Effects of Dietary Protein and Lipid Levels on Growth, Body Composition, Blood Biochemistry, Antioxidant Capacity and Ammonia Excretion of European Grayling (Thymallus Thymallus)

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Research

Keywords: Thymallus thymallus, Protein/energy ratio, Growth, Fatty acid composition, Ammonia excretion

DOI: https://doi.org/10.21203/rs.3.rs-458116/v1

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Abstract

**Background:** This study evaluated growth, body composition, blood biochemistry, antioxidant capacity, innate immunity and ammonia excretion of European grayling (*Thymallus thymallus*) fed diets containing different protein and lipid contents. Six diets were produced to contain 30, 40 or 50% protein with 10 or 20% lipid and fed to triplicate groups (100 fish per replicate) of fish (25.2 ± 0.28 g) to visual satiety twice daily for 12 weeks.

**Results:** Fish growth was enhanced (*P* < 0.05) as protein increased from 30 to 40% and plateaued thereafter. Enhancing protein and lipid content of diet led to reduced feed intake and improved feed efficiency. Moreover, protein efficiency ratio increased at higher lipid level while lower values were recorded at higher protein levels. Increasing dietary lipid content led to the enhancement of viscoseromatic index and intraperitoneal fat ratio. An interaction of protein and lipid was found on whole-body lipid, and muscle lipid was responsive to dietary lipid level. Muscle ARA, EPA and n-6 LC-PUFA contents increased by enhancing dietary protein level. Moreover, increasing fat content of diet led to enhanced muscle linoleic acid, linolenic acid, MUFA, n-6, DHA/EPA and n-6/n-3. However, EPA, DHA, n-6 LC-PUFA, n-3, n-3 LC-PUFA and EPA/ARA decreased at higher dietary lipid level. Serum triglyceride (TG) and lactate dehydrogenase (LDH) activity decreased as dietary protein level increased while an opposite effect was observed for cholesterol (CHO) concentration. Increasing fat content of diet led to enhanced serum TG, CHO and glucose concentrations and reduced alanine aminotransferase, aspartate aminotransferase and LDH activities. Serum malondialdehyde concentration was enhanced by increasing both dietary protein and lipid contents. Furthermore, serum myeloperoxidase activity was enhanced at higher dietary lipid level. Water total ammonium nitrogen (TAN) concentration was measured after 5 and 24 h of last feeding, and the results indicated the reduction of ammonia excretion as dietary lipid content increased.

**Conclusions:** These findings suggest that 40% dietary protein can support optimal growth of juvenile European grayling and increasing lipid content from 10 to 20% can improve feed utilization and reduce ammonia excretion to the rearing water.

**Background**

Maximizing fish growth performance while reducing the production costs is the key to profitable aquaculture. Feed cost accounts for over 50% of the total expenses [1], and protein is the most costly feed ingredient particularly in carnivorous fish feed which should contain 40-60% protein [2]. Accordingly, determination of nutritional requirements of any new species is necessary for formulation of cost-effective aquafeed.

Protein is not only essential for somatic growth but also required for tissues maintenance, and production of many key components such as hormones, enzymes and antibodies [3]. Fish utilizes protein as a source of energy preferentially to lipid and carbohydrate [4], but its catabolism for energy production rather than being used for tissue synthesis increases the feed cost and leads to higher excretion of ammonia to the rearing water [5] which adversely impacts fish feed consumption and growth [6]. The efficient utilization of dietary protein not only relies on quantity and quality of dietary protein but also on sufficient supply of lipid and carbohydrate as energy sources [7]. Considering the lower ability of carnivorous fish in metabolizing carbohydrates, lipids as energy-dense nutrient are better utilized as energy source by carnivorous fish [8]. On the other hand, lipids play crucial roles in fish growth and health by providing essential fatty acids [9], and participating in uptake, transport and metabolism of fat-soluble vitamins and carotenoids [10]. It has been shown that adequate lipid supplementation in the feed can prohibit using protein for energy production [9]. However, dietary lipid level could be increased up to a certain level beyond which undesirable effects could be attained such as lipid accumulation in the body and reduced growth performance resulting from the lack of essential nutrients due to reduced feed intake [11]. It is believed that evaluation of the optimal ratio of dietary protein to energy is a more logic way of determining fish protein demand than quantifying merely the crude protein requirement [2] for production of efficient and environmentally-friendly aquafeed.

European grayling (*Thymallus thymallus*) belongs to Salmonidae family which inhabits the waters of central, northern and north-eastern Europe. Its population has dwindled mainly due to the deterioration of its habitat, water contamination, bird depredation and overfishing [12–15]. Accordingly, conservation programmes have been started to rehabilitate its stocks through releasing cultured fingerlings. However, the lack of information about its nutritional requirements remains as a major constraint to formulation of nutritionally adequate feed for this species. This study aimed to assess growth, body composition, blood biochemistry, antioxidant activity, innate response and ammonia excretion of European grayling.

**Materials And Methods**

**Test diets**

Six diets were prepared with varying protein (30, 40 and 50%) and lipid (10 and 20%) contents with protein to energy ratios ranging from 13.6 to 24 g MJ⁻¹ (Table 1). Fish meal and soy protein concentrate were used as the protein sources and a mixture of fish oil and soybean oil were used as the lipid sources. The experimental diets were prepared following the procedures described in our previous study [16].

**Fish rearing**

Grayling larvae were obtained from local hatchery of Šumava Natural Park (Borová Lada, Czech Republic), and they were cultured for three months in an experimental Recirculating Aquaculture System (RAS) under controlled conditions at Faculty of Fisheries and Protection of Waters, University of South Bohemia (FFWP, USB) (Vodnany, Czech Republic). Prior to starting the trial, the fish were moved and kept in 450-l tanks in another RAS (FFWP, USB) for 4 weeks and fed a commercial diet (protein: 55%, lipid: 16%) to acclimatize them to the rearing conditions. Then, 1800 fish of similar size (25.2 ± 0.28 g) were distributed into eighteen 450-l tanks (100 fish/tank) containing 350 liters of freshwater and fed the test diets to satiation for 12 weeks. Water temperature, pH and dissolved oxygen (DO) concentration were recorded daily during the experiment and their values were 16.5 ± 0.03 °C, 7.05 ± 0.02 and 11.7 ± 0.05 mg l⁻¹, respectively. Water nitrite (NO₂⁻) and total ammonia (TAN) concentrations were measured three times a week and estimated at 0.44 ± 0.03 mg l⁻¹ and 0.27 ± 0.01 mg l⁻¹, respectively. The photoperiod was kept at a 12:12 light/dark cycle.
Sampling protocol

At the end of the feeding test, fish number and bulk weight were determined for estimation of survival rate and growth performance. Five intact fish from each tank were randomly captured and stored at -20 °C for proximate composition analysis. Also, dorsal muscle samples were collected from five fish per tank for proximate and fatty acid composition analyses. Blood was collected from six fish per tank with 3-ml syringes after anesthetizing (200 mg 1⁻¹ of 2-phenoxyethanol) and left to clot at 4 °C for 24 h. Then, the samples were centrifuged at 5000 × g for 10 min at 4 °C and the serum was separated and stored at -80 °C for analyses of blood biochemical, antioxidant and innate immune parameters. Total length, individual weight, and viscera, liver and intraperitoneal fat weights of 10 fish from each tank were recorded to estimate condition factor (CF), viscerosomatic index (VSI), hepatosomatic index (HSI) and intraperitoneal fat ratio (IPF). All the fish were starved for 24 h before handling to reduce stress on fish.

Analytical methods

Whole-body and muscle composition

Crude protein was measured based on the Kjeldahl technique [17], crude lipid according to Folch et al. [18], ash by combustion at 600 °C [17], and moisture content by drying at 110 °C [17]. Fatty acid methyl esters of the lipids were obtained by transmethylation [19], which were subsequently separated by gas liquid chromatography as explained earlier [20].

Blood biochemistry, immunity, and antioxidant activity

Serum biochemical indices including total protein (TP), triglyceride (TG), total cholesterol (CHO), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were measured using kits with a VET-TEST 8008 analyzer (IDEXX Laboratories Inc., Maine, USA). Superoxide dismutase (SOD) activity was determined using a SOD Assay Kit (Sigma, 19160). Commercial assay kits were used for measurement of catalase (CAT) activity (ThermoFisher Scientific, CA, USA) and malondialdehyde (MDA) concentration (Sigma-Aldrich, Missouri, USA). A turbidimetric method was used for determination of lysozyme activity according to Swain et al. [21]. MPO activity was determined as described by Quade and Roth [22]. Serum antiprotease activity was quantified based on Ellis [23] with some modifications [24].

Plasma ammonia concentration and total ammonia nitrogen (TAN) excretion to water

To evaluate the effects of dietary treatments on plasma ammonia concentration, blood was collected from caudal vein of three fish per tank using heparinized syringes after 5h of last feeding. Then plasma was separated by centrifugation at 5000 × g for 10 min at 4 °C and kept at -80 °C until analysis. Plasma ammonia concentration was determined by the VET-TEST 8008 analyzer using a commercial kit.

To assess the effects of dietary treatments on fish ammonia excretion, 5 fish were randomly captured from each tank and moved to eighteen 40-l glass aquaria containing 25 liters of freshwater. The fish were adapted to the new system for two days and fed with the same test diets. On the 3rd day, the uneaten feed and fecal materials were syphoned out after feeding, water flow was stopped, and TAN concentration was measured after 5 and 24 h. The TAN concentration was measured with a water analyzer (HANNA Instruments Inc., RI, USA) using kits. The following equation was used for the calculation of ammonia excretion [25]:

\[ \Delta N-NH_3 = \Delta N-NH_3 \times v / (m \times t) \]

where \( \Delta N-NH_3 \) is the change in total ammonia concentration during each test period; \( v \) is the water volume (l); \( m \) is the fish biomass (kg); and \( t \) is the test period (h).

Water temperature, pH and DO were measured at both sampling points and were evaluated at 16.4 ± 0.05 °C, 7.03 ± 0.01 and 8.02 ± 0.12 mg 1⁻¹ at 5 h, and 16.5 ± 0.08 °C, 7.41 ± 0.03 and 8.82 ± 0.04 mg 1⁻¹ at 24 h after last feeding.

Statistical analysis

All the data are shown as mean ± SE. Mean values of parameters were analyzed by one- and two-way ANOVAs to determine the significant differences due to the dietary levels of protein, lipid and their interaction. When ANOVA identified differences among groups, the difference in means was made with Tukey's test. Statistical significance was determined at \( P \leq 0.05 \). All statistical analyses were carried out using Statistica version 13.5.0.17 (TIBCO Inc., CA, USA).

Results

Growth of grayling juveniles was only responsive to dietary protein content (\( P \leq 0.05 \)) but not to the fat content or their interaction (Table 2). As dietary protein level elevated from 30 to 40%, growth performance enhanced significantly but no further improvement could be found at higher protein level. Feed intake (FI) decreased markedly as dietary protein and lipid contents increased. Feed efficiency (FE) was improved with enhancement of feed protein and lipid. The highest protein efficiency ratio (PER) was found at the 30% protein level and increasing dietary lipid content led to the remarkable enhancement of PER. No significant effect of dietary treatments could be found on fish survival rate which ranged from 98 to 100% (Table 2). CF and HSI remained unaffected whereas VSI and IPF increased drastically by enhancing dietary fat content (Table 3).

A significant interaction of protein and lipid was found on whole-body lipid content. Moreover, enhancing lipid content of feed led to reduced whole-body moisture content (Table 4). Dorsal muscle lipid content increased by the increment of feed fat content, and muscle ash increased as dietary protein increased (Table 5). Fatty acid (FA) composition analysis revealed that oleic acid (OA) (18:1n-9), palmitic acid (16:0), linoleic acid (LA) (18:2n-6) and docosahexaenoic
acid (DHA) (22:6n-3) were the dominant FAs in the dorsal muscle regardless of dietary treatments (Table 6). Increasing protein content of feed was accompanied with enhancement of 14:0, 14:1n-7, 15:0, 16:0, 16:1n-7, 16:2n-4, 17:0, 16:4n-3, 18:0, 18:1n-7, 18:2n-9, 18:2n-6, 18:4n-1, 20:1n-5, 20:2n-9, arachidonic acid (ARA) (20:4n-6), eicosapentaenoic acid (EPA) (20:5n-3), docosapentaenoic acid (22:5n-6), sum of saturated fatty acids (SFA) and n-6 long chain polyunsaturated FA (n-6 LC-PUFA). However, sum of monounsaturated FAs (MUFA), n-6, DHA/EPA and n-6/n-3 were decreased at higher protein levels. Muscle OA, LA, linolenic acid (LNA) (18:3n-3), 18:3n-1, 20.0, 20:1n-7, 20:2n-6, 20:3n-3, MUFA and n-6/n-3 contents, and ratios of DHA/EPA and n-6/n-3 increased at higher dietary lipid level. Whilst decreased EPA, DHA, n-6 LC-PUFA, n-3, n-3 LC-PUFA and EPA/ARA were found at higher dietary lipid level.

Increasing dietary protein level led to the reduction of serum TG concentration and enhancement of CHO level, and their values increased significantly at higher dietary lipid level. The lowest glucose level was found at 40% protein level which differed significantly from the other groups, and its value increased at higher lipid level. Serum ALT and AST activities were only affected by lipid level where lower activities were recorded at higher lipid level. Serum LDH activity decreased as feed protein and lipid contents increased. However, serum total protein level and ALP activity did not vary among different experimental groups (Table 7).

Numerically higher serum SOD, MPO and antiprotease activities were found at 40% protein level although the differences were not statistically different. Serum MDA concentration was significantly enhanced by increment of lipid level and increasing protein content from 30 to 50%. Moreover, increasing dietary lipid content led to the enhancement of serum MPO activity. No specific trends were observed for serum CAT and lysozyme activities (Table 8).

Water TAN concentration after 5 and 24 h of last feeding was increased at higher protein levels while increasing dietary lipid level reduced its concentration at both sampling points. Plasma ammonia concentration showed a similar trend to that of water TAN concentration without significant difference among treatments (Table 9).

**Discussion**

The results revealed that growth rate of grayling was influenced by protein content of feed and that 40% protein produces a comparable growth rate to the group fed the diet with 50% protein. Similarly, Lee and Kim [26] found no significant improvement in growth performance of masu salmon (Oncorhynchus masou Brevoort) when protein content was increased from 40 to 50%. Also, enhancing protein content of feed from 44 to 54% at 15 and 20% fat levels did not result in better growth performance of Arctic Char (Salvelinus alpinus L.) [27]. It has been shown that 17 to 26 g MJ⁻¹ is the desirable range of P/E ratio for the most fish species [8]. In the current study, the highest growth rate (266% WG) was obtained at 50% protein and 20% lipid levels with P/E ratio of 22.1 g MJ⁻¹ which was comparable to the growth rate of the group received the diet containing 40% protein and 20% lipid (257% WG) with P/E ratio of 18 g MJ⁻¹. Similarly, Azevedo et al. [28] found no significant improvement in growth performance of Atlantic salmon (Salmo salar) when P/E ratio increased from 18 to 20 g MJ⁻¹. Also, Green and Hardy [29] found similar growth performance for rainbow trout (Oncorhynchus mykiss) fed diets with P/E ratios of 18, 22 and 24 g MJ⁻¹. Moreover, a Manchurian trout (Brachymystax lenok) study showed that although the best growth rate occurs at 50% protein and 8% lipid with P/E ratio of 29.36 g MJ⁻¹, similar WG and SGR could be achieved at 45% protein and 16% lipid levels with P/E ratio of 23.68 g MJ⁻¹ [30].

Fish FI decreased at higher protein and lipid levels which agrees with studies on bagrid catfish (Pseudobagrus fulvidraco) [31], brown-marbled grouper (Epinephelus fuscoguttatus) [32] and hybrid grouper (Epinephelus x lanceolatus) [1]. This could be due to increased feed energy content as lesser feed would be consumed by fish to meet its energy requirement [6]. Moreover, when fish are offered diets with a protein content below the requirement level, they would consume more feed to gain sufficient protein needed for supporting growth and metabolism while at optimum or higher dietary protein levels lesser diet would be needed. Our results revealed the enhancement of FE as protein and lipid levels increased which is consistent with studies on masu salmon [33], Manchurian trout [30], brown trout (Salmo trutta fario) [34], Japanese seabass (Lateolabrax japonicus) [7] and black seabass (Centropomus striata) [35]. Xu et al. [30] showed that enhancing fat content of feed from 8 to 16% enhances PER in Manchurian trout at dietary protein levels of 40-45%. Moreover, Lee and Kim [33] showed the significant improvement of PER in masu salmon by increasing energy content of diet from 19 to 21 MJ kg⁻¹ at protein levels of 30, 40 and 50%. Similarly, in this study PER was improved by increasing dietary lipid level from 10 to 20% corresponding to 2 MJ kg⁻¹ increase in dietary energy content. Improvement of PER at higher dietary fat level has also been shown in pikeperch (Sander lucioperca) [36], Japanese seabass [7] and yellow drum (Nibea albiflora) [37]. On the other hand, in this study PER showed a decreasing tendency by increasing dietary protein level which is parallel to earlier findings in pikeperch [36], red-spotted grouper (Epinephelus akaara) [38] and hybrid grouper [1,10]. The underlying reason for the higher PER at lower dietary protein level could be the efficient utilization of protein at low protein levels [39]. However, no protein sparing effect of lipid could be found in this study as dietary lipid level did not influence fish growth performance. This is consistent with studies on sunshine bass (Morone chrysops × M. saxatilis) [40], red-spotted grouper [38], hybrid grouper [10] and hybrid snakehead (Channa maculata × C. argus) [41]. These findings may signify the lower ability of grayling juveniles in oxidizing lipids and using them as an energy source. This was confirmed by increased VSI and IPF at increased lipid level indicating the lipid accumulation in fish abdominal cavity rather than being oxidized for energy production.

Whole-body lipid content was impacted by interaction of protein and lipid and moisture content decreased by increasing fat content of diet. Moreover, muscle lipid concentration reflected the dietary fat content. Likewise, Lee and Kim [33] showed the reduction of whole-body moisture and increment of lipid content in masu salmon at increased dietary fat. Also, a rainbow trout study showed the enhancement of whole-body lipid and reduction of moisture content at increased lipid level [42]. Similar observations have been reported in Manchurian trout [30], giant croaker (Nibea japonica) [43] and Channa striata [44]. Ash content of muscle increased by increasing protein level and reflected the dietary ash content.

Dietary lipid level is one of the primary factors that influences the muscle fatty acid composition [45]. Increasing fat content of feed in this study resulted in reduced muscle ARA, EPA, DHA, n-6 LC-PUFA, n-3, n-3 LC-PUFA, and EPA/ARA ratio whereas an increasing trend was observed for LA, LNA, MUFA, n-6, and ratios of DHA/EPA and n-6/n-3. Similarly, Kim et al. [46] reported that higher fat content in the diet resulted in reduced EPA, DHA, n-3 PUFA and n-3 HUFA in far eastern catfish whole-body. A hybrid grouper study revealed only increased muscle LNA content at increased dietary fat level [1] which is consistent with the
Conclusions

These results suggest that 40% protein can support growth of European grayling with average body weight of 25 – 60 g, and increasing dietary fat content to 20% can enhance FE and PER. Moreover, increasing dietary fat content resulted in reduced stress indices such as ALT, AST and LDH activities, and decreased ammonia excretion to the rearing water.
Declarations

Ethics approval

All the experimental procedures were performed in compliance with valid legislative regulations in Czech Republic (law no. 166/1996 and no. 246/1992); the permit was issued to No. 2293/2015-MZE-17214 and No. 55187/2016-MZE-17214. All samplings were carried out with the relevant permission from the Departmental Expert Committee for Authorization of Experimental Projects of the Ministry of Education, Youth and Sports of the Czech Republic (permit no. MSMT 4394/2017-2).

Consent for publication

Not applicable.

Availability of data and materials

The datasets produced and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

There are no conflicts to declare.

Funding

This study was financially supported by the Ministry of Agriculture of the Czech Republic, project NAZV QK1920326, and by the Ministry of Education, Youth and Sports of the Czech Republic, project Biodiversity (CZ.02.1.01./0.0/0.0/16_025/0007370).

Authors contribution

Conceptualization, Validation, Visualization, Methodology: SR, KD, TP. Investigation: SR. Formal analysis, collection and interpretation of data: SR, MI. Software: OM. Manuscript drafting: SR. Review & editing: SR, KD, JK, TP. Funding acquisition, Project administration, Resources: TP.

Acknowledgments

The authors would like to express thanks to the technicians of Faculty of Fisheries and Protection of Waters for their contributions in running the feeding trial.

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### Tables

Table 1. Formulation and proximate composition of the experimental diets (% dry matter).

|                | P30L10 | P30L20 | P40L10 | P40L20 | P50L10 | P50L20 |
|----------------|--------|--------|--------|--------|--------|--------|
| Fish meal<sup>a</sup> | 32.0   | 32.0   | 46.0   | 46.0   | 60.0   | 60.0   |
| SPC<sup>b</sup>      | 4.00   | 6.00   | 8.00   | 10.0   | 12.0   | 14.0   |
| Wheat flour         | 46.85  | 34.85  | 29.85  | 17.85  | 12.85  | 0.85   |
| Brewer’s yeast      | 2.00   | 2.00   | 2.00   | 2.00   | 2.00   | 2.00   |
| Dextrin             | 4.00   | 4.00   | 4.00   | 4.00   | 4.00   | 4.00   |
| Fish oil            | 3.60   | 10.0   | 3.00   | 9.40   | 2.40   | 8.80   |
| Soybean oil         | 2.00   | 5.60   | 1.60   | 5.20   | 1.20   | 4.80   |
| Lecithin            | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   |
| Stay-C              | 0.05   | 0.05   | 0.05   | 0.05   | 0.05   | 0.05   |
| Mineral premix<sup>c</sup> | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Vitamin premix<sup>d</sup> | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Choline chloride    | 0.50   | 0.50   | 0.50   | 0.50   | 0.50   | 0.50   |
| Carboxymethylcellulose | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 | 3.00 |

**Proximate composition**

|                |          |          |          |          |          |          |
|----------------|----------|----------|----------|----------|----------|----------|
| Dry matter     | 91.0     | 90.9     | 89.9     | 91.7     | 89.1     | 90.0     |
| Protein        | 29.8     | 30.3     | 40.0     | 40.3     | 49.2     | 49.7     |
| Lipid          | 9.90     | 19.0     | 9.70     | 19.3     | 10.1     | 19.5     |
| Ash            | 3.65     | 3.60     | 5.00     | 4.99     | 6.42     | 6.38     |
| GE<sup>e</sup> | 20.2     | 22.3     | 20.4     | 22.4     | 20.5     | 22.5     |
| P/E ratio<sup>f</sup> | 14.8 | 13.6 | 19.6 | 18.0 | 24.0 | 22.1 |

<sup>a</sup>Fish meal Super Prime (Crude protein: 67.9%, crude fat: 9%).

<sup>b</sup>Soy protein concentrate (Crude protein: 62%, crude fat: 0.5%).

<sup>c</sup>Mineral premix (mg or g kg<sup>-1</sup> diet): NaF, 2 mg; KI, 0.8 mg; CoCl<sub>2</sub>·6H<sub>2</sub>O (1%), 50 mg; CuSO<sub>4</sub>·5H<sub>2</sub>O, 10 mg; FeSO<sub>4</sub>·H<sub>2</sub>O, 80 mg; ZnSO<sub>4</sub>·H<sub>2</sub>O, 50 mg; MnSO<sub>4</sub>·H<sub>2</sub>O, 25 mg; MgSO<sub>4</sub>·7H<sub>2</sub>O, 200 mg; zoelite, 4.582 g.

<sup>d</sup>Vitamin premix (mg or g kg<sup>-1</sup> diet): thiamin, 10 mg; riboflavin, 8 mg; pyridoxine HCl, 10 mg; vitamin B12, 0.2 mg; vitamin K3, 10 mg; inositol, 100 mg; pantothenic acid, 20 mg; niacin acid, 50 mg; folic acid, 2 mg; biotin, 2 mg; retinol acetate, 400 mg; cholecalciferol, 5 mg; alpha-tocopherol, 100 mg; ethoxyquin,
150 mg; wheat middling, 1.1328 g.

Calculated gross energy (GE) (MJ kg\(^{-1}\)) = based on combustion values of 23.6 MJ kg\(^{-1}\) for protein, 39.5 MJ kg\(^{-1}\) for lipid and 17.2 MJ kg\(^{-1}\) for carbohydrate.

P/E ratio (g MJ\(^{-1}\)) = Protein to energy ratio.

### Table 2. Growth, feed utilization and survival of European grayling (Thymallus thymallus) (25.2 ± 0.28 g) fed the experimental diets for 12 weeks.

| Diets    | Protein (%) | Lipid (%) | FBW  | WG   | SGR  | FI   | FE   | PER | SUR  |
|----------|-------------|-----------|------|------|------|------|------|-----|------|
| Individual treatment means |             |           |      |      |      |      |      |     |      |
| P30L10  | 30      | 10        | 82.9 | 225  | 1.40 | 80.1 | 0.70 | 2.36| 99.3 |
| P40L10  | 40      | 10        | 85.6 | 243  | 1.47 | 69.9 | 0.85 | 2.13| 98.0 |
| P50L10  | 50      | 10        | 89.2 | 257  | 1.52 | 63.8 | 0.99 | 2.02| 99.3 |
| P30L20  | 30      | 20        | 82.5 | 232  | 1.43 | 69.1 | 0.82 | 2.72| 99.3 |
| P40L20  | 40      | 20        | 89.4 | 257  | 1.51 | 64.0 | 1.02 | 2.52| 100 |
| P50L20  | 50      | 20        | 92.5 | 266  | 1.54 | 59.7 | 1.15 | 2.31| 100 |
| SEM     |          |           | 1.08 | 4.17 | 0.01 | 1.71 | 0.04 | 0.06 | 0.40 |
| Means of main effects |             |           |      |      |      |      |      |     |      |
| 30       |          |           | 82.7\(^b\) | 229\(^b\) | 1.42\(^b\) | 74.6\(^a\) | 0.76\(^c\) | 2.54\(^a\) | 99.0 |
| 40       |          |           | 87.5\(^a\) | 250\(^a\) | 1.49\(^a\) | 66.9\(^b\) | 0.93\(^b\) | 2.32\(^b\) | 99.0 |
| 50       |          |           | 90.8\(^a\) | 262\(^a\) | 1.53\(^a\) | 61.7\(^c\) | 1.07\(^a\) | 2.17\(^b\) | 99.7 |
| 10       |          |           | 85.9 | 242  | 1.46 | 71.3\(^A\) | 0.85\(^B\) | 2.17\(^B\) | 98.9 |
| 20       |          |           | 88.1 | 252  | 1.50 | 64.2\(^B\) | 0.99\(^A\) | 2.52\(^A\) | 99.6 |

Two-way ANOVA (P-value)

| Protein | Lipid | Interaction |
|---------|-------|-------------|
| 0.00    | 0.15  | 0.46        |
| 0.00    | 0.08  | 0.87        |
| 0.01    | 0.08  | 0.87        |
| 0.00    | 0.00  | 0.21        |
| 0.00    | 0.00  | 0.69        |
| 0.00    | 0.00  | 0.77        |
| 0.00    | 0.00  | 0.61        |

Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments (P < 0.05).

FBW: Final body weight (g).

WG: Weight gain (%) = [(final body weight – initial body weight) / initial body weight × 100].

SGR: Specific growth rate (%) = [ln final body weight – ln initial body weight) / days] × 100.

FI: Feed intake (g fish\(^{-1}\)) = dry feed consumed (g) / fish.

FE: Feed efficiency = weight gain / dry feed fed.

PER: Protein efficiency ratio = wet weight gain / total protein given.

SUR: Survival (%).

### Table 3. Organosomatic indices of European grayling (Thymallus thymallus) fed the experimental diets for 12 weeks.

| Protein | Lipid | Interaction |
|---------|-------|-------------|
| 0.00    | 0.15  | 0.46        |
| 0.00    | 0.08  | 0.87        |
| 0.01    | 0.08  | 0.87        |
| 0.00    | 0.00  | 0.21        |
| 0.00    | 0.00  | 0.69        |
| 0.00    | 0.00  | 0.77        |
| 0.00    | 0.00  | 0.61        |
Diets | Protein (%) | Lipid (%) | CF    | HSI   | VSI   | IPF   |
|-------|------------|----------|-------|-------|-------|-------|
| Individual treatment means |
| P30L10 | 30         | 10       | 1.07  | 0.84  | 10.7  | 2.42  |
| P40L10 | 40         | 10       | 1.03  | 0.81  | 10.2  | 2.41  |
| P50L10 | 50         | 10       | 1.04  | 0.78  | 9.65  | 2.07  |
| P30L20 | 30         | 20       | 1.03  | 0.87  | 11.8  | 3.07  |
| P40L20 | 40         | 20       | 1.04  | 0.81  | 11.4  | 3.68  |
| P50L20 | 50         | 20       | 1.10  | 0.82  | 11.7  | 3.86  |
| SEM   | 0.01       | 0.02     | 0.22  | 0.19  |
| Means of main effects |
| 30    | 1.05       | 0.85     | 11.3  | 2.75  |
| 40    | 1.03       | 0.81     | 10.8  | 3.05  |
| 50    | 1.06       | 0.80     | 10.7  | 2.96  |
| 10    | 1.04       | 0.81     | 10.1A | 2.30A |
| 20    | 1.05       | 0.83     | 11.6A | 3.54A |

Two-way ANOVA (P-value)

|        | Protein | Lipid | Interaction |
|--------|---------|-------|-------------|
| Protein| 0.58    | 0.83  | 0.00        |
| Lipid  | 0.51    | 0.53  | 0.95        |
| Interaction | 0.23 | 0.00  | 0.30        |

Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments (P ≤ 0.05).

CF: Condition factor = Body weight × 100 / (Body length)³

HSI: Hepatosomatic index = Liver weight × 100 / fish weight.

VSI: Viscerosomatic index = Viscera weight × 100 / fish weight.

IPF: Intraperitoneal fat ratio = Intraperitoneal fat weight × 100 / fish weight.

**Table 4.** Whole-body proximate composition (% dry weight) of European grayling (*Thymallus thymallus*) fed the experimental diets for 12 weeks.
| Diets    | Protein (%) | Lipid (%) | Moisture | Protein | Lipid | Ash |
|----------|-------------|-----------|----------|---------|-------|-----|
| P30L10   | 30          | 10        | 72.0     | 59.1    | 29.0  | 11.1|
| P40L10   | 40          | 10        | 71.8     | 58.7    | 29.1  | 12.2|
| P50L10   | 50          | 10        | 72.2     | 58.2    | 31.9  | 9.90|
| P30L20   | 30          | 20        | 71.1     | 56.7    | 31.8  | 10.9|
| P40L20   | 40          | 20        | 70.2     | 57.5    | 30.9  | 11.5|
| P50L20   | 50          | 20        | 70.6     | 58.9    | 30.0  | 11.3|
| SEM      |             |           | 0.23     | 0.34    | 0.41  | 0.26|

Means of main effects

|          | Protein | Lipid | Moisture | Protein | Lipid | Ash |
|----------|---------|-------|----------|---------|-------|-----|
| 30       | 71.5    | 57.9  | 30.4     | 11.0    |       |     |
| 40       | 71.0    | 58.1  | 30.0     | 11.9    |       |     |
| 50       | 71.4    | 58.5  | 30.8     | 10.6    |       |     |
| 10       | 72.0<sup>A</sup> | 58.7 | 30.0     | 11.1    |       |     |
| 20       | 70.6<sup>B</sup> | 57.7 | 30.8     | 11.3    |       |     |

Two-way ANOVA (P-value)

|          | Protein | Lipid | Interaction |
|----------|---------|-------|-------------|
| Protein  | 0.45    | 0.74  | 0.62        |
| Lipid    | 0.00    | 0.16  | 0.29        |
| Interaction | 0.65 | 0.17  | 0.03        |

Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments (P<0.05).

**Table 5.** Dorsal muscle proximate composition (% dry weight basis) of European grayling (*Thymallus thymallus*) fed the experimental diets for 12 weeks.
Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments ($P \leq 0.05$).

Table 6. Dorsal muscle fatty acid composition of European grayling (Thymallus thymallus) fed the experimental diets for 12 weeks (% total identified FA).
| Fatty acids | Diets      | SEM | Protein (%) | Lipid (%) | Two-way ANOVA (Pval) | Protein | Lipid | Inte |
|------------|------------|-----|-------------|-----------|----------------------|---------|-------|------|
|            | P30L10     | 19.9| 0.08        | 1.91b     | 2.00b                | 2.35a   | 2.22A | 1.96 | 0.01 |
|            | P30L20     | 1.82| 0.00        | 0.01b     | 0.02b                | 0.03a   | 0.02 | 0.01 |
|            | P40L10     | 1.99| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | P40L20     | 2.02| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | P50L10     | 2.67| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | P50L20     | 2.03| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 14:0-1n-7  | 0.02| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 14:0-1n-5  | 0.06| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 15:0-1n-5  | 0.23| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:0-1n-5  | 0.03| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:0-1n-7  | 15.1| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:1-1n-7  | 3.39| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:1-1n-5  | 0.10| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:2-1n-4  | 0.21| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 17:0-1n-4  | 0.18| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:3-1n-4  | 0.18| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:3-1n-3  | 0.10| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:3-1n-1  | 0.02| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:4-1n-3  | 0.15| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 16:4-1n-1  | 0.02| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:0-1n-9  | 3.31| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:1-1n-7  | 2.83| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:1-1n-5  | 0.13| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:2-1n-9  | 0.06| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:2-1n-6  | 10.8| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:2-1n-4  | 0.14| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:3-1n-6  | 0.24| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:3-1n-4  | 0.13| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:3-1n-3  | 2.33| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:3-1n-1  | 0.02| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:4-1n-3  | 0.80| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 18:4-1n-1  | 0.11| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 20:0-1n-9  | 0.18| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 20:1-1n-7  | 2.81| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 20:1-1n-5  | 0.17| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 20:2-1n-9  | 0.07| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 20:2-1n-6  | 0.62| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|            | 20:3-1n-9  | 0.04| 0.00        | 0.00      | 0.00                 | 0.00    | 0.00 | 0.00 |
|       | 20:3n-6 | 20:4n-6 | 20:3n-3 | 20:4n-3 | 20:5n-3 | 22:1n-11 | 22:1n-9 | 22:4n-6 | 22:5n-6 | 22:5n-3 | 22:6n-3 | SFA | MUFA | n-6 | n-6 LC-PUFA | n-3 | n-3 LC-PUFA | EPA + DHA | EPA/ARA | DHA/EPA | n-6/n-3 |
|-------|---------|---------|---------|---------|---------|-----------|---------|---------|---------|---------|---------|-----|------|----|--------------|-----|-------------|---------|--------|--------|---------|
|       | 0.24    | 0.49    | 0.21    | 0.44    | 4.24    | 1.58      | 0.36    | 0.08    | 0.29    | 1.41    | 16.0   | 21.0  | 39.4 | 12.9         | 1.91| 22.3        | 20.2   | 6.31    | 3.77   | 0.51    |
|       | 0.17    | 0.81    | 0.24    | 0.40    | 2.69    | 1.61      | 0.37    | 0.06    | 0.22    | 0.94    | 12.1   | 19.7  | 45.8 | 10.9         | 1.56| 16.3        | 14.8   | 5.50    | 4.44   | 0.69    |
|       | 0.23    | 0.55    | 0.19    | 0.45    | 5.01    | 1.31      | 0.33    | 0.09    | 0.36    | 1.58    | 18.7   | 22.5  | 26.5 | 10.9         | 2.04| 26.0        | 23.7   | 6.21    | 3.73   | 0.38    |
|       | 0.18    | 0.86    | 0.24    | 0.42    | 3.21    | 1.42      | 0.35    | 0.06    | 0.36    | 1.03    | 12.4   | 20.5  | 44.9 | 9.1          | 1.62| 26.0        | 15.6   | 5.82    | 3.87   | 0.38    |
|       | 0.19    | 0.65    | 0.17    | 0.43    | 5.24    | 1.25      | 0.30    | 0.08    | 0.35    | 1.50    | 16.9   | 26.6  | 36.1 | 11.5         | 1.95| 26.9        | 22.2   | 6.07    | 3.23   | 0.60    |
|       | 0.17    | 0.03    | 0.23    | 0.46    | 3.85    | 1.41      | 0.33    | 0.08    | 0.31    | 1.28    | 15.6   | 20.7  | 41.8 | 11.5         | 1.79| 25.0        | 19.5   | 5.90    | 4.05   | 0.46    |
|       | 0.01    | 0.58    | 0.01    | 0.01    | 0.23    | 1.59     | 0.01    | 0.00    | 0.01    | 0.06    | 0.68   | 20.3  | 42.6 | 13.2         | 0.04| 0.90        | 0.90   | 5.91    | 4.11   | 0.60    |
|       | 0.20    | 0.68    | 0.23    | 0.42    | 3.46    | 1.36     | 0.37    | 0.07    | 0.26    | 1.18    | 15.6  | 21.5  | 40.7 | 11.7         | 1.73| 0.96        | 0.96   | 6.02    | 4.11   | 0.49    |
|       | 0.20    | 0.76    | 0.22ab  | 0.44    | 4.11    | 1.33     | 0.34ab  | 0.08    | 0.30    | 1.31    | 15.6  | 23.6  | 39.0 | 10.3         | 1.83| 19.3        | 22.2   | 5.99    | 3.64   | 0.40    |
|       | 0.18    | 0.78    | 0.20b   | 0.44    | 4.55    | 1.38     | 0.32b   | 0.08    | 0.33    | 1.39    | 15.6  | 26.4  | 37.3 | 11.0         | 1.86| 21.7        | 22.8   | 9.99    | 3.58   | 0.40    |
|       | 0.22    | 0.56    | 0.19b   | 0.42    | 4.83    | 1.38     | 0.33    | 0.08    | 0.33    | 1.50    | 15.6  | 20.3  | 44.2 | 12.5         | 1.96| 21.7        | 24.2   | 6.20    | 4.12   | 0.41    |
|       | 0.17b   | 0.00    | 0.24A   | 0.42    | 3.25    | 1.48     | 0.00    | 0.00    | 0.25    | 1.08    | 14.8  | 13.4  | 44.2 | 12.5         | 1.66| 22.8        | 27.2   | 0.90    | 0.59A  | 0.00    |
|       |         | 0.00    |         |         |         | 0.01    | 0.00    |         |         |         | 0.00  | 0.00  | 0.01 | 0.00         | 0.00| 0.01        | 0.14   | 0.00    |        | 0.00    |
|       |         |         |         |         |         |         |         |         |         |         | 0.00  | 0.00  | 0.01 | 0.00         | 0.00| 0.01        | 0.05   | 0.00    |        | 0.01    |

Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments ($P \leq 0.05$).

**Table 7.** Serum biochemical parameters of European grayling (*Thymallus thymallus*) fed the experimental diets for 12 weeks.
| Diets | Protein (%) | Lipid (%) | TP   | TG   | CHO  | GLU  | ALT  | AST  | ALP  | LDH  |
|-------|-------------|-----------|------|------|------|------|------|------|------|------|
|       |             |           |      |      |      |      |      |      |      |      |
| P30L10 | 30          | 10        | 4.08 | 264  | 218  | 76.4 | 48.3 | 835  | 380  | 7704 |
| P40L10 | 40          | 10        | 4.38 | 256  | 242  | 75.6 | 32.2 | 719  | 344  | 5192 |
| P50L10 | 50          | 10        | 4.51 | 220  | 277  | 83.1 | 33.0 | 611  | 363  | 5767 |
| P30L20 | 30          | 20        | 4.48 | 473  | 279  | 103  | 28.3 | 592  | 386  | 4437 |
| P40L20 | 40          | 20        | 4.16 | 426  | 258  | 84.4 | 28.7 | 519  | 332  | 4389 |
| P50L20 | 50          | 20        | 4.49 | 379  | 277  | 96.2 | 34.3 | 605  | 350  | 3416 |
| SEM   |             |           | 0.07 | 23.2 | 6.11 | 2.73 | 2.16 | 33.4 | 8.02 | 330  |

Individual treatment means

Means of main effects

| Protein      | 4.28   | 368<sup>a</sup> | 248<sup>b</sup> | 89.9<sup>a</sup> | 38.3 | 713 | 383 | 6070<sup>a</sup> |
|--------------|--------|------------------|------------------|------------------|------|-----|-----|------------------|
| Lipid        | 4.27   | 341<sup>a</sup>  | 250<sup>b</sup>  | 80.0<sup>b</sup> | 30.4 | 619 | 338 | 4790<sup>b</sup> |
| Interaction  | 4.50   | 300<sup>b</sup>  | 277<sup>a</sup>  | 89.7<sup>a</sup> | 33.7 | 608 | 357 | 4591<sup>b</sup> |

Two-way ANOVA (P-value)

| Protein      | 0.27   | 0.00  | 0.01  | 0.03  | 0.20  | 0.25 | 0.09 | 0.00  |
|--------------|--------|-------|-------|-------|-------|------|------|-------|
| Lipid        | 0.70   | 0.00  | 0.00  | 0.00  | 0.04  | 0.02 | 0.68 | 0.00  |
| Interaction  | 0.17   | 0.11  | 0.01  | 0.07  | 0.06  | 0.20 | 0.85 | 0.00  |

Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments ($P \leq 0.05$).

TP: Total protein (g dl<sup>-1</sup>).
TG: Triglyceride (mg dl<sup>-1</sup>).
CHO: Total cholesterol (mg dl<sup>-1</sup>).
GLU: Glucose (mg dl<sup>-1</sup>).
ALT: Alanine aminotransferase (U l<sup>-1</sup>).
AST: Aspartate aminotransferase (U l<sup>-1</sup>).
ALP: Alkaline phosphatase (U l<sup>-1</sup>).
LDH: Lactate dehydrogenase (U l<sup>-1</sup>).

Table 8. Serum antioxidant and innate immune parameters of European grayling (Thymallus thymallus) fed the experimental diets for 12 weeks.
| Diets | Protein (%) | Lipid (%) | SOD | CAT | MDA | LYZ | MPO | AP |
|-------|-------------|-----------|-----|-----|-----|-----|-----|----|
| P30L10 | 30          | 10        | 49.1 | 36.4 | 170 | 12.7 | 1.33 | 30.2 |
| P40L10 | 40          | 10        | 57.7 | 34.2 | 213 | 12.2 | 1.45 | 35.8 |
| P50L10 | 50          | 10        | 62.4 | 36.8 | 243 | 12.9 | 1.47 | 33.6 |
| P30L20 | 30          | 20        | 55.6 | 38.5 | 254 | 12.7 | 1.75 | 30.2 |
| P40L20 | 40          | 20        | 58.3 | 37.2 | 227 | 12.4 | 1.70 | 34.4 |
| P50L20 | 50          | 20        | 49.2 | 36.4 | 274 | 12.4 | 1.53 | 33.2 |
| SEM   | 1.58        | 0.61      | 9.96 | 0.45 | 0.04 | 0.62 |

Means of main effects

|     | Protein | Lipid | SOD | CAT | MDA | LYZ | MPO | AP |
|-----|---------|-------|-----|-----|-----|-----|-----|----|
| 30  | 52.3    | 37.4  | 212b | 12.7 | 1.54 | 32.0 |
| 40  | 58.0    | 35.7  | 220ab| 12.3 | 1.57 | 35.1 |
| 50  | 55.8    | 36.6  | 258a | 12.6 | 1.50 | 33.4 |
| 10  | 56.4    | 35.8  | 208a | 12.6 | 1.42a| 33.2 |
| 20  | 54.4    | 37.3  | 252a | 12.5 | 1.66a| 33.8 |

Two-way ANOVA (P-value)

|     | Protein | Lipid | Interaction |
|-----|---------|-------|-------------|
|    | 0.22    | 0.43  | 0.02        |
|    | 0.53    | 0.23  | 0.53        |
|    | 0.04    | 0.01  | 0.14        |
|    | 0.95    | 0.94  | 0.95        |
|    | 0.63    | 0.90  | 0.95        |
|    | 0.11    | 0.59  | 0.16        |

Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments (P≤0.05).

SOD: Superoxide dismutase activity (% inhibition).
CAT: Catalase activity (U ml⁻¹).
MDA: Malondialdehyde concentration (nmol ml⁻¹).
LYZ: lysozyme activity (U ml⁻¹).
MPO: Myeloperoxidase activity (U ml⁻¹).
AP: Antiprotease activity (% trypsin inhibition).

**Table 9.** Blood plasma ammonia (NH₃ – μmol l⁻¹) and rearing water total ammonium nitrogen (TAN - mg kg⁻¹ h⁻¹) concentrations in European grayling (*Thymallus thymallus*) after 5 and 24 h of last feeding.
| Diets   | Protein (%) | Lipid (%) | Plasma 5h | Plasma 5h | Plasma 24h | Rearing water 5h | Rearing water 5h | Rearing water 24h |
|---------|-------------|-----------|-----------|-----------|-------------|------------------|------------------|------------------|
| P30L10  | 30          | 10        | 630       | 11.1      | 14.0        |                  |                  |                  |
| P40L10  | 40          | 10        | 595       | 13.5      | 14.9        |                  |                  |                  |
| P50L10  | 50          | 10        | 648       | 17.0      | 20.8        |                  |                  |                  |
| P30L20  | 30          | 20        | 503       | 9.80      | 10.9        |                  |                  |                  |
| P40L20  | 40          | 20        | 556       | 8.90      | 11.6        |                  |                  |                  |
| P50L20  | 50          | 20        | 625       | 11.5      | 15.3        |                  |                  |                  |
| SEM     |             |           | 20.2      | 0.72      | 0.91        |                  |                  |                  |

Means of main effects

|        | Protein (%) | Lipid (%) | Plasma | Rearing water |
|--------|-------------|-----------|--------|---------------|
| 30     |             |           | 566    | 10.5<sup>b</sup> | 12.4<sup>b</sup> |
| 40     |             |           | 576    | 11.2<sup>b</sup> | 13.3<sup>b</sup> |
| 50     |             |           | 637    | 14.3<sup>a</sup> | 18.1<sup>a</sup> |
| 10     |             |           | 624    | 13.9<sup>A</sup> | 16.6<sup>A</sup> |
| 20     |             |           | 562    | 10.1<sup>B</sup> | 12.6<sup>B</sup> |

Two-way ANOVA (P value)

|        | Protein | Lipid | Interaction |
|--------|---------|-------|-------------|
|        | 0.31    | 0.13  | 0.52        |
|        | 0.01    | 0.00  | 0.12        |
|        | 0.00    | 0.00  | 0.65        |

Values are mean of triplicate groups. Different superscript letters indicate significant difference among treatments (P < 0.05).