were carried out on each ovary. In case of small juvenile ovaries the size
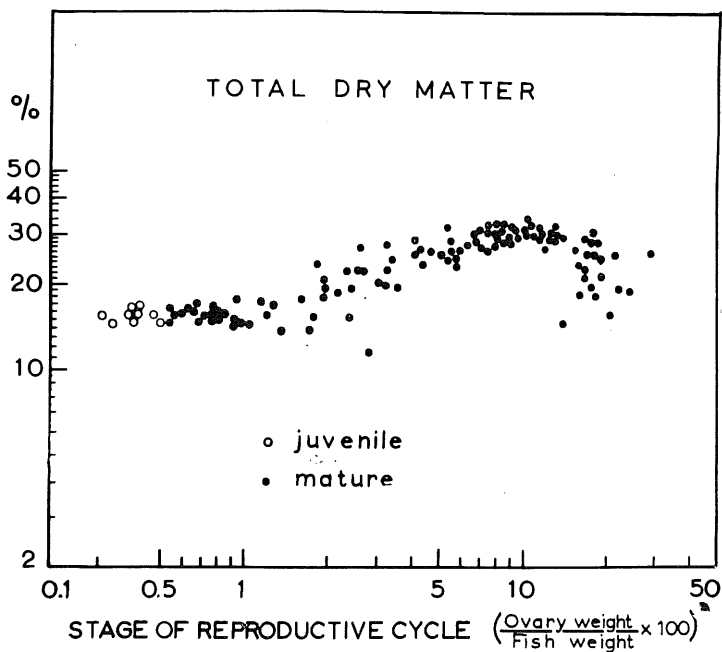often permitted only one or two different determinations, thus values

Fig. 1. Relation between percentage total dry matter and stage
of reproductive cycle in cod (*Gadus morrhua*).

from this stage were derived from different samples. In the previous paper (BRÆKKAN, 1958 a) the values for the vitamins were plotted and considered in relation to the weight of the ovaries. In the present paper the percentage ovary has been chosen, as it gives a more exact expression of the relation to maturation and regeneration, regardless of the size of the fish. Based on the observation of SIVERTSEN (1935, 1939), a percentage lower than 0.5 has been classified as juvenile. The results are plotted in Fig. 1—4. A log, log plotting has been applied. The graphs report all the single values. Calculations of the values in relation to the stages of the reproductive cycle are summarized in Table 1. The progress of maturation and regeneration has been divided into 7 stages in addition to the juvenile stage. The groupings < 0.5, 0.5—2, 2—4, 4—6, 6—8, 8—10, 10—13 and > 13 % were chosen to give a series of values over the whole range of the cycle. When the total dry matter was taken into consideration, it was found natural to put all the values for ovaries weighing more than 13 percent of the fish weight in one group. At this stage the dilution effect usually observed before spawning seems to have started, and the total dry matter decreases considerably.

The percentage total dry matter (Fig. 1 and Table 1) shows an increase from an average of 15.3 ± 0.52 in juvenile and 16.3 ± 0.38 in

Table 1. *The contents of vitamin B_6, vitamin B_{12}, biotin and dry matter inovaries from cod (Gadus morrhua) in to the stages of the reproductive cycle.*

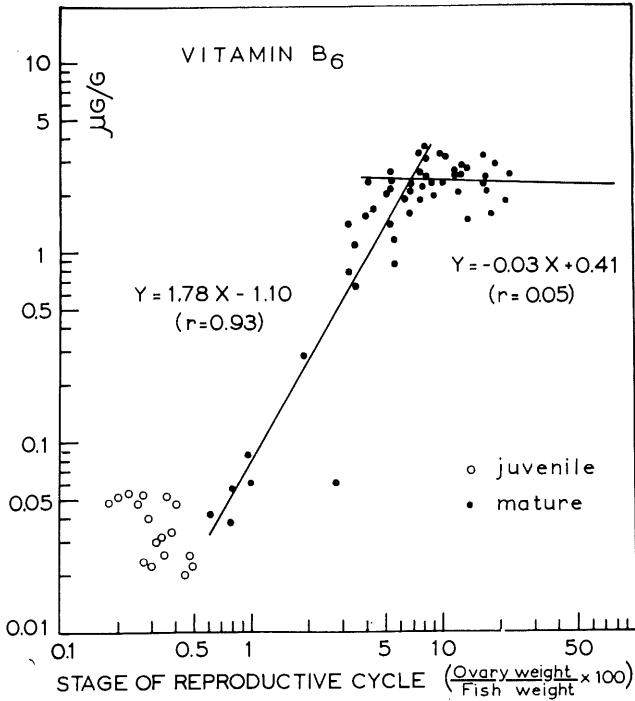| Stage of reproduction $\left(\dfrac{\text{Ovary weight}}{\text{Fish weight}} \times 100\right)$		I < 0.5	II 0.5 — 2.0	III 2.0 — 4.0	IV 4.0 — 6.0	V 6.0 — 8.0	VI 8.0 — 10	VII 10 — 13	VIII > 13
Total dry matter (%)	(Number samples)	(7)	(32)	(14)	(13)	(13)	(9)	(13)	(21)
	Min —max	14.3 —16.1	13.7 —23.9	11.4 —27.8	23.2 —32.7	26.8 —32.6	28.3 —32.3	26.9 —32.0	14.3 —31.2
	M $\pm s_M$	15.3 ± 0.52	16.3 ± 0.38	20.9 ± 1.15	26.4 ± 0.70	29.3 ± 0.51	30.3 ± 0.49	30.2 ± 0.33	23.9 ± 0.99
Vitamin B_6 μg/g wet weight	(Number samples)	(18)	(6)	(6)	(10)	(8)	(6)	(8)	(9)
	Min —max	0.02 —0.05	0.04 — 0.28	0.06 —1.39	0.85 —2.39	1.59 —3.49	1.94 —3.45	2.02 —3.11	1.44 —3.11
	M $\pm s_M$	0.04 ±0.003	0.09 ±0.04	0.71 ±0.20	1.79 ±0.18	2.55 ±0.22	2.59 ±0.25	2.56 ±0.12	2.23 ±0.19
μg/g dry matter	(Ave.)	0.226	0.577	3.40	6.78	8.70	8.54	8.49	9.35
Vitamin B_{12} μg/g wet weight	(Number samples)	(27)	(7)	(6)	(12)	(8)	(6)	(8)	(14)
	Min —max	0.195 —0.606	0.160 —0.390	0.135 —0.327	0.096 —0.327	0.067 —0.228	0.106 —0.221	0.105 —0.227	0.044 —0.151
	M $\pm s_M$	0.359 ±0.019	0.266 ±0.043	0.224 ±0.027	0.222 ±0.023	0.163 ±0.024	0.149 ±0.018	0.139 ±0.016	0.092 ±0.008
μg/g dry matter	(Ave.)	2.35	1.63	1.07	0.84	0.56	0.49	0.46	0.38
Biotin μg/g wet weight	(Number samples)	(27)	(8)	(6)	(12)	(8)	(6)	(8)	(15)
	Min —max	0.072 —0.191	0.066 —0.250	0.190 —0.250	0.162 —0.280	0.101 —0.305	0.169 —0.289	0.140 —0.215	0.073 —0.180
	M $\pm s_M$	0.119 ±0.005	0.141 ±0.023	0.220 ±0.011	0.206 ±0.010	0.218 ±0.023	0.222 ±0.022	0.171 ±0.010	0.132 ±0.008
μg/g dry matter	(Ave.)	0.78	0.87	1.05	0.78	0.74	0.73	0.57	0.55

Fig. 2. Relation between vitamin B_6 per g wet weight and stage of reproductive cycle in cod (*Gadus morrhua*)

$$\left(X = \log \left(\frac{\text{Ovary weight}}{\text{Fish weight}} \cdot 100 \right) \; ; \; Y = \log \mu\text{g vitamin } B_6 \text{ per g} \right).$$

ovaries in the first stages of maturation, to values of the order of 30 $\pm$ 0.5 when the percentage ovary is 6 to 13. It then decreases rapidly, and in the last stage ovaries larger than 13 percent of the fish weight show an average of 23.9 $\pm$ 0.99 %.

Vitamin B_6 (Fig. 2 and Table 1) shows a rapid increase from 0.04 $\pm$ 0.003 μg/g fresh weight in juvenile ovaries to 2.55 $\pm$ 0.22 μg/g in the 5th stage of the present grouping. In the following stages where the ovaries exceed 6 % of the weight of the fish, the values remain on this level. Calculated on the basis of dry matter, a similar trend is observed, from 0.266 μg/g in juvenile ovaries to 8.70 μg/g in stage V.

Vitamin B_{12} (Fig. 3 and Table 1) shows decreasing values from 0.359 $\pm$ 0.019 μg/g in the juvenile to 0.092 $\pm$ 0.008 μg/g fresh weight in the last stage just before spawning. Calculated on the basis of dry matter the decrease is most pronounced in the three first stages, from 2.35 to 1.07 μg/g. It further decreases more slowly to show 0.38 μg/g in the last stage before spawning.
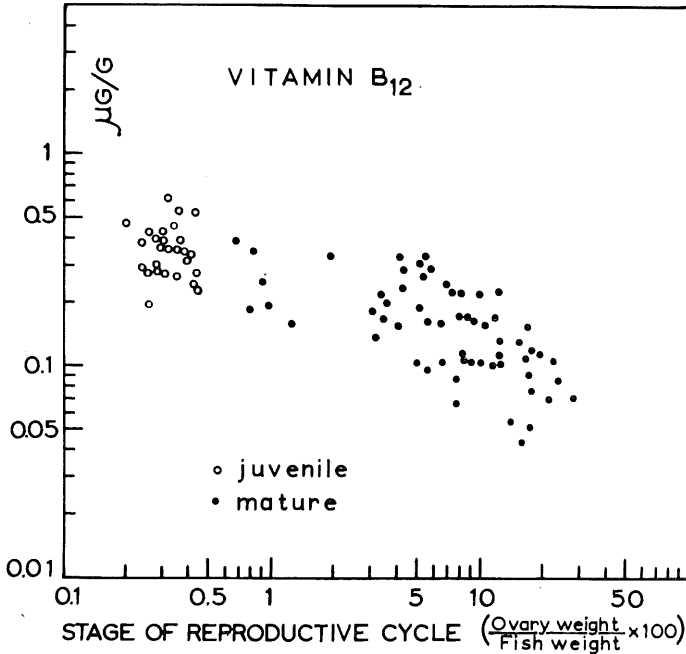
Fig. 3. Relation between vitamin B$_{12}$ per g wet weight and stage of reproductive cycle in cod (*Gadus morrhua*).

Biotin (Fig. 4 and Table 1) shows an increase from 0.119 $\pm$ 0.005 μg/g fresh weight in juvenile ovaries, to 0.220 $\pm$ 0.011 μg/g in the second stage of maturation. It remains at this level to decrease in the last two stages to 0.132 $\pm$ 0.008 μg/g. Calculated on the dry matter the values show a similar, but much less pronounced trend, the average being in the order of 0.75 μg/g.

DISCUSSION

The reproductive cycle in fish involves the mobilization of relatively large quantities of nutrients. During the development of the eggs in the ovary, the maternal body supplies these different substances. HOAR (1957) has discussed the problem of gonads and reproduction in fish, and pointed out that development of secondary sex characters in some cases require profound reorganization of tissues and development of new structures. The picture is further complicated as the sexual maturation and related changes frequently occur during shorter or longer periods of fasting. Thus during maturation there is usually a larger deposition of nutrients such as protein, fat and carbohydrate in the
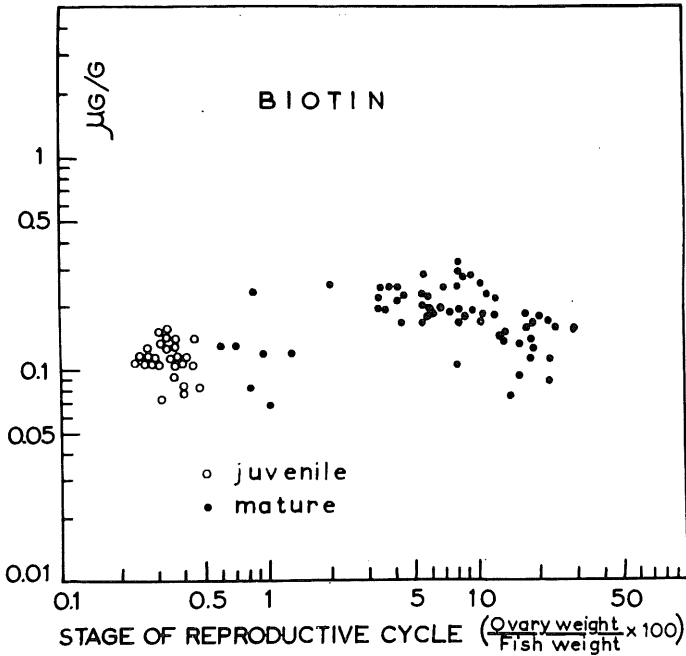
Fig. 4. Relation between biotin per g wet weight and stage of repro-
ductive cycle in cod (*Gadus morrhua*).

ovary and exhaustion of tissues of the maternal body. In the previous
paper (BRÆKKAN, 1958 a) was reported chemical analysis of percentage
moisture, protein and ash. It was pointed out that the increase in total
dry matter during maturation mainly constitutes an increase in the
protein content. In the present paper only values for total dry matter
are reported. As no comprehensive studies have previously been reported
on the relation between total dry matter and the reproductive cycle
of ovaries in cod, it was found of value to compile all our findings.
Altogether 122 analyses have been carried out and the results are re-
corded and summarized in Fig. 1 and Table 1. The total dry matter
increases during maturation from 15.3 % in juvenile ovaries to about
30 %, then decreases considerably just before spawning. PLACK et al.
(1961) have recently reported on the weight and dry matter of ovaries
in cod. They found an increase from 16 to 29 % during maturation,
but claim that this increase did not take place until the later stage of
the ripening process. The present findings from a considerably larger
number of samples over the whole reproductive cycle show the increase
to start at an early stage of the ripening process. Thus t-tests on the
results reported in Table 1 for the stages I—VIII gave the following

result. There is no significant difference between the juvenile ovaries in stage I and the first stage of maturation or regeneration (II). Comparisons between the stages II and III, III and IV, and IV and V show highly significant differences ($p < 0.001$). Between the stages V and VI, and between VI and VII the differences are not significant, while finally the difference between the stages VII and VIII is highly significant ($p < 0.001$). It may thus be concluded that the total dry matter of the ovaries in cod increases fairly steadily and significantly during regeneration and maturation, to reach a maximum of about 30 %, and just before the time of spawning it shows a significant decrease.

Information on the content of vitamins in the gonads of fish is slowly accumulating (LOVE et al. 1959). Most values, however, derive from nutritional studies, and thus only refer to the mature stages. The first extensive investigation of the relation between the vitamin content of ovaries and the reproductive cycle in fish was carried out by BRÆKKAN (1955, 1958 a). The present results represent an extension of these studies to vitamin B_6, and to additional investigations of vitamin B_{12} and biotin.

Vitamin B_6 (Table 1 and Fig. 2) shows a strong increase up to a certain stage of maturation or regeneration. The values then remain fairly constant up to the time of spawning. t-test applied to the values reported in Table 1 for the stages I to VIII gave the following results: Juvenile ovaries (I) and ovaries in the first stage of maturation (II) show no significant difference. The differences between the stages II and III, III and IV, and IV and V, are all significant, while the differences between the stages V and VI, VI and VII, and VII and VIII are not significant. Based on these calculations the values in Fig. 2 have been treated in 3 groups. One comprises the juvenile ovaries (I), one the stages of increase (II—V), and one the stages of maximum values (V—VIII). The juvenile ovaries are samples unevenly distributed over a small percentage range, and further treatment of these results has not been found justified. From stage I to stage V the increase is significant and rather strong, the regression coefficient being 1.78. From stage V to stage VIII the values are fairly constant, as the regression coefficient (− 0.03) does not deviate significantly from zero ($0.8 > p > 0.7$). The vitamin B_6 concentration per g fresh weight thus increases considerably during the first stages of maturation or regeneration. It should, however, be noted that the increase is from very small values up to values normally found in fish and fish products (BRÆKKAN and BOGE, 1960). Calculated on the basis of dry matter, the vitamin B_6 values show the same general trend, increasing from 0.226 to about 8.75 μg per g. It may be pointed out that vitamin B_6 does not decrease significantly at the very last

stages as observed for vitamin B_{12} and biotin (see below). Calculated on the basis of dry matter, even a slight increase is observed. To few values are reported for final conclusions to be drawn, but the possibility that vitamin B_6 is mobilized to participate in the embryonic development after spawning and fertilization should not be overlooked.

Vitamin B_6 as pyridoxal phosphate participates in the activation of amino acids and amines. Thus with the exception of its role in phosphorylase (CORI and ILLINGWORTH, 1957), all of the pyridoxal phosphate-dependent reaction so far known are reactions of amino compounds, and it is chiefly the reactions of α-amino acids which are dependent on this factor (SNELL, 1958). NICKERSON (1958) has reviewed and discussed the biochemistry of morphogenesis. He emphasized the importance of the protein synthesis, and pointed out that our present knowledge only permits discussion of a few steps in these processes. The first step actually involves the activation of α-amino acids. Thus a direct relation between vitamin B_6 and the processes of morphogenesis of the ovaries in cod seems indicated. The extreme increase in the protein synthesis during the maturation or regeneration of the ovaries demands the presence of increased amounts of enzymes of which vitamin B_6 is part.

Vitamin B_{12} shows a decrease with the progress of maturation or regeneration, thus the finding in the previous study was confirmed (BRÆKKAN, 1958 a). The close agreement between the results justifies to treat all values summarized to reach more final conclusions. t-test on the values reported in Table 1 for the stages I to VIII gave the following results: There is a significant difference between the stages I and II $(0.05 > p > 0.02)$. Between the stages II and III, III and IV, IV and V, and V and VI the differences are not significant, while the difference between VII and VIII is significant $(0.01 > p > 0.001)$. Between the stages II and IV, and V and VI the differences are not significant, while the differences between II and V $(0.05 > p > 0.02)$ and between IV and VI $(0.02 > p > 0.01)$ are significant. From this may be concluded that juvenile ovaries from cod contain significantly more vitamin B_{12} than ovaries in the progress of maturation. It is, however, a slight but significant decrease during this process, from 0.266 to 0.139 μg per g fresh weight in the present samples. At the very last stage before spawning the concentration decreases significantly. Calculated on the basis of dry matter, the trend is very similar. BRÆKKAN (1958 b, 1959) has reported values for vitamin B_{12} in ovaries from different fish. When his results are considered in relation to the present finding of a variation with the reproductive cycle, it is interesting to note that the highest values frequently are found in unripe ovaries. Thus the following values for some species were reported: Ripe ovaries from: cod (*Gadus morrhua*)

0.10, herring (*Clupea harengus*) 0.13, and for mackerell (*Scomber scombrus*) 0.30 µg vitamin B_{12} per g fresh weight. Unripe ovaries at unspecified stages: pollach (*Gadus pollachius*) 0.18, haddock (*Gadus aeglefinus*) 0.66, ling (*Molva molva*) 0.50 and torsk (*Brosmius brosme*) 0.70. Even higher concentrations are encountered, thus samples of ripening ovaries from salmon (*Salmo salar*) contained 0.64, 1.4 and 1.5, and from troug (*Salmo trutta*) 1.0 and 2.3 µg vitamin B_{12} per g. fresh weight (BRÆKKAN, unpublished). It may be pointed out that the male gonads ("soft roe") from this species do not show similar high values for vitamin B_{12}.

Biotin showed no variation of importance in the previous study with relatively few samples (BRÆKKAN, 1958 a). The trend is similar in the present study and the values of the same order, thus the results have been treated together. t-test on the values in Table 1 showed no significant difference between the stages I and II, or between juvenile ovaries and the first stage of the reproductive cycle. There is, however, significant differences between the values for stage II and III ($0.01 > p > 0.001$), and highly significant differences between VII and VIII ($p < 0.001$). Thus the biotin content seems to increase at the very early stage of maturation or regeneration, to remain fairly constant until just before spawning, when a significant decrease takes place. It may be pointed out that the values for biotin thus follow the same trend as those for total dry matter. This is further emphasized by the values for biotin calculated on the basis of dry matter, which show more moderate variation and generally values of the order 0.75 µg/g.

Studies on the importance of vitamins for the reproduction of different species are numerous. The various aspects of this problem have been treated in several reviews. LUTWAK—MANN (1958) has reviewed the problem of the dependence of gonadal function upon vitamin and nutritional factors. She gives a comparative account of the relation in different groups of species, and points out the incredible plasticity of the early embryonic tissues, the readiness with which they respond to alterations in the flow of nutrient material.

The previous (BRÆKKAN, 1958 a) and the present findings with regard to the relation between some B-vitamins and the reproduction cycle in cod is difficult to interprete conclusively. Some parallells, however, may be drawn and discussed. In birds the ovary itself has not been much studied, but its product the eggs. They lend themselves excellently to fruitful studies. The reproductive performance may be assessed by the rate of egg production, fertility and hatchability. Such studies are rather numerous, and it is interesting to note the similarity in the high concentrations of vitamins in hens eggs as well as in fish roes. The different vitamins in the hens diet seem to pass readily into the egg

in rather high proportions. The high values for vitamin B_{12} in fish ovaries should thus be considered in relation to the findings of DENTON et al. (1954) in hens. They found that in hens first depleted in vitamin B_{12}, 20—30 % of parenterally or orally administered vitamin B_{12} entered readily into the egg yolk. Even in insects studies of comparative interest have been carried out. Thus PAIN (1951) investigated the importance of B-vitamins for the ovarian development in worker honeybees to make them queens. As a parallell to the present findings for vitamin B_6 in cod ovaries, it is interesting to note that he claimed all vitamins investigated to be essential, but particularly vitamin B_6 influenced the progress of the processes. Finally may be pointed out some studies with the sea urchin. This marine invertebrate, which has served as model object in so many biological investigations, has not escaped vitamin studies. Here shall only be mentioned the studies of the metabolism of B-vitamins and related compounds in embryonic sea urchin development. BANHIDI and KAVANAU (1956) found correlations between pantothenic acid, and free amino acids. They suggested that in the sea urchin embryo the supply and withdrawal of free pantothenates may be a regulatory mechanism for reactions in which they or CoA participate. Thus an equilibrium between the concentration of free pantothenic acid and CoA is proposed. It may be recalled that BRÆKKAN (1955, 1958 a) reported the high values for pantothenic acid in cod ovaries to refer to the free water-extractable vitamin. KAVANAU and BANHIDI (1956) investigated niacin in sea urchin development and concluded that the high content of free niacin in sea urchin embryo may have some other physiological role besides serving as precursor for the pyridine nucleotide enzymes.

The present findings indicate the possible specific importance of some of the B-vitamins in reproduction in fish. Fishes should lend themselves very conveniently for reproductive studies. A planned set up with a large enough number of uniform experimental fishes should make it possible to collect samples from all the stages: gonadal development, fertilization and embryonic development. Thus controlled studies of the importance of vitamins and other nutritional factors for the reproduction in fish could be performed. Such studies should prove very important from the point of view of fish culture, which has gained increased importance as a mean of increasing food production.

SUMMARY

The relations between vitamin B_6, vitamin B_{12}, biotin and total dry matter and the reproductive cycle of the ovaries in cod has been investigated.

The stage of maturation or regeneration was expressed as the percentage ovary of the fish weight, and the cycle divided into 8 groups from the juvenile stage to the pre-spawning stage.

Vitamin B_6 showed a strong increase in the first stages, from 0.04 to 2.5 μg per g fresh weight. It remained on this level until spawning. Calculated on the basis of dry matter, the trend was the same and the corresponding values 0.23 to 8.5 μg per g.

Vitamin B_{12} showed a significant decrease from 0.359 μg per g fresh weight in juvenile to 0.266 in the first stage of maturation. It then decreased slowly but significantly to 0.139 to drop just before spawning to 0.092 μg per g fresh weight. The corresponding values calculated on total dry matter were 2.35, 1.63, 0.46 and 0.38 μg per g.

Biotin showed an increase in the first stages from 0.119 to 0.220 μg per g fresh weight. Just before spawning the concentration decreased to 0.132 μg per g fresh weight. The trend is similar to the change in dry matter, and calculated on this basis the values remained about 0.75 μg per g until the time of spawning, when it decreased to 0.55 μg per g.

Total dry matter increased significantly from 15.3 % to about 30 % to decrease significantly just before spawning.

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