



OPEN Effects of angler's groundbaits on fish physiology and growth

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Although angler's groundbaits (GBs) can be an important food resource for fish, we do not know much about the effects of GB consumption on the growth and health of fish. To fill this knowledge gap, we conducted a controlled, six-week long feeding trial (feeding ration: 2% of body weight) with common carp at 22 °C (*Cyprinus carpio*, mean initial body weight: 557 g) to test the effect of two GBs composed mostly of animal-derived ingredients (AN-GBs) and two plant-based GBs (PL-GBs) relative to one aquaculture feed, as a control (five treatment altogether). Consumption of PL-GBs resulted in lower growth rate than AN-GBs, presumably due to the low protein content. However, the unit biomass increment per unit nitrogen input was higher in PL-GBs. Although PL-GBs resulted in reduction of hepatic energy reserves, hepatosomatic index, viscerosomatic index, and body condition did not differ among the treatments. We did not find differences in expression of inflammatory cytokines in the liver. In conclusion, AN-GBs more effectively increases the carrying capacity of fisheries, but fish sequester a higher portion of nitrogen content of PL-GBs –PL-GB input can be more effectively counterbalanced by fish removal. Finally, the GB consumption does not pose a health risk to fish.

In the last decades, recreational fishing, especially angling, has become a popular outdoor activity, concerning approximately 10% of the population of industrialized countries^{1,2}. Recreational fishing is an important part of tourism having considerable positive economic effects^{3,4} and socio-cultural importance^{5,6}. However, recreational fishing also affect adversely fish, such as metabolic capacity and boldness^{7,8}, and aquatic ecosystems, one of which is the influence of ground-baiting^{9–12}.

In freshwaters, to maximize the catch, groundbaits (hereafter GB) are frequently introduced (i.e. ground-baiting) to attract fish (primarily cyprinids) to the fishing area^{9,13}. Traditionally used GBs are the particles (i.e. cereals and nuts) and their ground mixes supplemented with ground crackers and flavorings¹⁴. Beside them, the formulated GBs, boilies, and pellets have become popular in the last decades. Boilies are cooked, solid bait balls invented by specialized carp anglers and used originally to catch large common carp (*Cyprinus carpio*)^{10,15}. The pellets—based on the available data on their ingredients¹¹—are pelletized ground mixes or cylindrical boilies (the latter is often called “dumbbell”). Today, there are countless types of GBs available on the market with very variable compositions. They can consist almost solely of animal-derived ingredients (e.g. fish-, bone-, bloodmeal, and fish oil) or purely of plant (mostly cereals, seeds, nuts, and vegetable oil) material and they can be mixtures of both¹¹.

Although the amount of GB input can be high, knowledge on the effect of this external load (especially on fish) is still scarce. Mean daily GB input is usually between 1 and 3 kg^{11,12,15}. Baiting habit, however, differs considerably among anglers and angler groups, and annual GB use can be even several hundred kg per angler^{10,12}. In larger lakes, where angling activity is high, the total annual GB input can reach thousands of tons—e.g. in Lake Balaton, the yearly input can exceed two thousand tons¹¹. In relation to excessive GB use, the most frequently emerging concerns are around the related nutrient load, and its effect on water quality^{9–11,16}. Decomposition of non-consumed GBs, furthermore, can cause deoxygenation of sediment, and affect the benthic fauna^{17,18}. Although the importance of GB as food resource for fish varies in time¹⁹ and among ecosystems^{20,21}, stable

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isotope analyses revealed high contribution of GB to the diet of fish—it is averaged between 30 and 50%^{20,21}, but it can be almost 80%²⁰. Consequently, GBs can provide strong trophic subsidies and increase the carrying capacity of fisheries^{22,23}. Nevertheless, merely a few studies are available on effects of consumed GBs on the growth and health of fish^{10,24}.

Compared to granulated aquaculture feeds, GBs are not designed to provide balanced nutrition (i.e. providing macronutrients in appropriate amounts and ratios) for fish, but rather they are optimised to lure fish to the fish spot and keep them there actively foraging as long as possible¹⁰. Indeed, Niesar et al.¹⁰ revealed substantial differences in macronutrient content and accompanying effects of GBs on growth rate of fish. Although the classification of experimental GB was not based on the origin of ingredients (i.e. animal- vs plant-derived), Niesar et al.¹⁰ observed that plant material content—assessed by visual inspection—was associated negatively with macronutrient content (i.e. crude protein + crude fat) and growth rate of fish. Studies on aquaculture feeds are in accordance with this observation, and explain the adverse effect of plant materials on the growth rate by low protein content, the imbalance in essential amino acid profile, and impaired digestibility due to and presence of antinutritional factors (hereafter ANFs) in plant materials^{25,26}. In contrast, a study on GBs¹³ reported as high protein digestibility for GBs as for commercial aquaculture feeds even in the case of GB with less fish meal and more plant material. One possible explanation for this conflicting finding on digestibility is the heat treatment which is a common step in GB manufacturing¹⁰ and a widely use way to eliminate ANFs as well²⁷. Direct comparisons of macronutrient content and effect of animal derived and plant-based GBs on growth of fish can move towards a better understanding of environmental and economic impacts of GB use.

In the present study, we aimed to compare the effects of the animal-derived and plant-based GBs on the growth and health of fish using the common carp as a model species, which is among the most popular game fishes in inland recreational fisheries, particularly in Europe^{15,28–30}. More specifically, we fed experimental common carp stocks with either animal- or plant-based ground baits for six weeks and evaluated (i) their growth rate (ii) feed efficiency parameters such as feed conversion ratio (FCR), protein efficiency ratio (PER), energy efficiency ratio (EER). We predicted higher growth rate for GB composed from animal-derived ingredients. Since the fish relied exclusively on GB in the experiment, malnutrition associated health issues could occur in GB with inappropriate macronutrient contents and ratios. For instance, feeds high in fat and carbohydrate can lead to obesity, and lipid and glycogen deposition in the liver^{31,32}, resulting in oxidative stress, and inflammation³³. Also, fish feed with high plant material content can induce metabolic disorders in fish, leading to impaired growth and immune suppression^{25,26}. Therefore, we assessed the (iii) inflammatory cytokines expression, and (iv) lipid and glycogen deposition in the liver.

Results

Groundbait composition

Crude fat, starch, energy, non-protein energy (NPE) contents, and NPE:protein ratio varied considerably among the GBs, and even between the two animal-based and between the two plant-based products (Table 1). The phosphorus content of the control feed, and the two animal-based and one of the plant-based GBs were similar (slightly above 1% in dry weight), while PL-GB-2 contained much less (0.36%) phosphorus. Nitrogen and crude protein contents of GBs reflected the origin of their ingredients and were lower in PL-GBs. Similarly, essential amino acid contents—especially regarding the Methionine, Arginine, and Lysine—were lower in PL-GBs (Table 1).

Growth, condition and somatic indices

Although difference in final weight, and final biomass were marginally significant (final weight: LMM, $F_{4,10} = 2.74$, $p = 0.089$, $r^2_m = 0.13$, $r^2_c = 0.13$; final biomass: ANOVA, $F_{1,4} = 3.66$, $p = 0.044$) and final length did not differ among treatments (final length: LMM, $F_{4,10} = 1.87$, $p = 0.193$, $r^2_m = 0.09$, $r^2_c = 0.09$), individually tracked than in AN-GBs and control feed (Table 2). Namely, absolute growth rate (AGR), thermal growth coefficient (TGC) and weight gain (WG) were significantly lower in PL-GBs, than in AN-GBs and the control feed (AGR: LMM, $F_{4,10} = 15.91$, $p < 0.001$, $r^2_m = 0.46$, $r^2_c = 0.46$; TGC: LMM, $F_{4,10} = 27.81$, $p < 0.001$, $r^2_m = 0.60$, $r^2_c = 0.60$; WG: LMM, $F_{4,10} = 25.69$, $p < 0.001$, $r^2_m = 0.58$, $r^2_c = 0.58$). Difference in WG and TGC was observed between two AN-GBs, but not between AN-GB-1 and the control feed (Table 2). FCR, PER was higher in PL-GBs, than in AN-GBs and the control feed (FCR: ANOVA, $F_{1,4} = 256.6$, $p < 0.001$, PER: ANOVA, $F_{1,4} = 351.7$, $p < 0.001$). Further, differences in FCR were also observed between the two PL-GBs as well as between the two AN-GBs (Table 2). In PER values, differences occurred between two PL-GBs, but not between two AN-GBs (Table 2). Energy efficiency ratio of AN-GB-2 was higher than of other GBs and control feed (EER: ANOVA, $F_{1,4} = 99.94$, $p < 0.001$), but further differences was not observed among the treatments (Table 2). Body condition differed marginally among the treatments (K: LMM, $F_{4,10} = 2.61$, $p = 0.099$, $r^2_m = 0.12$, $r^2_c = 0.12$). Somatic indices did not differ among treatments (HSI: LMM, $F_{4,10} = 2.63$, $p = 0.098$, $r^2_m = 0.12$, $r^2_c = 0.12$, VSI: LMM, $F_{4,10} = 1.45$, $p = 0.288$, $r^2_m = 0.09$, $r^2_c = 0.17$; Table 2).

Immune responses in the liver

Compared to the control group, groundbait consumption did not induce significant changes in the expression of inflammatory factors (TNF- α : ANOVA, $F_{1,4} = 0.79$, $p = 0.544$, IL-1 β : ANOVA, $F_{1,4} = 1.29$, $p = 0.301$, IL-10: ANOVA, $F_{1,4} = 0.25$, $p = 0.908$, TGF- β : ANOVA, $F_{1,4} = 0.73$, $p = 0.581$; Fig. 1).

Liver histology

The hepatic energy reserves were affected by the origin of ingredients of GBs (inclusion of plant material) but not by macronutrient and energy content of GBs (Figs. 2 and 3). The total number of lipid droplets were two-times lower in liver samples derived from PL-GBs treatments than in AN-GBs and control feed (Kruskal–Wallis, $H = 16.53$, $P = 0.002$; Fig. 3). The mean lipid droplet size in PL-GB treatments were the half of the sizes observed

	Control	AN-GB-1	AN-GB-2	PL-GB-1	PL-GB-2
Water content (%)	8.06	7.87	16.31	14.11	13.85
Nutrient content (% in dry weight)					
Nitrogen	5.47	5.92	5.76	4.50	4.16
Phosphorus	1.13	1.07	1.12	1.02	0.36*
Energy (MJ/kg)	19.55	20.17	17.19	18.35	17.53
Energy (kcal/kg)	4669	4817	4106	4382	4187
NPE (kcal/kg)	1594	2297	1124	1527	1712
NPE:protein (kcal/mg)	0.49	0.77	0.39	0.70	0.89
Proximate composition (% in dry weight)					
Crude ash	5.28	5.45	8.33	6.17	5.75
Crude fat	7.46	12.03	10.26	13.03	7.71
Crude fiber	2.99	1.91	3.11	3.54	2.11
Starch	24.27	33.05	9.12	13.33	30.65
Crude protein	34.40	32.39	33.84	25.38	22.30
Amino acids (g/kg in dry weight)					
Asparagine	2.76	2.87	3.47	2.55	2.28
Threonine	1.27*	1.18*	1.34*	1.12*	0.97*
Seine	2.12	1.61	1.71	1.48	1.50
Glutamine	6.08	6.08	5.67	4.58	4.33
Proline	2.42	1.94	1.63	1.35	1.21
Glycine	2.18	1.67	1.83	1.24	0.89
Alanine	1.86	1.94	2.13	1.49	1.36
Cysteine	0.70	0.51	0.48	0.41	0.44
Valine	2.05	1.87	1.98	1.54	1.30*
Methionine	0.86	0.60*	0.76	0.54*	0.58*
Isoleucine	1.36	1.34	1.44	1.15	1.09
Leucine	2.70	2.85	2.92	2.17	2.12
Tyrosine	1.01	1.03	1.24	0.93	0.82
Phenylalanine	1.75	1.83	1.88	1.51	1.37
Histidine	0.97	1.19	1.03	0.64	0.49
Lysine	2.19	1.95*	2.24	1.16*	0.86*
Arginine	2.12	1.93	2.09	1.52*	0.69*

Table 1. Composition of groundbaits and control feed. AN-GB: groundbaits composed mostly of animal-derived ingredients, PL-GB: plant-based groundbaits, the control is a widely used aquaculture feed. Values with asterisk (*) are below the demand of common carp (*Cyprinus carpio*) based on the recommendation of the National Research Council (2011).

in AN-GBs and control feed (ANOVA, $F_{1,4}=20.85$, $p < 0.001$; Fig. 3). Similar differences were observed also in the density of lipid droplets (i.e. mean area covered by lipid droplets divided by the total area of view field, Kruskal–Wallis, $H = 18.22$, $p < 0.001$; Fig. 3).

Discussion

Although the use of GBs increases with increasing popularity of angling, and they can constitute an important food resource for fish^{19,21,23}, we do not know much about the physiological response of fish to GB consumption. Here, we highlight substantial differences in composition and nutritional value of GBs depending primarily on the origin of their ingredients—being composed mainly of animal or plant-originated materials. Two PL-GBs are less nutritious and resulted in reduced growth, but showed higher unit biomass increment per unit nitrogen input compared to AN-GBs. In contrast, fish fed with animal-originated GBs showed as high growth rate as control fish fed with aquafeed developed for aquaculture use. Despite of their unbalanced macronutrient content, exclusive consumption of GBs did not cause adverse immunological or histological alterations in the liver of the experimental common carp that could indicate a health issue.

Imbalanced plant-based diet, in general, can result in a reduced growth rate because of (i) its low protein content, (ii) deficiencies in essential amino acids (iii), presence of antinutritional factors in plant materials, and (iv) low phosphorus content (commonly present as phytate). Theoretically, each of these factors per se could explain lower growth rate³⁴, but the combination of these effects was more plausible in the present study. The protein contents of both tested PL-GBs were far less than the requirement of common carp (NRC recommends 32% crude protein³⁵). The energy content of these GBs were above the demand³⁵, thus it could potentially support growth through protein-sparing effect³⁶, this compensatory effect generally is not absolute. For instance, Fan et

	Control	AN-GB-1	AN-GB-2	PL-GB-1	PL-GB-2
Initial weight (g)	563 ± 160 ^a	541 ± 168 ^a	543 ± 144 ^a	534 ± 163 ^a	604 ± 149 ^a
Final weight (g)	966 ± 288 ^a	966 ± 271 ^a	861 ± 232 ^a	742 ± 201 ^a	815 ± 195 ^a
Final biomass (g)	4982 ± 243 ^a	4832 ± 397 ^a	4306 ± 484 ^a	3712 ± 752 ^a	4077 ± 347 ^a
Final length (cm)	36 ± 3 ^a	36 ± 3 ^a	34 ± 2 ^a	34 ± 3 ^a	35 ± 3 ^a
Survival (%)	100	100	100	100	100
WG	77.60 ± 22.76 ^a	81.51 ± 22.78 ^a	59.19 ± 16.85 ^b	40.49 ± 8.51 ^c	35.20 ± 7.87 ^c
AGR	9.86 ± 3.67 ^a	9.66 ± 3.08 ^a	7.22 ± 2.47 ^a	4.74 ± 1.07 ^b	4.79 ± 1.49 ^b
TGC	1.85 ± 0.47 ^a	1.88 ± 0.41 ^a	1.45 ± 0.34 ^b	1.03 ± 0.14 ^c	0.95 ± 0.21 ^c
FCR	1.25 ± 0.05 ^a	1.22 ± 0.04 ^a	1.57 ± 0.09 ^b	2.24 ± 0.07 ^c	2.48 ± 0.03 ^d
PER	5.83 ± 0.10 ^a	6.22 ± 0.06 ^b	6.09 ± 0.10 ^b	7.27 ± 0.03 ^c	8.09 ± 0.12 ^d
EER	0.43 ± 0.01 ^a	0.42 ± 0.004 ^a	0.50 ± 0.01 ^b	0.42 ± 0.002 ^a	0.43 ± 0.01 ^a
K	2.07 ± 0.26 ^a	2.09 ± 0.18 ^a	2.06 ± 0.26 ^a	1.88 ± 0.19 ^a	1.95 ± 0.20 ^a
HSI	3.18 ± 0.49 ^a	3.12 ± 0.54 ^a	2.66 ± 0.52 ^a	2.99 ± 0.54 ^a	3.16 ± 0.64 ^a
VSI	10.24 ± 2.04 ^a	11.81 ± 1.99 ^a	10.76 ± 1.96 ^a	10.57 ± 1.21 ^a	11.44 ± 1.77 ^a

Table 2. Mean and standard deviation of weight of common carp (*Cyprinus carpio*), growth parameters. WG: weight gain, SGR: specific growth rate, FCR: food conversion ratio, and somatic indices. K: Fulton’s condition factor, HSI: hepatosomatic index, VSI: viscerosomatic index. AN-GB: groundbaits composed mostly of animal-derived ingredients, PL-GB: plant-based groundbaits, the control is a widely used aquaculture feed. Values marked by different letters are statistically different at $p < 0.05$.

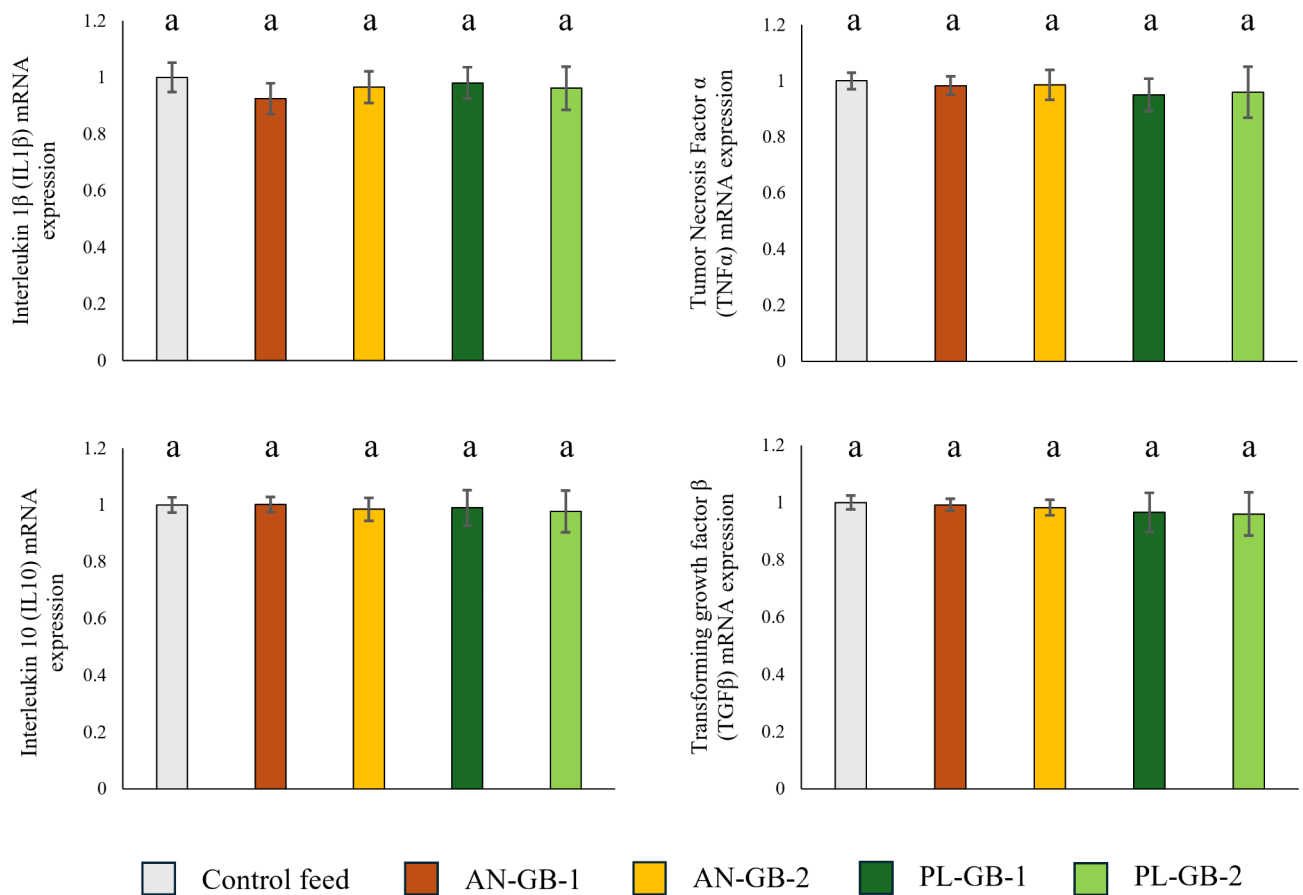


Fig. 1. Effects of different groundbaits on the expression of liver inflammation-related genes in common carp (*Cyprinus carpio*). AN-GB: groundbaits composed mostly of animal-derived ingredients, PL-GB: plant-based groundbaits, the control is a widely used aquaculture feed. Values marked by different letters are statistically different at $p < 0.05$.

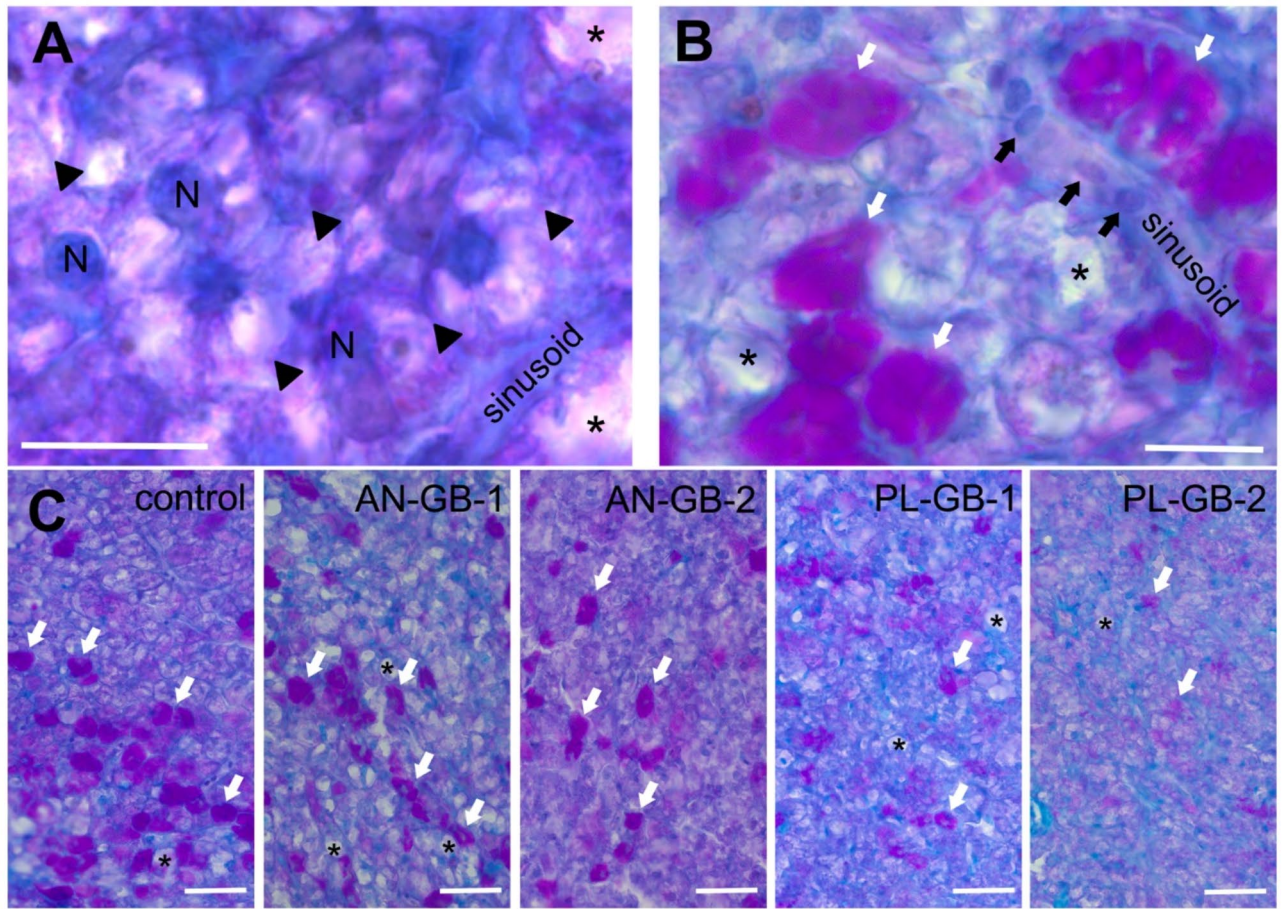


Fig. 2. Glycogen and lipids in liver cells (hepatocytes) highlighted by alcian-blue PAS staining (scale bar: 15 μm). (A) Glycogen granules are pink (black triangles). The cell nuclei (N) and membranes are blue. (B) Lipids droplets are purple (white arrows). Among the liver cells, blood cells (black arrows) are also visible in sinusoid. Vacuoles are marked by asterisks in liver cells. (C) Lipid droplets (white arrows) and vacuoles (asterisk) in representative liver samples of fish fed with different groundbaits (control, AN-GB-1, AN-GB-2, PL-GB-1, PL-GB-2; scale bar: 30 μm). AN-GB: groundbaits composed mostly of animal-derived ingredients, PL-GB: plant-based groundbaits, the control is a widely used aquaculture feed.

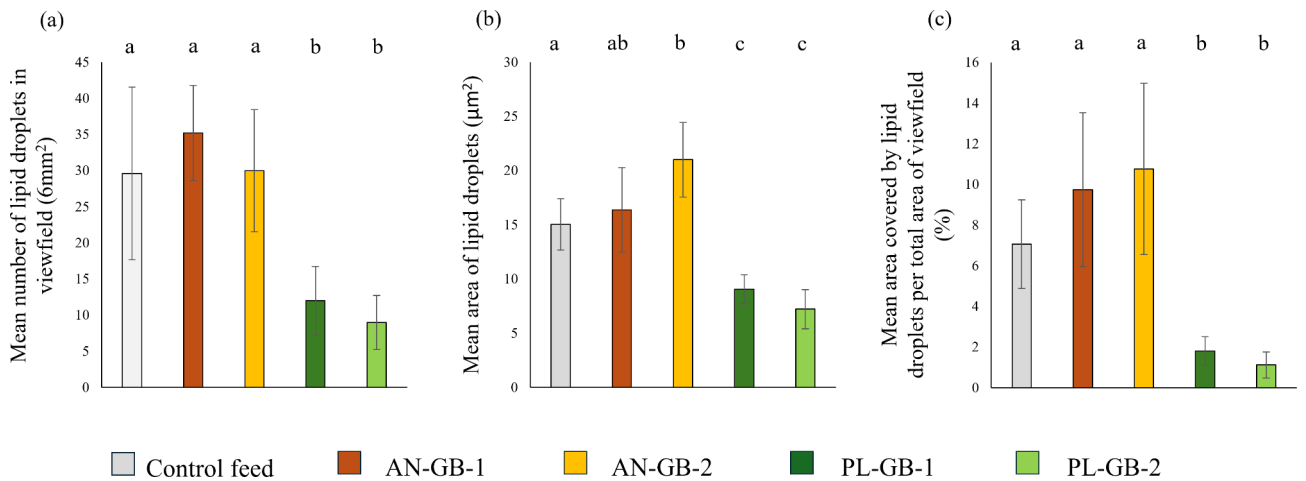


Fig. 3. Effects of different groundbaits on (a) number, (b) size, and (c) density of lipid droplets in the liver of common carp (*Cyprinus carpio*). AN-GB: groundbaits composed mostly of animal-derived ingredients, PL-GB: plant-based groundbaits, the control is a widely used aquaculture feed. Values marked by different letters are statistically different at $p < 0.05$.

al.³⁷ observed reduced growth in common carp reared on a 28% crude protein diet compared to those reared on a 30–32% crude protein diet even if the feeds were rich in energy. Also, Niesar et al.¹⁰ observed similar patterns and effect size to that revealed in our study: GB with lower protein content (19 vs 42% crude protein) resulted in two times lower growth rate in common carp despite the similar energy content (19.7 vs 21.4 MJ/kg). Based on our and previous^{10,13} findings, GBs composed mostly from plant materials contain much less protein, which is beyond the limits of protein sparing effects, consequently resulting lower growth rate. Further, the imbalance in essential amino acid profile (i.e. deficiencies especially in Lysine content) and the presence of ANFs could reduce the assimilation of proteins in plant-based GBs³⁴. The Lysine contents of PL-GBs were approximately the half of the recommended values³⁵, which could impair the growth performance³⁸. Also, the antinutritional factors can bind essential amino acids impeding assimilation³⁹. It has to be noted that the effect of amount (i.e. crude protein content of GB) and origin (animal vs plant originated ingredients) of protein on fish growth could not be distinguished because these features covaried in our study. The higher PER values in PL-GB, however, suggested the major role of low protein content in decreased growth. Finally, low, and unavailable phosphorus content could also contribute to reduced growth⁴⁰. The phosphorus content of one of the tested PL-GBs was obviously below the demand of common carp (which is 0.6–0.7% in water extractable form fed ad libitum^{41,42}). Although the other PL-GB theoretically contained enough phosphorus, we cannot be sure that this phosphorus was fully available for the fish. The plant materials contain phytic acid⁴³, which is rich in phosphorus but unavailable for common carp and other fishes that do not excrete phytase⁴⁴. Animal-based GBs—and aquaculture fish feeds in general as well—can also contain unavailable phosphorus forms, e.g. tri-calcium phosphate occurring in fishmeal^{45,46}. High growth rates, however, suggest that an appropriate proportion of phosphorus was available for fish in animal-based GBs.

Due to the imbalance in protein, lipid and carbohydrate content and/or impaired digestibility, foods with high inclusion level of plant-based ingredients can induce hepatic lipid and glycogen reduction, oxidative stress, and inflammation^{31,47}, reducing the immunity and anti-stress ability of fish⁴⁸—despite the fact that amylase activity is high in omnivorous fish, like common carp⁴⁹. On the contrary, the consumption of PL-GBs led depletion of hepatic energy reserves (lipid and glycogen) irrespectively from energy content of GBs—trends in differences in energy content, NPE, and NPE:protein ratio did not occur between AN-GBs and PL-GBs. Differences in carbohydrate (NFE) digestibility—which affect digestible energy—can occur among GBs¹³, but EER values did not suggest differences in energy availability between AN-GBs and PL-GBs. The reduction of hepatic energy reserves, however, did not affect the weight of the liver (there were no among-treatment differences in HSI), suggesting its lesser extent. While the previous studies on the effects of high-fat, high-carbohydrate, and high plant-protein diets on the liver health of common carp recorded substantial changes in the expression of inflammatory cytokines^{50,51}, similar patterns were not observed in our study. In contrast to the previous studies, which worked with larval and juvenile fish (body weight ranged between 1 and 50 g), we used much larger fish. Conflicting findings on the effect of plant-protein inclusion in feedstuffs on the health of fish can be associated with differences in the developmental stages of studied fish⁵². The tolerance against plant-based feed (i.e., nutrient imbalance, ANFs) can increase with increasing age of fish^{53,54}. The absence of innate immune response in our study suggests that adult common carp can be fed on less nutritious feed without considerable liver symptoms.

Under natural conditions, fish can supplement their diet with natural food resources^{21,23}, which potentially diminish the negative effects of GBs. Availability of natural food resources, however, can decrease with increasing fish biomass. In intensive fisheries, where both the abundance of fish, and angling activity is high, the quality of GBs fundamentally determines the fish carrying capacity, and thus, also the economic sustainability of fisheries. Besides the increase in catch rate, GBs should also promote the growth and health of fish. Larger fish increase the satisfaction of anglers⁵⁵, and thus, also the popularity of fishery. Based on our results, PL-GBs performed weaker than AN-GBs in this regard. Beside the economic aspects, however, reducing the environmental impacts of GB use are also a cornerstone for sustainable fisheries management. The external nutrient load, and accompanying eutrophication is among the most important environmental impacts related to GB use and recreational fisheries. Fish removal is an effective measure to counterbalance GB-induced nutrient inputs^{10,11}. Nutrients sequestered in fish bodies temporarily excluded from internal nutrient cycling, and can be removed through harvest; in contrast, the released (excreted and egested) nutrients can be accumulated in the recipient ecosystem and potentially accelerate the anthropogenic eutrophication. Therefore, the ecological impact of GB decreases with increasing fish yield per unit GB-induced nutrient input. Although the PL-GBs resulted in much slower growth rate, the unit biomass increment per unit nitrogen input (i.e. PER) was higher in PL-GBs than in AN-GBs. In natural conditions, where fish rely also on natural food resources, the difference in growth increment resulted by PL-GBs and AN-GBs is assumed to be less pronounced—exclusive GB consumption highly unrealistic in wild. Consequently, PL-GBs—with lower phosphorus content (like PL-GB-2)—have smaller environmental footprint^{10,15} but have less positive effects on carrying capacity of fisheries.

Highly digestible GBs with balanced nutrient content increase the growth rate of fish and also decrease the ground-baiting associated nutrient load. By relying on the achievements of the aquaculture feed industry, GB production could be improved from both economic and ecological point of view. Currently, the GB manufacturing, however, is mostly out of regulation; information about the composition, nutrient (both macro and micro) content, and production process are usually scarce, and largely depends on the producer's decision. Although a stricter regulation of manufacturing and using of GB can induce resistance form producers, sellers, and anglers (authors' personal experience), but it is necessary for sustainable fisheries and environmental management. Dissemination and education campaigns should be dedicated to eco-friendly GB production and GB use involving primarily GB manufacturers, but also sellers, fishery managers, and anglers. Such campaigns can highly increase the acceptance of alteration in regulations.

In conclusion, exclusive GB consumption does not cause health problems in age-cohorts of fish which are typically stocked in recreational fisheries. Groundbaits with high proportion of plant-material—primarily due to the lower protein content—resulted in lower growth rate, but fish sequester a higher portion of nitrogen content of PL-GBs. In intensive fisheries—where the fish biomass and angling activity are high, and the GBs are important food resources for fish –, using GBs that support the growth of fish (i.e. AN-GBs) increases the carrying capacity of and thus economic sustainability of fisheries. The ratio of unit biomass increments per unit nutrient input, however, is a cornerstone of environmental impact of GB use, because the nutrients sequestered in fish are excluded from internal nutrient cycling and can be removed by fish harvest. In vulnerable—shallow, small, nutrient poor and long water retention¹⁰—aquatic ecosystems, therefore, we recommend using plant-based GBs with lower phosphorus content.

Material and methods

Fish and culture conditions

A six-week long feeding trial was conducted in a recirculation aquaculture system (RAS) using common carp originated from pond aquaculture in accordance with the permit for the use of animals for scientific purposes (permit number: BE/25/4302-3/2017, issued by the Department of Food-security and Animal Health, Békés County, Hungary). Prior to the experiment, fish spent four weeks in quarantine tanks in RAS. Then, they were introduced into the experimental system—fifteen 1 m³ (1 × 1 × 1.2 m round-cornered, flow rate: 1.5 m³/h) tanks, 5 fish per tank—and tagged individually using PIT (passive integrated transponder) tags (12 × 2 mm, 0.1 g). One-week long acclimatization period was applied in the experimental system. During the quarantine and acclimatization period, the fish were fed with commercial fish feed with properly balanced nutrient content (Aller master, 8 mm, 0.3–0.35 g/particle, Denmark, composed with blood products, fish meal, grain products, marine by-products, non-marine by-products, processed animal proteins, single-cell proteins, vegetable oils, vegetable proteins, vitamins and minerals.)—which feed was also applied as control feed during the experiment—at 2% of fish biomass per day. At the beginning of the experiment, the total length (TL) and weight (W) of the fish (n = 75) were (mean ± SD) 31.2 cm (± 2.5 cm) and 557 g (± 155 g). This size group is frequently stocked in recreational fisheries^{29,30}. The initial body weight of fish did not differ significantly among the tanks (ANOVA, $F_{1,14} = 0.49$, $p = 0.675$). The mean (± SD) stocking density per tank was 2786 g/m³ (± 314 g) at the beginning and was 4382 g/m³ (± 633 g) at the end of the experiment. The dissolved oxygen and temperature were checked daily and were above 90% and 21.9 ± 0.9 °C, respectively. No mortalities occurred during the experiment.

Experimental design and feeds

Based on the feed supplied, five treatment types were applied in three replicates. Each replicate was run in separate tank with five fish (5 treatment × 3 replicates × 5 fish = 75 fish). In treatments (1) and (2) fish were fed with one of the two animal-based groundbait products tested (hereafter AN-GB-1 and AN-GB-2), which main ingredient is fishmeal. In treatments (3) and (4), fish were fed with one of the two plant-based groundbait products (hereafter PL-GB-1 and PL-GB-2). The initial selection of GB products was based on the description of products—the producers frequently advertise the PL-GBs as “carbohydrate boilie” (see Supplement material 1). After purchasing several products, the GBs have been undergone visual and olfactory inspection to verify ratio of animal- and plant-derived ingredients and choose the appropriate products. While treatment (5) served as control, with fish fed with widely used aquaculture feed (Aller master, 8 mm, Denmark). AN-GB-2 and the two PL-GBs were cut up to ensure similar size of feed and acceptability for fish. The applied feeding ration was 2% of wet body weight offered in two portions during the day. Body size (TL, standard length—SL, and W) was measured biweekly, and the amount of feed was adjusted accordingly. At the end of the experiment, we anesthetized all fish (n = 75) using phenoxethanol in accordance with AVMA Guidelines, and then, measured body, viscera and liver weight, body length (TL and SL) and took liver tissue samples.

Analysis of groundbait composition

Groundbaits were dried to constant weight at 60 °C (minimal duration was 48 h but lasted until we reach constant weight in a Memmert UFP 500 oven) and homogenized using mortar and pestle. To assess ash content, subsamples were ignited at 550 °C. Nitrogen content was measured by the Kjeldahl method. Phosphorus content was determined using microwave-assisted nitric acid-hydrogen peroxide digestion and subsequent ICP-OES (iCAP 6500 Duo View, Thermo Scientific) measurement. Ash, nitrogen, and phosphorus measurements were carried out in an accredited laboratory: Research Centre for Irrigation and Water Management, Laboratory for Environmental Analytics, Hungarian University of Agriculture and Life Sciences. The crude protein content of GBs was determined based on their nitrogen content (crude protein = 6.25 × nitrogen content¹). Amino acid profiles were determined with an automatic amino acid analyser (INGOS AAA400, Ingos Corporation, Czech Republic). Crude fiber, crude fat, and starch content were determined using acid-basic treatment⁵⁶, ether extraction⁵⁶, and polarimetric method, respectively, in accredited laboratory of UBM Feed Inc., Hungary. The gross energy content was determined using the Parr 6400 Automatic Isoperibol Calorimeter (Parr Instrument Co., Moline, IL, USA). We calculated the non-protein energy as NPE = (energy in starch + energy in lipid) and the ratio of NPE:protein = NPE/protein content of GB, expressed as kcal/kg and kcal/mg, respectively. To assess the energy contents of macronutrients, we used the following calorific values: 9.44 kcal for lipid, and 4.11 kcal for starch⁵⁷.

Analysis of growth, condition, and somatic indices

To evaluate the growth of fish, we determined the weight gain as $WG = (W_f - W_i) / W_i \times 100$, absolute growth rate as $AGR = (W_f - W_i) / \Delta t$, and thermal growth coefficient $TGC = [(W_f^{1/3} - W_i^{1/3}) / (T \times \Delta t)] \times 1000$, where W_f is final body weight in grams, W_i initial body weight in grams, T is water temperature (°C), and Δt is period of rearing in

days^{58,59}. We also calculated the final biomass for each tank. The feed conversion ratio was calculated as $FCR = F / (W_f - W_i)$, where F is the total weight of feed intake. Protein efficiency ratio was calculated as $PER = (W_f - W_i) / P$, where P is the protein consumed in grams. Energy efficiency ratio was calculated as $EER = (W_f - W_i) / GE$, where GE is the gross energy consumed in grams determined through calorimetric method. Fulton condition factor (K) was assessed using the formula $K = W \times 100 / SL^3$. To evaluate adiposity, hepatosomatic index $HSI = W_{liver} / W_{fish}$ and viscerosomatic index $VSI = W_{viscera} / W_{fish}$ were calculated.

Tissue sampling and analysis of gene expression

Liver samples were taken from two fish per tank for gene expression analysis. For each sample, 100 mg of liver was collected and placed in 1 mL of RNAlater (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for one day at 4 °C, followed by storage at –20 °C until analysis.

Gene expression analysis included the assessment of innate (non-specific) immune response (tumor necrosis factor alpha (TNF- α), interleukin-1 beta (IL-1 β), interleukin-10 (IL-10), and transforming growth factor beta (TGF- β)) by using real-time quantitative PCR (qPCR) and β -actin as an internal reference gene. Total RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The quantity of the RNA was measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1.5% agarose gel) and the purity was by measuring the OD260/OD280 absorbance ratio (> 1.95). The cDNA was generated from 1000 ng of total RNA using an iScript cDNA Synthesis Kit (BioRad Laboratories, Hercules, CA, USA) following the suggested protocol of manufacturer. The product of the first-strand cDNA synthesis was diluted to 10 \times and stored at –20 °C until the quantitative RT-PCR (qPCR) runs. The qPCR reactions were carried out using a LightCycler 96 instrument and the FastStart Essential DNA Green Master qRT-PCR kit (Roche, Basel, Switzerland). The primers used are presented in Table S1. The qPCR reaction was carried out in a final volume of 20 μ L consisting of 10 μ L of master mix (2 \times), 1 μ L of each primer (10 μ M), 5 μ L of cDNA (reverse transcription reaction mix), and 3 μ L of nuclease-free water. The thermal profile for all reactions was 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. The specificity of the reactions was checked via melting curve analysis, and no mispriming or primer dimers were found. All reactions were carried out in triplicate. The mean threshold cycle (Ct) values were calculated, and the qPCR data were analysed using the method described by Pfaffl⁶⁰. The efficiencies of qPCR reactions were determined using standard curves and serial dilutions, which had been made from cDNAs of liver samples. These cDNAs were diluted to 10 \times , 30 \times , 90 \times , 270 \times and 810 \times . Quantitative PCR reactions were carried out on these dilutions with all primer pairs in triplicates. Standard curves were drawn for each primer pair by plotting Ct values against the log₁₀ of different dilutions of cDNA sample solutions. Efficiencies (E) were calculated from the slopes of the standard curves using the equation $E = 10(-1/\text{slope})$.

Liver histology

The liver samples were dissected for identification of hepatic energy reserves (i.e. lipid and glycogen). A same pieces of liver samples were fixed in 4% paraformaldehyde (PFA) solution diluted in 0.1 M phosphate buffer (PB, pH=7.6) for 24 h at room temperature, then washed thoroughly (6 \times 15 min) in PB (0.1 M, pH=7.6), and cryoprotected in 20% sucrose solution for 1 h and then in 30% sucrose solution overnight at room temperature. After the incubation, the samples were embedded into Cryomatrix (#6,769,006, Thermo Fisher Scientific) and series of alternating cryostat Sects. (16 μ m) were mounted onto Superfrost ultra plus slides (#J3800AMNZ, Thermo Fisher Scientific) and stained with ready-to-use alcian-blue (#101,647, Sigma-Merck) Periodic acid-Schiff (PAS, #101,646, Sigma-Merck) staining following the manufacturer protocol. The microphotography was taken with a Leica Flexacam C1 digital camera coupled to a Zeiss Axioplane compound light microscope. To determine the density of the lipid droplets, we counted the number droplets and the area covered by them in relation the total area of view field with fixed view filed size (60 \times 100 μ m). We also measured the mean area of lipid droplets. Measurements were conducted on photography in ImageJ software.

Statistical analysis

Linear mixed-effects models (LMM) with tank id. as random effect were used to test the differences in final weight, final length, growth parameters (WG, ARG, TGR), condition, and somatic indices (K, HSI, VSI) among the treatments. We calculated both r^2_m (marginal r-squared, the effect size without considering the random factor, i.e. tank identity) and r^2_c (conditional r-squared; the effect size when the random factor is included). Where the treatments had significant effect ($p < 0.05$), subsequent Tukey HSD post-hoc tests were applied. Prior to analysis, data was checked for their data distribution and, if needed, log(x), or (\sqrt{x}) transformed to improve normality. To assess the influence of GBs on the expression of inflammatory cytokines and differences in final biomass, FCR, PER, EER, and density and size of the lipid droplets among the treatments, we used one-way analysis of variance (ANOVA) or Kruskal–Wallis H test and subsequent post-hoc tests (Tukey HSD or Mann–Whitney pairwise comparisons) depending on the variance homogeneity. We tested the homogeneity of variances with the Bartlett test. Bonferroni correction on p values was applied in post-hoc tests. Analyses were performed by using Statistica 12.0 (Statsoft, Tulsa, OK, USA) software and in R environment⁶¹.

Data availability

The datasets generated during the study are available from the corresponding author on reasonable request.

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Author contributions

D.L.F.: Conceptualization, Investigation, Formal analysis, Writing—original draft. L.A.: Investigation, Writing—review & editing. L.A.: Investigation, Methodology, Writing—review & editing. B.H.-K.: Investigation, Funding acquisition, Writing—review & editing. Z.J.S.: Investigation, Writing—review & editing. Z.P.: Investigation, Methodology, Writing—review & editing. F.T.: Investigation, Writing—review & editing. Z.V.: Investigation, Writing—review & editing. A.S.: Investigation, Methodology, Funding acquisition, Writing—review & editing. A.M.: Conceptualization, Investigation, Formal analysis, Writing—original draft.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

Experiments were performed in accordance with the permit for the use of animals for scientific purposes (permit number: BE/25/4302-3/2017, issued by the Department of Food-security and Animal Health, Békés County, Hungary). Also, the method used to euthanize fish (i.e. immersion in 2-phenoxyethanol solution) are in accordance with the recommendations of American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals⁶². The study was carried out in compliance in accordance with the relevant guidelines and regulations and the ARRIVE guidelines.

Additional information

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