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RESEARCH PAPER

Dietary available phosphorus restriction and skeletal integrity in stomachless fish *Gnathopogon caerulescens* and *Carassius auratus*: the effects of ferric chloride supplementation

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Abstract

Phosphorus is abundant in most feed ingredients and is classified into two forms: available and unavailable phosphorus. Available phosphorus is absorbed in the intestines of fish and used for various physiological needs. Any excess available phosphorus is excreted in urine, which is water-soluble and directly stimulates the growth of algae and macrophytes in surrounding water bodies. Therefore, minimizing the available phosphorus content in feeds to match the exact requirement level of fish is necessary to reduce the environmental impacts of aquaculture and promote its sustainable development. This study aimed to develop a technology to minimize the available phosphorus content in aquaculture feeds using ferric chloride as a phosphorus-binder. In a soybean meal-based diet devoid of inorganic phosphorus, the dietary addition of ferric chloride had no measurable effect on bone ash content or bone formation in experimental fish: *Gnathopogon caerulescens* and *Carassius auratus*, both stomachless fish belonging to the cyprinid family. However, in a purified diet containing a normal concentration of inorganic phosphorus, the dietary addition of ferric chloride decreased the bone ash content of the fish (p<0.01). Similarly, in a commercial diet containing inorganic phosphorus, dietary ferric chloride decreased the bone ash content of the fish (p<0.01). In conclusion, in diets containing soluble inorganic phosphorus, supplementing with ferric chloride can reduce the available phosphorus content, thereby decreasing the phosphorus burden on surrounding water bodies. However, when reducing the dietary available phosphorus content, it is advisable to occasionally monitor the bone ash content or check for bone deformities in cultured fish to prevent clinical deficiencies.

Introduction

Phosphorus (P) is an essential nutrient present in significant amounts in many feed ingredients. The phosphorus in feed can be classified into two forms: available P and unavailable P. Fish can absorb only the available P. Once absorbed from the intestinal tract, available P is used for various necessary functions within the body. However, any excess P that is not reabsorbed by the kidneys is excreted in urine (Bureau and Cho, 1999;

Sugiura et al., 2000). This urinary P is water-soluble and directly promotes the growth of phytoplankton and aquatic plants in the surrounding waters of aquaculture farms. In other words, excess available P in feed is a primary cause of environmental pollution, leading to eutrophication and harmful algal blooms such as red tides.

As global aquaculture production of fish and shellfish rapidly increases, developing technology to reduce the environmental impact of aquaculture has become an urgent research area to ensure the industry's sustainability (FAO, 2022; Nathanailides et al., 2023). Available P can react with various substances, transforming into unavailable P. Substances that facilitate this transformation are known as P binders. By using a P binder to "remove" available P from feed, the release of water-soluble P in aquaculture wastewater would decrease, thereby mitigating environmental pollution.

Various P binders, mainly used as medications for patients with renal failure (Sekar et al., 2018; Doshi and Wish, 2022) and in sewage treatment plants (Morse et al., 1998; Bashar et al., 2018; Bunce et al., 2018), include calcium compounds, aluminum compounds, and ferric compounds. It was assumed that adding these substances to feed could reduce the available P content easily and cost-effectively. However, to the best of our knowledge, there are no precedents for such experiments in fish. Therefore, the present experiments were conducted to test the hypothesis that ferric chloride, a P binder, could decrease the available P content in feed. The effect was evaluated by examining the skeletal ash content (bone density) and skeletal deformations in experimental fish, which serve as reliable indicators of the P status of fish.

Materials and Methods

Fish

Gnathopogon caerulescens is a small cyprinid gudgeon endemic to Japan, known locally as honmoroko. In its natural habitat, it has a lifespan of about three years and can grow up to 13 cm in total length. It is considered a minor aquaculture species in Japan. *Carassius auratus*, commonly known as the goldfish, is another cyprinid fish bred worldwide as an ornamental fish, with various breeds exhibiting different body shapes and colors. In the present study, we used the common goldfish, which retains the original wild shape. Although both species are omnivorous, *G. caerulescens* is more zoophagous than *C. auratus.*

Experiment 1 (Effects of ferric chloride in a practical diet):

Five test diets were prepared using practical ingredients (Table 1). Soybean meal was autoclaved at 120°C for 10 minutes before use. Blood meal consisted of spray-dried porcine blood cells (AP301, APC Japan, Inc.). Feather meal was purchased locally (grade unknown). Ferric chloride anhydrous ($FeCl_3$; Hayashi Pure Chemical Ind. Ltd., Osaka, Japan) dissolved in tap water was added to the basal diet (Diet 1a) to create Diets 1b through 1e. The same amount of tap water was added to Diet 1a. All diets were pelleted and dried at room temperature. After drying, the pellets were top-coated with liquid fish hydrolysate (1 g/100 g, dry weight basis), dried again, placed in plastic bags, and stored at 4°C until feeding. Vitamins and minerals were not supplemented due to the short experimental duration.

The P-binding equivalent of ferric chloride was calculated (Table 1), assuming a 1:1 stoichiometry—one mole of P binds to one mole of Fe forming insoluble $FePO_4$. Thus, the values indicate the maximum amount of P that may be trapped by the supplemental ferric ion (see Discussion for details). The total P and available P content of the experimental diets (Table 1) were calculated using literature values reported for each feed ingredient (Sugiura, 2018) and the present diet composition. The P availability values determined for stomachless fishes were used for these calculations. The supplemental ferric chloride was not considered when calculating the available P content.

G. caerulescens (mean body weight 0.35 g) and *C. auratus* (mean body weight 1.30 g) were stocked together in 20-L aquaria (30 fish per tank, 1 tank per diet). The fish were reared with recirculating filtered water (25 ± 1°C) with a 9.5-hour photoperiod using LED lights (a short photoperiod was applied to suppress gonadal development). Total P concentration in the recirculating water ranged from 0-0.2 ppm (as P). Fish were fed their respective experimental diets

for 30 days, 4 times daily, by hand to apparent satiation.

After rearing, 8 fish from each group and species were analyzed. Small or thin fish suspected of poor feed intake were excluded (the same applied to the following experiments). Growth rates and feed efficiency were not recorded due to the short rearing period (the same applied to the following experiments). Analyses were conducted for vertebral ash content (%, fat-free dry basis) and skeletal deformity.

The method for measuring vertebral ash content was as follows: the entire fish was immersed in water at approximately 80°C to denature soft tissues, after cooling down the vertebrae were carefully removed. The vertebrae were washed with tap water, soaked overnight in a methanol-chloroform mixture (1:1, v/v) for defatting, and dried at 105°C for 3 hours. The entire dried vertebrae (including neural and hemal spines; excluding ribs) were ashed at 550°C for 20 hours, and the ash content (% per dry weight) was determined. Due to individual differences in feed intake (growth) within the same tank, the minimum ash content (among 8 fish per tank) is also shown in the table. Individuals with deformed ribs or spines (neural and hemal spines) were counted and classified into three categories: frankly deformed, slightly deformed, and not deformed.

Table 1 Diet composition (upper rows) and the composition of *Gnathopogon caerulescens* and *Carassius auratus* fed their respective diets (lower rows)

Diet	1a	1b	1c	1d	1e
Soybean meal (g)	50	50	50	50	50
Blood meal (g)	10	10	10	10	10
Feather meal (g)	15	15	15	15	15
Wheat flour (g)	20	20	20	20	20
Canola oil (g)	5	5	5	5	5
$FeCl_3$ anhydrous (g)	0	0.25	0.5	1	2
P-binding eq.[1]	0.000	0.048	0.096	0.191	0.383
Total-P (%)	0.41	0.41	0.41	0.41	0.41
Available-P (%)	0.17	0.17	0.17	0.17	0.17
Gnat body wt (g), mean[2]	1.24	1.18	1.17	1.08	1.20
Feeding activity[3]	3-3	3-2	3-3	3-3	3-3
Mortality (#fish)[4]	1	2	0	4	3
Bone ash (%), mean[5]	34.5	36.3	33.7	32.4	33.1
Bone ash (%), min[5]	29.7	32.4	21.4	24.1	24.4
Rib-def (F:S:N)[6]	5:3:0	6:2:0	6:2:0	6:2:0	4:4:0
Sp-def (F:S:N)[6]	1:2:5	0:1:7	0:2:6	0:3:5	2:2:4
Cara body wt (g), mean[2]	3.55	3.07	3.83	3.44	3.49
Feeding activity[3]	2-2	2-2	2-2	2-2	2-2
Mortality (#fish)[4]	0	0	0	0	0
Bone ash (%), mean[5]	36.8	36.2	40.5	39.7	42.2*
Bone ash (%), min[5]	29.0	31.6	37.3	36.1	35.7
Rib-def (F:S:N)[6]	1:7:0	0:6:2	5:3:0	4:4:0	2:6:0
Sp-def (F:S:N)[6]	0:1:7	0:1:7	0:2:6	0:2:6	0:0:8

[1] P-binding equivalent (g/100g diet): P (g/100g diet) that can be trapped by Fe(III).

[2] *Gnat*: *Gnathopogon caerulescens*, *Cara*: *Carassius auratus.*

[3] Feeding activity (by observation): 0 (no feeding), 1 (low), 2 (moderate), 3 (active). The hyphened numbers indicate the first half and the second half periods.

[4] Mortality: the cumulative number of dead fish.

[5] Bone ash%: Mean represents the average of 8 fish. Values with asterisks are significantly different from Diet 1a (*p<0.05; **p<0.01). Min represents the lowest value in each treatment group.

[6] The number of fish with rib deformity (Rib-def) or neural-hemal spine deformity (Sp-def): F (frank), S (slight), or N (no) deformity.

Experiment 2 (Effects of ferric chloride in a purified diet):

Four test diets were prepared (Table 2). The basal diet (Diet 2a) was a purified low-P diet containing only 0.07% total P. Diets 2b through 2d included inorganic P in the form of KH_2PO_4, with ferric chloride added to Diets 2c and 2d. Vitamins were not supplemented, except in Diet 2d, to minimize potential reactions with added ferric ions. Additionally, lard was used as the fat source because it contains no vitamin E, which could react with ferric ions. Other diet manufacturing conditions followed those in Experiment 1. *G. caerulescens* (mean body weight 0.67 g) and *C. auratus* (mean body weight 1.41 g) were stocked together in 20-L aquaria (30 fish per tank, 1 tank per diet). The fish were fed their respective test diets for 30 days. Other rearing conditions were the same as in Experiment 1.

After rearing, 6 fish from each group and species were analyzed. The analysis items and methods were the same as in Experiment 1.

Table 2 Diet composition (upper rows) and the composition of *Gnathopogon caerulescens* and *Carassius auratus* fed their respective diets (lower rows)

Diet	2a	2b	2c	2d
Egg white powder (g)	60	60	60	60
Starch (g)	30	30	30	30
Cellulose (g)	3.5	3.5	3.5	3.5
Lard (g)	5	5	5	---
Vitamin mix (g)	---	---	---	5
KH_2PO_4 (g)	0	1.5	1.5	1.5
$FeCl_3$ anhydrous (g)	0	0	2	2
P-binding eq.[1]	---	---	0.38	0.38
Total-P (%)	0.07	0.41	0.41	0.41
Available-P (%)	0.05	0.39	0.39	0.39
Gnat body wt (g), mean[2]	1.95	2.60	2.20	2.29
Feeding activity[3]	2-1	2-3	1-1	1-2
Mortality (#fish)[4]	3	1	3	0
Bone ash (%), mean[5]	31.1	46.3	36.7**	33.9**
Bone ash (%), min[5]	25.5	43.3	28.8	26.7
Rib-def (F:S:N)[6]	2:4:0	0:0:6	0:4:2	3:2:1
Sp-def (F:S:N)[6]	3:3:0	0:0:6	0:1:5	2:1:3
Cara body wt (g), mean[2]	3.15	4.04	3.08	4.76
Feeding activity[3]	2-1	2-3	1-1	1-2
Mortality (#fish)[4]	0	1	0	0
Bone ash (%), mean[5]	39.4	50.1	48.6	43.6**
Bone ash (%), min[5]	36.5	48.3	44.5	38.9
Rib-def (F:S:N)[6]	1:2:3	0:2:4	0:2:4	2:4:0
Sp-def (F:S:N)[6]	0:0:6	0:0:6	0:0:6	0:1:5

[1-6] See footnotes of Table 1.

Experiment 3 (Effects of ferric chloride in a commercial diet):

Six test diets were prepared (Table 3). The basal diet (Diet 3a) was a commercial diet for common carp, containing 1.57% total P and 0.34% available P (based on *in vitro* analysis by Satoh et al., 1997). Diets 3b through 3f included either ferric chloride anhydrous ($FeCl_3$) or ferric ammonium citrate (Fe(III)NH_3-citrate; Fujifilm Wako Pure Chemical Co., Osaka, Japan) at levels of 2-4%. Other diet manufacturing protocols followed those in Experiment 1.

G. caerulescens (mean body weight 0.63 g) were stocked in 20-L aquaria (20 fish per tank, 1 tank per diet). The fish were fed their respective test diets for 30 days. *C. auratus* was not tested in Experiment 3, as this species was found to be less sensitive to dietary treatments based on the results of Experiments 1 and 2. Other rearing conditions and analytical methods followed those in Experiment 1. The mean values of the bone ash

content were compared using Dunnett's test (Experiments 1-3), with significant differences determined at the 5% level.

Table 3 Diet composition (upper rows) and the composition of *Gnathopogon caerulescens* fed their respective diets (lower rows)

Diet	3a	3b	3c	3d	3e	3f
Commercial diet (g)	100	100	100	100	100	100
$FeCl_3$ anhydrous (g)	0	2	4	---	---	---
Fe(III)NH_3-citrate (g)	0	---	---	2	4	8
P-binding eq.[1]	---	0.38	0.77	0.19	0.39	0.78
Total-P (%)	1.57	1.57	1.57	1.57	1.57	1.57
Available-P (%)	0.34	0.34	0.34	0.34	0.34	0.34
Gnat body wt (g), mean[2]	2.07	2.00	1.80	2.31	2.47	2.02
Feeding activity[3]	3-2	2-2	2-1	3-3	3-3	3-3
Mortality (#fish) [4]	0	0	0	0	0	0
Bone ash (%), mean[5]	47.2	44.1	39.0**	44.3	43.1	44.1
Bone ash (%), min[5]	44.8	36.0	30.6	38.2	37.5	39.2
Rib-def (F:S:N) [6]	1:5:2	5:1:2	1:6:1	6:1:1	5:3:0	1:5:2
Sp-def (F:S:N) [6]	0:0:8	0:0:8	0:2:6	0:0:8	0:0:8	0:0:8

[1-6] See footnotes of Table 1.

Results

Experiment 1 (Ferric chloride does not decrease available P in a practical diet):

In the practical diet, supplemental ferric chloride up to 2% was apparently ineffective as a P-binder in reducing dietary P absorption in both species, as their bone ash content and the incidence of skeletal deformity were similar across dietary treatments (Table 1; Fig. 1-2). Despite the apparent ineffectiveness of supplemental ferric chloride, all dietary groups of fish exhibited clinically low bone ash content as well as bone deformities in ribs and spines (hemal and neural), especially at their growing ends. The types of bone deformities were similar among dietary treatments, with no instances of scoliosis or lordosis identified. *G. caerulescens* tended to have lower bone ash content and a higher incidence of bone deformities compared to *C. auratus*. Significantly higher bone ash content (p<0.05) was recorded in *C. auratus* fed Diet 1e. Dietary ferric chloride, up to 2%, did not appear to affect feed intake or mortality in the fish.

Experiment 2 (Ferric chloride decreases available P in a purified diet):

In the purified diet, supplemental ferric chloride at 2% reduced dietary P absorption in *G. caerulescens*, as indicated by bone ash content (p<0.01) and bone deformity (Table 2; Fig. 1-2). In *C. auratus*, the effect was less pronounced but still tended to reduce P absorption, with Diet 2d reducing bone ash content (p<0.01). Feeding activity was high in the P-supplemented diet (Diet 2b), whereas it was noticeably low in the P-deficient groups (Diet 2a, 2c, 2d), especially during the second half of the feeding duration. In *G. caerulescens*, bone deformity was prevalent in the P-deficient groups, whereas it was absent in the P-supplemented group. In *C. auratus*, a similar pattern was observed, although the response was less clear compared to *G. caerulescens*.

Experiment 3 (Ferric chloride decreases available P in a commercial diet):

In the commercial diet, supplemental ferric chloride up to 4% reduced bone ash content (p<0.01) and increased bone deformity in *G. caerulescens* (Table 3; Fig. 1). However, in the same diet, supplemental ferric ammonium citrate was less effective in decreasing bone ash content, with no significant reduction observed (p>0.05). Nonetheless, severe bone deformities, particularly rib deformities, were frequently recorded in this group of fish. Feed intake was higher in fish fed ferric ammonium citrate-supplemented diets compared to those fed ferric chloride-supplemented diets. The commercial diet itself was apparently deficient in available P content, as evidenced by the bone ash content and the occurrence of bone deformities.

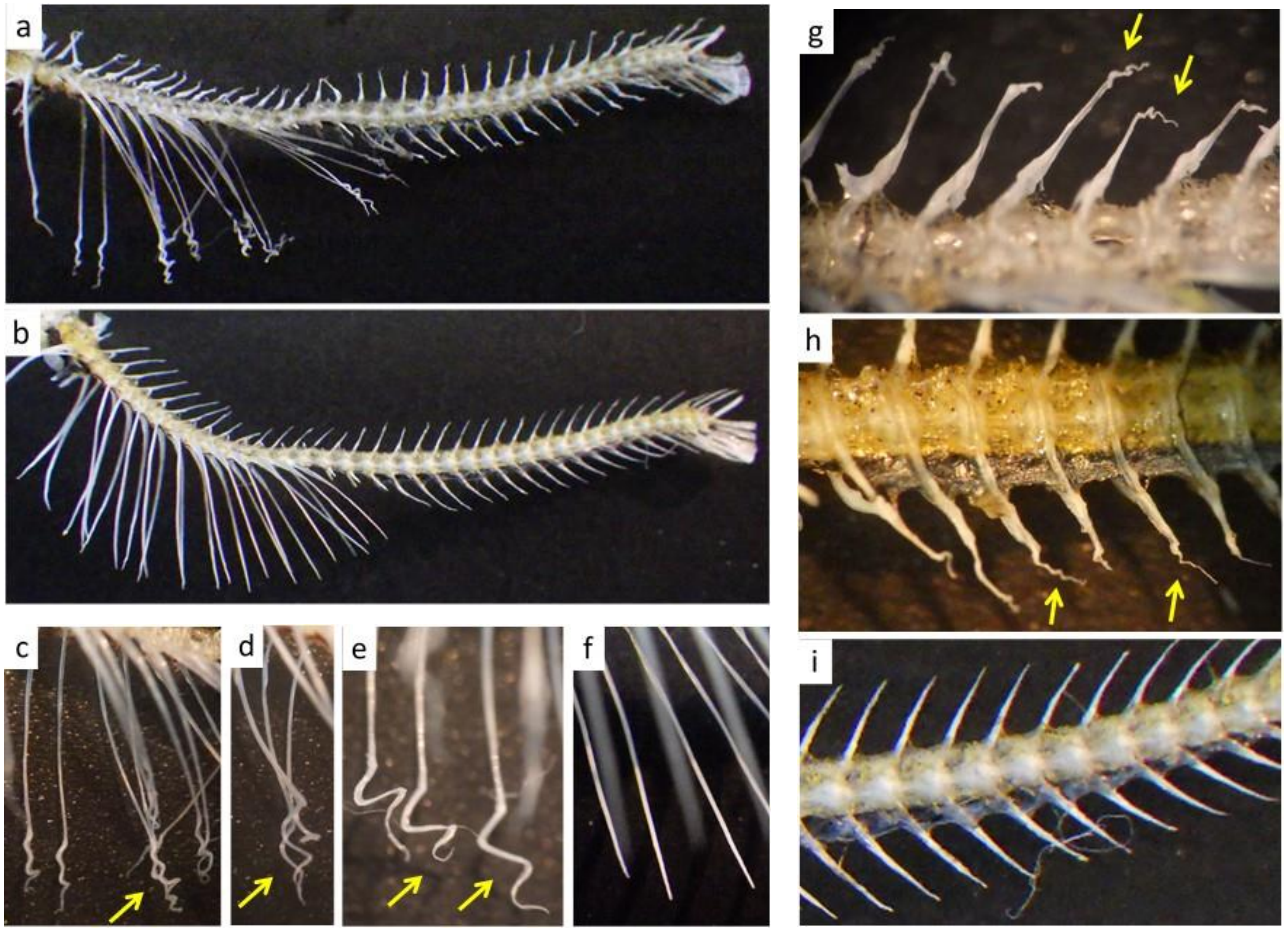

Figure 1. *Gnathopogon caerulescens*. (a) Skeleton of P-deficient fish fed Diet 1a, (b) Skeleton of normal fish fed Diet 2b, (c) Ribs of P-deficient fish fed Diet 1a: Yellow arrows indicate the wavy shape of the growing ends, (d) Ribs of P-deficient fish fed Diet 1b, (e) Ribs of P-deficient fish fed Diet 3d, (f) Ribs of normal fish fed Diet 2b, (g) Neural spines of P-deficient fish fed Diet 1e: Yellow arrows indicate the kinky shape of the growing ends, (h) Hemal spines of P-deficient fish fed Diet 3c, (i) Hemal and neural spines of normal fish fed Diet 2b (lint appeared accidentally at the bottom center).

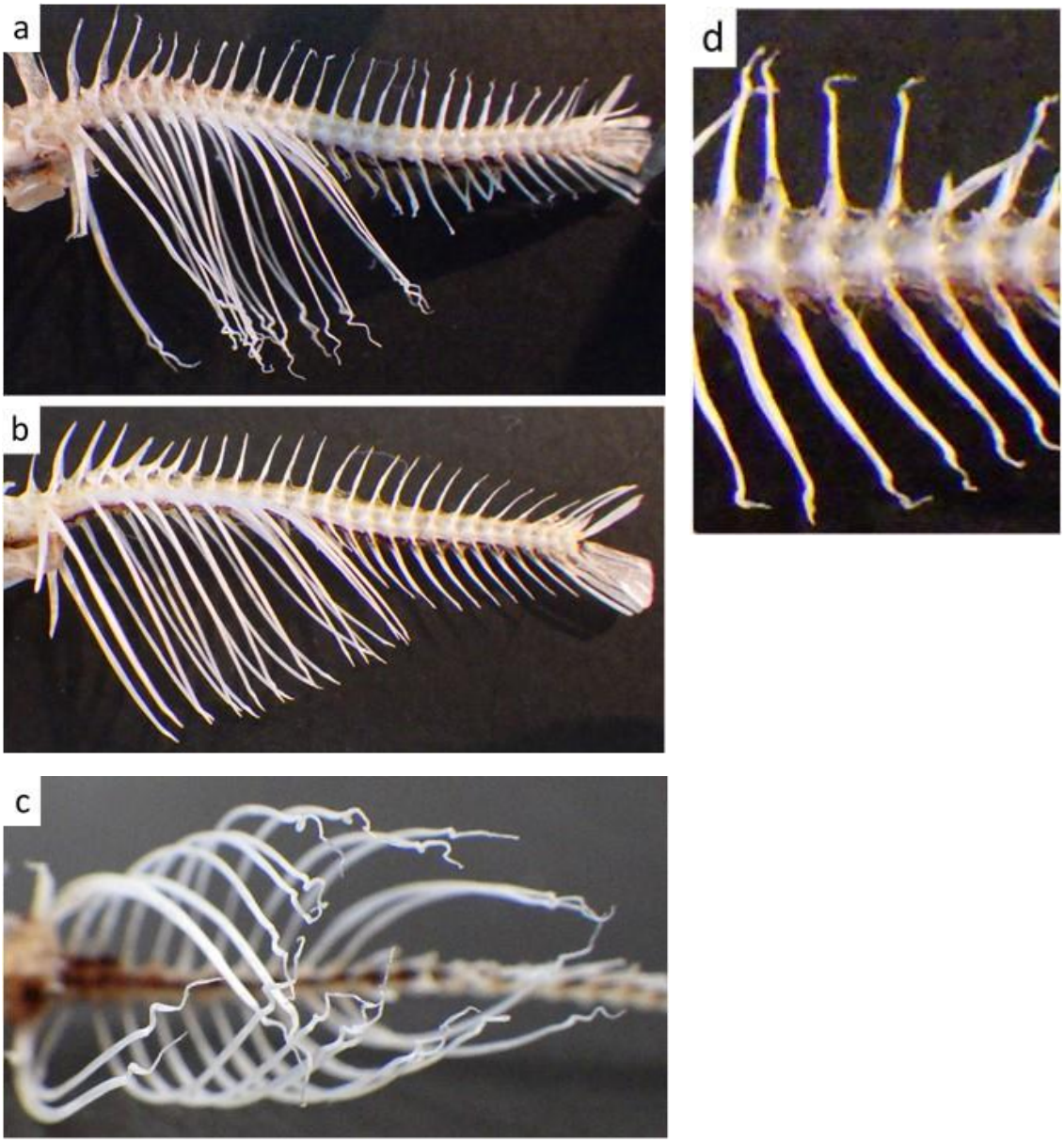

Figure 2. *Carassius auratus*. (a) Skeleton of P-deficient fish fed Diet 2d, (b) Skeleton of normal fish fed Diet 2b, (c) Ribs of P-deficient fish fed Diet 2d; ventral view: Note the wavy shape of the growing ends, (d) Hemal and neural spines of P-deficient fish fed Diet 1c: Note the kinky shape of the growing ends.

Discussion

As initially hypothesized, dietary supplementation of ferric chloride was expected to reduce dietary P absorption by fish and consequently reduce bone ash content. However, in Experiment 1, there was no apparent response in bone ash content to supplemental ferric chloride. The reason for this was unclear, but it was further hypothesized that the present diet (available P 0.17%) might contain little free inorganic P that could be bound or trapped by ferric ions. The diet, which contained 50% soybean meal, is known to contain various P compounds such as phytates, phosphoproteins, and phospholipids, but may contain little free inorganic P available to react with ferric chloride (Banaszkiewicz, 2011; Noack et al., 2012). To test this hypothesis, Experiment 2 was conducted using a semi-purified diet, where most of the P was present as a free inorganic form. In this diet, supplemental ferric chloride was highly effective in decreasing bone ash content, suggesting that ferric chloride reduced dietary P absorption by the fish. The response was more pronounced in *G. caerulescens* than in *C. auratus*, likely due to the size difference between the fish species (smaller fish generally respond faster to dietary treatments than larger fish). Fish fed Diet 2d, which contained vitamins instead of lard, had slightly lower bone ash content than those fed Diet 2c in both species. This could be attributed to enhanced

growth rate (feed intake) resulting from the omission of lard and consequent increase in protein intake.

As evident from Table 2, the P-binding equivalent of Diets 2c and 2d was nearly equal to the available P content of the diets. Nonetheless, the bone ash content of fish in those dietary groups was higher than that of fish fed Diet 2a, indicating that portions of the available P (free inorganic P) in those diets were not bound to the supplemental ferric ions.

In a study with rats, Hsu et al. (1999) determined, based on *in vivo* P absorption data, the P-binding capacity of ferric ions (P bound per gram of Fe^{3+}), which was only 181 mg for ferric chloride and 101 mg for ferric ammonium citrate. This suggests that more than two-thirds of the supplemented ferric ions were not bound to P, indicating that substantially higher amounts of ferric ions would be required to trap most of the free inorganic P in the diet.

Researchers in sewage treatment technology also report detailed conditions for P removal, including optimum pH, concentrations of each reactant, and mixing intensity. They also indicate substantially higher Fe/P ratios for optimal removal of orthophosphates (Fytianos et al., 1998; Caravelli et al., 2010; Szabó et al., 2008).

Since Experiment 2 verified the effect of ferric chloride in trapping free inorganic P, Experiment 3 was conducted to confirm the P-binding effect of ferric ions using a commercial diet known to contain free inorganic P.

In the commercial diet, supplemental ferric chloride significantly decreased the bone ash content; however, the effect of ferric ammonium citrate was only moderate and statistically insignificant based on bone ash content. Despite this, fish fed ferric ammonium citrate showed a high rate of bone deformity, likely due to higher feeding activity (and thus higher growth rate), resulting in pronounced rib deformities especially at the growing ends. These deformities were similar to those reported previously in P-deficient salmonid species (Sugiura, 2018).

According to our previous data, the normal bone ash content of *G. caerulescens* of similar body sizes fed a P-sufficient diet was 52% (manuscript in preparation). Hence, in the present experiment, fish fed the basal commercial diet had notably lower bone ash content, suggesting that the basal commercial diet was insufficient in available P content. The low ash content was associated with bone deformities. Since the fish used in this experiment were small juvenile fish, the basal commercial diet, which was formulated for adult carp, must be insufficient in available P to support the rapid growth of juvenile fish that typically show better feed conversion compared to adult fish (Shearer, 1988; Eya and Lovell, 1997).

Although both ferric chloride and ferric ammonium citrate are commonly used as food additives for humans, the amount used as an iron source is much smaller than the amount required as a P-binder (Whittaker, 1998; Teucher et al., 2004). High dietary levels of ferric chloride could depress feed intake due to its highly acidic nature and chlorine-like smell (cf. The Merck Index). In Experiment 3, fish fed diets supplemented with ferric ammonium citrate showed noticeably higher feeding activity than those fed diets supplemented with ferric chloride, suggesting that ferric chloride might negatively affect feed palatability. In Experiment 1, the bone ash content was significantly higher in *C. auratus* fed Diet 1e compared to those fed the basal Diet 1a. This paradoxical result may not be incidental, as fish fed Diets 1c and 1d also exhibited higher bone ash content, although the differences were not statistically significant. This could also be linked to feed intake in fish fed Diet 1e, although daily observations did not detect any reduction in feed intake for these fish. In the rat study mentioned above, 4.4% $FeCl_3 \cdot 6H_2O$ (which is equivalent to 2.6% $FeCl_3$ anhydrous) or 5% ferric ammonium citrate was supplemented to their diet (Hsu et al., 1999). The rats well-accepted both diets, which is different from our present observation in fish. But, the authors also reported that ferric chloride was more potent than ferric ammonium citrate in lowering intestinal P absorption. This is in agreement with our present study.

Conclusion

For commercial or farm-made feeds containing an excess amount of available P, supplementation with ferric iron can reduce the available P in the diet and consequently reduce the soluble P excretion into the environment. Since large fish consume most feed in commercial aquaculture,

and since large fish require significantly less P in their diet compared to small juvenile fish, adjusting the dietary available P content could help protect the aquatic environment surrounding fish farms. When reducing dietary available P content using P-binders, occasional monitoring of the bone ash content (bone density) of cultured fish is recommended to safeguard against clinical P deficiency. Early P deficiency can also result in rib deformities at their growing ends, with similar deformities observed in the neural and hemal spines. Scoliosis or lordosis of the vertebral column may be absent except in cases of extreme P deficiency. P-deficient fish may appear externally normal even under severe P deficiency.

Ethical statement

The author declares that the present study was conducted in accordance with the guidelines approved by the Field-Experiment Committee of the University of Shiga Prefecture.

Informed consent

Not available

Conflicts of interest

There is no conflict of interests for publishing of this study.

Data availability statement

The author declares that data are available from the author upon reasonable request.

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