Dietary low-glucosinolate rapeseed meal affects thyroid status and nutrient utilization in rainbow trout (*Oncorhynchus mykiss*)

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Two rapeseed (*Brassica napus*) meals, RM1 and RM2, with two levels of glucosinolates (GLS; 5 and 41 μmol/g DM respectively) were incorporated at the levels of 300 and 500 g/kg of the diets of juvenile rainbow trout (*Oncorhynchus mykiss*) in replacement of fish meal, and compared with a fish-meal-based diet. A decrease in the digestibility of the DM, protein, gross energy and P was observed with high-rapeseed meal (RM) incorporation. In trout fed on RM-based diets, growth performance was reduced even after only 3 weeks of feeding. Feed efficiency was adversely affected by RM and GLS intake. Protein and energy retention coefficients were significantly lower in fish fed on the diet containing the higher level of GLS. P retention was significantly lower with all the RM-based diets than with the fish-meal diet. Irrespective of the degree of growth inhibition, fish fed on RM-based diets exhibited similar typical features of hypothyroid condition due to GLS intake, expressed by lower plasma levels of triiodothyronine and especially thyroxine and a hyperactivity of the thyroid follicles. This hypothyroidal condition led to a strong adjustment of the deiodinase activities in the liver, the kidney and the brain. A significant increase of the outer ring deiodinase activities (deiodinases type I and II respectively) and a decrease of the inner ring deiodinase activity (deiodinase type III) were observed. It is concluded that the observed growth depression could be attributed to the concomitant presence of GLS, depressing the thyroid function, and of other antinutritional factors affecting digestibility and the metabolic utilization of dietary nutrients and energy.

Rainbow trout: Rapeseed: Growth: Glucosinolates: Thyroid function

Rapeseed is primarily grown for its high oil content (400–450 g/kg seed) but the defatted meal is also used as a source of protein for mammals and poultry. Rapeseeds are nevertheless known to contain antinutritional factors, such as a high fibre content, tannins, phytic acid and glucosinolates (anionic β-thio-D-glucopyrranosides encountered in the leaves, seeds, flowers and roots of a large variety of plants, such as rapes). The incorporation of rapeseed meals (RM) containing high levels of glucosinolates (GLS) to animal feed leads to reduced feed intake, enlarged thyroid, reduced plasma thyroid hormone levels and, occasionally, organ abnormalities (liver and kidney) and even mortality (Bunting, 1981; VanEtten & Tookey, 1983).

On account of its good amino acid profile, many studies since the seventies have focused on the incorporation of RM into fish feed (Yurkowski *et al*. 1978; Teskeredzic *et al*. 1995; Webster *et al*. 1997). A comprehensive review has also been made by Higgs *et al*. (1996). In brief, it has been shown that the deleterious effects of the high levels of fibre and of the GLS restrict the level of incorporation of RM into fish feeds to approximately 200 g/kg (Yurkowski *et al*. 1978; Hardy & Sullivan, 1983; Hilton & Slinger, 1986; Leatherland *et al*. 1987; Gomes & Kaushik, 1989; Gomes *et al*. 1993; Higgs *et al*. 1996). However, significant improvements have been made to reduce the levels of GLS below 20 μmol/g and to improve the nutritional value of RM by genetic selection of cultivars (Vermorel *et al*. 1986) and by the use of new processing techniques (Bell, 1993).

Studies carried out by Vermorel & Baudet (1987) in terrestrial animals and by Hardy & Sullivan (1983) in trout have shown that thyroidal disturbances (decreasing plasma triiodothyronine (T_3) and thyroxine (T_4) levels) caused by

Abbreviations: ADC, apparent digestibility coefficient; D, deiodinase; IRD, inner ring deiodination; GLS, glucosinolates; ORD, outer ring deiodination; RM, rapeseed meal; rT_3, 3,3′,5′-triiodothyronine; T_3, triiodothyronine; T_4, thyroxine.

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RM ingestion occurred at a level of incorporation lower than the level which led to growth depression. Higgs *et al.* (1982) and Hardy & Sullivan (1983) suspected the existence of a compensatory effect, likely to happen through an adjustment of the deiodinase activities. In all vertebrates, T_4 is the main secretory product of the thyroid follicles even though T_3 is the biologically active hormone. Outer ring deiodination (ORD) converts T_4 into T_3 and reverse T_3 (3,3′,5′-triiodothyronine (rT_3) into 3,5-diiodothyronine (T_2). Inner ring deiodination (IRD) converts T_4 into rT_3 and degrades active T_3 to T_2. The ORD reaction is the activating pathway of T_4 metabolism as it produces T_3, while the IRD reaction is considered to be the inactivating pathway as it degrades T_4 and inactivates T_3 (Kühn *et al.* 1993). Three types of deiodinases have been characterized in mammals (Leonard & Visser, 1986). Type I deiodinase (D1) can catalyse both ORD and IRD and has a substrate preference for rT_3, but can also convert T_4 into T_3. Type II deiodinase (D2) performs only ORD, and prefers T_4 as the substrate. Type III deiodinase (D3) has exclusive IRD activity and deiodinates T_3 preferentially. Recent studies have demonstrated that deiodinating activities similar to the mammalian D1, D2 and D3 are also found in five teleosts, including rainbow trout (*Oncorhynchus mykiss*) (Mol *et al.* 1998). Generally, in studies with fish, only the hepatic T_4 ORD (D2) reaction in liver has been considered, which may neglect an important part of the peripheral thyroid metabolism.

In this present study, two RM with different levels of GLS (5 to 41 mol/kg DM) were incorporated each at two levels (300 and 500 g/kg) in the diets of rainbow trout. The aim of the study was to determine a possible relationship between thyroidal-axis status (plasma levels of thyroid hormones, deiodinase activities, i.e. D1, D2 and D3 in different tissues and the activity of thyroid follicles) and growth and nutrient and energy utilization in rainbow trout.

Materials and methods

Two different rapeseed meals (*Brassica napus*) (provided by the Centre Technique Interprofessionnel des Oléagineux Métropolitains (CETIOM), Paris, France) with different levels of GLS were used. Rapeseeds were first subjected to an intense dehulling treatment in order to reduce their fibre content. Two different oil-extraction processes were then used. The first consisted of a pressure-cooking (97°, 5000 kPa) under wet conditions. Any remaining oil was extracted by six consecutive hexane washings in percolation, followed by removal of solvent by steam injection (105°, 600 kPa). Subsequent grinding produced rapeseed meal 1 (RM1). The method used to obtain rapeseed meal 2 (RM2) consisted of a direct oil extraction. Rapeseeds were subjected to a double pressing, and oil was removed by eight consecutive hexane washings. The removal of solvent was done using steam injection (80°, 300 kPa). The thermal treatment applied to RM1 significantly decreased the total GLS level to 5 mol/kg DM, compared with RM2 which contained 41 mol/kg DM (analyses were carried out by the Laboratoire d'Analyse du Cetiom, Orléans, France). Six different GLS were identified in RM1. Of these, progoitrine (2·2 mol/kg DM) and gluconapine (1·2 mol/kg DM) were the most important. Ten different GLS were identified in RM2. Progoitrine (17·2 mol/kg DM), gluconapine (6·9 mol/kg DM), glucobrassicanapine (2·2 mol/kg DM), sinalbine (4·5 mol/kg DM) and glucobrassicine (7·6 mol/kg DM) were the major components. A conversion factor of 0·432, based on the molar weight of each GLS and their average proportion in RM, can be applied to convert the GLS values expressed as mol/kg to g/kg. The chemical composition of these two RM is shown in Table 1. Their amino acid profiles meet the requirements of rainbow trout (Tables 2 and 3).

Diets and digestibility measurements

For the growth study, five experimental diets were formulated (Table 3), containing respectively no rapeseed (control diet), 300 and 500 g RM1/kg (R1-300 and R1-500 diets), 300 and 500 g RM2/kg (R2-300 and R2-500 diets).

The apparent digestibility coefficients (ADC) of the diets were measured using the indirect method developed by Choubert (1999) with diets containing Cr_2O_3 (10 g/kg) as an inert tracer. Ten tanks (60 litres capacity with a flow rate of 5 litres/min, 16·5°) with twenty fish in each (body weight about 100 g each) were adapted to the experimental conditions (12 h light–12 h dark) and fed on one of the experimental diets for 7 d before the start of faecal collection. Faecal samples were collected continuously during 7 d from each tank using the apparatus developed by Choubert *et al.* (1982). After freeze-drying, the faeces (two samples per

Table 1. Chemical composition of the fish meal* and rapeseed meals†

	Fish meal	Rapeseed meal 1	Rapeseed meal 2
Dry matter (g/kg DM)	902	906	948
Ash (g/kg DM)	173	78	76
Crude protein (g/kg DM)	693	453	446
Crude fat (g/kg DM)	98	18	13
Phosphorus (g/kg DM)	21	16	16
Starch (g/kg DM)	–	52	45
N-free extracts (g/kg DM)‡	–	399	420
Energy (kJ/kg DM)	2060	1970	1940
Phytic acid (g/kg DM)	–	4·2	4·4
Glucosinolates (mol/kg DM)	–	5·1	40·7

DM, dry matter.
* Norwegian herring meal.
† For details of the oil-extraction processes used to prepare rapeseed meals 1 and 2, see above.
‡ The amount of N-free extracts (lignin, non-starch polysaccharides, oligosaccharides) was estimated as follows: N-free extracts (g/kg dry matter) = 100 – (ash + crude protein + crude fat + starch).

Table 2. Essential amino acid composition (g/16 g nitrogen) of the fish meal* and rapeseed meal† as compared with the requirements of trout

Amino acid	Trout requirements‡	Fish meal	Rapeseed meal 1	Rapeseed meal 2
Arg	3·6	5·0	6·8	7·9
Lys	5·3	5·8	6·6	6·6
His	1·6	2·5	2·6	3·3
Ile	2·4	4·3	3·8	4·3
Leu	4·4	7·3	4·5	6·1
Val	3·2	5·4	3·7	4·3
Met	1·8	2·4	1·9	2·3
Cys	0·9	0·9	2·5	2·5
Phe	3·2	4·0	4	3·8
Tyr	2·0	3·2	4	4·2
Thr	3·3	3·6	4·9	5·6
Trp	0·6	1·1	1·3	1·3

* Norwegian herring meal.
† For details of the oil-extraction processes used to prepare rapeseed meals 1 and 2, see p. 654.
‡ Requirements according to the National Research Council (1993).

Table 3. Ingredients and chemical composition of diets used in the growth experiment

	Diets					Trout requirements
	Control	R1-300	R1-500	R2-300	R2-500	
Ingredients (g/kg)						
Rapeseed meal 1*	–	30	50	–	–	
Rapeseed meal 2*	–	–	–	30	50	
Fish meal†	53	35	20·5	35	21	
Soluble fish protein concentrate	3	3	3	3	3	
Flaked maize	32	18	10·5	18	9	
Fish oil	9	11	13	11	14	
Vitamin mixture‡	1	1	1	1	1	
Mineral mixture§	1	1	1	1	1	
Binder (sodium alginate)	1	1	1	1	1	
Chemical composition						
Dry matter (g/kg)	940	942	942	933	941	
Ash (g/kg DM)	104	92	82	92	83	
Phosphorus (g/kg DM)	15	15	14	15	14	
Crude protein (g/kg DM)	400	402	387	402	389	
Amino acids (g/kg DM)						
Arg	19	22	23	23	25	14
Lys	22	24	24	24	24	21
His	10	10	10	11	11	6
Ile	17	17	15	17	17	10
Leu	31	27	23	29	26	18
Val	21	19	17	20	19	13
Met	9	9	8	9	9	7
Cys	4	6	7	6	7	4
Phe	16	16	15	16	15	13
Tyr	12	14	14	14	15	8
Thr	14	16	17	17	18	13
Trp	4	5	5	5	5	2
Crude fat (g/kg DM)	161	169	171	159	158	
Starch (g/kg DM)	228	123	722	125	562	
N-free extracts (g/kg DM)‖	107	214	288	222	314	
Gross energy (kJ/kg DM)	2140	2190	2200	2190	2230	
Digestible protein (g/kg DM)¶	356	363	349	362	336	
Digestible energy (g/kg DM)¶	180	182	179	178	174	
Digestible protein : digestible energy value (mg/kJ)	19·7	19·9	17·9	17·8	17·4	
Glucosinolates (mol/kg DM)**	–	1·4	2·3	11·6	19·3	

DM, dry matter.
* For details of the oil-extraction processes used to prepare rapeseed meals 1 and 2, see p. 654.
† Norwegian herring meal, 700 g crude protein/kg.
‡ National Research Council (1993).
§ Mineral mixture contained (/kg diet): calcium carbonate 1·12 g, magnesium oxide 0·62 g, ferric citrate 0·1 g, potassium iodide 0·2 mg, zinc sulfate 0·2 g, copper sulfate 0·15 g, manganese sulfate 0·15 g, dibasic calcium phosphate 2·5 g, cobalt sulfate 1 mg, sodium selenite 1·5 mg, KCl 0·45 g, NaCl 0·2 g.
‖ Includes lignin, non-starch polysaccharides and oligosaccharides and were estimated as follows: N-free extracts (g/kg) = 100 − (ash + crude protein + crude fat + starch).
¶ Calculated from apparent digestibility coefficient data of the digestibility experiment (Table 4).
** Calculated from the glycosinolate content of the rapeseed meals (see Table 1).

diet, each sample corresponding to a pool of 7 d of defecation from each tank) were analysed for Cr_2O_3, protein, fat, energy, ash and P content. The ADC values of DM, nutrients and energy in the different experimental diets were calculated according to Maynard & Loosly (1969):

$$\text{ADC of DM} = 1 - (\text{dietary } Cr_2O_3/\text{faecal } Cr_2O_3),$$

$$\text{ADC of nutrient or energy} = 1 - (\text{dietary } Cr_2O_3/\text{faecal } Cr_2O_3) \times (\text{faecal nutrient or energy/dietary nutrient or energy}).$$

Growth study

Fifteen outdoor tanks (500 litres) were used at the INRA experimental fish farm (Donzacq, Landes, France) during spring 1995. Tanks were supplied with spring water (17 ± 1°, flow rate 10 litres/min). Fifty rainbow trout with an average body weight of 20 g were randomly allocated to each tank. After a 1-week adaptation period, one of the five diets were fed to each group of three tanks of fish for 9 weeks. Food was distributed twice a day by hand, to apparent satiety. Fish were weighed and the voluntary feed intake was recorded every 3 weeks.

At the end of the growth study, a series of tissue and blood samplings were performed on fish fed on the respective diets. In order to minimize the possibility of time and tank effects, samples of fish were taken from the tanks at two different times over a 4 d period (from day 70 until day 73), according to a randomly scheduled sequence. In addition, to keep disturbance to the fish at minimum, the same tanks were never sampled more than once every 24 h and nocturnal samplings were conducted without using any light. In total, this series of sampling resulted in five fish per tank 8 h before the morning meal (at 00.00 hours) and five others 4 h after the morning meal (at 12.00 hours), i.e. thirty fish per dietary treatment.

Blood samples of all sampled fish were obtained from vessels near the caudal peduncle using a heparinized syringe. Blood was immediately centrifuged and plasma was stored at −20°. The liver, the brain and the kidney of all sampled fish were dissected following blood sampling, frozen in liquid N_2, and stored at −80°. Ten additional fish were taken on day 73 at 12.00 hours from both control and the R2-500 dietary treatment for histological studies. The lower jaw was dissected from each fish immediately after blood sampling, and fixed in Bouin's fluid.

Chemical analysis of ingredients, diets, faeces and whole body

Whole fish body samples (three pools of ten fish from the beginning of the experiment and one pool of five fish per tank at the end of the experiment) were homogenized and freeze-dried. Analyses of composition of ingredients, diets, freeze-dried faeces and whole-body samples were made following the usual procedures: DM after drying at 105° for 24 h; ash by combustion at 550° for 12 h; protein (N × 6·25) by the Kjeldahl method after acid digestion; gross energy in an adiabatic bomb calorimeter (IKA C-4000, Cofralab, Colomiers, France); fat after extraction with light petroleum (40°–60°) by the Soxhlet method and total P by spectrophotometric analysis of the phosphovanadomolybdate complex after mineralization and acid digestion (ISO/DIS 6491 method). Phytic acid content was determined according to Davies & Reid (1979) (analysis performed by the Department of Animal Production Sciences, University of Udine, Italy). Starch was measured by an enzymic method (Thivend *et al.* 1972) using glucoamylase and glucose oxidase. Cr_2O_3 in the diet and faeces was determined according to Bolin *et al.* (1952). For essential amino acids, the ingredients were hydrolysed with 6 M-HCl at 110° for 24 h and the chromatographic separation and analysis of the amino acids were performed after orthophthaldehyde (Sigma P 1378, St Quentin, France) derivatization of amino acids using HPLC (HPLC-Varian Model 5000, Varian, Limerick, Ireland, C_{18} Aminotag column, Varian, Limerick, Ireland) following a modified procedure of Gardner & Miller (1980).

Thyroid status

Assay for thyroid hormones in plasma. Plasma thyroid hormone (T_4 and T_3) levels were measured with a radioimmunoassay described by Boeuf & Prunet (1985) and modified by Martinez *et al.* (1995). The detection limits for the T_4 and T_3 radioimmunoassays were 1·25 and 0·62 ng/ml respectively. The specific binding obtained was 46 % for T_4 and 67 % for T_3, and the non-specific binding was 10 % for T_4 and 9 % for T_3. These percentages were estimated at a hormone level of 80 ng/ml. The intra-assay CV was 10 % for both T_4 and T_3.

Deiodinase assay. A previous study (Mol *et al.* 1998) allowed us to determine the different types of deiodinases present in rainbow trout (D1, D2 and D3) and the tissues with the highest level of enzymic activity (liver, brain and kidney). Deiodinase activities were measured as described in that paper.

The microsomal fractions were prepared as described previously (Mol *et al.* 1998). Tissues were homogenized in five volumes of buffer (0·25 M-sucrose, 10 mM-HEPES, pH 7·0, 1 mM-1,4-dithiothreitol) and centrifuged for 20 min (4° at 25 000 ***g***). The supernatant fraction was then centrifuged for 60 min (4° at 100 000 ***g***). The resulting pellet and fluffy upper layer were resuspended together in three volumes of buffer (0·1 M-phosphate, pH 7·0, 2 mM-EDTA, 1 mM-1,4-dithiothreitol), snap-frozen in portions and stored at −80°. All procedures were carried out on ice. Protein concentrations were determined with the BCA protein assay reagent (Bio-Rad, Nazareth, Belgium) using bovine serum albumin as a standard.

D1 activity was measured in the kidney (*n* 30 per dietary treatment), while D2 activity was measured in the liver (*n* 30 per dietary treatment) and D3 activity was measured in the brain (brains of the fish sampled in each tank and at each sampling time are pooled, *n* 6 per dietary treatment). In brief, an adequate amount of microsomal protein (1 mg/ml for each assay except in the case of liver D2: 0·25 mg/ml) suspended in sodium phosphate buffer (0·1 M, 2 mM-EDTA, 1 mM-1,4-dithiothreitol, pH 7·1) was incubated with a similar amount of substrate–cofactor solution (kidney D1 and liver D2: 1 h at 30°; brain D3: 1 h at 37°). 3·70 kBq of the preferred radioactive (^{125}I) substrate was added in the substrate–cofactor solution (D1: rT_3 (0·1 μM); D2: T_4 (1 nM); D3: T_3 (10 nM)) and 1,4-dithiothreitol as cofactor

(30 mM for D1; 50 mM for D2; 10 mM for D3 in brain). Each sample was tested in duplicate, together with blanks, containing no protein, to measure non-enzymic degradation of the tracer. For the D1 and D2 enzyme assays, the reaction was stopped on ice by addition of bovine serum albumin (50 g/l) and release of radioiodine by ORD of outer ring-labelled rT_3 or T_4 was estimated (γ-counter, Hewlett-Packard, Groningen, The Netherlands) after precipitation of protein-bound iodothyronines with TCA (100 g/l). For the D3 assay, reactions were stopped by addition of methanol and iodothyronine products were analysed by HPLC (Eelkman Rooda *et al*. 1989). The deiodinase activity is expressed as fmol substrate converted/mg protein per minute.

Thyroid follicle histology. Lower jaws were kept in Bouin's fluid for 18 d. Jaws were decalcified in TCA (50 g/l) for 7 d, and after dehydration, the tissues were embedded in paraffin. Serial sections (20 μm) of longitudinally-oriented lower jaws were made. One section out of ten was kept and stained according to Gabe (1968) by alcian blue (pH 2·6), Groat's haematoxylin and orange G. Histological examinations were made with a semi-automatic image analyser (VIDS-IV, Systèmes Analytiques, Compiègne, France). The volume occupied by the total thyroid follicles was calculated from areas observed on serial sections in seven fish. The quantity of follicles was estimated using a screen (ninety-six equidistant dots; magnification ×60) which was moved over the entire area of each section. The number of dots situated on a follicle was counted and the corresponding volume (mm^3) was calculated by:

$$V = (N \times S \times E \times C)/96,$$

where N is the total number of the dots counted on the successive sections in each fish, S is the surface area of the screen (mm^2), 96 is number of dots in the screen, E is the thickness of the sections (mm), C is the interval between two sections examined (mm).

The mean height (distance between basal and apical face of cell; magnification ×500) of 100 follicle epithelial cells was also measured in ten fish.

Data analysis

The effect of dietary treatments was analysed by one-way ANOVA ($P < 0{\cdot}05$), and when appropriate, means were compared by the Tukey's multiple range test. When only two series of data were compared, the Student's *t* test was performed ($P < 0{\cdot}05$). Pearson's correlation coefficients (r_S; $P < 0{\cdot}005$) or regression coefficients (r^2; $P < 0{\cdot}05$) were used to assess the relationship between the different variables.

Results

Digestibility of components of experimental diets

The ADC of protein and fat were significantly lower in the R2-500 diet than in the other RM-based diets (Table 4). However, it was not different from the control diet. A significant inverse relationship was found between the GLS content of the diet and the ADC of the DM (r^2 0·97; $P < 0{\cdot}05$). The ADC of energy was significantly lower in the R2-500 diet compared with all the other diets. The ADC of starch was high (> 0·88), and its variation was associated mainly with the dietary starch content (r^2 0·86; $P < 0{\cdot}05$). The ADC of P was low in all diets (< 0·34) and especially with the R2-500 diet which contained the highest quantity of RM2.

Growth performance, feed intake and feed efficiency

The incorporation of RM in the diet caused a significant decrease in growth performance already after three weeks of feeding (Fig. 1(A)). The voluntary feed intake was adversely affected only over the first 3 weeks of feeding (Fig. 1(B)). After 6 weeks the feed intake of groups fed on the RM-based diet was similar to, or greater than that of fish fed on the control diet. The decrease in daily growth index (Table 5) was accompanied by a decrease in feed efficiency. Taking into account the different levels of dietary incorporation of rapeseed meal, these decreases were stronger with RM2, containing a higher amount of GLS, than with the heat-treated one (RM1). When the daily growth index and the feed efficiency were plotted against GLS intake (see Fig. 2 for feed efficiency), there was a strong decrease even at a low level of GLS intake (30–47 μmol/kg average-fish-body-weight and per d), with a plateau up to an intake level of 242 μmol/kg average-body-weight per d and a further decrease in daily growth index and feed efficiency was observed for higher levels of GLS intake (422 μmol/kg average body weight per d).

Table 4. Apparent digestibility coefficients of the nutrients and energy in the experimental diets by rainbow trout (Mean values and mean squared errors for forty fish per group)

	Diets*					MSE between groups	MSE within groups
	Control	R1-300	R1-500	R2-300	R2-500		
Dry matter	0·78^{c}	0·76^{c}	0·76^{c}	0·73^{b}	0·69^{a}	0·213	0·009
Protein	0·89^{ab}	0·90^{b}	0·90^{b}	0·90^{b}	0·86^{a}	0·055	0·014
Fat	0·91^{ab}	0·94^{b}	0·95^{b}	0·93^{b}	0·86^{a}	0·254	0·068
Gross energy	0·84^{b}	0·83^{b}	0·81^{b}	0·81^{b}	0·78^{a}	0·109	0·016
Starch	0·88^{a}	0·94^{b}	0·99^{c}	0·91^{b}	0·98^{c}	0·418	0·017
Phosphorus	0·33^{b}	0·33^{b}	0·34^{b}	0·34^{b}	0·15^{a}	1·414	0·226

MSE, mean squared error.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different (one-way ANOVA and Tukey's multiple range test; $P < 0{\cdot}05$).

* For details of composition of diets see Table 3; the glucosinolate content of the diets were (mol/kg dry matter): R1-300 1·4, R1-500 2·3, R2-300 11·6, R2-500 19·3.

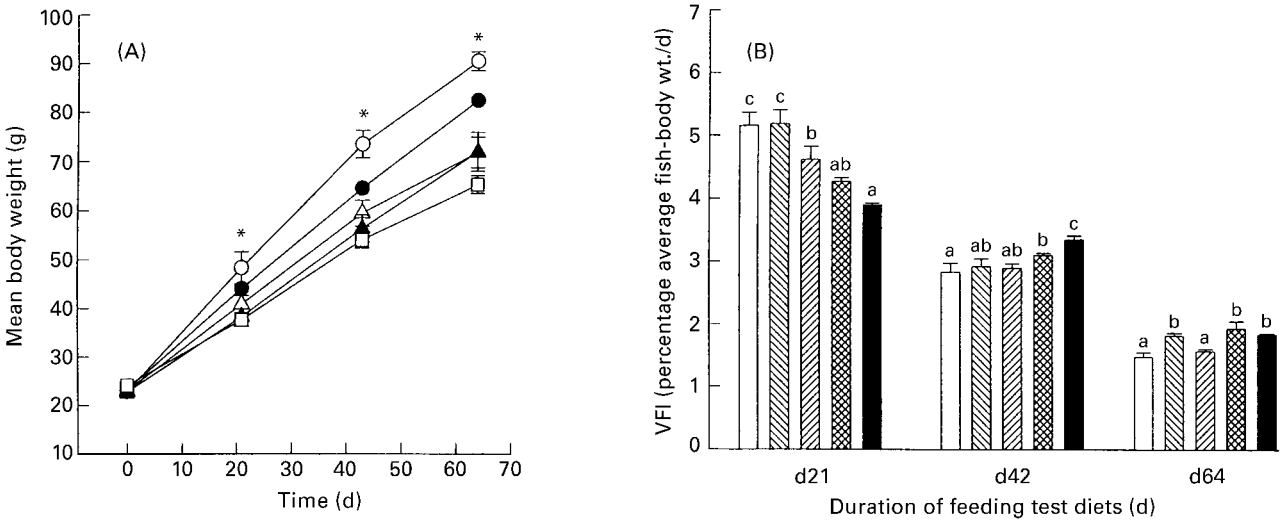

Fig. 1. Effect of diets containing 0, 300 or 500 g rapeseed meal on (A) mean body weight and (B) voluntary food intake (VFI) of rainbow trout. For details of the oil-extraction procedures used to prepare rapeseed meals 1 and 2 (R1 and R2) see p. 654, and for details of composition of diets see Tables 1–3. The diets were: (A), (–○–), control; (–●–), R1-300; (–△–), R1-500; (–▲–), R2-300; (–□–), R2-500; and (B), (□), control; (▨), R1-300; (▨), R1-500; (▨), R2-300; (■), R2-500. Values are means (*n* 150) with standard deviations shown by vertical bars. *$P < 0.05$ (one-way ANOVA); [a,b,c] mean values with unlike superscript letters were significantly different (one-way ANOVA and Tukey's multiple range test).

Protein and energy retention were significantly lower in fish fed on the R2-500 diet than in those fed on the other diets (Table 5). P retention was significantly lower in all fish fed on the RM-based diets than in fish fed on the control diet irrespective of the kind of RM and the level of incorporation. The decrease in P retention was correlated with an increase in RM intake (r_S –0·80; $P < 0.001$).

Thyroidal status

No significant effect of sampling time (diurnal and nocturnal samplings) was detected in plasma levels of T_3, T_4, and deiodinase activities. Ingestion of RM-based diets induced a significant decrease (about 40 %) of the plasma T_3 level and an even more important decrease (about 80 %) of the plasma T_4 level, regardless of the RM used and its level of incorporation (Fig. 3).

As shown in Table 6, the $T_3 : T_4$ ratio was considerably higher (about 300 %) in fish fed on RM-based diets than in fish fed on the control diet. D2 activity in the liver and D1 activity in the kidney were increased by 600 % and 50 % respectively, while D3 activity was inhibited in the brain (about 60 %) in fish fed on RM-based diets.

Changes in thyroid follicle activity, as determined through histological studies, are shown in Figs 4 and 5 and Table 6. The thyroid follicles were mainly located in the lower jaw of trout, scattered alongside the ventral aorta near the first brachial arches (Fig. 4). The volume of total thyroid tissue was significantly higher in fish fed on the R2-500 diet (about 270 %) than in fish fed on the control diet (Fig. 5(A)

Table 5. Growth, feed intake, nutrient and energy retention of rainbow trout fed on the experimental diets for 64 d† (Mean values and mean squared errors for 150 fish per group)

	Diets*					MSE between groups	MSE within groups
	Control	R1-300	R1-500	R2-300	R2-500		
Growth performance and feed utilization							
Final body weight (g)	90·6[d]	82·5[c]	71·9[b]	71·8[ab]	65·2[a]	302·4	13·2
Biomass gain (% IBW)	284[d]	252[c]	214[b]	208[b]	167[a]	6033	208
Daily growth index‡	2·6[d]	2·3[c]	2·1[b]	2·0[b]	1·8[a]	0·295	0·004
Voluntary feed intake (% of body weight/d)§	2·0[a]	2·1[bc]	2·1[ab]	2·0[ab]	2·2[c]	0·016	0·002
Feed efficiency ratio‖	0·99[d]	0·86[c]	0·80[b]	0·81[bc]	0·68[a]	0·0374	0·0009
Retention (% of intake)¶							
Protein	36·0[b]	31·7[ab]	32·5[b]	32·3[b]	27·1[a]	30·5	8·0
Energy	38·6[b]	36·6[b]	34·9[b]	35·6[b]	27·6[a]	51·8	7·8
Phosphorus	28·8[b]	22·0[a]	21·2[a]	21·8[a]	19·1[a]	40·2	6·4

MSE, mean squared error; IBW, initial body weight.
[a,b,c,d] Mean values within a row with unlike superscript letters were significantly different (one-way ANOVA and Tukey's multiple range test; $P < 0.05$).
* For details of composition of diets see Table 3; the glucosinolate content of diets were (μmol/g dry matter); R1-300 1·4, R1-500 2·3, R2-300 11·6, R2-500 19·3.
† The initial body weight of the trout was 23·2 (SD 0·6) g. The fish were kept at a temperature of 17 ± 1°.
‡ Daily growth index = 100 × ((final body weight)$^{0.333}$ – (initial body weight)$^{0.333}$)/duration.
§ Voluntary feed intake = 100 × dry feed intake (g)/((initial body weight + final body weight)/2) × duration.
‖ Feed efficiency ratio = wet wt. gain (g) : dry feed intake (g).
¶ Retention = 100 × (final body weight × final carcass nutrient content – initial body weight × initial carcass nutrient content)/nutrient intake.

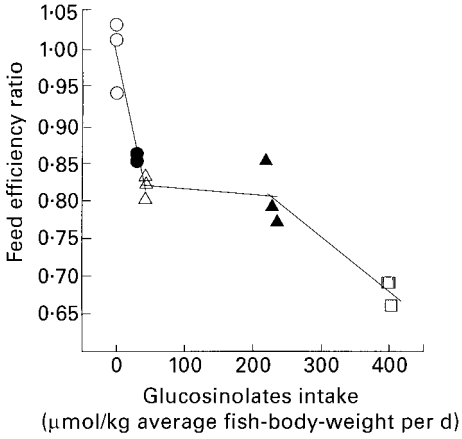

Fig. 2. Relationship between glucosinolate intake and feed efficiency ratio in rainbow trout fed on diets containing 0, 300 or 500 g rapeseed meal/kg over the whole growth period of 64 d. For details of the oil-extraction procedures used to prepare rapeseed meals 1 and 2 (R1 and R2) see p. 654, and for details of the composition of the diets see Tables 1–3. Each symbol represents one replicate. The diets were: (○), control; (●), R1-300; (△), R1-500; (▲), R2-300; (□), R2-500. No statistical analysis of the three straight lines drawn on the figure was made due to the small number of data.

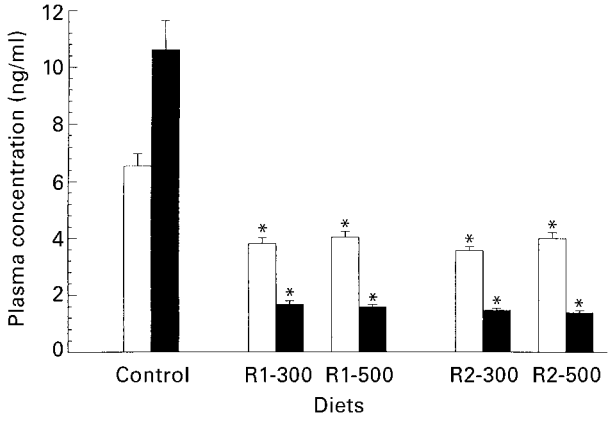

Fig. 3. Plasma concentrations of triiodothyronine (□) and thyroxine (■) in rainbow trout fed on diets containing 0, 300 or 500 g rapeseed meal/kg at the end of the experimental feeding period for each diet. For details of the oil-extraction procedures used to prepare rapeseed meals 1 and 2 (R1 and R2) see p. 654, and for details of the composition of the diets see Tables 1–3. Values are means (n 40 per group) with standard deviations represented by vertical bars. Mean values were significantly different from those of the control group: *$P < 0{\cdot}05$ (one-way ANOVA and Tukey's multiple range test).

and 5(B), Table 6). The height of the epithelial follicle cells was significantly higher (200 %) in fish fed on the R2-500 diet, indicating increased follicle activity (Fig. 5(C) and 5(D)).

Discussion

Detailed information on the goitrogenic activity of dietary RM in birds and mammals, including human subjects is available (see Mawson *et al*. 1994*b*). Data from terrestrial animals as well as from fish (Yurkowski *et al*. 1978; Higgs *et al*. 1982; Hardy & Sullivan, 1983; Hilton & Slinger, 1986; Leatherland *et al*. 1987; Hossain & Jauncey, 1988; Teskeredzic *et al*. 1995; Webster *et al*. 1997) have shown the role of GLS in thyroidal disturbances. In fact, the toxicity is not directly caused by the GLS, but by the GLS breakdown products such as isothiocyanates, thiocyanate anions, oxazolidinethiones and nitriles, mainly as a result of the activity of myrosinase, a specific plant hydrolytic enzyme. In intact plant tissues, the enzyme is stored separately from the GLS substrates in specific cells (idioblasts) (Grob & Matile, 1979). Contact between the two is a result of mechanical injury of the plant tissue as it occurs during the processing of RM.

Our data indicate that the content of intact GLS in the diet in itself may not be a good indicator of the potential deleterious effects of RM in fish. To reduce the GLS content and to denature the myrosinase during the processing of meal, it is necessary to apply high temperatures during the oil-extraction process. The difficulty then is to succeed in the degradation of the GLS without the production of toxic by-products. The two RM used in our study have been

Table 6. Effects of dietary glucosinolate intake on thyroid function in rainbow trout: thyroid follicular activity, triiodothyronine : thyroxine ratio and peripheral deiodinase activity

(Mean values and mean squared errors)

	Diets*					MSE between groups	MSE within groups
	Control	R1-300	R1-500	R2-300	R2-500		
Thyroid follicular activity†							
Thyroid volume (mm^3)	$2{\cdot}8^{a}$	ND	ND	ND	$10{\cdot}3^{b}$	200	46
Epithelial cell height (μm)	$4{\cdot}3^{a}$	ND	ND	ND	$13{\cdot}0^{b}$	339·92	0·57
$T_3 : T_4$	0·6	2·3	2·6	2·4	2·9		
Deiodinase activities (fmol/mg protein per min)‡							
D1 in kidney (fmol rT_3)	$145{\cdot}4^{a}$	$224{\cdot}9^{bc}$	$262{\cdot}0^{d}$	$206{\cdot}2^{b}$	$232{\cdot}3^{c}$	45·8	1·4
D2 in liver (fmol T_4)	$10{\cdot}1^{a}$	$72{\cdot}9^{b}$	$73{\cdot}2^{b}$	$71{\cdot}4^{b}$	$72{\cdot}0^{b}$	37306	82
D3 in brain (fmol T_3)	$22{\cdot}2^{b}$	$8{\cdot}3^{a}$	$9{\cdot}3^{a}$	$10{\cdot}7^{a}$	$6{\cdot}7^{a}$	216	43

MSE, mean squared error; ND, not determined; T_3, triiodothyronine; T_4, thyronine; rT_3, reverse triiodothyronine; D, deiodinase.
[a,b,c] Mean values within a row with unlike superscript letters were significantly different (one-way ANOVA and Tukey's multiple range test or Student's t test; $P < 0{\cdot}05$).
* For details of the composition of diets see Table 3; the glucosinolate content of the diets were (mol/kg dry matter): R1-300 1·4, R1-500 2·3, R2-300 11·6, R2-500 19·3.
† Thyroid volume was measured on seven fish and the epithelial cell height on ten fish.
‡ n 15, except for deiodinase 3 activity in the brain where n 3 (pooled).

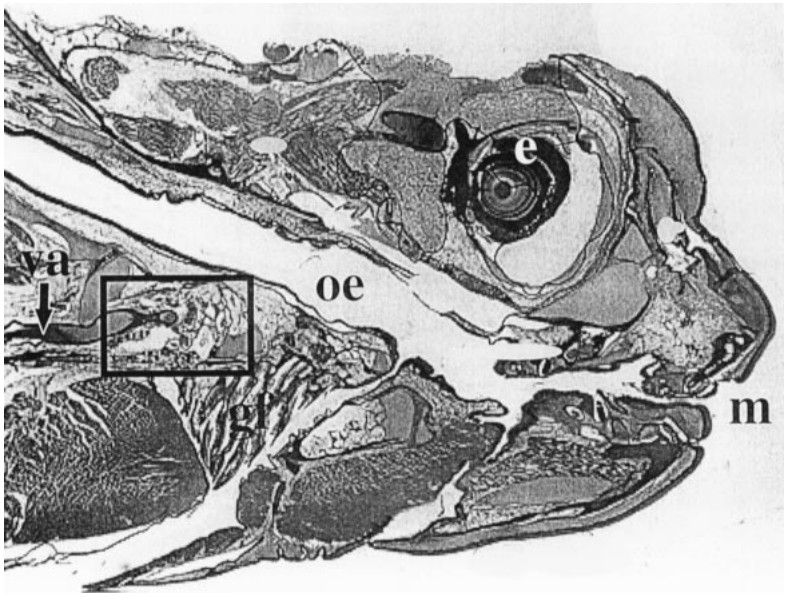

Fig. 4. Sagittal section of a rainbow trout head after a period of starvation (1 week; hypothyroidal fish). Large follicles are scattered throughout a highly vascularized region of the lower jaw adjacent to the ventral aorta (see in the square). The histological features suggest a very low activity of the follicles: very large follicles with large, homogenous colloids. va, Section of the ventral aorta; gf, section of gill filaments; oe, oesophagus; m, mouth; e, eye. Magnification ×5.

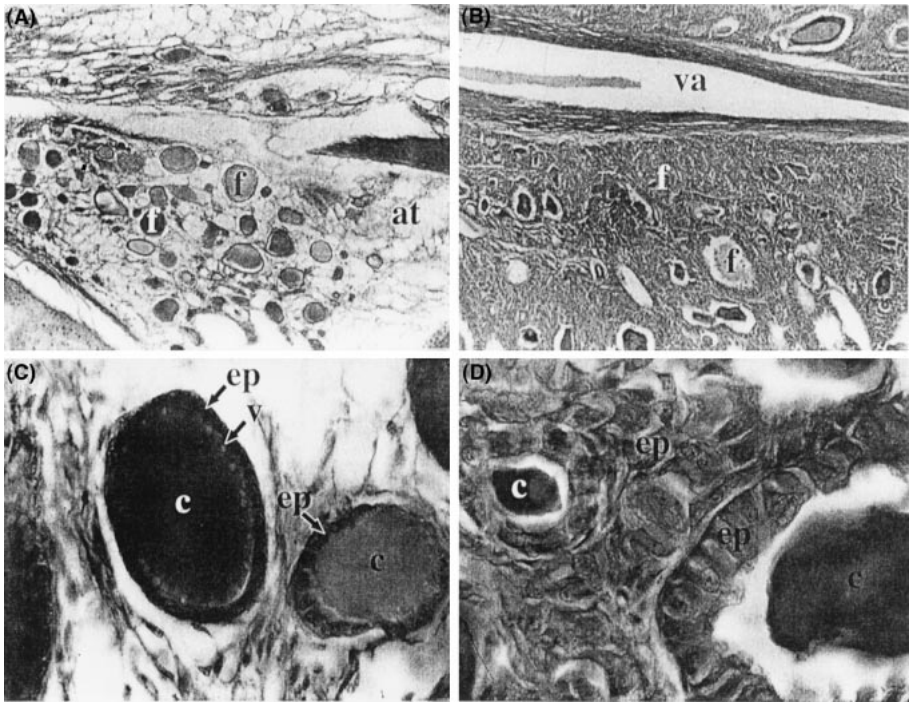

Fig. 5. Thyroid follicles in rainbow trout. (A) Thyroid follicles in a rainbow trout fed on a control diet for 73 d. The thyroidal tissue is not very dense, the follicles (f) are spread into adipose tissue (at) (magnification ×60). (B) Thyroid follicles of a rainbow trout fed on a diet containing 500 g rapeseed meal (R2)/kg diet (R2-500) for 73 d. Thyroidal tissue is very dense (f) and the proximity to the ventral aorta (va) is evident (magnification ×60). (C) Unstimulated thyroid tissue of a rainbow trout fed on a control diet for 73 d. The follicle epithelial cells (ep) are cuboidal in appearance, and the colloid (c) within the lumen is homogenous. The nucleus : cytoplasm ratio of the epithelial cells is high. Vesiculation (v) of the colloid is evident in this follicle (magnification ×500). (D) Stimulated thyroid tissue of a rainbow trout fed on diet R2-500 for 73 d. The follicle epithelial cells (ep) are very large and are columnar. The colloid (c) is partly or wholly depleted. The nucleus : cytoplasm ratio of the epithelial cells is small (magnification ×500). For details of the oil-extraction procedure used to prepare R2 see p. 654, and for details of the composition of the diets see Tables 1–3.

subjected to two different oil-extraction processes including different thermal conditions. In addition to the different quantities and profiles obtained in GLS, different quantities and profiles of active breakdown products could be obtained either during the process or later on if enough myrosinase remained active (Nugon-Baudon & Rabot, 1994).

In this present study, a deleterious effect of dietary RM incorporation on thyroidal status and fish growth is shown, even at an incorporation level of 300 g heat treated RM/kg diet with a very low content of GLS (1·4 μmol/g diet). In the previous studies on fish (Yurkowski *et al*. 1978; Higgs *et al*. 1982; Hardy & Sullivan, 1983; Hilton & Slinger, 1986; Leatherland *et al*. 1987; Hossain & Jauncey, 1988; Teskeredzic *et al*. 1995; Webster *et al*. 1997), the dietary amount of toxic compounds was expressed either as the amount of intact GLS, as the amount of intact GLS plus GLS breakdown products or as the amount of GLS breakdown products alone. As a result, the lowest level of toxic compounds inducing thyroidal disturbance or growth depletion varied greatly between studies. Nevertheless, our results are generally in accordance with those obtained by Yurkowski *et al*. (1978), Hardy & Sullivan (1983) and Leatherland *et al*. (1987) in rainbow trout, who determined a critical level of toxic compounds in the range of 1–2 mol/kg diet using different types of processed RM. Amongst terrestrial animals, ruminants are considered to be less sensitive than single-stomached animals (for reviews see Mawson *et al*. 1994*a*, *b*): growth, feed intake and feed conversion are reduced by a dietary GLS content of approximately 3–4 mol/kg diet in the rat, 0·6–3 mol/kg in the young pig, 4–10 mol/kg in growing poultry, and 7–15 mol/kg in young calves; thyroid function is affected by dietary levels of 0·5–4 mol/kg in the rat, 2–3 mol/kg in swine, 1·4 mol/kg in growing poultry, and 8 mol/kg in calves.

Irrespective of the type of RM used, we observed the typical features corresponding to a hypothyroid condition in trout. Lower T_3 and T_4 levels were accompanied by thyroid tissue hyperactivity, i.e. an increase in the volume of the thyroid tissue and epithelial follicle cell height (hyperplasy and hypertrophy). Moreover, these cells became columnar with the colloid partly or completely depleted. Our results also clearly demonstrate an effect of the hypothyroidal condition on the *in vitro* deiodinase activities. *In vitro* D2 activity in the liver and D1 activity in the kidney were elevated, both offering the possibility for an increased T_3 production from the available T_4 by ORD. In addition, there was a decrease in *in vitro* D3 activity in the brain, reducing the degradation of T_3 by IRD. It is not easy to extrapolate to the *in vivo* situation, but these results suggest that deiodinases compensate for the lack of bioactive T_3, as suspected earlier by Higgs *et al*. (1982) and Hardy & Sullivan (1983) in fish and in terrestrial animals (Mawson *et al*. 1994*b*). The *in vivo* potential role of each enzyme in the adjustment of the plasma thyroid hormones levels is difficult to establish, because their activity depends on the substrate and cofactor availability, the intracellular pH and the temperature. Nevertheless, the response of the D3 activity in the brain, given the size of this organ, seems to be a localized response and acts probably to protect the brain against T_3 levels which are too low.

The relationship between hypothyroidism and poor growth is established in mammals including human subjects (Boyages *et al*. 1989; Hetzel, 1994). However, the relationship between the thyroid disturbances induced by the metabolites of GLS and growth performance of fish has not been clearly demonstrated. Indirect evidence was given by Leatherland *et al*. (1987), who showed that a dietary T_3 supplement in trout fed on RM-based diets led to an improved growth performance. In the present study, the lower growth rate of fish fed on the RM-based diets compared with that of the control fish was accompanied by low feed utilization. When this effect is related to dietary GLS intake, data show two thresholds of sensitivity. Indeed, ingestion of very low amounts of GLS, corresponding to a dietary content of GLS of 1·4 mol/kg DM, led to a decrease of both growth rate and feed efficiency, but this effect was not further exacerbated with increasing dietary GLS levels up to 11·6 mol/kg. However, a higher dietary content of GLS (19·3 mol/kg DM) led to a stronger decrease of growth and feed efficiency. But the hypothesis of the existence of these thresholds of sensibility cannot be assured with the present data, because first, the amount of toxic derivatives of GLS were not measured here, and second, the effect of the other antinutritional factors present in the two rapeseed meals tested must be taken into account. The poor growth performance observed could be due to: (1) thyroid disturbances caused by increased levels of GLS. It is known that thyroid hormones play a role in the regulation of fish growth through intermediary metabolism (metabolic utilization of energy, amino acids and possibly carbohydrates; see Leatherland, 1994). Besides, several authors (Harvey *et al*. 1988; Luo & McKeown, 1991; Farchi-Pisanty *et al*. 1995; Melamed *et al*. 1995) have shown a thyroidal regulation of growth hormone metabolism, hypothyroid condition possibly leading to a decrease of GH release. As a matter of fact, a deleterious effect of isolated isothiocyanates on the digestive utilization of nutrients, together with thyroid disturbances, has been reported in carp (*Cyprinus carpio*) (Hossain & Jauncey, 1988). In the rat (Bille *et al*. 1983; Vermorel & Baudet, 1987; Roland *et al*. 1996), isolated progoitrine, sinigrin and sinalbine impaired also the thyroid metabolism and protein digestibility and retention. A low level of bioactive T_3 may interfere with the digestive capacities of trout, given the effect of a treatment with thyroid hormones on the digestive function of rats (Hodin *et al*. 1992) and of red sea bream (*Chrysophrys major*) (Woo *et al*. 1991). In our study, the digestibility of the DM, protein, lipid, energy and P was significantly reduced when the diet contained 500 g untreated RM/kg, i.e. with the higher content of GLS. Nutrient and energy retentions were also reduced in fish fed on this diet. In addition, results of the present study show a dissociation between plasma T_3 levels and growth. It seems that there was a threshold of thyroidal sensitivity to the GLS breakdown products even at an incorporation level of 300 g heat-treated RM/kg. Two major hypotheses can be formulated. First, the compensatory effects of hypertrophy of thyroidal tissue and of the deiodinase activities may result in sufficient circulating levels in T_3 (about 4 ng/ml) to support normal growth. In such case, the goitrogenic activity of GLS breakdown products would play a minor or no role at all in the poor growth performance observed. Second, thyroidal disturbance could

only be partially responsible for the lower growth rate, and thus, the differential response would be due to the direct effects of other antinutritional factors; (2) a direct effect of GLS on feed utilization. Indeed, GLS can interfere with liver function, because they induce alterations of the hepatic detoxification system as shown in mammals (Rabot *et al*. 1993; Williamson *et al*. 1996). An increase of the weight of liver and kidneys related to the amount of GLS ingested has been shown in growing pigs (Bourdon & Aumaître, 1990). However, no work conducted in fish clearly show this effect, and in the present study, no liver or kidney abnormality was observed in trout; (3) a high dietary fibre content as generally found in RM (Higgs *et al*. 1996). The dehulling of the rapeseed allows only a partial decrease in the fibre content, with average levels of acid detergent fibre and neutral detergent fibre both remaining at 124 g/kg DM (A Quinsac and D Ribailler, personal communication). Hilton & Slinger (1986) found that dietary fibre can depress mineral bioavailability as well as decrease the intestinal transit time, with consequent effects on digestibility of DM, energy and P; (4) a high content of phytic acid and/or tannins. The concentration of phytic acid in the two RM were high (42–44 g/kg DM), as generally found in this protein source (24–57 g/kg DM; Higgs *et al*. 1996). Elevated levels of these compounds are known to depress the mineral bioavailability as well as activity of digestive enzymes in fish (Spinelli *et al*. 1983). RM are also known to have a high tannin content (Fenwick, 1982), which can depress protein digestibility.

It is interesting to notice that in apparent contradiction of what is observed in terrestrial animals (Mawson *et al*. 1993) and also in a previous study with rainbow trout (Hilton & Slinger, 1986), our data do not show that dietary incorporation of RM leads to a decrease in feed intake. It was only decreased during the first 3 weeks of feeding, but the fish quickly became accustomed. This might be related to reduced levels of tannins and sinapine, known to affect the organoleptic properties of this ingredient, in these new cultivars of rapeseed.

The findings of this present study show that, despite their low content of GLS, the rapeseed meals tested here cannot be used in practical rainbow trout diets at 300 g/kg without deleterious effects. These data are in agreement with those of Hardy & Sullivan (1983), Hilton & Slinger (1986), Leatherland *et al*. (1987), Gomes & Kaushik (1989), Abdou Dade *et al*. (1990), McCurdy & March (1992) and Gomes *et al*. (1993). Despite an important compensatory effect through the deiodinases, the levels of T_4 and T_3 in the plasma were reduced. The lower growth performance observed, especially with the higher level of RM, could be caused by the concomitant effect of the hypothyroid condition, the direct action of GLS and the action of the other antinutritional factors.

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