Influence of feeding procedure on biomarkers in caged rainbow trout (*Oncorhynchus mykiss*) used in environmental monitoring

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Biomarkers in feral fish can be a useful tool for environmental monitoring of aquatic ecosystems. Drawbacks, however, are that suitable fish species are not always available and that natural variations can bias the results. An alternative strategy is to use farmed fish placed in cages. There is, however, still a risk that factors other than pollution level could have an impact on the biomarkers and the observed responses in the fish. The present study evaluates the effects of feeding procedure on biomarkers in caged fish. Two feeding rations (2% and 8% weekly feeding) have been examined for a large number of biomarkers in caged rainbow trout (*Oncorhynchus mykiss*). Significant effects of feeding rations were found on hepatic ethoxyresorufin-O-deethylase (EROD) and catalase activity, PAH metabolites in bile, plasma ion concentrations and the presence of immature red blood cells. The influence on EROD activity and PAH metabolites seems to be caused by elevated uptake of pollutants when feeding ratios are higher. The effects on other biochemical and physiological variables are more likely caused by stress due to insufficient feeding. According to these results, valid comparison of fish groups in environmental monitoring requires standardized feeding levels.

Introduction

The aquatic environment is the ultimate sink for many pollutants, which can have an impact on aquatic ecosystems as well as on human health. It is therefore of great importance to have an appropriate methodology for environmental monitoring as well as ecological risk assessment of aquatic ecosystems. Laboratory tests where single species are exposed to single chemicals for a short time-period (*e.g.* 48 h EC50 on *Daphnia magna*) have for a long time constituted the foundation of ecotoxicological research.[1] This is a useful approach for screening a large number of chemicals. The ecological relevance is, however, low. Many of the compounds that contaminate the aquatic environment are unknown,[2] modified by abiotic as well as biotic factors[1] or simply not bioavailable. Furthermore, the main focus of pollution control has shifted from control of point sources and known chemicals to control of diffuse sources and complex mixtures of chemicals.[3] Therefore, new methods in environmental monitoring that integrate the effect of all pollutants in environmentally realistic experiments would be beneficial. The use of biomarkers is one possible way to achieve this.[4] Numerous studies have been performed where biomarkers in feral fish have been analyzed.[5–10] Drawbacks with this approach, however, are that suitable fish species may not be available and that migratory behaviour could affect the representativeness of the exposure. Furthermore, the sensitivity to environmental toxicants can be affected by factors such as age and reproductive status,[11] feeding[8,12,13] and temperature.[14] The use of farmed fish, placed in cages, may reduce these effects and secure the specificity of the water exposure.[15–17] In relatively calm waters, net cages can be used to cage the fish. This is a cheap and reliable method. For sites with streaming water, it may be better to use a flow through tank system. This requires access to electricity and is somewhat more unreliable as there is always a risk of technical failure. A previous study has shown that net cages and flow through tanks can be used and compared in monitoring studies.[18]

There are four possible routes for uptake of pollutants in fish; through feeding and the digestive tract, *via* contact between gill tissues and water, diffusion through the skin and by drinking water.[19] Fish that are kept in cages will not be able to feed like feral populations. Therefore exposure will be mainly through contact between ambient water and gill-tissue.[20] This can be considered as an advantage when monitoring water quality, but a disadvantage in ecotoxicological studies where factors like biomagnification are of interest. If the fish is fed commercial fish feed during the experiment, there is an obvious risk that the feed could be a source of contaminants. This has been shown in farmed salmon, which contained elevated levels of several toxicants, *e.g.* PCDDs (polychlorinated dibenzodioxins) and PCBs (polychlorinated biphenyls), probably due to contaminated fish oil present in the fish diets used by the aquaculture industry.[21–23]

The aim of this study was to evaluate the effect of feeding in caged rainbow trout (*Oncorhynchus mykiss*) on the following physiological and biochemical variables; body mass index (BMI), liver somatic index (LSI), hepatic ethoxyresorufin-O-deethylase (EROD) activity, glutathione S-transferase (GST), glutathione reductase (GR), catalase activity, fixed wavelength fluorescence of 2-, 4- and 5-ringed polycyclic aromatic hydrocarbon (PAH)-metabolites in bile, blood

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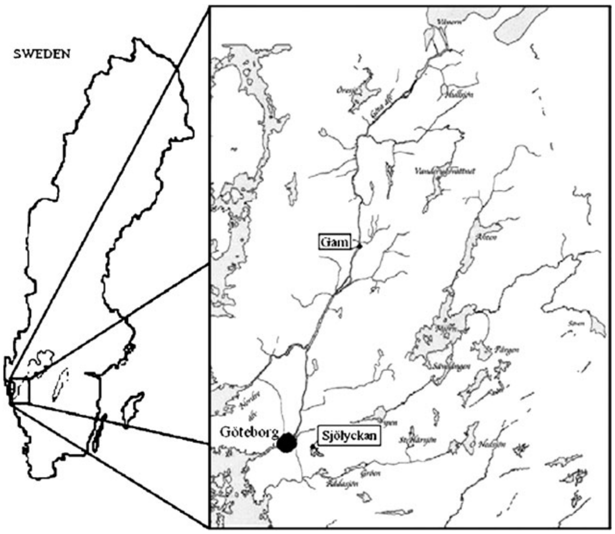

Fig. 1 Map of experimental sites.

glucose, haemoglobin (Hb), hematocrit (HT), concentration of ions (Ca^{2+}, Cl^-, K^+, Na^+) and lactate in blood plasma and relative abundance of white blood cells (lymphocytes, granulocytes and thrombocytes) and immature red blood cells. These variables were chosen to reflect vital physiological functions that are known to be affected by toxic effluents, *e.g.*, growth and energy metabolism (BMI, LSI), liver function and detoxification (EROD, GST, LSI), immune defence (white blood cells) and haematology (red blood cells, plasma ions).

Materials and methods

Two experiments were conducted at two sites (Garn and Sjölyckan) in western Sweden during 2004. Garn is located along the river Göta älv some 50 km north of the city Göteborg, and Sjölyckan is located at Lake Delsjön about 5 km east of Göteborg (Fig. 1). The river Göta älv is a frequently trafficked waterway for industry and Lake Delsjön is a recreational area in the outskirts of Göteborg without any boat traffic. At Garn, two plastic tanks were placed just next to the river Göta älv. At Sjölyckan, two net cages were placed in a large basin (approximately 20*30 m) belonging to a fish farm and connected to Lake Delsjön. This lake is a reservoir for drinking water to the city Göteborg and the lake is continuously fed with water from the river Göta älv. In total, lake Delsjön consists of approximately 90% Göta älv water and 10% from the natural water shed of the lake. At the time of the experiments, the basin at Sjölyckan was not in use for fish farming. At both sites, the fish were fed twice-weekly using commercial fish feed (Orion, 4 mm pellets). The fish were bought at the fish farm Antens Laxodling AB and transported in aerated tanks to the experimental sites. The transport time was approximately 45 min to Garn and 1 h 30 min to Sjölyckan.

Experimental design

In the first experiment (feeding), starting 24th of September 2004, rainbow trout of an initial mean weight of 300 g were used. The fish were kept outdoors in two PVC tanks (400 l) at Garn and in two net cages ($\approx$1000 l) at Sjölyckan. The experimental water was pumped from the river with a flow rate of approximately 11.5 l min^{-1} and a water exchange rate of 1.7 changes h^{-1}. A total of 14–15 fish were put into each tank or net cage, resulting in stocking densities of 11 kg m^{-3} and 4.4 kg m^{-3} for tanks and net cages, respectively. At each site, one cage (tank or net) was fed 4% and one was fed 1% (feed weight/fish weight) twice weekly, resulting in weekly feeding rations of 8% and 2% (Table 1). Sampling took place after 28 days.

Table 1 Experimental set-up for the two experiments

Experiment	Unit	Weekly feeding (%)	Temperature/°C	Site
	Tank 1	2	12	Garn
	Tank 2	8		
Feeding	Net cage 1	2	14	Sjölyckan
	Net cage 2	8		
Starvation	Net cage 1	2	6	Sjölyckan
	Net cage 2	0		

In a second experiment (starvation), starting 8th of November 2004, fish with an initial weight of 320 g were placed in two net cages and two PVC tanks as in the feeding experiment. Due to technical problems, however, the experiment could not be finished in the PVC tanks at Garn. Therefore, only the net-cages at Sjölyckan were sampled, with a total of 17 fishes in each cage (density 6.4 kg m^{-3}). The fish in one of the net cages were fed 2% weekly and the fish in the second net cage were not fed (Table 1). Feeding was performed twice weekly. The experiment lasted for 30 days before sampling. Measurements of enzyme activities in the cytosolic liver fraction (GST, GR and catalase) were not performed in this experiment.

The fish used in both experiments were from the same batch. Feeding levels for rainbow trout vary greatly between biomarker studies. Typical ratios are 1% to 10% weekly,[24–27] but it is also common that the fish are starved during the experiment.[16,28] In the present study, the feeding levels were set to 2% and 8%. The lower water temperature in the second experiment reduces the appetite of the rainbow trout, therefore only the lower feeding level (2%) was used for the fed fish group. To minimize variations in feeding due to dominant behavior, the fish were hand fed so that feed pellets were distributed as equally as possible among the fish in the cages.

Sampling and analytical methods

Each fish was killed by a blow to the head and 1 ml of blood was taken into a syringe. Weight and length were recorded and the fish was opened for bile and liver samples. The processing time of each fish was approximately five minutes.

Hematocrit, hemoglobin and blood glucose were measured directly. Blood smears were prepared and stained using the method of Giemsa and Pappenheim for differential counting of blood cells[29] and sent for microscopic examination according to Undritz[30] and Lehmann and Stürenberg.[31] Plasma concentrations of ions and lactate were measured using an

EML™105 Electrolyte Metabolite Laboratory (Radiometer, Copenhagen, Denmark).

The procedure for EROD measurement is described by Hanson *et al.*[18] Cytosolic liver samples were prepared as previously described by Förlin.[32] GR measurements were modified from Cribb *et al.*[33] GST was measured as previously described by Habig *et al*,[34] adapted to a microplate reader.[35] Catalase measurement was modified from Aebi.[36]

Measurements of PAH metabolites in bile were preformed using a semi-quantitative FF method[37,38] on pooled bile samples (n = 4–5). Measurements were conducted at the excitation/emission wavelength pairs (nm) 290/335, 341/383 and 380/430 to discriminate between 2-, 4- and 5-ring PAH metabolites, respectively.[38] The different measurements will henceforth be denoted FF2, FF4 and FF5.

Sampling procedure and analytical methods for blood, liver and bile variables are described in more detail in Hanson *et al.*[18] In the present study, however, storage time for plasma samples was up to 30 h in dry ice followed by 6–7 weeks in a −20 °C freezer before measurements.

Statistical treatments

The feeding experiment was analyzed using two-way analysis of variance (ANOVA) with the factors feeding (8% and 2%) and site (Garn and Sjölyckan). In the starvation experiment, a one-way ANOVA was used for the feeding factor (2% and 0%). In studies on caged fish there is always a risk of random environmental factors or hierarchical feeding that may affect single cages. For this reason, it is advisable to use a nested experimental design with replicate cages for each treatment. In an orthogonal two-way ANOVA, however, these problems are also avoided as long as no interactions are found. The benefit of a two-way ANOVA, using different sites and types of cages, is that the results can be interpreted in more general terms compared to a nested ANOVA using only one site and one type of cage. The lack of replication in the starvation experiment due to technical failure is, however, problematic. Therefore, the results of the starvation study are only presented briefly and not discussed in detail. In both experiments, Cochrans C was used to test for heterogenic variances.[39] When heterogenic variances were found, log-transformation was used.

Results

Feeding experiment

No fish died during the experiment. The result of the ANOVA showed that BMI, LSI, EROD and catalase activity, bile concentration of 2-ringed PAH metabolites, plasma concentration of Ca^{2+} and Na^+ and the proportion of immature red blood cells differed significantly between feeding groups (Fig. 2). Furthermore, significant differences between sites were found for bile concentration of 4-ringed PAH metabolites, plasma concentration of Na^+ and Ca^{2+} and the proportion of thrombocytes in the blood (not shown). EROD and GST activity as well as bile concentration of 2-ringed PAH metabolites were log-transformed to avoid heterogeneity. All results of the feeding experiment are shown in Table 2.

Starvation experiment

No fish died during the experiment. According to the ANOVA, plasma concentration of Ca^{2+} and the proportion of immature red blood cells were significantly higher in the starved group compared to the fed fish. The results of the starvation experiment are shown in Table 3.

Discussion

The aim of the present study was to investigate the importance of equal feeding levels for caged rainbow trout used in environmental monitoring. The results showed that over an exposure period of four weeks, several biomarkers differed

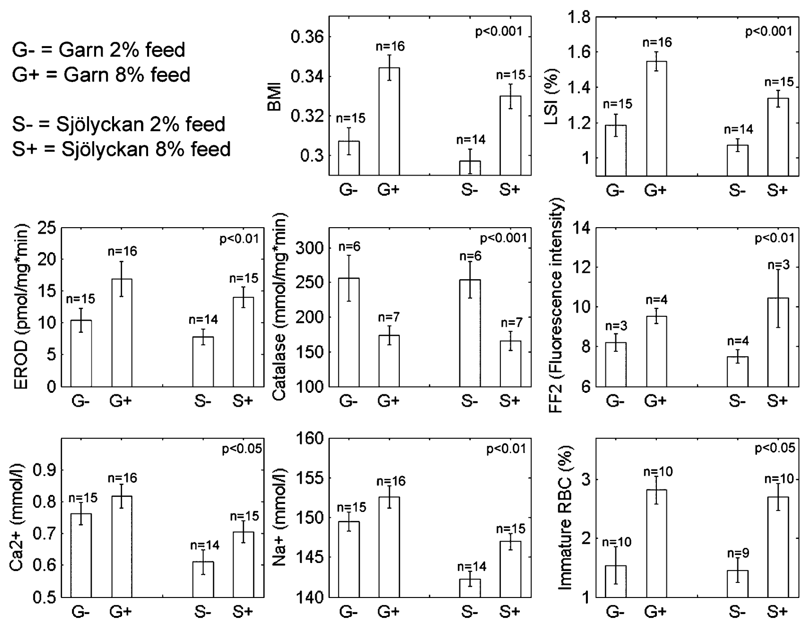

Fig. 2 Mean (±SE) for all variables showing significant effects, according to the ANOVA, in the feeding experiment.

Table 2 Results for all variables in the feeding experiment. Mean values, standard error (SE) and number of fish (*n*) are from pooled data from both sites

Variable	Feeding level	Mean	SE	*n*
BMI***/g cm^{-2}	2%	0.302	0.0046	29
	8%	0.337	0.0040	31
LSI*** (%)	2%	1.13	0.040	29
	8%	1.44	0.037	31
Glucose/mmol l^{-1}	2%	5.73	0.19	29
	8%	5.70	0.11	30
Hb/mmol l^{-1}	2%	4.95	0.10	29
	8%	5.00	0.10	29
HT (%)	2%	30.3	0.80	29
	8%	30.2	0.70	28
K^+/mmol l^{-1}	2%	3.23	0.090	29
	8%	3.10	0.075	31
Na^+ **/mmol l^{-1}	2%	146	1.02	29
	8%	150	1.00	31
Ca^{2+} */mmol l^{-1}	2%	0.689	0.029	29
	8%	0.764	0.027	31
Cl^-/mmol l^{-1}	2%	115	0.94	28
	8%	114	1.17	29
Lactate/mmol l^{-1}	2%	1.89	0.26	28
	8%	2.00	0.30	29
2-Ringed PAHs** (fluorescence)	2%	7.65	0.23	7
	8%	9.91	0.61	7
4-Ringed PAHs (fluorescence)	2%	2.27	0.31	7
	8%	2.37	0.17	7
5-Ringed PAHs (fluorescence)	2%	0.723	0.079	7
	8%	0.847	0.050	7
EROD**/pmol mg^{-1} min^{-1}	2%	9.13	1.14	29
	8%	15.5	1.62	31
GST/mmol mg^{-1} min^{-1}	2%	0.334	0.017	12
	8%	0.328	0.010	14
GR/mmol mg^{-1} min−1	2%	9.10	0.55	12
	8%	9.11	0.44	14
Catalase***/mmol mg^{-1} min−1	2%	255	20.3	12
	8%	170	9.35	14
Lymphocytes (%)	2%	1.60	0.087	19
	8%	1.49	0.113	20
Granulocytes (%)	2%	0.911	0.099	19
	8%	0.795	0.049	20
Thrombocytes (%)	2%	1.05	0.060	19
	8%	1.03	0.077	20
Immature RBC* (%)	2%	1.49	0.19	19
	8%	2.76	0.16	20

$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Table 3 Results from the starvation experiment

Variable	Feeding level	Mean	SE	*n*
BMI/g cm^{-2}	0%	0.320	0.014	16
	2%	0.342	0.015	17
LSI (%)	0%	1.35	0.065	16
	2%	1.52	0.053	17
Glucose/mmol l^{-1}	0%	5.13	0.26	16
	2%	4.91	0.20	17
Hb/mmol l^{-1}	0%	5.28	0.16	16
	2%	5.55	0.17	17
HT (%)	0%	28.4	1.06	16
	2%	30.9	1.51	16
K^+/mmol l^{-1}	0%	4.13	0.25	15
	2%	4.24	0.27	16
Na^+/mmol l^{-1}	0%	158	1.55	15
	2%	159	1.74	16
Ca^{2+}*/mmol l^{-1}	0%	1.14	0.042	15
	2%	0.981	0.058	15
Cl^-/mmol l^{-1}	0%	123	1.24	15
	2%	125	1.72	13
Lactate/mmol l^{-1}	0%	0.988	0.13	16
	2%	1.11	0.083	13
2-Ringed PAHs (fluorescence)	0%	12.2	2.65	3
	2%	9.32	1.95	3
4-Ringed PAHs (fluorescence)	0%	4.43	1.35	3
	2%	3.45	1.00	3
5-Ringed PAHs (fluorescence)	0%	1.82	0.51	3
	2%	1.46	0.43	3
EROD/pmol mg^{-1} min^{-1}	0%	6.36	1.54	16
	2%	6.42	1.38	17
Lymphocytes (%)	0%	1.03	0.12	10
	2%	0.922	0.083	9
Granulocytes (%)	0%	0.630	0.060	10
	2%	0.489	0.031	9
Thrombocytes (%)	0%	0.700	0.077	10
	2%	0.811	0.11	9
Immature RBC* (%)	0%	0.620	0.093	10
	2%	0.344	0.077	9

$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

significantly between feeding rations. The significant difference in EROD activity between the 8% and 2% feeding groups is noteworthy as EROD is known to be a good measure of exposure to several groups of toxicants, *e.g.* PAHs, dioxin-like compounds and planar PCBs. This difference may be a result of inhibition due to metabolic reorganization in response to changes in the nutritional state. This has been shown in rainbow trout that have been starved for twelve weeks, while no significant differences were found after six weeks.[40] In the feeding experiment in this study, however, the lower feeding group was not completely deprived of food (2% weekly feeding) and the experiment lasted for only four weeks. Therefore, it is more likely that the fish are exposed to higher levels of EROD inducers when feeding ratios are higher, either through contaminants in the feed or increased uptake from the water. It has been shown that dioxin-like compounds are present in the fish oil used to produce fish feed.[21,22,41] Furthermore, Forsberg[42] found that the metabolic rate and oxygen consumption in Atlantic salmon increased when feeding rates were higher, which could increase the uptake of pollutants from the ambient water *via* gill tissues. This is in agreement with the higher concentration of 2-ringed PAH metabolites in bile found in the higher feeding group. Because of the low bioaccumulation rate of PAHs,[43] it is not likely that this is caused by contaminated feed. Whatever the ultimate cause for the increase in EROD-activity with higher feeding ratio, this needs to be considered in the design of a monitoring program using caged fish.

Differences in feeding level also caused other effects. One example is the differences in ratio of immature red blood cells. This difference was significant in both experiments, but of contradictory nature. In the feeding experiment, the fish in the 2% feeding group that were fed less had significantly lower ratio of immature blood cells. This can probably be explained by reduced blood cell production. This is supported by the findings of Rios *et al.*,[44] suggesting that red blood cells are not replaced in starved Trahira (*Hoplias malabaricus*). In the starvation experiment, however, the fish that were starved had a significantly higher ratio of immature red blood cells compared to the fed fish. Furthermore, the ratios of immature red blood cells were lower in the starvation experiment compared to the feeding experiment. The diverging response of the ratio of immature red blood cells is hard to explain. However, the study clearly shows that the feeding level strongly affects this blood variable. The significant differences in plasma Na^+ and Ca^{2+} concentrations between feeding levels are also most likely an effect of starvation rather than an effect of elevated uptake of contaminants. It has, for example, been shown that starved trout are unable to alleviate the ionoregulatory disturbance at low pH.[45] The higher catalase activity in the low feeding groups is in accordance with earlier studies showing that feed deprived fish have experienced elevated levels of oxidative stress.[46,47] In addition, Zn-deficiency in rainbow trout has been linked to an increase in oxidative stress.[48]

To reduce the risks of bias in the data, feeding should be standardized so that all cages receive equal amounts of feed. However, this does not completely solve the problem. As feeding behavior varies with individuals, some fish within each cage are likely to eat more than others. This may increase the variance within treatments and thereby reduce the statistical power of the experiment. It is therefore desirable to avoid excessive feeding. It is also important to make sure that the availability of natural food sources does not bias the results when caged fish are used in environmental monitoring. When net cages are used, keeping the fish in the water column eliminates the risk of feeding from the bed and a small mesh size reduces the availability of food items that passes through the cage. In flow through tank systems, the size of the particles that can pass through the pump should be taken into consideration. The use of a standardized feeding regime, where all treatments receive equal amounts of feed, will reduce most of these problems, regardless of whether they are caused by elevated exposure, starvation stress or some other sort of bias resulting from the feeding level. However, if there are contaminants in the feed, analyzing time trends over several years will be problematic as a significant trend could be caused by a change in contamination level of the feed. Therefore, chemical analysis for contaminants should be performed on feed used in long term biomonitoring. However, one of the strengths with the use of biomarkers for environmental monitoring is that they respond to many types of chemicals, known or unknown. When it comes to contaminated fish feed, this will be a problem as it is possible that there will be contaminants in the feed that are not among the ones being analyzed. An alternative solution is to avoid feeding. This would, however, reduce the maximum length of the exposure time, and valuable information on the contamination load may be missed.

The significant differences found between sites are not in focus in this study. It is, however, interesting to note that the methodology was able to identify several differences between sites. One example of this is the significant difference in 4-ringed PAH metabolites in bile. This is not very surprising as the PAH exposure is likely to differ between a frequently trafficked river and a boatless lake. The difference in holding conditions between the two sites may complicate the interpretation of the site effects. A previous study has, however, shown that net cages and plastic tanks are comparable for the variables used in the present study.[18]

Conclusions

The present study shows that the feeding rations have an impact on several biomarkers in caged rainbow trout. In environmental monitoring with caged fish, it is therefore important to standardize the feeding procedure so that all cages receive equal amounts of feed. Hand feeding is preferable to distribute the feed in the cages. To be able to analyze time trends, feeding should be avoided or performed using well examined, low contaminated feed.

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