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Effects of Dietary Protein and Lipid Ratios on Growth Performance, Nutrient Utilization, and Survival in Larval Fish Under Controlled Rearing Conditions

Michael Aina
Department of Biological Sciences, Florida Atlantic University, Boca Raton, Florida, USA

* Corresponding Author: **Michael Aina**

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Abstract
Optimal dietary protein and lipid proportions during early fish ontogeny are shaped by protein deposition, non-protein energy supply, digestive maturation, and the physiological consequences of nutrient excess. This paper develops a controlled-rearing factorial design and a reproducible computational experiment to examine joint dietary protein–lipid effects on specific growth rate, feed conversion ratio, protein efficiency, and larval survival. A 3 × 3 design spans 40%, 45%, and 50% protein with 8%, 12%, and 16% lipid, yielding 45 independent synthetic tanks (five per diet), each followed at days 7, 14, 21, and 28. Tank-held-out linear, ridge, random forest, and histogram gradient-boosting models were compared for specific-growth-rate estimation. In the artificial experiment, the 50% protein/16% lipid treatment yielded the largest mean day-28 specific growth rate (3.965% day−1) and survival (94.08%), while the 50%/12% combination produced the lowest feed conversion ratio (1.340). Random forest achieved the smallest held-out RMSE (0.170), but these differences reflect programmed response functions rather than actual larval physiology. A new conceptual figure links diet composition, larval digestive and energetic processes, water-quality controls, and measured outcomes. The study provides a transparent computational template for designing species-specific protein–lipid feeding trials, with explicit attention to tank-level replication, uncertainty, competing nutritional endpoints, and biological validation.

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Keywords: Fish Larvae; Dietary Protein; Dietary Lipid; Protein-To-Lipid Ratio; Specific Growth Rate; Feed Conversion; Survival; Factorial Design; Response Surface; Aquaculture Nutrition

1. Introduction

1.1. Larval nutrition and the protein–energy balance

Early fish larval development couples high rates of tissue synthesis with limited digestive capacity and rapidly changing nutrient requirements. Protein contributes substrates for growth and enzyme synthesis, whereas lipid supplies metabolizable energy, essential fatty acids, and membrane constituents. Adequate dietary lipid may spare amino acids from oxidation, but excessive lipid can depress feed intake or alter deposition; likewise, insufficient protein may constrain tissue growth even when non-protein energy is plentiful (Hamre *et al*., 2013; NRC, 2011; Tocher, 2010) [16, 32, 44]. A protein-to-lipid ratio should therefore be treated as a convenient summary of two independently manipulable nutrient factors rather than a physiologically sufficient requirement.

1.2. Biological significance of dietary protein

Larval feeding requirements are distinguished from juvenile and adult requirements by intense developmental change, high protein turnover, digestive immaturity, and often incomplete acceptance of formulated microdiets. Species-specific amino-acid needs determine whether a diet with seemingly high crude protein supports growth. Studies of young fish have shown that

increasing crude protein is not invariably beneficial: beyond the anabolic optimum, metabolic costs, nitrogen excretion, and ingredient limitations may offset greater supply. Controlled comparisons should therefore maintain amino-acid balance and identify the protein source instead of attributing all outcome variation to nominal protein percentage.

1.3. Lipid supply, phospholipids, and larval development

Early fish larval development couples high rates of tissue synthesis with limited digestive capacity and rapidly changing nutrient requirements. Protein contributes substrates for growth and enzyme synthesis, whereas lipid supplies metabolizable energy, essential fatty acids, and membrane constituents. Adequate dietary lipid may spare amino acids from oxidation, but excessive lipid can depress feed intake or alter deposition; likewise, insufficient protein may constrain tissue growth even when non-protein energy is plentiful (Hamre *et al*., 2013; NRC, 2011; Tocher, 2010) [16, 32, 44]. A protein-to-lipid ratio should therefore be treated as a convenient summary of two independently manipulable nutrient factors rather than a physiologically sufficient requirement.

1.4. Statement of the research problem

Published studies often examine protein or lipid gradients separately, whereas an interaction between these macronutrients could shift the diet associated with the best growth, survival, and feed conversion. Further, repeated sampling of larvae in shared tanks creates clustered observations. Treating each time point as an independent replicate inflates inferential precision. A stronger research design therefore distinguishes tank-level experimental units, repeated growth measurements, and distinct endpoints with potentially incompatible optima.

1.5. Objectives and hypotheses

The principal objective is to construct a technically transparent framework for analysing how dietary protein, lipid, and their interaction relate to larval growth, feed utilization, and survival under controlled rearing conditions. Specific objectives are to evaluate a 3 × 3 factorial design; compare day-28 outcomes across diets; fit interpretable response surfaces; compare predictive estimators on withheld tanks; and define an empirical validation protocol. H1 concerns a positive but nonlinear protein response; H2 concerns a lipid-related intermediate optimum for feed conversion; and H3 concerns a protein × lipid interaction. The synthetic model tests computational coherence, not biological support for these hypotheses.

1.6. Scope of inference

The design targets a hypothetical larval fish rather than a named species, preventing unjustified transfer of optimal inclusion levels. Simulated environmental variables vary only slightly around assumed targets. All effects are set through artificial generating equations. Accordingly, recommended treatment combinations and fitted predictive associations cannot be used as feed recommendations without replicated feeding trials using identified larvae, measured diet composition, and species-specific nutritional constraints.

2. Literature Review

2.1. Frameworks for larval fish nutritional requirements

Hamre *et al*. (2013) [16] review the bottlenecks that complicate larval feeding, including nutrient delivery, lipid classes, digestive development, and microdiet physical properties. The National Research Council (2011) [32] provides a foundational nutrient-requirement reference while noting important interspecies variation. Early life stages cannot be treated as simply scaled-down juveniles, because digestive processing, endogenous reserves, mouth gape, and feed particle residence time change rapidly.

2.2. Protein requirements, amino-acid adequacy, and nitrogen utilization

Protein adequacy concerns indispensable amino-acid supply and digestible nitrogen rather than crude protein alone. Work on amino-acid requirements and protein metabolism emphasizes that surplus amino acids can be oxidized when non-protein energy, amino-acid balance, or feed intake are suboptimal. Francis *et al*. (2001) [13] and Gatlin *et al*. (2007) [14] highlight the implications of ingredient quality and antinutritional constituents. A suitable experiment should hold protein source composition as constant as practicable across treatments while documenting changes required to achieve target protein levels.

2.3. Lipid effects and protein sparing

Lipid provides energy and essential fatty acids, but requirements vary with species, ontogeny, and lipid class. Morais *et al*. (2005) [30] experimentally examined protein:lipid ratio and lipid nature in teleost larvae and found important metabolic implications. Hamre *et al*. (2013) [16] identify phospholipid supply as especially relevant during early development. For this reason, a nominal 12% or 16% lipid diet cannot be considered equivalent across studies unless fatty-acid composition and energy density are also reported.

2.4. Direct experimental evidence on protein and lipid gradients

A factorial analysis of three-spot cichlid larvae used four protein and two lipid levels and reported favorable outcomes at 45% protein and 22% lipid under that study's conditions (Álvarez-González *et al*., 2019; Idowu, *et al*., 2025) [1, 20]. Work on rohu larvae found an advantageous 9% lipid treatment among four diets formulated at 50% protein, illustrating species- and protocol-specific effects (Optimization of inclusion level of lipid in larval diet of Labeo rohita, 2024) [36]. A 2024 co-feeding investigation of hilsa larvae observed differences among protein treatments, emphasizing the role of plankton provision and early-life feeding context (Chattopadhyay *et al*., 2024) [7]. These studies motivate experimentation rather than extrapolation.

2.5. Controlled rearing conditions as confounders and effect modifiers

Water temperature alters metabolic demand and gastric emptying, whereas dissolved oxygen constrains aerobic metabolism and can depress feed intake. Stocking density, feeding frequency, light regime, tank hydrodynamics, and pathogen burden may influence measured survival

independent of diet. Any inference that protein-to-lipid ratio caused altered growth requires random assignment of diets to tanks, reproducible water-quality management, and independent monitoring of survival and feed utilization.

2.6. Growth and nutrient-use metrics

Specific growth rate (SGR) measures log-scale growth per day and is useful when initial sizes are comparable. Feed conversion ratio (FCR) expresses dry feed consumed per wet weight gained, though larval feed losses and live-feed co-feeding can make estimates unreliable. Protein efficiency ratio (PER) is normally weight gain divided by protein intake, not a percentage (James, *et al*., 2025) [23]. Protein retention and apparent digestibility require additional measurements or indirect markers. Survival requires explicit accounting of mortality, sample removals, and censoring during the study.

2.7. Statistical methods for factorial rearing trials

Two-way factorial models can evaluate main effects and interaction, but a significant interaction requires simple-effects interpretation rather than isolated main effects. When individuals share a tank, the tank not the individual fish is the randomized experimental unit. Repeated observations should be modelled with tank random intercepts or other correlated-error structures. Response-surface terms may identify nonlinear optima but should not be extrapolated beyond the tested treatment domain. Bootstrap intervals should resample tanks rather than individual records.

2.8. Predictive algorithms for nutritional response modelling

Linear and ridge regressions supply transparent baselines, while tree ensembles approximate nonlinear interactions without assuming a fixed parametric response. Random forests reduce variance through aggregation; gradient-boosted trees combine sequential learners. Prediction performance must be assessed using tanks absent from training, avoiding information leakage from repeated measures. A large R^2 in a simulation may reflect the encoded generating equation, not a model capable of generalizing across species or hatcheries.

2.9. Nutritional interpretation and research gap

A single protein-to-lipid mass ratio maps multiple pairs of actual percentages to the same number and therefore loses information about absolute inclusion. Protein and lipid should enter the statistical model independently with an interaction term; the ratio can be shown descriptively. Prior work supports factorial nutrition studies but leaves open the need for reproducible multivariate workflows that compare growth, utilization, and survival jointly while retaining tank-level experimental independence.

2.10. Conceptual framework

Figure 1 integrates the proposed pathway from factorial diet construction through controlled rearing, biological measurements, and inferential evaluation. It explicitly separates manipulated diet composition from monitoring variables and measured endpoints. Its feedback arrow acknowledges that final formulation choices require species-specific feeding validation, water-quality audit, and iterative refinement rather than automatic selection from a simulated score.

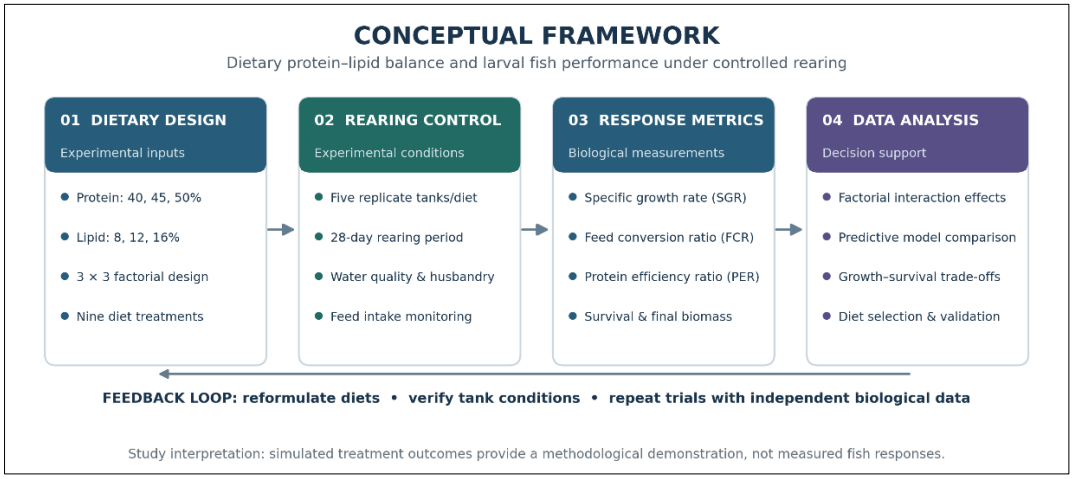


Fig 1: Factorial dietary composition, controlled larval rearing, biological measurement, and statistical inference.

3. Materials and Methods

3.1. Design and study setting

A 3 × 3 factorial design crossed three crude-protein levels (40%, 45%, 50% of dry feed) with three total-lipid levels (8%, 12%, 16%). Five independently simulated tanks per treatment provided 45 experimental units. Each tank contributed measures at days 7, 14, 21, and 28 (180 observations). Dietary treatments and tank values were generated by a fixed seed (202508). No specific species, hatchery, sampling season, or geographical location was represented.

3.2. Diet characterization and manipulation

The design assumes a common ingredient matrix and consistent vitamin, mineral, and essential fatty-acid sufficiency; however, no experimental feed was manufactured or chemically analyzed. A physical implementation would produce nine experimental diets, verify crude protein and lipid by recognized proximate methods, assay energy and amino-acid composition, and document microdiet particle stability. Diet percentages were treated as independent design factors, while P:L ratio was calculated for illustration.

3.3. Rearing environment and randomization

The simulation uses temperature centered on 27 °C, dissolved oxygen on 6.5 mg L−1, and pH on 7.4 with small tank-level deviations. A real experiment should randomize tank positions and feeding assignments, establish initial size and developmental stage, monitor ammonia and salinity where applicable, and document any mortalities or removals. Five tanks per diet provide replication at the unit of intervention, not five independently measured animals.

3.4. Primary and secondary endpoints

Primary outcomes are day-28 specific growth rate and survival; secondary outcomes include FCR, a simulated protein-use proxy, and longitudinal body mass. Because the simulated protein-use variable is a constructed percentage-like index rather than true PER, it must not be interpreted as an experimentally measured protein efficiency ratio. Empirical work should compute PER from weight gain divided by protein intake and include digestibility coefficients where feasible.

3.5. Synthetic response process

The generator encodes a nonlinear response to protein and lipid, an interaction term, tank-specific noise, and small environmental deviations. Day-28 SGR is expressed through a quadratic response centered on 45% protein and 12% lipid; survival is mapped through a logistic function of the same diet factors. Coefficients are selected to produce an informative statistical benchmark rather than fitted to published feeding outcomes. This approach allows accurate reproduction but inevitably favors nonlinear estimators when the encoded relationship is nonlinear.

3.6. Statistical estimation and comparison

A factorial regression with protein, lipid, squared terms, and an interaction represents the specified response surface. For predictive benchmarking, linear regression, ridge regression, random forest, and histogram gradient boosting are fitted on the same features and evaluated on a group-held-out subset of tanks. RMSE, MAE, and R^2 are recorded, with errors interpreted on the simulated SGR scale. A real analysis should fit a mixed-effects structure or cluster-robust estimator and report uncertainty intervals over tank replicates.

3.7. Reproducibility and analytical safeguards

The complete Python script, tank-level records, factorial summary, and model results are released as supplemental files. The script avoids splitting repeated observations from one tank between train and test sets. No feed conversion, survivorship, or growth metric was adjusted after comparison. All generated figures are derived from the released CSV values and can be recreated with standard scientific Python libraries.

3.8. Native Microsoft Word mathematical formulation

The following numbered equations define the principal quantities. The equations are stored as editable Word mathematical objects (OMML); the experiment script specifies the complete synthetic generator and estimator settings.

$$RPL = \frac{P}{L}$$

P and L denote dietary crude protein and lipid percentages on the same dry-matter basis.

$$SGR = \frac{100[ln(Wf) - ln(Wi)]}{t}$$

Wi and Wf denote initial and final mean larval weights, and t is the interval in days.

$$FCR = \frac{Dry\ feed\ consumed}{Wet\ weight\ gain}$$

The true FCR requires measured feed intake, accounting for uneaten feed and losses.

$$PER = \frac{Wet\ weight\ gain}{Protein\ intake}$$

Protein efficiency ratio is dimensionless and is not equivalent to the simulated percentage-like proxy.

$$S = \frac{100\ Nfinal}{Ninitial}$$

This simplified survival expression requires adjustment if larvae are deliberately removed for sampling.

$$g(P, L) = \beta 0 + \beta 1\ P + \beta 2\ L + \beta 3\ P^2 + \beta 4\ L^2 + \beta 5\ PL + \varepsilon$$

The cross-product PL identifies interaction beyond the separate main effects.

$$RMSE = \sqrt{[(1/n)\ \Sigma_i\ (\hat{y}_i - y_i)^2]}$$

The intended RMSE is the square root of the mean squared error; software evaluation uses its exact implementation.

4. Results

All reported results in Section 4 are generated by the fixed-seed simulation. They should not be described as measured responses of live fish. Day-28 treatment means are based on five simulated tanks per diet; test accuracy is assessed on tanks excluded from training.

Table 1: Simulated day-28 factorial diet responses (n = 5 tanks per treatment).

Protein/lipid (%)	SGR (%/day)	FCR	Protein-use proxy	Survival (%)
40/8	0.724	1.980	2.29	80.14
40/12	1.689	1.675	9.08	87.15
40/16	1.196	1.724	10.98	87.00
45/8	2.107	1.681	12.64	87.30
45/12	3.438	1.482	20.43	92.16
45/16	3.437	1.441	20.66	93.01
50/8	2.072	1.640	15.70	87.77
50/12	3.562	1.340	23.03	93.45
50/16	3.965	1.504	22.81	94.08

4.1. Specific growth-rate response

The synthetic SGR varies nonlinearly over the factorial grid. The 50% protein/16% lipid combination produces the highest mean value, 3.965% per day, compared with 0.724% per day at 40%/8%. The apparent advantage follows the programmed response function and cannot be generalized. Figure 2 displays all nine cells and makes the interaction visible. An

empirical conclusion about a true optimum would require confidence intervals, response-surface estimation, and independent replicate trials.

4.2. Feed conversion across treatment combinations

Simulated FCR is lowest at 50% protein/12% lipid (1.340) and is highest at 40%/8% (1.980). Because lower FCR is preferable, the growth-maximizing combination is not also the FCR-minimizing combination. This divergence highlights the danger of optimizing a single performance measure. Feed conversion in real larval work would also be sensitive to feed losses, microdiet sinking, and the use of live prey.

4.3. Survival response and nutritional risk

Survival in the simulated day-28 tanks ranges from approximately 80.14% at 40%/8% to 94.08% at 50%/16%. The response is smooth because mortality probabilities were generated from a logistic diet-response rule. In a live larval experiment, tank-level survival would be a binomial or beta-binomial outcome rather than an exactly continuous percentage, and losses unrelated to dietary treatment would need to be recorded.

4.4. Growth dynamics throughout the rearing period

The repeated day-7, day-14, day-21, and day-28 weight trajectories show diverging patterns as diet-dependent growth accumulates. Figure 5 therefore conveys a biologically interpretable consequence of even modest differences in daily SGR. These are generated longitudinal profiles rather than individual larval measurements. A real analysis would treat repeated observations within each tank as dependent and should not equate technical subsampling with independent replication.

4.5. Comparative predictive performance

Four estimators were evaluated under a tank-held-out design. Random forest achieved RMSE 0.170 and R^2 0.971; ridge regression achieved RMSE 0.395 and R^2 0.842. Histogram boosting and ordinary linear regression were intermediate. The result is expected because the programmed diet response contains quadratic and interaction terms poorly captured by a purely linear predictor. The performance numbers demonstrate algorithmic fit to the artificial generator, not universal superiority of random forests for larval growth.

4.6. Protein-to-lipid ratio and multidimensional decision making

A scatter display relating ratio to survival clarifies that absolute protein and lipid concentrations are not recoverable from their ratio. For instance, ratios derived from different nutrient percentages can yield different survival probabilities in the generator. The final graphic relates the artificial protein-use proxy to survival, illustrating how biological objectives may trade off. Selecting a treatment for commercial use would require nutrient retention, deformity rates, whole-body composition, and economic outcomes in addition to growth and survival.

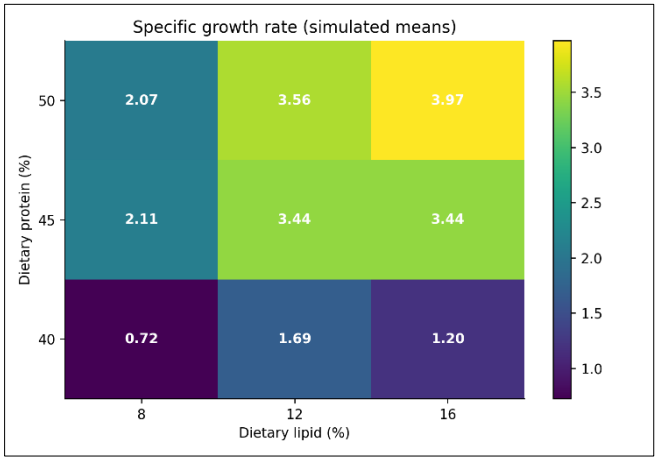


Fig 2: Synthetic SGR response across protein and lipid treatment combinations.

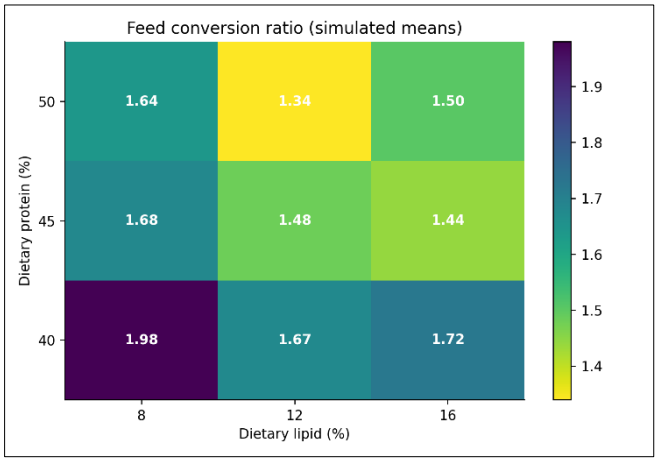


Fig 3: Synthetic FCR response (lower values indicate better conversion).

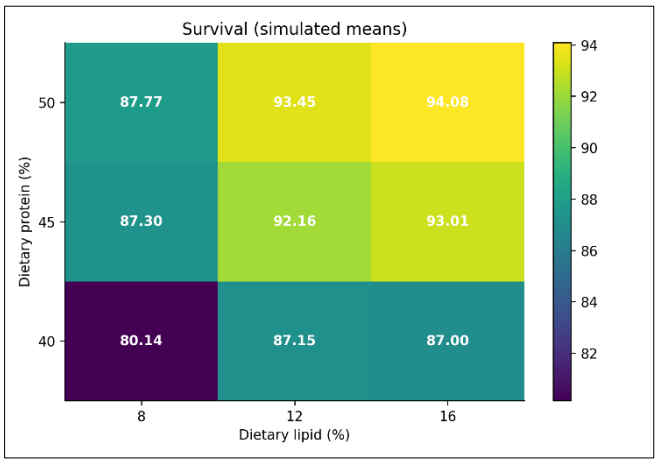


Fig 4: Day-28 simulated survival over the nine-cell factorial design.

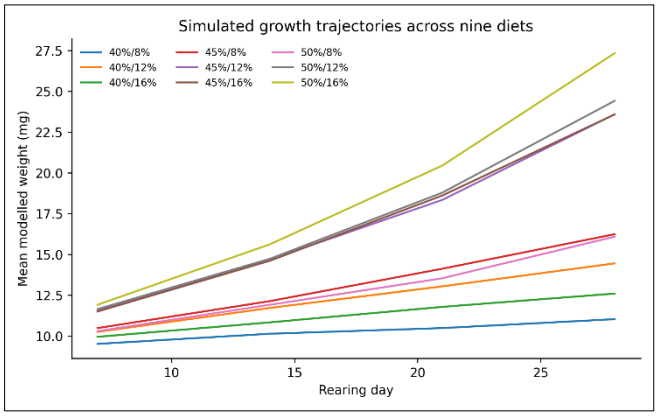


Fig 5: Modelled growth trajectories for the nine dietary treatments.

Table 2: Tank-held-out prediction of simulated SGR.

Model	RMSE	MAE	R^2
Linear	0.427	0.376	0.815
Ridge	0.395	0.347	0.842
Random forest	0.170	0.144	0.971
Hist gradient boost	0.296	0.260	0.911

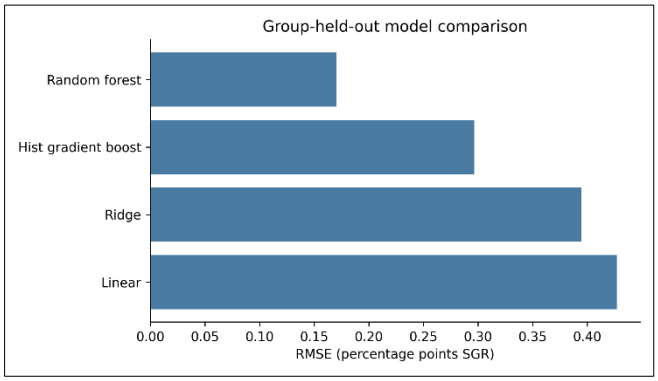


Fig 6: Held-out RMSE comparison across predictive estimators.

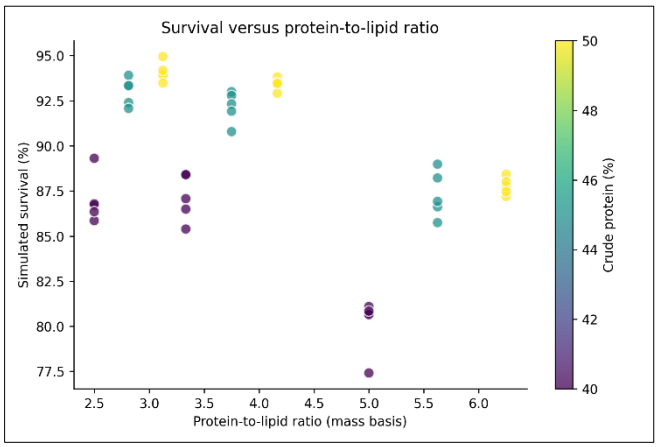


Fig 7: Simulated survival versus dietary protein-to-lipid mass ratio.

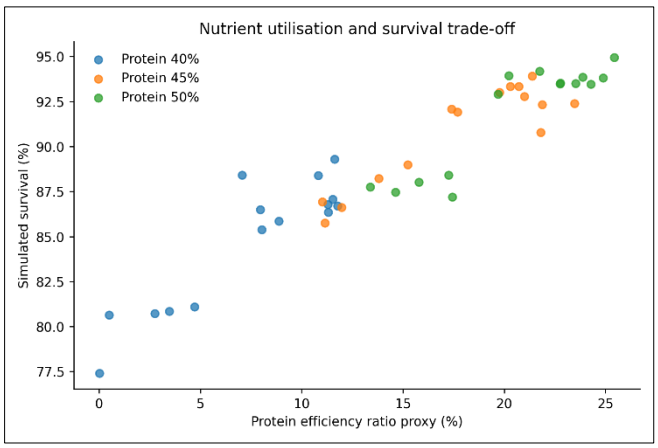


Fig 8: Joint behaviour of the simulated protein-use proxy and survival.

5. Discussion

5.1. Interpretation in relation to larval fish physiology

The simulation illustrates a recognizable nutritional logic: dietary protein can become limiting at low inclusion, while lipid may reduce amino-acid oxidation and support energetically demanding growth. Nevertheless, lipid supplies more than bulk energy, and essential fatty acids and phospholipids cannot be replaced by any lipid source. Morais *et al*. (2005) [30] demonstrated that protein:lipid ratio and lipid nature jointly influence larval fatty-acid absorption and metabolism. This distinction is essential when translating percentage gradients into species-specific feed design.

5.2. Interpretation of growth versus utilization endpoints

A treatment can maximize SGR without minimizing FCR or maximizing protein retention. In this constructed experiment, 50%/16% produces the greatest growth and survival, while 50%/12% produces the lowest FCR. A real feeding trial should prespecify whether the primary outcome is growth, survival, feed economy, or a joint objective; otherwise, researchers may selectively emphasize a favorable measure. Nutritional interpretation should also separate true PER from protein-retention efficiency and from any simulated proxy.

5.3. Why species and developmental stage matter

The diet combinations in this benchmark resemble plausible experimental gradients but have no demonstrated optimality for any particular larval species. Available feeding studies vary widely in species, age, protein sources, live-feed supplementation, lipid class, and rearing temperature. For example, findings reported for cichlid larvae, rohu larvae, and hilsa larvae differ, which underscores the difficulty of pooling nominal protein and lipid inclusion levels without modeling contextual variables (Álvarez-González *et al*., 2019; Chattopadhyay *et al*., 2024) [1, 7].

5.4. Strengths and weaknesses of predictive comparisons

The predictive analysis respects tank-level grouping and provides transparent baselines. However, it evaluates a single nonlinear data-generating mechanism with no genuine measurement drift, unseen species, or rearing-system shift. The strong random-forest performance is therefore an internal consistency result. Publication-quality validation would repeat the experiment across independent hatchery cohorts, compare a prespecified quadratic mixed model, examine residual patterns, and provide bootstrap uncertainty intervals.

5.5. Controlled-rearing validity and ethics

Real experiments must record animal husbandry, welfare endpoints, and applicable ethics approvals. Random assignment is necessary to distinguish nutritional effects from tank position, initial body size, or water-quality differences. Growth sampling should avoid damage and stress; mortality observations must distinguish background losses from disease or handling events. Under such a protocol, the tank remains the appropriate unit for dietary treatment comparisons, and repeated observations require correlated-data methods.

5.6. Limitations and implications

The present evidence is synthetic and deliberately constrained. The generator excludes disease outbreaks, feeding interruptions, nutrient leaching, phospholipid class, fatty-acid profiles, digestive enzyme maturation, and real mortality events. It also uses a protein-use index that is not measured PER. Thus, none of the numeric outcomes proves a causal dietary effect. The contribution is instead a reproducible research design and an analysis framework that can be adapted after authenticated larval feeding measurements become available.

5.7. Recommendations for empirical replication

The next study should use a clearly identified larval species, defined post-hatch age, analytically verified diets, a balanced 3 × 3 factorial allocation, at least five independently randomized tanks per treatment where power analysis permits, and daily water-quality logs. Essential fatty acid adequacy and ingredient digestibility should be checked. SGR, measured FCR, PER, protein retention, deformities, and survival should be collected under a preregistered primary endpoint. A mixed model should evaluate diet factors, while binomial mortality models should be fitted at the tank level.

6. Conclusion

An integrated analysis of protein and lipid levels provides a defensible framework for examining growth, utilization, and survival in larval fish. In the reported artificial benchmark, outcomes differed across the nine treatment combinations and revealed an apparent divergence between the diet associated with maximum growth and the diet associated with minimum FCR. Nonlinear prediction also outperformed a linear baseline. These findings describe only the specified simulation; they do not establish a dietary requirement for any live fish species. A biological experiment using controlled, analytically verified diets and true tank-level replication remains necessary before feeding recommendations or causal conclusions can be made.

Appendix A. Data dictionary and reproducibility

The released file synthetic_tank_observations.csv contains dietary protein and lipid, calculated ratio, tank ID, sampling day, simulated temperature, dissolved oxygen, pH, growth, FCR, a protein-use proxy, survival, and individual body weight. The experiment.py script regenerates all outputs with NumPy seed 202508 and uses GroupShuffleSplit to hold entire tanks out of predictive training. The file factorial_summary.csv contains day-28 means across the five tanks per diet; model_comparison.csv records held-out prediction scores. Numerical results are artificial and should not be cited as measured hatchery results.

Appendix B. Field validation and publication checklist

Before actual larval feeding experiments, investigators should complete a power analysis on tank-level outcomes; obtain animal ethics approvals where applicable; characterize experimental feeds by proximate analysis and fatty-acid assays; control initial larval age and weight; randomize tanks; record temperature, dissolved oxygen, pH, and nitrogenous wastes; weigh feed inputs and losses; establish sampling and mortality procedures; and preregister primary outcomes. External validation should compare at least two rearing cohorts, use mixed-effects models, cluster bootstrap confidence intervals, and report uncertainty around optimal diet levels.

Appendix C. Statistical analysis plan for real larval feeding trials

A prespecified statistical analysis plan should begin with the primary unit of randomization. Diets are applied to tanks and not independently to every sampled larva. The analysis dataset should therefore include an unambiguous tank identifier, a diet identifier, baseline larval size, dates of sampling, total numbers initially stocked, and reasons for removal or mortality. The preplanned principal comparison should estimate adjusted mean SGR differences across the nine treatment combinations, with time included when measurements are repeated. Fixed dietary terms should contain protein percentage, lipid percentage, and their interaction. If curvature is plausible, centered quadratic terms should be included to reduce collinearity. The mixed-model error specification must acknowledge shared tank environments and within-tank longitudinal correlations.

With five tanks per diet, statistical power is sensitive to both the residual variance and the magnitude of diet-by-diet interactions. Investigators should avoid selecting a complex model merely because it has the highest apparent fit. Repeated-sample measurements that are destructive must be documented as such, whereas non-destructive sampling can be analyzed with a repeated-measurement covariance structure. Normality assumptions should be evaluated for tank-level continuous residuals, not individual observations treated as independent. If the data are strongly skewed, transformations, generalized mixed models, or robust alternatives may be necessary. For mortality counts, fit a binomial mixed model with initial stocking or at-risk count as the denominator and tank as the clustered unit.

Treatment contrasts should reflect clearly articulated biological hypotheses, such as whether increasing lipid from 8% to 12% changes outcomes at each level of protein, or whether added protein contributes more when lipid supply is sufficient. A joint Wald or likelihood-ratio test may assess interactions, but specific contrasts should still be reported with 95% confidence intervals and relevant units. Multiplicity correction should be selected before examining the data when multiple post-hoc comparisons are expected. The reporting table should distinguish raw treatment means from model-adjusted estimates. Response-surface optima should only be calculated within the tested domain and should include profile or bootstrap uncertainty intervals.

Because survival, FCR, and SGR are not interchangeable, inference for one outcome should not be used to establish claims about another. For example, an apparently favorable SGR treatment could show no improvement in feed conversion or could incur excess deformities. A weighted composite endpoint could support practical diet selection, but weights must be justified using biological and economic priorities, and sensitivity analyses should show whether changing those weights changes the recommended diet. Where survival loss causes missing subsequent growth data, the missingness mechanism should be investigated rather than assuming missing values are random.

Appendix D. Reporting standards and nutrient quality assurance

A future experiment should report the fish species, strain where relevant, age in days post-hatch, baseline wet weight

or length, larval density, tank volume, water source, photoperiod, temperature, dissolved oxygen, pH, salinity, total ammonia nitrogen, and handling regime. Each dietary treatment should have a unique code and be linked to complete formulation tables showing protein sources, lipid sources, mineral premix, vitamin premix, binder, and processing method. The physical properties of microdiets—including particle size, buoyancy, water stability, and leaching—should be measured because these influences the amount of dietary nutrient actually ingested.

Chemical analyses should establish crude protein on an appropriate nitrogen-to-protein conversion basis, total lipid, ash, moisture, energy, and fatty-acid composition. Ideally, indispensable amino-acid concentrations should also be verified. If dietary protein is increased by changing ingredient proportions, the design must acknowledge that ingredients carry non-protein components and bioactive compounds that may confound the response. To isolate energy sparing, researchers should consider dietary digestible energy rather than lipid percentage alone and should describe whether diets are intended to be isoenergetic or differ intentionally in energy density. For larval fish, phospholipid classes and n-3 long-chain polyunsaturated fatty acids may be especially relevant.

Feed delivery should be documented daily as dry mass offered, timing of meals, live feed co-feeding if any, and estimates of uneaten particles. Researchers should explain how FCR is measured in larvae when lost microfeed is difficult to recover. If a direct measurement cannot be supported, the reported indicator should not be mislabeled as observed feed conversion ratio. Similarly, a true protein efficiency ratio requires both wet biomass gain and protein intake, while apparent digestibility requires a validated indicator or collection protocol. A detailed data dictionary can prevent confusion among these endpoints and improve reproducibility across hatcheries.

Data transparency should include raw tank-level outcomes, not only means and standard deviations. Practical quality controls include replicate chemical assays, instrument blank checks, certified or secondary reference materials, documented calibration intervals, and storage-condition logs. Unexpected mortalities or interruptions should be reported whether or not they support the hypothesized optimum. Analytical scripts should regenerate each figure from the raw dataset, and the manuscript should disclose software versions, random seeds, and model-tuning procedures. These safeguards are particularly important in studies with relatively few independent tanks per diet.

Appendix E. Interpretation limits for protein-to-lipid ratios

Protein-to-lipid ratios are mathematically compact but can be misleading if they are treated as independent biological treatments. A diet with 40% protein and 8% lipid has a mass ratio of 5, whereas a diet with 50% protein and 10% lipid would also have ratio 5, despite substantially different absolute nutrient and energy supply. Because the present design varies protein and lipid independently, the preferred inferential representation includes both percentages and an interaction term. Ratio-based regression alone discards information and creates a risk of denominator-driven artifacts.

For feed formulation, the protein-to-energy ratio can sometimes be more biologically informative than a mass protein-to-lipid ratio, but only when energy values and digestibility are estimated appropriately. Gross-energy calculations do not account for species-specific differences in nutrient availability. An increase in lipid percentage may alter total digestible energy, fatty-acid balance, pellet characteristics, and voluntary feed consumption. Researchers should therefore avoid describing all lipid effects as protein sparing unless protein oxidation or nitrogen retention has been directly assessed.

The illustrative optimal treatments in this simulation differ across growth, conversion, and survival. Under actual hatchery conditions, a protein level that increases growth could increase feed costs or nitrogen discharge. A lipid level that reduces FCR could increase carcass lipid deposition. A sustainability-focused evaluation should consider larval health and welfare, production efficiency, ingredient availability, feed price, nitrogenous emissions, and downstream juvenile performance. These broader outcomes can be linked to a multi-objective optimization study after genuine empirical response functions are fitted.

Appendix F. Pre-registered validation questions and expected deliverables

An empirical study can be organized around a limited set of preregistered questions. First, is the protein × lipid interaction detectable for specific growth rate after accounting for tank-level clustering? Second, do lipid gradients modify feed conversion in a manner compatible with protein sparing, or are observed differences better explained by altered feed intake? Third, are the apparent growth-optimal and survival-optimal treatments consistent with acceptable deformity rates and larval stress? Fourth, do diet effects persist after adjustment for baseline size and modest variation in temperature and dissolved oxygen? Fifth, can the response surface predict a separate rearing cohort better than a simple mean-treatment benchmark? These questions translate the simulation into testable biological hypotheses without presuming their answers.

Each planned result should have a matching evidence product: a verified diet-composition table; a diagram of the randomized tank assignment; a water-quality summary; a tank-level response table; a longitudinal growth figure with uncertainty bars; a factorial interaction plot; mortality counts and survival confidence intervals; and a summary of nutrient-use endpoints with measurement definitions. The primary model should be presented before exploratory machine-learning models. Where performance is assessed on a second hatchery or cohort, all preprocessing should be trained on the development data only, and the external set should not be used for tuning.

The article should also report plausible alternative explanations. For example, a lipid-rich feed may have changed water stability, nutrient leaching, or ingestion rate independently of dietary energy supply. A higher-protein formulation may contain more fishmeal or different levels of palatability enhancers. Tank-to-tank differences in microbial water quality may alter survival, and handling stress during sampling may affect small larvae disproportionately. Such alternatives should be investigated analytically and through recorded husbandry observations before claiming a specific physiological mechanism.

Finally, the decision criterion for recommending a feed must be explicit. One hatchery may prioritize survival and robust juvenile quality, whereas another may prioritize cost per

surviving fish, time to a target size, or protein retention. A transparent choice should report the consequences of alternative outcome weights and should reject diets that violate animal welfare or nutritional constraints regardless of their predicted composite score. The simulation can support software testing and study planning, but it cannot substitute for controlled feeding evidence or establish a species-specific requirement.

Appendix G. Reproducibility, uncertainty, and sensitivity protocol

Independent reproduction requires a clean execution environment and clear separation between generated observations and fitted results. The accompanying Python script should be treated as the authoritative source for the artificial response process. It initializes a NumPy random generator, constructs a balanced grid of dietary treatments, assigns identifiers to five tanks per treatment, and generates four repeated observations per tank. The analysis then creates a day-28 aggregate and partitions observations by tank identifier so that no records from a held-out tank enter model fitting. The same split is used for each predictive estimator. This workflow is preferable to random record-level partitioning, which would allow highly similar repeated observations from a single tank to appear in both data partitions and exaggerate out-of-sample performance.

Repeated simulation should vary the random seed while preserving the prespecified diet grid, measurement schedule, and synthetic response equations. At least 100 independent replicates would permit estimation of the Monte Carlo distribution of treatment differences, predictive model errors, and the fraction of replicates selecting each diet as optimal under competing objectives. Means and 95% Monte Carlo intervals could then be reported in a supplementary table. Such intervals quantify uncertainty induced by the simulator only; they should not be interpreted as biological confidence intervals for real larvae. Uncertainty about the form of the generating mechanism would require altering the assumed response coefficients and perhaps adding non-monotonic temperature interactions, feed rejection, and mortality clustering.

For transparent sensitivity analysis, investigators should independently perturb the quadratic lipid coefficient, the protein × lipid interaction coefficient, tank-level random effect variance, and the baseline mortality log odds. The resulting changes in the highest-ranked diet and the relative predictive performance of models can identify conclusions that rely heavily on arbitrary assumptions. Further, comparisons should include a quadratic regression with the correct interaction terms: its omission would otherwise create an unfair advantage for nonlinear ensemble methods in a generator known to contain curvature. Model rankings should be accompanied by computational cost and interpretability criteria, since a small statistical improvement may be insufficient to justify maintenance complexity in hatchery decision support.

A formal reporting checklist should distinguish measured values, calculated indices, simulated quantities, and projected decisions in the table captions and prose. For example, protein and lipid percentages in the current benchmark are nominal treatment inputs; survival percentages are generated from a logistic function; the protein-use variable is a constructed proxy; and final weight values are derived from the programmed growth relationship. Nothing in the current data provides actual feed consumption, protein retention, endocrine status, nutrient digestibility, or deformity frequency. These exclusions should remain explicit in the abstract, Methods, Results, Discussion, and any derivative presentation.

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