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Humoral immune parameters in Atlantic cod (*Gadus morhua* L.) II. The effects of size and gender under different environmental conditions

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Abstract

The effects of size and gender on several humoral immune parameters in cod were examined under different environmental conditions. Serum samples were collected from wild cod of different sizes. Two samplings were undertaken: In the spring in relatively cold waters off the north west coast of Iceland and in the fall in relatively warm waters off the west coast of Iceland. Most of the parameters increased with increasing cod size, except the haemolytic activity which decreased. Higher serum protein levels were seen in cod sampled in the fall than in the spring. In cod sampled in the spring there was an apparent difference between specimens < 75 cm in length and the larger specimens with respect to haemolytic activity and iron concentration. None of the parameters were influenced by the gender of the cod. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introdution

Atlantic cod (*Gadus morhua* L.) is an economically important fish species and there is a growing concern for its survival in some ocean areas due to climatic changes and increasing pollution [8,9,17]. In this context it is important to know how environmental changes might affect the immune system of cod, and hence its ability to combat diseases, at different stages in their life history and seasonal cycle.

The immune system of Atlantic cod (*G. morhua* L.) has not been extensively examined but different studies have indicated some unusual features. Cod serum contains a relatively high level of natural antibodies [23,31] and the immunoglobulin (IgM) concentration is high [18,23]. Most fish species exert an adequate humoral antibody response against various antigens, although often of limited protective value [11,22]. Attempts to induce humoral antibody response in cod have been unsuccessful in spite of giving protection against infection [12,31,33].

In view of this it is believed that in cod, as in many other fish species, non-specific immune factors, as well as the cellular immune system, are important to their

Abbreviations: Asa, *Aaeromonas salmonicida* ssp. *achromogenes*; BSA, bovine serum albumin; ECP, extracellular product; Glyc, glycogen; LPH, *Limulus polyphemus* hemocyanin; Pf, *Pseudomonas fluorescens*; ssDNA, single stranded DNA; Thyr, thyroglobulin; TNP, trinitrophenyl.

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immune defence [1]. Only limited studies have been made of these components of the immune system of cod [2,16,23,36].

Studies have shown how environmental and seasonal changes can influence various physical and biochemical parameters in cod [21]. It is also well documented that environmental changes affect the immune system of fish [5,20,28,39]. The object of this study was to examine if and how certain immunologically important humoral parameters of cod are influenced by size and gender under different environmental and seasonal conditions.

2. Materials and methods

2.1. Fish

Wild cod of different sizes (20–100 cm) were sampled at sea by trawling. From four to 20 fish (average 12) were sampled for each 10 cm size interval. Two samplings were carried out. The first sampling (110 fish) was in late spring, 15–19 June 1995, off the north west coast of Iceland (Vestfirðir), at near-bottom sea temperatures from -0.1 to 4.1°C. This was a cold winter and spring. Normally the near-bottom mean temperature in this area in June is about 5.1°C [35]. The second sampling (91 fish) was in the fall, from 2 to 6 October 1996, off the west coast of Iceland (Faxaflói), at near-bottom sea temperatures from 6.1 to 10.0°C. These temperatures were close to the expected mean value for this area in October (8.3°C) and just below the seasonal maximum near-bottom temperature of 9.3°C in August [35].

2.2. Sampling

The wet weight and fork length of the fish were recorded, sex determined and otoliths collected for age determination. Blood samples, collected from the caudal aorta were allowed to clot at 22°C for 1–2 h and then at 4°C overnight. Serum, collected after centrifugation at $750 \times g$ for 10 min, was divided into several aliquots and stored at -20°C.

2.3. Protein concentration

A protein assay kit (Pierce, USA) was used for protein estimation following the manufacturer's instructions. The kit was based on the analytical method described by Bradford [7] with bovine serum albumin (BSA) used as the standard protein.

2.4. Immunoglobulin (IgM) concentration

The total immunoglobulin (IgM) concentration in cod serum was measured using the capture-ELISA described by Pilström and Petersson [31] and Israelsson and co-workers [18], with minor modifications to incubation times as described previously [23].

2.5. Natural antibody activity

The antibody activity in cod serum, against eight antigens, was measured using the double sandwich-ELISA previously described [23]. The following antigens were used (see abbreviations): TNP-BSA (containing 21 residues of TNP per BSA molecule), LPH (Sigma, St. Louis, MO, USA), ssDNA (from calf thymus, Sigma, USA), Thyr (bovine, Sigma, USA), BSA (Sigma, USA), Glyc (from *Mytilus edulis*, Sigma, USA), ECP-Asa and ECP-Pf. The last two antigens were prepared from bacteria, isolated from Atlantic salmon, by the modified overlay method [15].

2.6. Haemolytic activity

The haemolytic activity of serum was measured using a modification of the method described by Yano [38] for measuring complement activity by the alternative pathway as described previously [23].

2.7. Lysozyme activity

A turbidimetric assay was used [29] with some modifications [23]. In view of previous negative results for cod serum [23] approximately 10% of the samples from each group were analysed for lysozyme activity.

2.8. Anti-protease activity

A modification of the method described by Ellis [10] was used as described previously [23].

2.9. Iron binding capacity

The total iron content (TI, $\mu g\ ml^{-1}$) and unsaturated iron binding capacity (UIBC, $\mu g\ ml^{-1}$) were determined using a kit (Sigma no. 565) which is based on the method by Persyn et al. [30] as described previously [23]. Total iron binding capacity (TIBC $\mu g\ ml^{-1}$) and percentage iron saturation (% I) were calculated from these values.

2.10. Data handling

The StatView™ analysis system for Macintosh (Apple Computer) was used for statistical analysis. Most of the data showed non-parametric distribution. The Spearman Rank Correlation test was used for analysis of correlation between parameters. The unpaired t-test was used for comparative analysis of different parameters of the same size range between the two groups. The criteria for significance was set at $P \leq 0.05$ in all instances.

3. Results

3.1. Health status, age, size and gender

The health status of the wild cod appeared to be good and no external or internal signs of infection were observed.

Analysis of the otoliths showed that the age ranged between 2 and 9 years for cod sampled in the spring and 1 and 8 years old for cod sampled in the fall. In both groups about 75% of the fish was < 75 cm long (< 5–6 years old). A good correlation (Rho > 0.995) was obtained between age, length and weight. Until the age of 6 years, cod sampled in the fall, was bigger than cod of the same age sampled in the spring, corresponding roughly to a year's growth. After the age of 6 years the mean size was fairly similar for both groups (Fig. 1).

Of the 110 fish from the spring sampling 57 were females and 36 males, whereas 17 were omitted (human error). Of the 91 fish from the fall sampling 43 were females and 48 males. Statistical analysis showed that none of the parameters analysed were influenced by the gender of the cod.

3.2. Protein concentration

The serum protein concentration varied from 18.5 to 51.0 μg ml^{-1} and 23.5 to 55.5 μg ml^{-1} in cod sampled in the spring and in the fall, respectively.

The protein concentration increased with increasing size (Fig. 2). This correlation was statistically significant in both groups (Rho ≥ 0.415).

The lower concentration observed in cod sampled in cold water was statistically significant (unpaired t-test: $t \geq -2.0$) within each 10 cm size range.

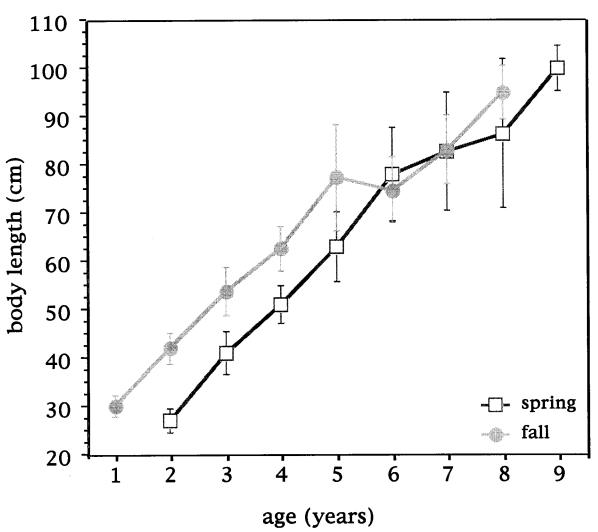

Fig. 1. The mean body length (cm) for each age group of cod sampled in the spring and in the autumn. The bars indicate S.D.

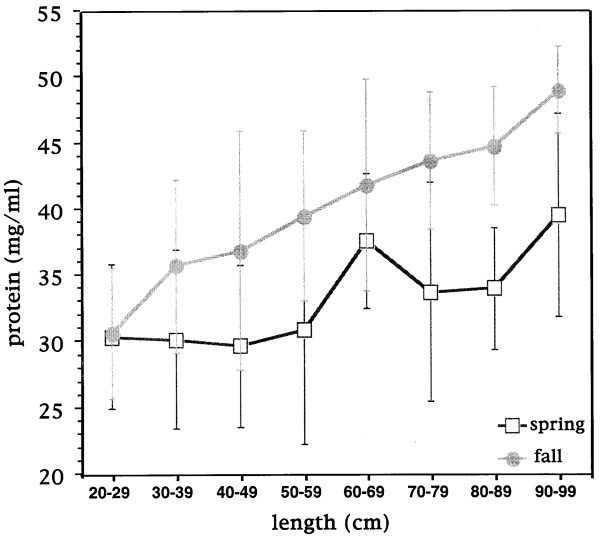

Fig. 2. The mean serum protein concentration (mg ml^{-1}) of each size group of cod sampled in the spring and in the autumn. The bars indicate S.D.

3.3. Immunoglobulin (IgM) concentration

The total serum IgM concentration varied from 1.2 to 42.5 and 1.2 to 44.0 mg ml^{-1} in cod sampled in the spring and in the fall, respectively. The mean IgM content was about 33% of the serum proteins.

The serum IgM concentration increased with increasing size (Fig. 3). This correlation was statistically significant in both groups (Rho ≥ 0.347). When IgM was expressed as a percentage of the protein concentration (not shown) the positive correlation between IgM and size was maintained.

Although the IgM level was generally higher in cod sampled in the fall (Fig. 3), this difference was not statistically significant.

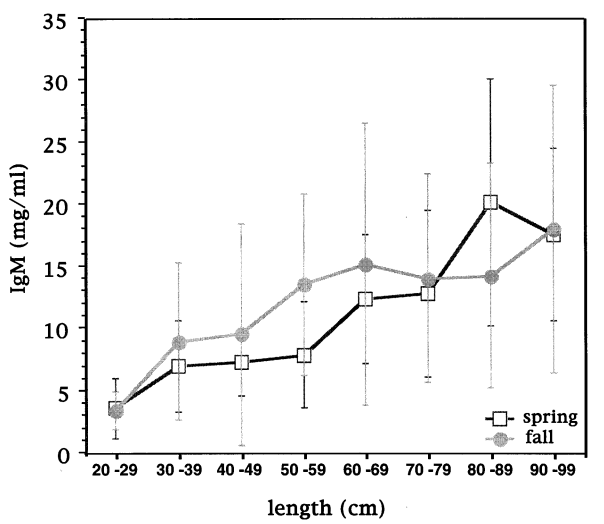

Fig. 3. The mean serum immunoglobulin (IgM) concentration (mg ml^{-1}) of each size group of cod sampled in the spring and in the autumn. The bars indicate S.D.

Table 1
Natural antibody activity (optical density at 405 nm) of cod sampled in the spring and in the autumn[a]

Activity against:	Cod sampled in the spring			Cod sampled in the fall		
	Mean ± S.D.	Correlation with size		Mean ± S.D.	Correlation with size	
		Rho	*P*		Rho	*P*
TNP-BSA	1.06 ± 0.611	0.732	<0.0001	1.193 ± 0.465	0.473	<0.0001
LPH	0.231 ± 0.159	0.525	<0.0001	0.370 ± 0.241	0.390	0.0004
ssDNA	0.309 ± 0.181	0.645	<0.0001	0.382 ± 0.191	0.535	<0.0001
Thyr	0.175 ± 0.099	0.426	<0.0001	0.226 ± 0.140	0.538	<0.0001
Glyc	0.143 ± 0.082	0.257	0.0075	0.169 ± 0.138	0.599	<0.0001
ECP-Asa	0.098 ± 0.073	−0.017	0.8557	0.163 ± 0.119	0.305	0.0041
ECP-Pf	0.094 ± 0.059	0.236	0.0141	0.145 ± 0.109	0.421	<0.0001
BSA	0.108 ± 0.109	−0.069	0.4755	0.139 ± 0.086	0.279	0.0084

[a] The table shows the mean values, S.D. and Spearman Rank correlation with body length.

3.4. Natural antibody activity

A strong antibody activity was observed against TNP-BSA, about 45 and 65% of the samples collected in the spring and in the fall, respectively showed values exceeding 1.0. Relatively high antibody activity was also seen against LPH, ssDNA and Thyr.

On the other hand, the antibody activity against Glyc, BSA and the bacterial antigens, ECP-Asa and ECP-Pf, was relatively low. However, a few individuals sampled in the fall gave OD values exceeding 0.8 against the ECP-Asa, ECP-Pf and Glyc.

The natural antibody activity increased with increasing size except the anti ECP-Asa and anti BSA activity of cod sampled in the spring (Table 1).

The natural antibody activity was slightly higher in fish sampled in the fall than in fish sampled in the spring (Table 1) but this difference was not statistically significant.

Correlation analysis of the antibody activity against the eight antigens (not shown) gave variable but in most cases significant correlation ($Rho \geq 0.221$) especially in cod sampled in the fall. There was exceptionally good correlation between the activity against LPH, ssDNA and Thyr ($Rho \geq 0.684$) on the one hand and between the activity against Glyc, ECP-Asa, ECP-Pf and BSA ($Rho \geq 0.612$) on the other hand. This grouping of antibody activity was particularly evident in cod sampled in the spring.

The correlation between antibody activity and IgM concentration was also examined (not shown). This correlation was in most cases significant ($Rho > 0.202$). However the correlation was more marked in cod sampled in the spring than in cod sampled in the fall, especially the correlation between IgM and anti TNP-BSA activity ($Rho = 0.661$ and 336, respectively).

3.5. Haemolytic activity

The mean haemolytic activity was around 60% in both groups. Overall the activity decreased with increasing size. This negative correlation was statistically significant in cod sampled in the spring ($Rho = -0.414$) but not in cod sampled in the fall ($Rho = -0.102$). However, when the mean haemolytic activity was plotted against length (Fig. 4) it was evident that the haemolytic activity of cod sampled in the spring was relatively high ($> 80\%$) in the younger cod (< 75 cm, < 5–6 years old), but fell off sharply to below 40% in the older fish. The haemolytic activity of cod sampled in fall fluctuated around the 60% level over the size range examined.

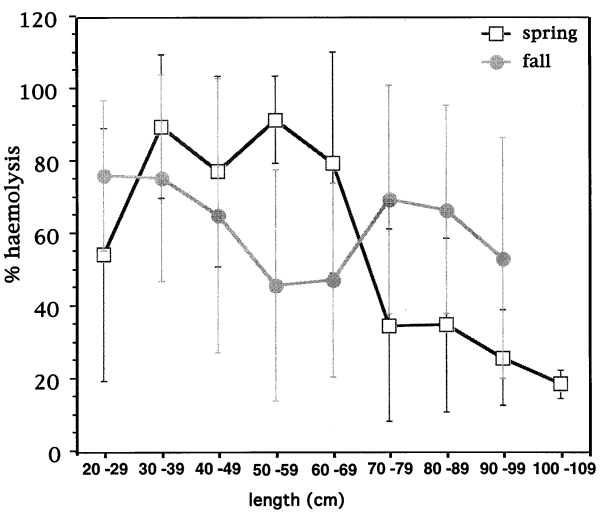

Fig. 4. The mean serum haemolytic activity (percentage haemolysis) of each size group of cod sampled in the spring and in the autumn. The bars indicate S.D.

3.6. Lysozyme activity

No lysozyme activity was detected in the samples tested. Control Sea bass serum showed lysozyme activity of about 1280 units ml^{-1}.

3.7. Anti-protease activity

The anti-protease activity varied from 12.5 to 70.0% and from 26.2 to 70.0% in cod sampled in the spring or in the fall, respectively.

The level of anti-protease activity increased slightly with increasing size and the increase was gradual over the whole size range. This correlation was significant in cod sampled in the fall (Rho = 0.307) but not in cod sampled in the spring (Rho = 0.150).

Although the overall mean activity was lower in cod sampled in the spring than in cod sampled in the fall this difference was not statistically significant.

3.8. Iron binding capacity

Both groups showed similar range in their serum iron content and binding capacity: the total iron content (TI) varied from 0 to 1.5 μg ml^{-1}, unsaturated iron binding capacity (UIBC) from 5.3 to 10.5 μg ml^{-1}, total iron binding capacity (TIBC) from 5.5 to 16.7 μg ml^{-1} and percentage iron saturation from 0 to 15.9%.

In cod sampled in the spring TI, TIBC and percentage iron saturation increased significantly with increasing size (Rho > 0.244). This trend was primarily due to a relatively low TI level of 0.20–0.25 μg ml^{-1} in the younger cod (< 75 cm, < 5–6 years old) followed by a sharp increase to a TI level of 0.4–0.5 μg ml^{-1} in the older cod (Fig. 5a). The UIBC on the other hand was fairly levelled (7–8.5 μg ml^{-1}) over the whole size range (Fig. 5b) and did not increase significantly with increasing size (Rho = 0.182).

In cod sampled in the fall the values of all the iron parameters, including TI, were fairly constant over the whole size range and corresponded to those of the older cod sampled in the spring (Fig. 5a). TI (and %*I* saturation, not shown) did not show significant correlation with size whereas the UIBC (and TIBC, not shown) level increased significantly with increasing size (Rho = 0.463). This increase was gradual over the whole size range (Fig. 5b).

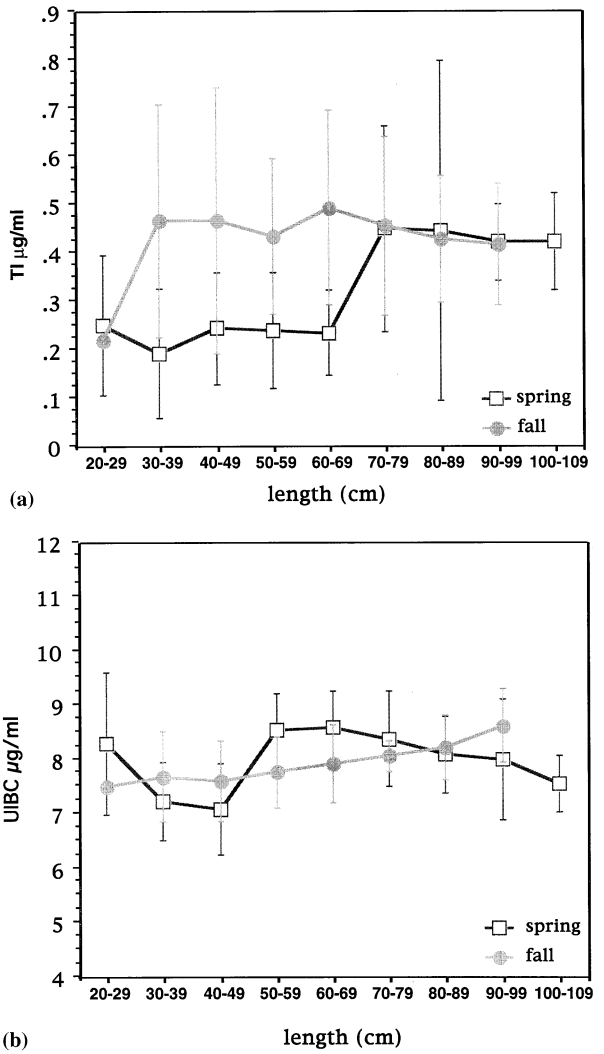

Fig. 5. The iron parameters of each size group of cod sampled in the spring and in the autumn, (a) TI, total iron concentration (μg ml^{-1}), (b) UIBC, unsaturated iron binding capacity (μg ml^{-1}). The bars indicate S.D.

4. Discussion

The aim of this study was to examine whether certain immune parameters were affected by the size and gender of cod under different environmental and seasonal conditions.

The two groups did not represent different stocks of cod since considerable mixing of the Icelandic cod population takes place during spawning [19] and the Icelandic cod shows little genetic variation [3]. However, the two groups varied with respect to other important factors. The sea temperature was different, both in the period preceding and during sampling. The long term mean annual near-bottom temperature (over a period of 25 years) of the two localities on the other hand differed by only 1.7°C, these being 4.8°C at Vestfirðir and 6.5°C at Faxaflói [35]. The season and hence the time from spawning was also different in the two groups. Spawning, which takes place in late March till early May, primarily off the south west coast of Iceland, but also in other inshore areas [19], is a period of stress and temporary depletion for the sexually mature cod which could affect immune and other blood parameters in fish [4,21,26]. The different geographical

locations and food sources available may also have been influential.

None of the parameters tested, including the growth rate, were influenced by the gender of the cod. Sexual maturity was not determined but would have been of interest in view of the results obtained. On the other hand, most of the parameters were influenced by the size of the cod.

Cod, like the majority of fish species, continues to grow throughout its life but the rate of growth generally decreases with increasing age [21]. Several studies have shown that the growth rate of cod is influenced by temperature [21,23,34]. The difference between the two groups in length vs age (Fig. 1) may be explained by the difference in sampling time within the year since the main growing period of Icelandic cod is during the warm period from June to October [19].

In both groups, the total serum protein concentration increased with increasing size of cod. An increase in serum protein concentration with increasing size has been reported during the early development of fish [13,27]. The higher serum protein levels observed in cod sampled in the fall compared to cod sampled in the spring may reflect better nutritional conditions during fall than spring [19,21].

The serum IgM concentration increased with increasing size in both sub groups. An increase in IgM level with increasing age has been reported for several fish species and has been claimed to reach an optimum and constant level at sexual maturation [13,20,25,27]. This does not seem to be the case in cod. The different environmental or seasonal conditions did not appear to influence the immunoglobulin level. The mean percentage IgM level was relatively high (33%) and in a few individuals appeared to approach 100%. To some extent these high values reflect a discrepancy between the two analytical methods. However, as much as 85% IgM was verified in the serum of these individuals by gel filtration and polyacrylamide gel electrophoresis analysis (Magnadóttir, unpublished data).

As in our previous study the correlation analysis indicated two types of antibody response. One type of response, against TNP-BSA, LPH, ssDNA and Thyr was also relatively higher and tended to show better correlation with the IgM concentration than the other type of response, against Glyc, ECP-Asa, ECP-Pf and BSA. Antigens like TNPBSA, ssDNA and Thyr, sometimes referred to as auto-antigens, have been used in other studies of natural antibody response in fish and mammals [14,24,37]. Whether this natural antibody population arises in response to conventional antigen stimulus or as a result of a faulty regulatory system is not certain. As was mentioned in our previous study high level of auto-antibodies are associated with immune diseases in mammals but in fish it may reflect a primitive regulatory system [23]. One could speculate on whether this high level of IgM concentration and natural antibody response contributes or compensates for the poor specific antibody response by cod.

BSA, Glyc and the bacterial antigens probably do not function as auto-antigens. The relatively strong activity observed in a few individuals which did not correlate with high IgM level, especially against the bacterial antigens, suggests a specific antibody response. This is contrary to the fact that it has proved very difficult to induce specific antibody response in cod [12,33]. However, this may indicate that under natural conditions cod may produce a low level of specific antibody response. The haemolytic activity of cod sampled in the spring was relatively high in the younger cod but low in the older cod. This difference may reflect an adaptation of the more endemic younger population to the very low temperatures experienced by this sub group. Higher haemolytic activity at lower temperatures has been indicated in other studies [23,32]. Similarly, the older, more migratory cod have recently come from higher environmental temperatures of the spawning ground further south. Stress experienced during the spawning period or associated with the migration could on the other hand adversely affect the haemolytic activity in the older cod [1]. The serum iron concentration, however, was relatively low in smaller cod, sampled in the spring, whereas the larger fish showed levels comparable to those of cod sampled in the fall. Reduction in these parameters is generally a sign of acute phase reaction [1] and may reflect the response of the younger, endemic fish to extremely low temperatures (≤ 0°C).

In cod sampled in the fall such a difference between the younger and older fish was not observed and when for example a positive correlation was obtained between size and haemolytic activity or iron binding capacity, this was indicated by a gradual increase over the whole size range examined. This group, sampled in October at favourable environmental temperature, must have recovered from the last spawning season.

Both groups of cod showed a relatively constant anti-protease level over the size range examined. Other studies have shown that individual or species difference in serum antiprotease activity of fish, kept under similar environmental conditions, is limited [6].

It has been shown that the anti-protease activity of fish serum deteriorates when stored at -20°C [10], the slight difference between the anti-protease activity of the two groups was therefore probably influenced by the longer storage time of the serum from cod sampled in the spring. This was the only parameter that appeared to be sensitive to storage.

As in our previous study no lysozyme activity was detected in the serum of wild cod.

The present study has shown that humoral immune parameters of cod are influenced by its size and that the

size factor probably exerts its influence throughout the life history of cod. The results also show that the immune parameters of wild cod are influenced by other biological and environmental factors, of prime importance being the cod's maturity and the seasonal cycle as well as the environmental temperature.

Compared to our previous study on captive cod [23] the present study presented several interesting differences. Wild cod sampled in the spring, of roughly the same size as the captive cod kept at 1°C (44–52 cm), differed by having relatively lower serum protein concentration, anti-protease activity, total iron concentration and iron binding capacity but higher IgM levels and haemolytic activity. On the other hand, in wild cod sampled in the fall, of the same size as the captive cod, kept at 7°C (51–65 cm), these parameters were fairly similar. The underlying reasons for these differences probably vary but they again indicate the increased prominence of non-specific humoral parameters at the lower temperature limits of cod [23].

The results of the present and our previous study [23] emphasise the complex relationship between the environment, the biological state and the immune system of cod.

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