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ANIMAL SCIENCE

Influence of particle size on the efficiency of semipurified diets in juvenile zebrafish

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Abstract: Standardizing feeding protocols for zebrafish (*Danio rerio*) is essential to improve experimental reproducibility. This study evaluated the effects of semi-purified diet particle size (159, 256, 362, 462, and 550 µm) on the performance of juvenile zebrafish. A total of 225 fish (85.8 ± 1.3 mg) were randomly distributed in a completely randomized design with three replicates per treatment (15 fish per tank). Fish were fed twice daily at 5% body weight for 49 days using a semi-purified diet containing 35% crude protein. Weight gain increased quadratically ($p < 0.05$) with particle size, peaking at 514 µm. Feed conversion was optimized at 515 µm while survival was unaffected ($p > 0.05$). Fish fed 159 and 256 µm particles had 23-32% lower weight gain than those fed 362-550 µm particles, which performed similarly ($p > 0.05$). The estimated optimal particle size (~515 µm) represented 2.0-2.1% of fish total length (16.3 ± 0.9 mm), although sizes from 1.5 to 2.3% (362-550 µm) showed no significant differences. The findings demonstrate that diets with a particle size of approximately 2% of fish length maximize growth and feed efficiency, providing a practical guideline for standardizing feeding protocols in zebrafish research.

Key words: Standardized diet formulation, Nutritional requirements in zebrafish, Dietary particle size, Zebrafish model.

INTRODUCTION

The zebrafish (*Danio rerio*), an omnivorous cyprinid, has become a key model organism in various research fields (Canedo et al. 2022, Trigueiro et al. 2020). However, the lack of standardized protocols for husbandry, feeding and environmental conditions can lead to inconsistent results across laboratories, thereby complicating interlaboratory comparisons and generalization of findings (Brenes-Soto et al. 2019, Frederickson et al. 2021, Licitra et al. 2024).

Feed presentation is a critical aspect of feed management, directly influencing weight gain and feed efficiency. Physical characteristics such as particle size, color, texture, and density affect the fish's ability to detect, capture, and ingest food (Ramos-Júdez & Duncan 2022). Despite widespread recognition of the need for nutritional standardization (Siccardi et al. 2009, Watts & D'Abramo 2021), particularly particle size, within zebrafish feeding protocols. The significance of appropriate particle sizing has been documented across multiple aquaculture applications (Martins et al. 2020), yet systematic evaluation in zebrafish remains limited despite the species' growing importance in biomedical research. A commonly cited guideline for many fish species suggests that optimal feed particle size ranges from 25% to 50% of the fish's mouth gape (Jobling et al. 2001).

However, this general recommendation has not been rigorously validated for zebrafish under controlled laboratory conditions using semi-purified diets. Larger-than-optimal pellets may facilitate feed detection but can hinder capture and ingestion due to increased handling time and nutrient leaching (Azaza et al. 2010, Knights 1983). Conversely, undersized particles hinder food detection, reduce net energy gain, and impair digestive efficiency through accelerated intestinal transit (Hossain et al. 2000, Busti et al. 2022). Consequently, inappropriate particle sizes can impair growth, worsen feed efficiency, degrade water quality, and compromise overall rearing efficiency.

The lack of standard diets, such as those available for rodents (NRC 1995), remains a critical challenge to consolidating zebrafish as a model organism (Monteiro et al. 2024, Hillman et al. 2024). In this context, the adoption of semi-purified diets has been recommended as an intermediate step toward the development of standardized feeds for zebrafish (Watts & D'Abramo 2021). This approach aims to replace generic commercial diets (Fowler et al. 2019, Siccardi et al. 2009) or those formulated based on the nutritional requirements of other species (Kaushik et al. 2011), until specific knowledge gaps regarding zebrafish nutritional are addressed (Monteiro et al. 2024). Semi-purified diets are composed of highly refined ingredients and are free from antinutritional factors, unlike conventional ingredient-based feeds (NRC 1995), thereby reducing the risk of adverse health effects in zebrafish (Watts et al. 2016). However, these diets are also associated with higher production costs and lower palatability, which may negatively affect feed intake, growth, and feed efficiency (NRC 2011).

Formulating semi-purified diets with particle sizes suited to zebrafish oral anatomy poses practical challenges, given typical laboratory equipment constrains (Wilson 2012, Brenes-Soto et al. 2019). Particle size is often adjusted post-pelleting via mechanical fragmentation (Fernandes et al. 2016, Martins et al. 2020). However, this process introduces pellet fractures that increase water penetration and nutrient leaching. While binders are incorporated to enhance pellet integrity, their efficacy is limited by fragmentation damage. The problem is especially acute for semi-purified diets, which often contain highly water-soluble ingredients such as crystalline amino acids, vitamins, and dextrin. Thus, identifying optimal particle sizes is essential not only to maximize nutrient retention and minimize leaching, but also establish reproducible feeding protocols that improve interlaboratory comparability.

Accordingly, this study aimed to determine the optimal particle size of a semi-purified diet for juvenile zebrafish reared under controlled laboratory conditions.

MATERIALS AND METHODS

Ethical approval

All experimental procedures were approved by the Ethics Committee on Animal Use of the Palotina Campus, Federal University of Paraná (CEUA/Palotina; Protocol 11/2022).

Fish acquisition and acclimation

Wild-type juvenile zebrafish (*Danio rerio*) were obtained from a commercial ornamental fish supplier (Aqua Paraná, Toledo, PR, Brazil) and underwent quarantine and acclimation to laboratory conditions. Fish were housed in a circular fiberglass quarantine tank (1 m^3) equipped with a biological filter, temperature control (28 ± 1.9°C), and continuous aeration to maintain dissolved oxygen levels above 5.0 mg/L. Water quality parameters, including pH (7.5 ± 0.0), total ammonia nitrogen (< 0.1mg/L), and nitrite (< 0.1 mg/L), were monitored weekly and remained within optimal ranges for the species (Mocho 2024) throughout the acclimation period.

Experimental diet preparation

A semi-purified experimental diet was formulated (Table I). Ingredients were weighed, homogenized, moistened, pelleted into 2 mm diameter pellets, and dried in a forced-air oven at 55°C for 16 hours. Pellets were cooled in a desiccator and mechanically fragmented using a commercial-grade grinder. The resulting particles were sieved with an electromagnetic shaker using mesh sizes of 600, 500, 425, 300, 212, and 106 µm. Mean particle size was calculated as the arithmetic mean between the upper and lower mesh sizes retaining particles: 600-500 µm (550 µm), 500-425 µm (462 µm), 425-300 µm (362 µm), 300-212 µm (256 µm), and 212-106 µm (159 µm) (Azaza et al. 2010). Particles retained on the 600 µm sieve or that passed through the 106 µm sieve were discarded. Diets were stored in labeled airtight containers at 4°C until use.

Table I. Ingredients and chemical composition of semi-purified diet for zebrafish.

Ingredients	%
Casein	42.00
Corn starch	18.93
Gelatin	7.00
Dicalcium Phosphate	6.20
Dextrin	5.00
Soybean Oil	5.00
Fish Oil	5.00
Cellulose	3.00
Vitamin and Mineral Premix[1]	3.00
Soy lecithin	2.50
Carboxymethylcellulose	2.00
Choline chloride	0.20
Betaine	0.15
Butylated Hydroxytoluene	0.02
Analyzed chemical composition	
Moisture (%)	4.7
Crude protein (%)	34.9
Ether extract (%)	10.9
Neutral detergent fiber (%)	8.3
Ash (%)	7.5
Gross energy (kcal/kg)	4,607

Vitamin and Mineral Premix (Tectron, Toledo, Paraná, Brazil). Guaranteed levels per kilogram of product: Vitamin A: 1,000,000 IU; Vitamin D3: 500,000 IU; Vitamin E: 20,000 mg; Vitamin K3: 500 mg; Vitamin B1 (Thiamine): 1,900 mg; Vitamin B2 (Riboflavin): 2,000 mg; Vitamin B6 (Pyridoxine): 2,400 mg; Vitamin B12 (Cobalamin): 3,500 mg; Folic Acid: 200 mg; Calcium Pantothenate: 4,000 mg; Vitamin C (Ascorbic Acid): 25 g; Biotin: 40 mg; Niacin: 5,000 mg; Iron (Fe): 12.5g; Copper (Cu): 2,000 mg; Manganese (Mn): 7,500 mg; Zinc (Zn): 25 g; Iodine (I): 200 mg; Selenium (Se): 70 mg.

Feeding transition protocol

During the first 17 days of quarantine, fish were fed to satiation twice daily with a commercial flake diet (Novo Color - JBL; 43% crude protein minimum, 8.3% lipids minimum, 1.9% fiber maximum, 8.1% ash maximum), maintaining the feeding regimen used by the producer from which the fish were acquired. On day 18, a subsample of 10% of the initial population was weighed to initiate the transition to the experimental diet. The semi-purified diet used during this transition was fragmented but not sieved, to avoid fish acclimation to specific particle sizes before the experiment. Fish were fed at 5% biomass per day for six days, with the following ratios: 75:25% (commercial diet to semi-purified diet) for two days, 50:50% for two days, and 25:75% for two days. After the transition, fish were exclusively fed the semi-purified diet at 5% biomass/day.

Experimental facilities and management

Following complete acclimation, fish were fasted for 24 h prior to the start of the trial. A total of 225 fish (mean weight: 85.8 ± 1.3 mg; standard length: 16.3 ± 0.9 mm) were randomly assigned to 15 polycarbonate aquaria (3 L each), with 15 fish per aquarium. All aquaria were connected to a recirculating water system in which the mechanical and biological filtration compartment had a total volume of 300 L. The system provided continuous aeration and operated under an 11:13 h light:dark photoperiod. A 6000 L/h pump with flow control ensured a flow rate of 8.4 L/h per experimental unit.

Fish were assigned to a completely randomized design with five treatments (particle sizes: 159, 256, 362, 462, and 550 μm), each three replicates. Feeding occurred twice daily (08:00 and 16:00) at 5% group biomass/day for seven weeks. Weekly biometric measurements were performed to monitor growth and adjust feed rations. For measurements, fish were fasted for 16h, anesthetized with 50 mg/L benzocaine, weighed, and counted.

Water pH (7.4 ± 0.2), total ammonia nitrogen (0.02 ± 0.06 mg/L), and nitrite (0.0 ± 0.0 mg/L) were assessed as acclimation protocols. Daily temperature (28.0 ± 1.7°C) and dissolved oxygen (5.4 ± 0.9 mg/L) were measured using a multiparameter meter. A malfunction in the climate control system on day 37 temporarily increased water temperatures to 29.4-31.1°C for eight days. To minimize stress, biometric measurements were skipped on day 42. Temperatures exceeded 30°C on only four of the affected days. All other water parameters remained within the optimal range for the species (Mocho 2024).

Performance evaluation

At the end of the experiment, fish were assessed following the weekly biometric protocol. The following performance metrics were calculated: weight gain (mg/fish) = final body weight – initial body weight; specific growth rate (%/day) = 100 × [(ln(final body weight) – ln(initial body weight)]/experimental days; feed conversion ratio (g:g) = total feed intake/weight gain; and survival rate (%) = 100 × (final fish count/initial fish count). Total length of the fish was measured from the tip of the snout to the end of the caudal fin using a digital caliper.

Statistical analysis

Data normality was evaluated using the Shapiro-Wilk test, and homogeneity of variances was assessed using Bartlett's test, to validate ANOVA assumptions. One-way ANOVA was applied, followed by Tukey's test for multiple comparisons when significant differences were detected. Regression analysis was performed to determine the predicted optimal particle size. Among the tested models, the quadratic regression yielded the best fit, as evidenced by the lowest residual sum of squares (Shearer 2000). All statistical analyses were conducted using SAS version 9.1, with a significance threshold set at $p < 0.05$.

RESULTS

Dietary particle size had no significant effect on survival rates ($p > 0.05$; Table II). Growth performance did not differ significantly ($p > 0.05$) among fish fed pellets ranging from 362 to 550 μm throughout the experiment period (Figure 1a). In contrast, diets with smaller particles sizes resulted in reduced growth, although differences were not statistically significant ($p > 0.05$) until day 35. By day 49, fish fed the 256 μm diet achieved intermediate mean weight, significantly higher than those in the 159 μm group but lower than all other groups ($p < 0.05$), which remained statistically similar to each other ($p > 0.05$).

The greatest weight gain (Figure 1b) was observed at an estimated particle size of 514 μm ($r^2 = 0.804$), while the lowest feed conversion ratio (Figure 1c) occurred at 515 μm ($r^2 = 0.697$). The highest final total length (Figure 1d) was

Table II. Productive performance parameters of zebrafish fed semi-purified diet with different particle sizes after 49 days.

Parameter	159 µm	256 µm	362 µm	462 µm	550 µm	*p-value*
Initial weight (mg $fish^{-1}$)	86.7±4.0	85.0±2.0	87.0±3.5	86.0±1.0	86.7±2.5	0.91
Final weight (mg $fish^{-1}$)	149.7±4.2 ^{B}	186.3±40.1 ^{B}	267.7±39.4 ^{A}	283.7±19.6 ^{A}	275.3±22.0 ^{A}	<0.01
Weigh gain (mg $fish^{-1}$)	63.0±4.6 ^{B}	101.3±38.6 ^{B}	180.7±40.3 ^{A}	197.7±20.5 ^{A}	188.7±24.5 ^{A}	<0.01
Specific growth rate (% BW day^{-1})	1.1±0.1 ^{C}	1.6±0.4 ^{BC}	2.3±0.3 ^{AB}	2.4±0.2 ^{A}	2.4±0.2 ^{A}	<0.01
Initial total length (mm)	16.3±0.5	15.5±1.0	16.0±1.3	16.8±0.8	16.6±0.3	0.44
Final total length (mm)	20.4±0.5 ^{B}	21.6±0.7 ^{B}	24.4±1.1 ^{A}	25.1±0.4 ^{A}	24.2±0.2 ^{A}	<0.01
Total length gain (mm)	4.1±0.1 ^{B}	6.1±0.3 ^{A}	8.4±1.8 ^{A}	8.3±0.8 ^{A}	7.6±0.5 ^{A}	<0.01
Feed conversion rate (mg:mg)	4.6±1.3 ^{A}	3.8±0.9 ^{AB}	2.1±0.3 ^{B}	2.0±0.2 ^{B}	2.0±0.1 ^{B}	<0.01
Survival ratio (%)	93.3±6.5	80.0±20.0	95.7±7.5	93.0±0.0	95.3±4.0	0.36

Values are expressed as mean ± standard deviation (n=3). BW: body weight. Values in the same row with different superscripts are significantly different according to Tukey's honestly significant difference post-hoc test (P < 0.05).

observed at an estimated particle size of 481 µm (r^2 = 0.856), while the greatest total length gain (Figure 1e) occurred at 438 µm (r^2 = 0.798).

DISCUSSION

Particle size plays a critical role in zebrafish feeding behavior, influencing diet palatability, capture efficiency, and ingestion (Hasan & Macintosh 1992). Licitra et al. (2024) recommended dietary particle sizes of 200-400 µm for juveniles (50-305 mg body weight), consistent with protocols used by Martins et al. (2020) for zebrafish aged 30-60 days post-fertilization (dpf). However, the present results contradict this recommendation, as diets containing 256 µm particles significantly impaired growth performance. Growth improvement was observed only with particle sizes ≥362 µm, with maximal weight gain and optimal feed conversion ratio (FCR) at 514 µm and at 515 µm, respectively. Notably, the recommendation by Licitra et al. (2024) was based on a systematic review of studies using heterogeneous commercial diets, underscoring the need for empirical validation of feeding protocols to strengthen the utility zebrafish as a model organism (Hillman et al. 2024).

Ease of ingestion may not be the primary factor driving prey selection, larger particles may reduce predation-associated energy expenditure (Busti et al. 2022, Khadka & Rao 1986). Energy density correlates directly with pellet volume (Zarzar et al. 2022), and fish consuming smaller particles must exert greater foraging effort to achieve the same intake.

Feeding regime (*ad libitum* or restricted) also impacts particle availability. Excessive provisioning of small particles over short intervals can increase waste due to leaching, as physical capture and ingestion rates are limited within given timeframes (Tabachek 1988). Larger particles, while eliciting faster detection responses (Ramos-Júdez & Duncan 2022), may require prolonged handling, increase aqueous exposure time and promote nutrient leaching. In recirculating systems, uneaten feed is often lost due to water flow, leading to waste and environmental impacts (Linnér & Brännäs 1994, Hasan & Macintosh 1992).

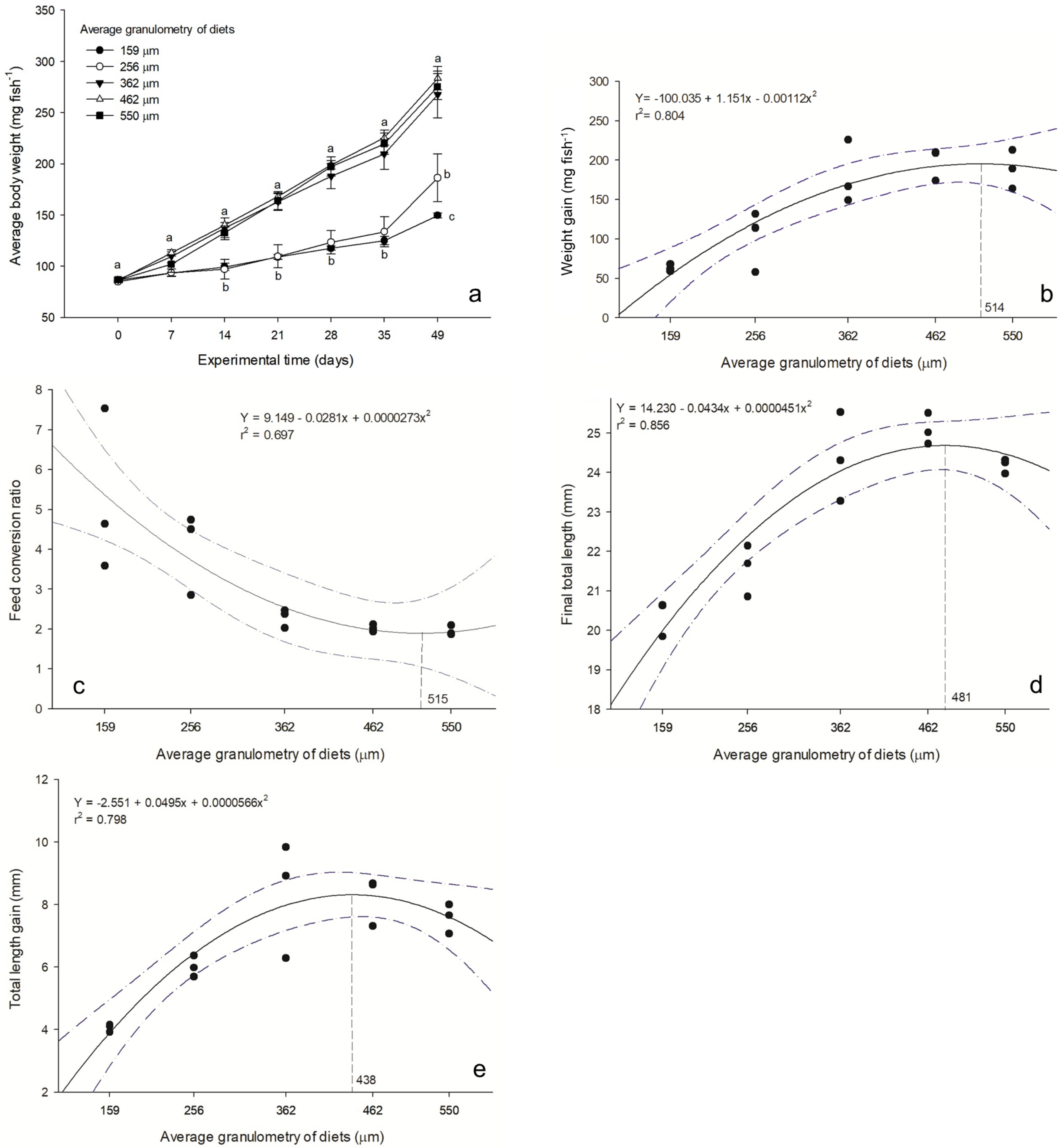

Figure 1. Growth performance and biometric parameters of juvenile zebrafish fed semi-purified diets with varying particle sizes. (a) Body weight evolution over the 49-day experimental period; different letters indicate significant differences between treatments at specific time points (Tukey's test, $P < 0.05$). (b) Weight gain, (c) Feed conversion ratio, (d) Final total length, and (e) Total length gain as a function of diet particle size. In panels b-e, solid lines represent the quadratic regression model, curved dashed lines indicate the 95% confidence intervals, and vertical drop lines highlight the optimal particle size estimated by the model. Values are presented as mean ± standard deviation (n=3).

Feed efficiency varies with particle size across fish species (Azaza et al. 2010, Hossain et al. 2000, Zarzar et al. 2022), possible due to effects on digestive transit. Smaller particles are evacuated from the digestive tract more rapidly, reducing nutrient absorption efficiency (Jobling 1987). However, species-specific adaptations may alter this pattern. For example, Busti et al. (2022) found that gastric evacuation contributes less to digestibility than intestinal processes in gilthead sea bream, which exhibit chewing behavior when fed 4-6 mm diameter pellets. In zebrafish, a stomach less species, particle size effects are confined to intestinal transit. Despite the larger surface area of smaller particles enhancing enzymatic access (Azaza et al. 2010), the accelerated passage through the intestine likely reduced FCR (Busti et al. 2022, Hossain et al. 2000), as observed in this study.

Growth response to dietary particle size results from a multifactorial interplay between evacuation time, net energy gain per particle, and individual particle availability. The slightly higher albeit non-significant, mortality ($p > 0.05$) observed in fish fed 256 µm particles likely reduced intraspecific competition (Mattila & Koskela 2018), enhanced feed access and uniform distribution (Zarzar et al. 2022). Zebrafish regulated intake effectively across a 1.5-fold particle size range (362-550 µm). Understanding the relationship between oral anatomy and particle size remains central to nutritional studies (Dabrowski et al. 1983, Dabrowski & Bardega 1984), as mouth gape is a key predictor of optimal prey or pellet dimensions.

As fish grow, net energy gain per unit handling time becomes the primary criterion for prey selection (Khadka & Rao 1986). Thus, fish length can be used to dynamically adjust particle size, since optimal size scale with body size (Hasan & Macintosh 1992). For example, a strong correlation ($r = 0.967$) exists between mouth gape and total length in common carp (*Cyprinus carpio*) by Dabrowski et al. (1983). Another advantage of using total length instead of measuring mouth gape is that, for many species, it can be performed without the need to sacrifice individuals, aligning with the basic principles of animal use in research (Canedo et al. 2022). In this study, particle sizes corresponding to 1.5-2.3% of total length supported optimal growth, peaking at 2.0-2.1%. These values are within the range found for common carp fry with values of 1.7-3.7% of total length reported Hasan & Macintosh (1992).

Despite its ease of measurement, total length does not adequately reflect zebrafish development at more advanced stages. The species exhibits rapid growth between 9 and 51 days post-fertilization (dpf), followed by a plateau in length at approximately 65 dpf (Gómez-Requeni et al. 2010). These authors report that, during this phase, muscle hyperplasia becomes minimal and redirects a significant portion of dietary energy toward lipogenesis and gonadal maturation, resulting in increased body weight without a proportional gain in length. In the present study, positive allometry was observed in zebrafish. This finding explains why the particle-size estimates required to maximize total length (481 µm) and length gain (438 µm) were slightly smaller than those associated with the greatest weight gain (514 µm). Hillman et al. (2024) corroborate our results and suggest that length-only assessments may underestimate true growth differences. Therefore, we recommend that zebrafish feeding protocols include a multidimensional growth analysis to ensure applicability across diverse scientific fields.

Fish growth is a key performance for diet evaluation in aquaculture. However, growth rate declines as fish grow, necessitating feeding practice adjustments for each zebrafish life stage (Licitra et al. 2024). To ensure proper feed intake, it is essential to consider the physical and chemical characteristics of the diet, including particle size, which should be tailored to the developmental stage of zebrafish (Watts et al. 2012). The ideal particle size is based on the measurement of mouth gape (Goolish et al. 1999) and varies according to life stage, impacting not only growth but also survival of larvae, juveniles, and adults. In zebrafish, the juvenile stage encompasses body weights ranging approximately from 50-70 mg to 350-500 mg (Licitra et al. 2024). In this context, juveniles fed semi-purified diets containing 35% crude protein with particle sizes greater than 362 μm exhibited specific growth rates of 2.3-2.4% body weight per day. Conversely, adult zebrafish with an initial body weight of 350 mg, fed higher-protein semi-purified diets (45% CP) under restricted feeding regimes (5% BW/day), showed lower specific growth rates ranging from 1.07 to 1.63% BW/day (Vural et al. 2021). These findings highlight that beyond nutritional composition and feeding regimen, optimizing feed particle size according to developmental stage is critical to maximizing feed intake and growth performance, particularly in juveniles, whose physiological development imposes limitations on ingesting larger particles (Lawrence 2007, Siccardi et al. 2009).

Semi-purified diets generally yield lower growth rates than those formulated with standard ingredients (e.g., fish meal, soybean meal). Juvenile zebrafish fed laboratory-formulated practical diets exhibit variable growth rates. For example, Carneiro et al. (2020) reported growth rates of 1.55-1.66% BW/day in juvenile zebrafish (initial BW = 160 mg) fed practical diets containing 33% crude protein (CP). Similarly, Silva et al. (2021) registered lower growth rates (0.46-1.15% BW/day) in juvenile zebrafish (initial BW = 290 mg) fed 51% CP practical diets compared to the current findings. Physical (e.g., size, shape), chemical (e.g., nutrient profile), and nutritional factors (e.g., energy density) modulate nutrient intake and assimilation (Watts & D'Abramo 2021), despite higher nutrient levels than those in the semi-purified diet used here. However, optimized feeding protocols combined with semi-purified diets of appropriate particle size achieved species-typical growth rates, highlighting the critical role of efficient dietary nutrient acquisition.

Therefore, documenting the particle size of diets administered to zebrafish is a critical consideration in experimental designs. Numerous studies document the manufacturing particle size, nutritional composition, and ingredient profiles of zebrafish diets (Carneiro et al. 2020, Silva et al. 2021, Vural et al. 2021). However, given equipment limitations in laboratory-scale production, reported pellet sizes often exceed the ingestion capacity of adult *D. rerio* (Licitra et al. 2024). Thus, while rarely methodologically detailed, pellets were likely fragmented to match species-specific ingestion capacity, as observed in prior work (Fernandes et al. 2016, Martins et al. 2020). However, grinding method, duration, force, diet texture, and pellet cohesion may yield particles outside the optimal size range for the species.

In summary, particle size in semi purified diets significantly influences growth and feed efficiency in juvenile zebrafish, highlighting the critical importance of standardizing this parameter in experimental protocols. Beyond the direct nutritional implications, these results underscore how this dietary characteristic may introduce uncontrolled inconsistencies in scientific outcomes. Thus, a systematic documentation of particle size specifications in zebrafish experiments is recommended. This practice would mitigate experimental variability in zebrafish feeding protocols, which critically influence growth and development (Watts et al. 2016, Watts & D'Abramo 2021), and enhance reproducibility across laboratories. This approach directly supports refinement principles in zebrafish husbandry (Canedo et al. 2022) by ensuring that fish receive optimized nutrition tailored to their physiological requirements, promoting welfare and enhancing scientific rigor. Implementing evidence-based feeding protocols that incorporate standardized granulometric specifications and nutritional composition advances zebrafish utilization as a biological model, strengthens its translational applicability, and minimizes methodological inconsistencies among research institutions (Watts et al. 2016, Watts & D'Abramo 2021).

CONCLUSIONS

In conclusion, the use of semi-purified diets with a particle size ranging from 362 to 550 µm is recommended for juvenile zebrafish (initial weight of ~85 mg). To maximize overall performance, the formulation should predominantly consist of particles near 515 µm.

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Data availability

The datasets generated and analyzed in this study are available from the corresponding author upon justified request.