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RESEARCH ARTICLE

Effects of chemical stress and food limitation on the energy reserves and growth of turbot, *Scophthalmus maximus*

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Abstract The objective of the present study is to examine the growth and energetic performance of juvenile turbot after exposure to contaminated sediment and during the subsequent recovery period with or without food limitation. We designed a two-step experiment by first exposing juvenile turbot to harbour sediment for 26 days and then transferring them to clean sea water with different frequencies of feeding for 35 days. Without food limitation, fish previously exposed to contaminated sediment compensated for weight, length and lipid reserve losses; we did not record any differences in size, Fulton's K condition index and triacylglycerol/sterol (TAG/ST) ratio after the 35-day depuration period compared to the reference fish. This result could be related to the compensatory growth mechanism observed in a wide range of fish species following a period of growth depression. With food limitation during the 35-day depuration period, recovery growth was not sufficient to restore length and weight values similar to the reference fish. Moreover, turbot previously exposed to contaminated sediment and subsequently fed twice or once a week exhibited extremely low TAG/ST ratios, but the reference fish submitted to the same restrictive feeding conditions did not. This study indicates that juvenile fish affected by chemical pollution can improve their biological performance if pollution events are followed by a period of abundant food. However, if pollution events occur during periods of food scarcity, e.g. in winter, storage of energy reserves will be compromised.

Keywords Multiple stressors · Pollution · Food availability · Fish growth · Lipid reserves

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Introduction

A common objective for all marine species and life stages is to allocate available energy to maximize individual survival. In particular, in juvenile fish, energy reserves are critical in determining growth and overall population size (Gibson 1994). Reserves are typically acquired in times of abundant food supply or where environmental conditions support adequate physiological performance of the individual (Hurst 2007). The presence of simultaneous stressors throughout the year may compromise the storage of these energy reserves (Calow 1991; Adams 1999; McGeer et al. 2000). This is particularly true in temperate latitudes, where the phenology of the seasons generates large annual variations in light, temperature, oxygen and food resources. This variation poses significant trade-off challenges for living organisms as they have to respond according to the abiotic and biotic factors of their environment (Shuter et al. 2012). Thus, increased metabolic demand for biological maintenance and stress-resisting systems may result in decreased health status (Calow 1991; Lemly 1993; Congdon et al. 2001). In particular, increased winter mortality resulting from exhaustion of energy reserves has been reported during the early life stages of a variety of species (Shuter et al. 2012).

Among the stresses juvenile marine fish have to cope with, chemical contaminants adversely affect their biological performance, impair their immune function and may even threaten their survival (Van der Oost et al. 2003). As a consequence,

efforts have been devoted to assessing how pollutants affect fish populations. Investigating the effects of pollutants typically involves studying only chemical stressors as variation factors. However, pollution will rarely operate in isolation of other abiotic and biotic environmental influences. Interactions between pollutants and other environmental stressors are poorly understood, yet they are crucial for defining the capacity of fish to adapt to their environment.

In a previous study, we exposed juvenile turbot to contaminated sediment for 21 days (Kerambrun et al. 2012). The results showed that contaminated turbot exhibited decreased growth, morphometric indices and lipid indices following exposure. Like many authors of ecological studies, we concluded that considering these reductions in fish growth and energetic status, the chances for turbot to survive in nursery grounds were probably compromised (Suthers 2000; Adams 2002; Gilliers et al. 2006). However, in field conditions, fish are subjected to many other biotic and abiotic factors that could add positive or negative effects to those induced by chemical contaminants. For example, the lipid reserves of juvenile fathead minnows (*Pimephales promelas*) are negatively impacted by metal mining effluents in winter conditions (4 °C and 8/16 h light/dark) but not in summer conditions (20 °C and 16/8 h light/dark) (Driedger et al. 2010). Similarly, variations in food availability could interfere with the effects of pollutants on juvenile fish, since they could modulate the amount of available energy that fish require to maintain basic metabolic processes and repair damaged biological systems.

The objective of the present study is to examine the growth and energetic performance of juvenile turbot after exposure to contaminated sediment and during the subsequent recovery period with or without food limitation. We designed a two-step experiment by first exposing juvenile turbot to harbour sediment for 26 days and then transferring them to clean sea water with different frequencies of feeding for 35 days. We then assessed growth, RNA/DNA ratios, lipid and morphometric indices.

Materials and methods

Sediment exposure conditions

The sediment exposure assay was almost as described in a previous study (Kerambrun et al. 2012). The present study adds a recovery period and then focuses on it. Briefly, sediments were sampled from two stations in a harbour in northern France (Boulogne-sur-Mer stations B and C) and from a reference site close to the harbour. Two-month-old turbot, *Scophthalmus maximus*, (weight 3.91±0.42 g) were acclimatized at 14.8±0.1 °C for 2 weeks and fed with commercial fish food (Le Gouessant; Turbot Label Rouge, 55 % proteins, 12 % lipids) once a day at approximately 1 % of the total fish weight. Before the beginning of the experiment, each fish was anaesthetised in a 200 μg L^{-1} 2-phenoxyethanol solution, weighed (0.01 g accuracy), measured for total length (0.1 mm accuracy) and individually marked (Visual Implant Tag, 1.2 mm×2.7 mm, Northwest Marine Technology).

The experimental device for the 26-day assay consisted of a static water system made of 37-L glass tanks containing 5 L of sediment and 25 L of clean sea water. The assay was performed in triplicate for the three different types of sediment, in the same conditions as acclimatization. Twenty tagged fish were randomly distributed into each tank (60 per treatment).

During sediment exposure, fish mortality was 1 % for the reference condition, 21 % for the B condition and 59 % for the C condition. After the 26-day exposure period, 14 fish of the reference condition, 12 of the B condition and 10 of the C condition were equally sampled from triplicate tanks. They were identified (tagged), weighed and measured. Their muscle was sampled and preserved at −80 °C. The remaining fish were transferred into clean sea water as described in the next section.

Recovery period in clean sea water

A 35-day recovery period in clean sea water was undertaken following exposure to contaminated sediment. Eight 37-L glass tanks containing 5 L of reference sediment and 25 L of clean sea water were used. Considering the differences in mortality rates between treatments, the number of fish was adjusted as described in Table 1 to a density of 12 or 13 fish per tank. Fish from the reference, B and C conditions were fed once a day for three tanks or once a week for three other tanks. An additional feeding frequency of twice a week was also studied for two tanks with fish from the reference and B conditions.

No mortality was observed during the recovery period. After 35 days of the recovery period, therefore 61 days after the beginning of the experiment, all fish were sampled. They were identified (tagged), weighed and measured, and their muscle was sampled as previously.

Table 1 Repartition of fish previously exposed to sediment REF, B or C and the feeding frequency applied during the 35-day recovery period among the eight experimental tanks

No. of tank	Number of fish among exposure conditions				Feeding frequency
	REF	B	C	Total	
1	5	4	3	12	Once a day
2	5	4	3	12	
3	5	4	3	12	
4	7	6	0	13	Twice a week
5	8	5	0	13	
6	5	4	3	12	Once a week
7	5	4	3	12	
8	5	4	3	12	

Biological analysis

Growth index

Turbot-specific growth rates in weight (% per day) were estimated as

$$GW = 100(lnW_2 - lnW_1)/(t_2 - t_1),$$

where W_1 and W_2 are values of fish total body weight at times t_1 (beginning of the experiment) and t_2 (time of collection). Similarly, specific growth rates in length were estimated as

$$GL = 100(lnL_2 - lnL_1)/(t_2 - t_1),$$

where L_1 and L_2 are values of fish total length at times t_1 and t_2, respectively.

Condition indices

We estimated three condition indices in all fish: RNA/DNA ratio and triacylglycerols/sterols (TAG/ST) ratio, as indicators of nutritional status, and Fulton's K condition index as an indicator of the general well-being of the fish. This latter morphometric index assumes that heavier fish, for a given length, are in better condition. We calculated Fulton's K condition index with the following formula:

$$K = 100\left(W/L^3\right),$$

where W is the body mass (mg) and L is the total length (mm).

The procedure used to determine RNA and DNA concentrations in the individual fish was based on Caldarone et al. (2001). On the day of analysis, each frozen muscle was extracted in 1 % *N*-lauroylsarcosine Tris-EDTA buffer to dissociate the nucleoproteins. The standards and *N*-lauroylsarcosine-extracted samples were mixed with ethidum bromide in a 96-well microplate. Total fluorescence in each sample was measured first. RNA was then enzymatically digested by adding RNase, and RNA values were determined by subtracting the values of the second reading from total fluorescence. DNA values were determined from the fluorescence remaining after RNase treatment.

Total lipids in each individual fish were measured on lyophilised muscle (0.07 g). Lipid extraction was conducted using the method of Bligh and Dyer (1959), slightly modified as described by Meziane and Tsuchiya (2002). Lipids were extracted using a mixture of water/chloroform/methanol (1:1:1, *v*/*v*/*v*). TAGs and STs were separated from other lipids by thin layer chromatography (TLC).

Statistical analysis

Statistical analyses were performed with XLSTAT 2007. As our data did not comply with the parametric assumption of normality (Shapiro-Wilk tests) and homogeneity of variance (Levene tests) after various transformation techniques were tested, the non-parametric Kruskall-Wallis test and Mann-Whitney U test for post hoc pairwise comparisons were used to analyse differences in biological parameters.

Results

Effects of contaminated sediment on turbot physiological responses

Growth parameters and condition indices at the beginning of the experiment (t_0) and after 26 days of exposure are presented in Table 2.

Growth rates were significantly lower in weight and in length in turbot exposed for 26 days to sediments B (GW 0.10±0.15 % day^{-1}; GL 0.13±0.03 % day^{-1}) and C (GW 0.03±0.12 % day^{-1}; GL 0.10±0.03 % day^{-1}) compared to the reference (GW 1.01±0.07 % day^{-1}; GL 0.36±0.02 % day^{-1}).

We observed no significant difference in condition indices (K, RNA/DNA and TAG/ST ratios) between t_0 and t_{26} in turbot exposed to reference sediment. The K index was significantly lower in fish exposed to sediments B (1.41±0.03 mg mm^{-3}) and C (1.39±0.04 mg mm^{-3}) compared to t_0 (1.50±0.01 mg mm^{-3}) and also compared to reference fish (1.48±0.02 mg mm^{-3}) for condition C. Similarly, the lipid index based on the TAG/ST ratio was significantly lower in fish exposed to sediments B (0.76±0.07) and C (0.60±0.03) compared to t_0 (1.39±0.08) and reference fish (1.73±0.12). On the other hand, we observed no significant difference between the treatments as regards to RNA/DNA ratios.

Turbot physiological responses after the 35-day recovery period

Growth performance

Regarding the control sediment, when turbot were fed once a day, growth rates in weight and in length were not significantly different from those measured after 26 days of exposure and then 35 days of recovery (Fig. 1). Whatever the treatment (REF, B or C), GW and GL significantly decreased when the fish were fed twice and once a week compared to the fish fed once a day. We observed no significant difference in these parameters between the two kinds of restrictive feeding conditions in the reference or contaminated fish.

Table 2 Mean (±SE) of the specific growth rate in weight (GW) and length (GL), the Fulton's condition index (K), the RNA/DNA and TAG/ST ratios of turbot at t_0 and after the 26-day exposure to the three sediments REF, B and C

		GW	GL	K	RNA/DNA	TAG/ST
t_0				1.50±0.01	2.45±0.11	1.39±0.08
t_{26}	REF	1.01±0.07	0.36±0.02	1.48±0.02	2.59±0.08	1.73±0.12
	B	0.10±0.15[b]	0.13±0.03[b]	1.41±0.03[a]	2.38±0.15	0.76±0.07[a,b]
	C	0.03±0.12[b]	0.10±0.03[b]	1.39±0.04[a,b]	2.69±0.09	0.60±0.03[a,b]

[a,b] represents significant difference ($p<0.05$) compared to t_0 and reference, respectively

Whatever the feeding frequency during the recovery period, fish previously exposed to contaminated sediment no longer exhibited significantly lower growth rates in weight and in length compared to reference fish (Fig. 1). GL of fish exposed to sediments B and C returned to values similar to reference fish whether the fish were fed once a day, twice or once a week. For GW, we found differences among treatments according to feeding frequencies. When the fish were fed once a day, GW was significantly higher in the fish previously exposed to sediments B (GW 1.25±0.09 % day^{-1}) and C (GW 1.34±0.09 % day^{-1}) compared to reference fish (GW 0.72±0.14 % d^{-1}) whereas there was no significant difference when they were fed twice or once a week.

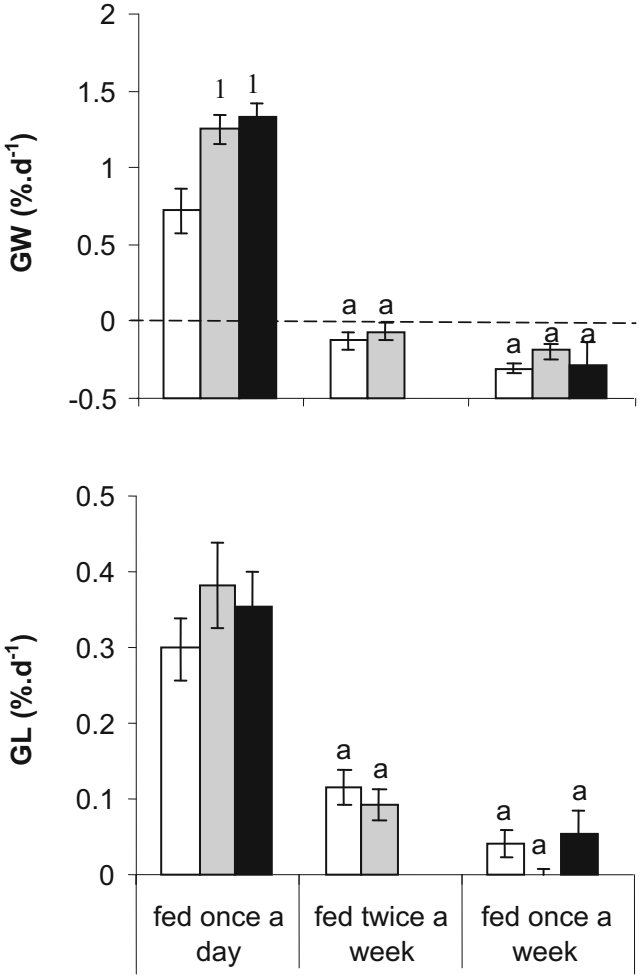

Fig. 1 Comparison of specific growth rate in weight (GW) and length (GL) of turbot exposed for 26-days to the three sediments: Ref (*white bar*), B (*grey bar*) and C (*black bar*) after the 35-day recovery period in clean sea water fed once a day, twice a week or once a week. Data represent mean (±SE). *1* represents significant difference ($p<0.05$) compared to reference of the same sample time and *a* compared to fish fed once a day among the same treatment

These results about fish growth during the recovery period can be visualised by weight and length trajectories throughout the experiment (Fig. 2). The decrease in GW and GL during contaminated sediment exposure resulted in significantly lighter (B=W 3.31±0.68 g; C=W 3.35±0.56 g) and smaller (B=L 61.4±3.1 mm; C=L 62.1±2.5 mm) turbot than the reference (W 4.06±0.67 g; L 64.8±2.8) after the 26-day exposure period. When fed once a day during the 35-day recovery period, contaminated turbot returned to weights and lengths similar to reference fish (mean values=W 5.54±1.9 g; L 71.9±0.8 mm). When turbot were fed once a week, their weight (B=W 2.85± 0.35 g; C=W 2.50±1.32 g) and length (B=L 60.8±2.1; C=L 62.4±2.8) remained significantly lower in contaminated fish after the 35-day recovery period compared to reference fish (W 3.61±0.71 g; L 64.9±3.5). We found similar variations in weight and length between the fish previously exposed to sediment B and the reference fish when fed twice a week during the recovery period (data not shown).

Condition indices

When the fish were fed once a day, in the reference treatment, the three condition indices (K, RNA/DNA and TAG/ST ratios) were not significantly different after 26 days of exposure and 35 days of recovery (Fig. 3). When the fish were fed twice and once a week, all condition indices significantly decreased compared to the fish fed once a day, whatever the treatment (REF, B or C). We observed no significant difference in these parameters between the two kinds of restrictive feeding conditions whatever the treatment is.

As for growth parameters, fish previously exposed to contaminated sediment exhibited no significant decrease in the

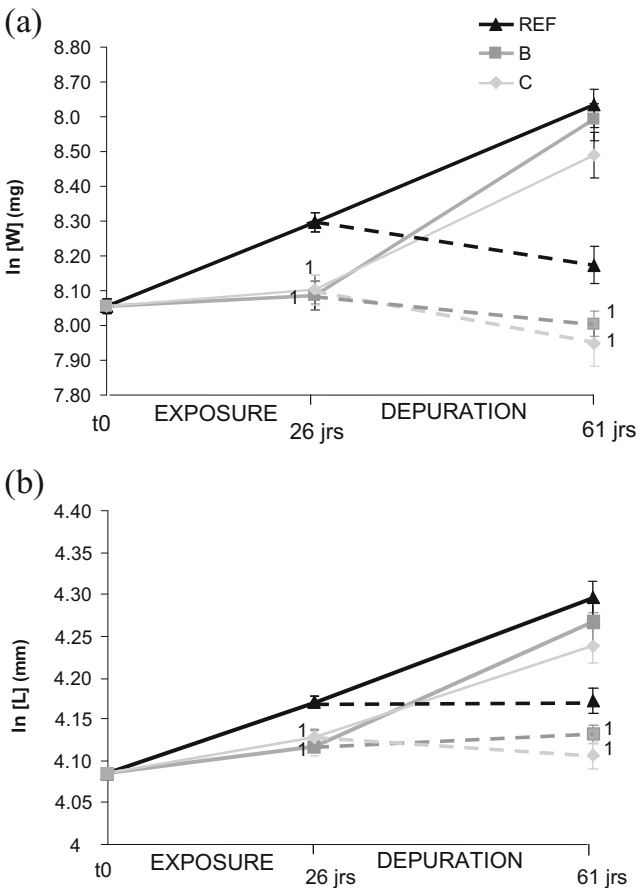

Fig. 2 Weight (**a**) and length (**b**) trajectories of turbot exposed for 26 days to the three sediments Ref, B, and C following by a 35-day recovery period in clean sea water fed once a day (*full line*) or once a week (*stippled line*). *1* represents significant difference ($p<0.05$) compared to reference of the same sample time

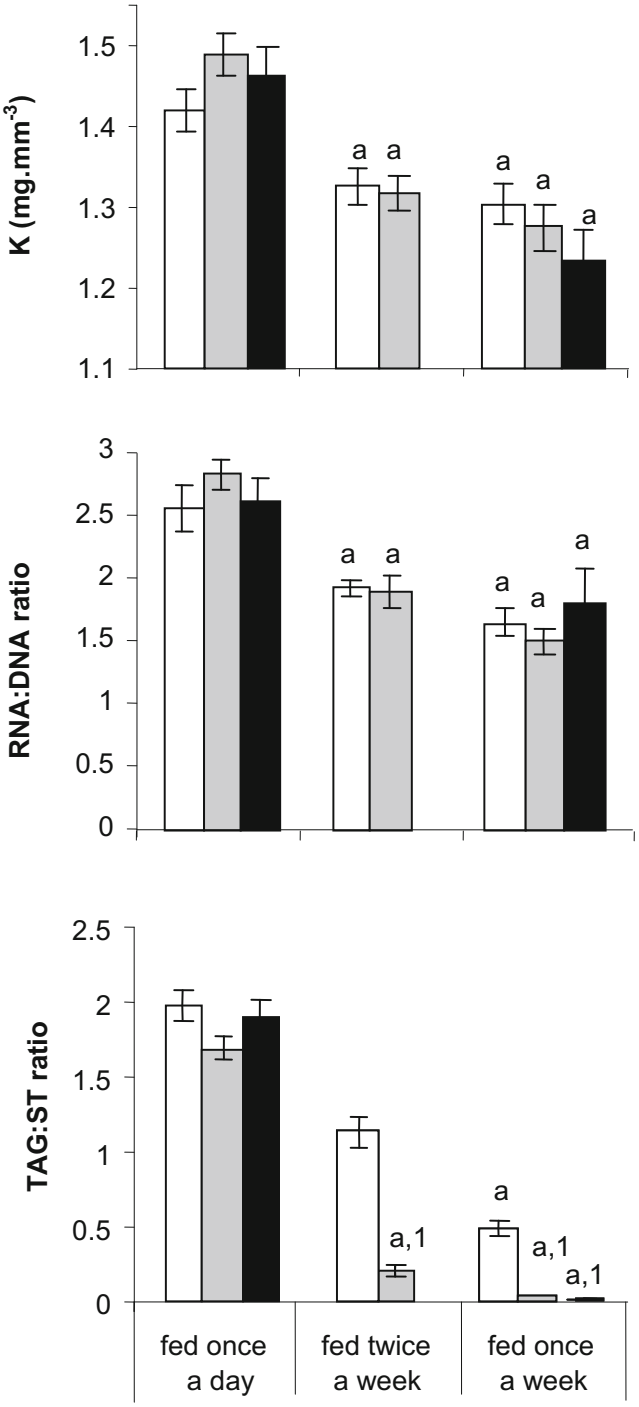

Fig. 3 Comparison of Fulton's condition index (K), the RNA/DNA and TAG/ST ratios of turbot exposed for 26 days to the three sediments Ref (*white bar*), B (*grey bar*) and C (*black bar*) after the 35-day recovery period in clean sea water fed once a day, twice a week or once a week. Data represent mean (±SE). *1* represents significant difference ($p<0.05$) compared to reference of the same sample time and *a* compared to fish fed once a day among the same treatment

Fulton's K condition index compared to reference fish whatever the feeding frequency is during the recovery period (Fig. 3): K index values were similar in reference and treated fish whether they were fed once a day, twice or once a week.

Similarly to the exposure period, we found no significant difference in RNA/DNA ratios between treatments after the 35-day recovery period whatever the feeding frequency is.

The lipid index was the only parameter that displayed significant differences among treatments between the feeding frequencies. When contaminated turbot were fed once a day, they recovered similar TAG/ST ratios to the reference fish (mean TAG/ST ratio 1.85±0.19) after the 35-day recovery period. However, when they were fed twice a week, TAG/ST ratios were significantly lower in turbot previously exposed to sediment B (0.21±0.03) compared to reference fish (1.14±0.10). Similarly, when contaminated turbot were fed once a week, the ratio significantly decreased by about 10-fold in B fish (0.04±0.01) and by about 50-fold in C fish (0.01±0.01) compared to reference fish (0.49±0.05).

Discussion

Our study consisted of exposing juvenile turbot to two contaminated sediments with similar exposure conditions as in a

previous study (Kerambrun et al. 2012), with an additional recovery period. We did not record any fish mortality in our previous study, but we found 21 % mortality with sediment B treatment and 59 % with sediment C treatment in the present one. This result could be explained by higher contaminant concentrations in sediment, especially higher PAH values (Kerambrun et al. 2013): In the present study, total US EPA's 16 priority PAHs amounted to 2.24 mg kg^{-1} in sediment B and 10.2 mg kg^{-1} in sediment C versus respectively 1.27 mg kg^{-1} and 2.44 mg kg^{-1} in the previous one. Metal concentrations were similar in the two studies except Mn in sediment B (700 mg kg^{-1} in the first experiment and 1,758 mg kg^{-1} in the present one). Moreover, juveniles were younger (2 months vs 4 months old) and so potentially more sensitive to contamination. For example, George et al. (1996) reported that the sensitivity of the early life stages of *S. maximus* following exposure to cadmium decreased with age. In the same way, based on fish EC50 data, Hutchinson et al. (1998) found that juveniles were more sensitive than adults for 92 % of the substances they tested. The apparent variability in sensitivity between life stages may be due to several factors such as surface area/volume ratio, greater uptake of toxicants from the environment, under-developed homeostatic mechanisms to deal with toxicants, immature immune systems and under-development of organs (liver and kidney) that play an important role in detoxifying and eliminating toxicants (Mohammed 2013).

As previously, growth rates in weight and in length, K indices and TAG/ST ratios significantly decreased in the fish exposed to the two contaminated sediments for 26 days compared to reference fish. The effects of contamination on growth and condition indices appeared slightly lower in the present study than in the previous one. In the present study, mortality events probably occurred in fish with the lowest physiological performance, and thus, surviving fish exhibited higher tolerance to contamination than the mean of all turbot analysed in the previous, mortality-free study. We observed no difference in RNA/DNA ratios among treatments. This biochemical index is a useful and reliable indicator of the nutritional status and growth of larval and juvenile fish (Clemmesen 1988; Buckley et al. 1999). However, individual nucleic acid determinations have revealed extensive and unexplained inter-individual variability in RNA content and in RNA/DNA ratios in fish reared under identical conditions (Clemmesen 1988; Robinson and Ware 1988; Mathers et al. 1993). This variation in fish metabolism may have limited the observation of differences between exposed and reference fish. Moreover, many of these studies were carried out on larval fish or very young fish. Fish age is known to influence RNA/DNA variability, so this may have been the case. For example, decreased RNA concentrations have been observed with age in rainbow trout (*Oncorhynchus mykiss*) (Perago et al. 2001). In addition, in cases of exposure to contaminants, an increase in RNA content might be related not only to the induction of protein detoxification systems but also to an increase in the protein turnover rate, with subsequent high RNA/DNA ratios but decreased growth rates (Fonseca et al. 2009).

We applied a 35-day depuration period after the exposure period to analyse the physiological recovery of turbot after being weakened by chemical contaminants. Under optimal feeding conditions, growth was no longer impaired in contaminated fish compared to reference fish. Moreover, in fish previously exposed to contaminated sediment, we no longer observed differences in size and K index after the 35-day depuration period. Therefore, they made up for their loss in weight and length. This result could be related to the classical compensatory growth (CG) process observed in a wide range of organisms including fish (Abdalla et al. 1988; Ali et al. 2003; Critser et al. 1995; Wu et al. 2006). It consists of faster fish growth following a period of growth depression as compared to their normally growing counterparts (Sevgili et al. 2012). In fish, CG can be elicited through a number of ways such as starvation, feed restriction, exposure to low or high temperature and hypoxia (Eroldoğan et al. 2006; Hayward et al. 1997; Huang et al. 2008; Miglavs and Jobling 1989; Person-Le Ruyet et al. 2003). As observed in the present study, CG was more frequent with fish weight than fish length. Some authors suggest that in energetically restricted fish, priority is given to replenishing lost mass rather than structure (Álvarez and Nicieza 2005; Bavčević et al. 2010).

The depletion of lipids in fish exposed to contaminated sediment, with low values of TAG/ST ratios, was no longer there following the 35-day depuration period with optimal feeding. This result suggests that the metabolic process associated with compensatory growth was not related to overutilization of lipids. Increased rates of food consumption or a hyperphagic response are a common phenomenon considered as the main mechanism of the CG response following feed restriction or starvation (Ali et al. 2003; Eroldoğan et al. 2006; Cho et al. 2012). Other authors also suggest enhanced food conversion efficiency (Qian et al. 2000; Myszkowski 2013). In turbot, when excess feeding followed a severe feed restriction period, the fish displayed compensatory growth that fully compensated for lost growth and became hyperphagic during the re-feeding period (Saether and Jobling 1999).

To our knowledge, no study has reported any compensatory growth following chemical pollution so far. A commonly observed sublethal response of organisms chronically exposed to chemical contaminants is a change in their energy allocation (Rowe et al. 2001). Maintenance costs associated with the response to chemical toxicants lead to reduced growth and a decrease in overall fish condition (Tanaka et al. 2007) that could be stronger than during starvation (Kerambrun et al. 2012). In a previous study, we analysed metal and PAH detoxification during the depuration period (Kerambrun

et al. 2013). While Cd, Cu and Pb accumulated in turbot liver after 26 days of exposure to sediments B and C, they no longer accumulated after the 35-day depuration period. Similarly, PAH metabolites detected in turbot after exposure returned to values similar to reference fish after 35 days in clean sea water. Considering these results, turbot were able to compensate for their losses in weight, length and lipid reserves despite the cost associated to metal and PAH detoxification.

Indices of juvenile fish health are used in ecological studies, assuming that reductions in fish growth and energetic status could dramatically decrease their chances for survival in nurseries (Suthers 2000; Adams 2002; Gilliers et al. 2006). The present investigation sheds new light on this aspect by suggesting that even though growth and condition were impaired in fish exposed to chemical stress, the fish recovered size, lipid status and condition similar to uncontaminated fish after a depuration period. Thus, surviving fish weakened by chemical contaminants can recover good physiological performance when back into a clean environment with optimal living conditions. This means that their survival may not be compromised even with delayed growth and low lipid reserves.

In their natural habitats, fish may be subjected to periods of food limitation (Navarro and Gutierrez 1995). That is why we also focused on the effects of food restriction during the depuration process. With food limitation during the 35-day depuration period, growth rates in length and in weight in turbot previously exposed to the two contaminated sediments and in reference fish were similar. However, this recovery growth did not appear sufficient to restore length and weight values similar to reference fish. This indicates that compensatory growth was not induced in that particular case of restricted feeding conditions. Moreover, based on the lipid index, turbot previously exposed to contaminated sediment and subsequently fed twice or once a week displayed lower lipid reserves than reference fish also submitted to these restrictive feeding conditions. Lipids are the first reserves to be mobilized in many fish species when food becomes scarce (Sargent et al. 1989; Van Dijk et al. 2005). The combined decrease in triacylglycerol content during chemical contamination and then during food limitation led to lipid depletion, with TAG/ST ratios close to zero. These results are in line with those of Blanquet and Oliva-Teles (2010) in which juvenile turbot were able to compensate for lost growth after being fed restricted rations on a daily basis but were unable to compensate for lost growth if still deprived of feed even for 1 day. The most likely explanation for the lack of compensatory growth is that with restrictive food, fish cannot have a hyperphagic response. Moreover, with food restriction, bioaccumulation of Cd, Cu and Pb was still observed in turbot liver following a 35-day recovery period (Kerambrun et al. 2013). Thus, following chemical contamination, a nutritional stress renders fish unable to correctly detoxify metals, which could be explained by the lipid depletion we observed.

Conclusion

Energy reserves are typically acquired in times of abundant food supply or when environmental conditions support adequate physiological performance of the individual (Hurst 2007). For most species, individuals accumulate energy over the spring-summer-fall period and deplete it in winter (Shuter and Post 1990). The results of the present study show that additional stresses, such as pollutants, could interfere with this classical process. One interesting result of this study is that individuals previously affected by pollution can recover good physiological status and reserve storage capacity if the pollution event is followed by a period of abundant food. However, if it occurs along with restrictive feeding conditions, as in winter, the storage of these energy reserves will be compromised. Thus, normal winter conditions of reduced food availability combined with chemical stress decrease the ability of an individual to store sufficient energy to survive.

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