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Diagnosis of stress in the European eel[1]

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Introduction

During the past several years, the eel has become an important fish for cultivation in intensive aquaculture systems. In Japan, 80 000 tonnes of eels are produced for human consumption annually. Elvers are raised to a marketable size in warm water. In other countries, as well, efforts are made to raise these valuable food fishes commercially and achieve optimal results. The methods applied during cultivation naturally create conditions very different from those to which the eels are exposed in their natural environment. The high stocking density produces permanent confrontations between individuals. The physical, chemical, and biological conditions of the artificial habitat show large periodic fluctuations. There are no possibilities for the eels to escape situations of stress. There is evidence to assume that the fishes find such living conditions to be injurious and react with stress effects in the form of disturbances to their physical health.

It is well known that teleosts placed under stress show a set of physiological reactions, that is, in principle, the same as that occurring in mammals (Gronow, 1974). The so-called "General Adaptation Syndrome" (GAS) normally serves to enable the organism to react in case of danger of threatening situations. Selye (1976) divided the GAS into three general phases: alarm, resistance, and exhaustion. The first is characterized by a whole set of hormonally controlled physiological changes which serve to provide the organism with instant energy. First, excitation of the sympathetic nervous system leads to a release of adrenalin and nor-adrenalin from the adrenal medulla, promoting the heart activity, increasing blood glucose, influencing kidney function, and affecting the water balance of the body.

At the same time the hypothalamus-hypophysis-adrenal cortex system is activated, bringing about an endocrine stimulation leading to such conditions as increases in gluconeogenesis in liver, in fatty acids, in catabolic protein metabolism, and in immuno-suppression. In many places along the metabolic pathways, complicated interactions and feedback mechanisms link both sets of reactions.

The object of our research was to find out how the European eel reacts to stress. For this purpose, we used the behavior pattern by which the eels establish social rank as the stressor. If two individuals are placed in a basin where no hiding places are available, sooner or later a conflict will occur in the form of threatening gestures, ramming, or biting, and one of the fishes will become dominant (α-eel) and the other, subordinate (β-eel). More than 100 stressed individuals were examined using over 20 physiological and morphological methods *simultaneously* (*Peters et al.*, 1980).

Some of the values recorded showed no difference between stressed and unstressed animals. Others showed alterations and some of them turned out to be suitable indices of the stress effects (Fig. 25). This study reports only these methods and discusses their value as stress indicators for the eel. To do this, the symptoms of stress were compared with those found in diseased fishes.

Experimental animals

The eels used in our investigations (106 individuals) had an average length of 40 cm and weight of 100 g. They were obtained from the Aquaculture Station of the Bundesforschungsanstalt für Fischerei in Emden, northern Germany. For the experiments, 50 liter aquaria were used in a warmwater (22° ± 0·5°C) circulation system (Peters et al., 1980). We investigated three groups of eels.

1. Controls (C): these fishes lived isolated individually in tanks for four weeks.
2. Stressed eels (S): to produce the stress situation, the pronounced hierarchical behavior of the eels was exploited. This proved to be a reliable stressor. When two eels were kept together in one container, a hierarchy was sometimes established within a few hours. The subordinate animals proved to be under great stress by the end of the experiment (5 to 10 days).

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Stress reaction in Anguilla anguilla

increase	decrease	no reaction
leucocrit granulocyte ct.	blood volume leucocyte ct. lymphocyte ct.	hematocrit * hemoglobin* erythrocyte ct.*
cortisol blood glucose blood lactate	liver glycogen	total protein total cholesterol
IR cell diam. epid. permeability gastric muscle diam.	spleen weight stomach diam. gastric epithelium cell diam. surface	IR nuclei diam.

Figure 25. Stress reaction in *Anguilla anguilla*. Good stress indicators have been underlined. (* if not measured immediately after blood removal.)

3. Diseased eels (U): the third group of fish used in this study was afflicted with ulcers that had developed in the experimental system. These eels were part of a stocking density experiment. Preliminary results showed that some alterations in the parameters investigated probably occurred due to these experimental conditions. The eels characterized as "ulcer eels" in the study, however, are only those which were severely affected. Within three weeks, crater-like lesions developed on the head, trunk, and tail of the fish. The sores appeared as flattened epidermal lesions that quickly spread to the corium and musculature. The cause of the disease was not investigated; the fish probably suffered from a bacterial infection similar to red disease.

For examination, the experimental eels were gently emoved from the tanks and killed in neutralized MS 22 (1 g/l). The methods used are described in the ollowing section. For statistical evaluation, Student's -test was used (Computer Wang 600).

hysiological and hematological methods nd results

)btainable blood quantity

lethod. Removal of blood was performed immediately fter narcotization of the fishes and opening of the body cavity. A 0·65 mm cannula was inserted horizontally from the anterior end into the bulbus arteriosus and all obtainable blood was slowly drawn out. Raising the tail facilitated the removal of the blood from the large veins. The relative blood quantity was calculated as follows:

$$\frac{\text{abs. blood quant.} \times 100}{\text{total weight}}$$

Results. Subordinate eels had a statistically significant smaller quantity of removable blood than the unstressed ones. It is not very probable that the absolute quantity of blood in the stressed fishes is reduced. We rather think that this phenomenon results from change in blood pressure. Eels with ulcer disease from which blood was removed yielded an average quantity greater than that from the controls (Table 18).

Leucocyte count (LC)

Method. In order to count the leucocytes, less than 5 µl of blood were required. The sample was diluted with stain solution (according to Lehmann and Stürenberg, 1974) in calibrated mixing pipettes. The stained cells were counted in a Neubauer hemocytometer.

Results. The LC showed a definite decrease in the stressed animals, compared with the controls. An increase was found, however, when the fish were afflicted with ulcers.

able 18. Physiological and hematological parameters in control, stressed, and diseased eels.

ivestigated parameters	Control eels			Stressed eels			Ulcer eels		
	n	$\bar{x}$	s.d.	*n*	$\bar{x}$	s.d.	*n*	$\bar{x}$	s.d.
eucocyte count (10^3)	17	120·60±	31·40	23	81·90±	22·70	16	153·00±	14·60
eucocrit (volume %)	18	1·60±	0·50	19	2·00±	0·80	16	2·40±	1·47
lood glucose (nmol/l)	18	5·72±	1·68	19	13·42±	8·51	16	13·39±	7·24
ver glucose units (µmol/g wet wt)	18	385·40±	207·80	28	167·00±	155·60	16	104·80±	89·60
ood volume obtained (10^{-2} ml/g)	17	1·18±	0·37	24	0·80±	0·34	15	1·36±	0·33

Leucocrit (Lct)

Method. The proportion of white cells in the blood by volume was determined by standard micro-methods. Capillary tubes filled with whole blood were centrifuged (Microfuge HC 102) at 10 000 *g* for five minutes. The leucocrit was determined under a dissecting microscope using a calibrated micrometer (McLeay and Gordon, 1977).

Results. In β-eels the leucocrit reacted inversely with the LC. Stressed eels had significantly increased leucocrits. Eels afflicted with ulcer disease showed an even greater increase in white blood cell volume than the stressed eels.

Blood smear analysis (white cell line)

Method. The proportional representation of the different kinds of leucocytes was investigated using smears stained according to Pappenheim. In each preparation, 200 to 400 leucocytes were counted at random and classified as thrombocytes, lymphocytes, or granulocytes. The relative proportions found were converted to absolute values based on the LC.

Results. In blood smears, it was observed that stressed eels possessed fewer lymphocytes but more granulocytes than controls. As granulocytes are much larger in volume than lymphocytes, the inverse relationship between LC and Lct, mentioned above, is explained. In the blood of fishes afflicted with ulcer disease, the total leucocyte count as well as the leucocrit was increased. In the blood smears, granulocytes had become more numerous, while the number of lymphocytes remained normal.

Blood glucose

Method. Glucose determination was carried out by the hexokinase/glucose-6-phosphate dehydrogenase method on blood from which the protein had been removed with perchloric acid (Boehringer test set). The photometric measurements were carried out using a semimicro technique at a wavelength of 334 m.

Results. The blood glucose concentration was significantly increased by stress. Eels infected with the disease also showed clear symptoms of hyperglycemia.

Liver glycogen

Method. Immediately after blood removal, the liver was excised and instantly deep frozen using liquid nitrogen. The glycogen content was determined using the method of Keppler and Decker (1974). After quantitative analysis of the glucose in the homogenized organ, the homogenate was hydrolysed with amyloglucosidase, and the amount of free glucose was subtracted from the total. The determination was performed using the HK/G-6-DH method.

Results. Stressed eels had smaller quantities of liver glycogen than unstressed ones. The same was true for the ulcer-diseased eels.

Summing up the physiological and hematological findings, the following set of changes in parameters tested is typical of eels under stress from social confrontations: significant decreases in leucocyte count, blood volume, and liver glycogen content, together with increases in leucocrit and blood glucose concentration.

Fishes afflicted with the ulcer disease also show three of these symptoms (leucocrit, glucose, glycogen), but an opposite effect is observed in the other two parameters (blood volume, leucocyte count) (Fig. 26).

It must be mentioned here that the eels with ulcer disease may have been under an extra stress due to the experimental conditions. They were taken from a stocking experiment conducted under a similar set of conditions as the stress investigation. In any event the effects of disease mask or superpose the symptoms of stress.

From this we can conclude that the evaluation of only one or two clinical symptoms can lead to a false diagnosis. Only by monitoring several blood and metabolic parameters is it possible to distinguish between stress and acute disease.

Even the investigation of several parameters in an individual fish allows no unequivocal stress diagnosis. On an individual basis, the stress response in terms of blood glucose and cortisol content does not manifest itself in a way that the data can be directly correlated,

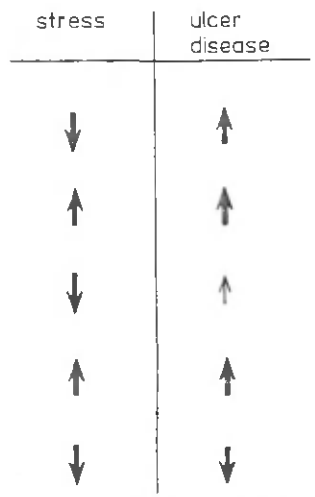

Figure 26. Physiological and hematological alterations in stressed and diseased eels.

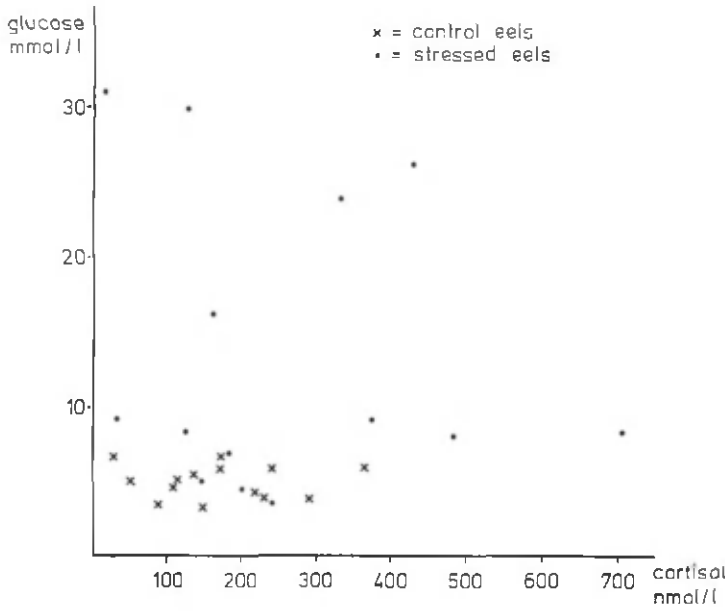

Figure 27. Blood glucose and cortisol level of unstressed (control) and stressed individuals.

though the average values of both are significantly increased (Fig. 27).

This indicates that the individual has a large degree of freedom in its physiological and hematological reactions to stress. In addition, it indicates that the strict sequence "alarm phase, resistance phase, exhaustion phase" or the duration of each can be greatly modified by the individual, that is, the stress response differs very much from fish to fish. In practice, a diagnosis of stress based on physiological and hematological methods can be made only when a representative group of fishes is investigated, and its members are very similar in background.

Morphological and morphometric methods and results

Stress experiments lasted over a period of 5 to 10 days. It was assumed that this time period was long enough for morphological and morphometric changes in the organs of the stressed animals to occur. Material for histological examination was obtained from the interrenal organ, the kidney, the liver, and the stomach of stressed eels. Diseased fishes could only be evaluated as random samples and are therefore not included in the graphs.

Method. Immediately after the body cavity was opened, the entire intestinal tract was removed and fixed in 4 % formalin. Four to five mm pieces of the above-mentioned organs were excised and embedded in paraffin. Sections 5 to 7 µm thick were stained with Azan or HE. Some parameters were measured using a Visopan projection microscope (Reichert) others by an ASM (Leitz).

Results. The reactions of the organs were determined according to the physical appearance of the tissues and the size of the cell nuclei. The second of these parameters can provide an indication of over- or under-secretion by the glands. Concerning the stomach the mucous vacuoles of the gastric epithelium were also measured.

The stress to which the β-eels were exposed did not lead to a significant reaction of the liver and kidney tissue in our experiments (Fig. 28). The tissue structure and the size of the nuclei were not essentially changed. Even in the unstressed fish, both of these tissues showed a great heterogeneity, possibly due to the different histories of the fishes. There was also no trend detected in the size of the cell nuclei in the interrenal organ (Fig. 29). However, the number of nuclei per unit area, which can provide an indication of the cell size, was clearly reduced in the stressed eels. This corresponded to the findings of investigations on mammals, in which the adrenal organ enlarged in response to stress.

Much more obvious than the findings on the liver, kidney, and interrenal organ are those changes that stress brings about in the stomachs of eels. In these fish stress leads to a shrinking of the whole stomach which in

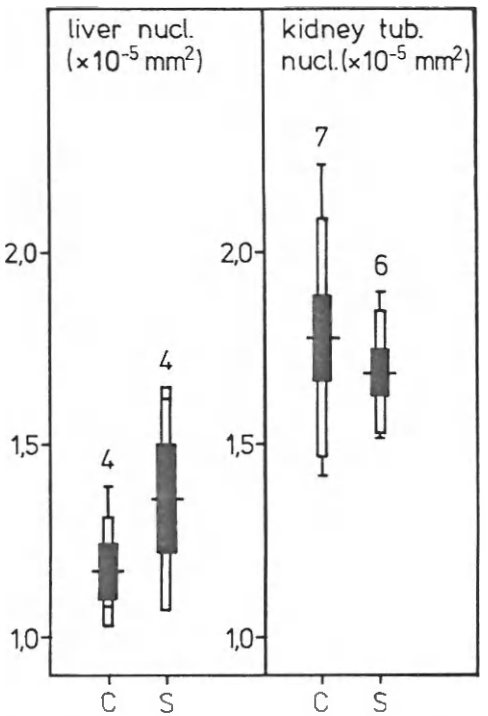

Figure 28. Surface area of cell nuclei in liver and kidney of unstressed (C) and stressed (S) eels.

our experiments apparently reached a maximum degree: all of the stomachs under stress have a minimum diameter (Fig. 30). This contraction is accompanied by a thickening of the circular and longitudinal musculature. At the same time the surface of the gastric epithelium shrinks distinctly. Figure 31 shows the macroscopically visible changes of the stomach epithelium. The folds of the mucosa, that are deeply convoluted in healthy fishes, become more shallow under stress. In some places they are apparent only as creases or have disappeared.

Additional details of this process can be found by histological examination. Very few gastric pits remain after the fish has been exposed to chronic stress. The gastric glands are reduced; and they lose their compactness and ability to stain. The diameter of the mucous cells also decreases significantly.

Under higher magnification, it is evident that the acidophilic mucoid portion of the epithelial cells is reduced. In some places, the mucous epithelium is totally destroyed. In the surface layer, large vacuoles or cavities become evident in which cell debris and remains of the nuclei can be identified. These cavities are possibly the products of degenerative or necrotic processes. In stressed eels, the nuclei of the gland cells are extremely polymorphic and often pycnotic. Almost everywhere, the cell integrity disappears, and the tissue structure disintegrates.

These findings seem to show that the functional capability of the stomach is greatly disturbed when the eels are under stress. From studies with mammals it is

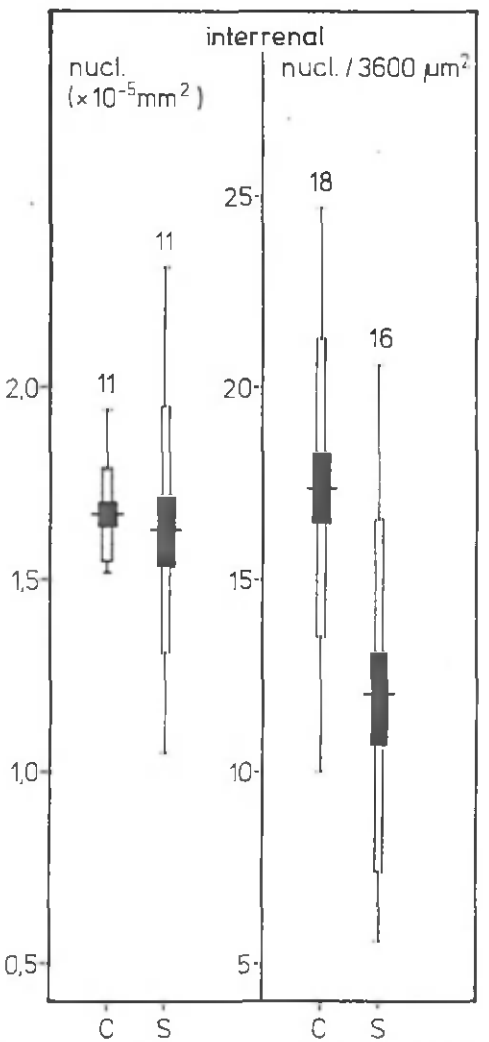

Figure 29. Surface area of cell nuclei per unit area in the interrenal organ of unstressed (C) and stressed (S) eels.

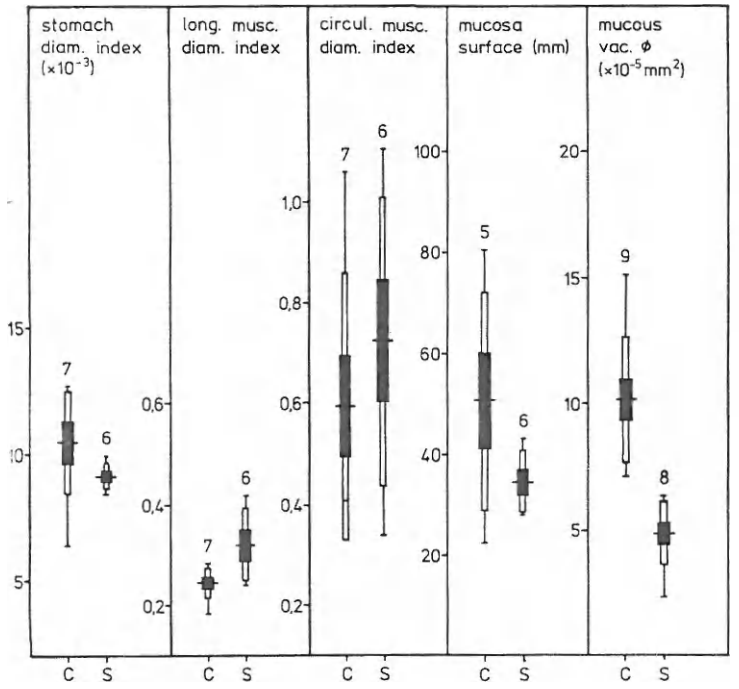

Figure 30. Morphometric data of the stomachs of unstressed (C) and stressed (S) eels.

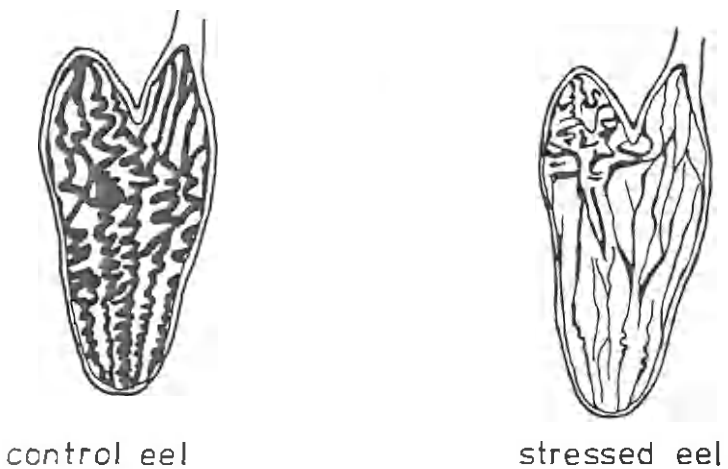

Figure 31. Macroscopic view of the stomach wall of unstressed (control) and stressed eels. Structural changes are apparent.

known that a reduction in vitality of the mucosa diminishes the protection against self digestion. In man, peptic ulcers are among the most characteristic manifestations of the stress (Selye, 1976). A similar response of the teleost stomach has not been reported previously.

These morphological alterations of the stomach of socially stressed eels indicate that the fish are placed under long-term stress. Only when the neuro-endocrine reaction series with all its reversible results continues unabated or is continually repeated do organic changes result.

None of these symptoms were found in ulcer-diseased eels studied for comparison. Their stomach epithelia showed no alterations when compared with the controls.

Discussions and clonclusions

It is well known from mammals and fish that stress results in an elevated cortisol level (Fagerlund, 1967; Schreck et al., 1976: Strange, 1977) which brings about a glyconeogenesis in the liver (Storer, 1967; Butler, 1968).

Adrenalin, on the other hand, causes glycogenolysis in liver and muscle which leads to hyperglycemia (Mazeaud et al., 1977). Comparing the diseased eels with the stressed ones, it can be seen that a similar reaction occurred in both groups. The infection may therefore result in an energy mobilization just as stress does.

Glancing back at the prerequisites for reliability of the physiological and hematological study methods, it seems that the morphological and morphometric parameters have a clear advantage for diagnosing a long-lasting resistance phase. The findings present an integrated set of evidence about the long-term burden on a fish, independent of the momentary state of experiment or fluctuations in the daily activity cycle. It is clear that a short-term stress, and signs of the exhaustion phase, can be detected through physiological parameters. However, a long-term stress, that is, one lasting uninterrupted for several weeks, should be diagnosed only on the basis of a broad spectrum of clinical parameters in a group of fish, or on morphological and morphometric data. It is such a form of stress as this, that we often encounter in aquaculture.

Summary

Under natural conditions, stress plays the role of a regulatory mechanism important for the survival of the individual and the species. In captivity, this regulatory mechanism loses its biological purpose. Because the animals cannot escape, stress becomes a physiological burden. In our experiments with European eels, stress effects were brought about by intraspecific aggression.

Pairs of experimental eels and isolated controls were kept in 50-l tanks in a warm-water circulation system. Fights between the members of each pair always occurred, which led to one becoming subordinate within a few hours. To determine the amount of stress placed on the fish, more than 20 physiological and morphological parameters were measured. The values were compared with corresponding data obtained from eels afflicted with ulcer disease. We obtained the following results:

1) intraspecific aggression, in this case battles for rank, produced great stress in the subordinate animal, leading to death in extreme situations;
2) the following parameters proved to be good indicators of stress: decreases in the obtainable blood volume, lymphocyte count, spleen weight, liver glycogen content, stomach diameter; an atrophy of the gastric epithelium; and increases in the leucocrit, the granulocyte count, and the blood glucose concentration;
3) diseased eels showed changes in some of the parameters mentioned above opposite to those caused by stress, but several similar changes also occurred.

Therefore, long-term stress should be diagnosed based on the simultaneous appearance of numerous physiological and hematological symptoms or on the occurrence of morphological or morphometric alterations.

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