PhD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: Federico Conti

Title of PhD research: Use of flavorings to improve the palatability of aquafeeds used in aquaculture: A multidisciplinary approach to better understand teleost physiological responses.

Name of PhD supervisor: Prof. Ike Olivotto

Research lab name: Reproductive and Developmental Biology Lab, DiSVA

Cycle:
[**X**] XXXVII
[ ] XXXVIII

PhD Curriculum:
[**X**] Marine biology and ecology
[ ] Biomolecular Sciences
[ ] Civil and environmental protection

DISVA instrumentation labs/infrastructure eventually involved in the project:
[ ] Actea Mobile Laboratory
[ ] Advanced Instrumentation lab
[**X**] Aquarium
[ ] MassSpec lab
[ ] MaSBiC
[ ] Simulation/informatics lab
[ ] Other. Please, indicate: .....

ABSTRACT (1000 characters, including spaces):
The use of alternative protein sources in aquafeed formulations affects both feed digestibility and palatability. Vegetable ingredients are the most widely used in aquaculture and specific production processes are applied to make them more digestible, especially by carnivorous fish. However, low palatability issues remain unsolved, leading to poor feed intake and imbalances of ecosystem and economic losses. In fact, the release of a high amount of nitrogen and phosphorous due to feed wastage causes eutrophication and impacts the farm economics since feed represents 50-70% of the aquaculture sector's costs. In this context, in order to improve feed palatability, natural feed attractants are used resulting again unsustainable and often scarcely effective. In the present project, the effects of synthetic flavors produced through standardized processes, more sustainable and widely used for human consumption, have been tested through a multidisciplinary approach in the rearing of different fish species. Specifically, the effects of three different synthetic flavors, have been assessed in zebrafish (*Danio rerio*) rearing, considering the whole life cycle from larvae to adults. The results will allow to evaluate, in a comparative study through the same dietary trial, the physiological responses of the European seabass (*Dicentrarchus labrax*), a commercially relevant species for Mediterranean aquaculture.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

The conventional Fish Meal (FM) and Fish Oil (FO) used in aquafeed formulations, considering their optimal protein contents and well-balanced amino acid profile, represent the ideal ingredients to ensure a proper fish growth and welfare [1]. However, the use of marine-derived protein sources presents several economic and ecological drawbacks due to their increasing cost and poor sustainability [2]. In this context, in the last decades more sustainable ingredients were included in aquafeed formulations, mostly represented by plant-derived ones. Nevertheless, vegetable meals (e.g., soybean meals, corn meals) besides resulting in poor digestibility due to presence of non-digestible carbohydrates and anti-nutritional factors, lack in palatability, affecting a proper feed intake and the fish health status [3,4]. Part of the digestibility problems has been addressed by improving the ingredients processing methods (e.g., protein hydrolysates or inclusion of feed supplements such as butyrate) or replacing them with more sustainable alternatives (e.g., poultry by-product, insect, and crayfish meals or proteins derived from microbial biomasses), despite palatability issues still remain [5,6]. This results in a large feed wastage that has both an economic and an environmental impact, leading to eutrophication of aquatic ecosystems and important economic losses [7]. Currently, to overcome this problem, natural substances named feed attracts are regularly included in fish diets to improve palatability, stimulating fish feed-seeking and ingestion. However, these ingredients are mainly obtained from marine sources (e.g., anchovy meals, squid meals, shrimp meals), posing further unsustainability issues [8]. In addition, due to their natural origin, their attractive effect is highly variable depending on the raw material composition, freshness, and processing methods [9]. Other substances with noteworthy attractive effects are molecules such as free amino acids, nucleotides, nucleosides, betaine, taurine, that however present different attractive effects depending on the fish species considered, as well as the fish life cycle, their concentration, and the interaction with other molecules present in the feeds, leading to controversial results [10].

- SCIENTIFIC AIMS

The present PhD project wants to explore the application of synthetic flavors as a novel and sustainable alternative to the natural feed attractants, widely used for human food consumption and produced through standardized processes. This will allow to increase attractiveness and palatability of commercial feed formulations, reducing at the same time the above-mentioned issues by improving fish physiological responses, in favour of farm productivity and profitability. Through a multidisciplinary approach, fish feed intake, growth rate, and welfare will be assessed through a set of laboratory techniques. Initially, the dietary trial was conducted on the zebrafish (*Danio rerio*) experimental model, considering the whole life cycle; after this phase, the same trial will be conducted on a commercially relevant species, the European seabass (*Dicentrarchus labrax*), providing important results about the possible use of attractive synthetic flavors for the aquafeed production.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

Three different synthetic flavors (two attractive – F1, F2; one repulsive – F3), previously identified through a behavioral test performed on zebrafish (*Danio rerio*) larvae, have been added to a commercial diet for zebrafish, to investigate possible dietary effects on zebrafish early development. This allowed to assess the flavors' potential role as feed attractants within aquafeed. Through a multidisciplinary approach, growth performances, feed ingestion and fish health status were evaluated applying biometric and feed intake analyses, histopathological analyses of the liver and intestine, and molecular analyses of genes involved in the regulation of growth, appetite, brain dopaminergic activity, immune and stress. At this phase, analyses were performed on both the larval and juvenile stage of zebrafish (21- and 60-day post-fertilization – dpf, respectively).

Methods.

Experimental diets. Starting from a commercial diet for zebrafish (Zebrafeed, Sparos), used as control diet (CTRL), four experimental diets were prepared as follows: (i) PG diet – control diet with 1% Propylene Glycol

(solvent of the flavors); (ii) F1 diet – control diet with 1% F1 attractive flavor; (iii) F2 diet – control diet with 1% F2 attractive flavor; (iv) F3 diet – control diet with 1% F3 repulsive flavor.

Experimental design. Six experimental groups (in triplicate) were set up: (i) CTRL group, fish fed control diet; (ii) PG group, fish fed PG diet; (iii) F1 group, fish fed F1 diet; (iv) F2 group, fish fed F2 diet; (v) F3 group, fish fed F3 diet; (vi) ROT group, fish fed the two attractive diets (F1 and F2), each administered singularly in a weekly rotation scheme. Starting from day 5 dpf to the end of the trial (60 dpf), zebrafish were fed the experimental diets, provided at a feeding rate of 3% body weight (BW). Finally, fish were sampled after a lethal dose of MS222 (1 g/L, Merck KGaA), at 21 and 60 dpf.

Biometry. Ten larvae per tank (in triplicate) were randomly collected at 3 dpf to measure the initial body weight (IBW). Then, 20 larvae and 20 juveniles per group (both in triplicate) were randomly collected at 21 and 60 dpf, respectively, to measure the final body weight (FBW). The specific growth rate (SGR) was calculated as follows: $SGR = [(\ln FBW - \ln IBW) / t] \times 100$; (t, number of feeding days – 17 and 57 for larvae and juveniles, respectively).

Feed intake. Fifteen juveniles per tank (in triplicate) were fed the experimental diets (3% BW); then, uneaten feed was recovered 15 minutes post-administration by siphoning and eventually dried in an oven, overnight at 40 °C for quantification.

Histological analysis. Five larvae per tank (in triplicate), while liver and whole intestine from 5 juveniles per tank (in triplicate) were collected. After the paraffin embedding steps, sections of 5µm cut with a microtome were stained with Mayer hematoxylin and eosin Y, to evaluate potential alterations in tissue architecture and occurrence of inflammatory events in both the intestinal tract and the hepatic parenchyma. For the morphometric evaluation of mucosal folds height, undamaged and non-oblique folds were measured using ZEN 2.3 software (Zeiss).

Molecular analysis. Total RNA extraction from 3 larvae per tank (in triplicate), and from brain, liver, and intestine samples from 9 juveniles per tank (in triplicate), was performed followed by the cDNA synthesis using 1 µg of RNA. Real-Time quantitative PCRs (qPCRs) were performed to quantify the gene expression using, as housekeeping genes, the ribosomal protein L13 (*rpl13*) and actin-related protein 2/3 complex subunit 1a (*arpc1a*) to standardize the results. The relative quantification of genes involved in growth (*igf1*, *mstnb*), appetite (*ghrl*, *npy*, *lepa*), brain reward system (*drd1b*, *drd2a*, *drd3*), stress and immune responses (*nr3c1*, *il1b*, *il10*, *litaf*), was performed.

Total lipid analysis. Five larvae and five juveniles per tank (in triplicate) were collected and subdued to the lipid extraction using chloroform:methanol (2:1) as solvents. The amount of total lipids was gravimetrically quantified, and the results were expressed as g/100 g of whole fish.

Statistical analysis. Data from each analysis were checked for normality (Shapiro–Wilk test) and homoscedasticity (Levene's test). Then, data were analyzed through ANOVA followed by Tukey's multiple comparison post hoc test, performed using the software package Prism 8 (GraphPad software version 8.0.2, San Diego, CA, USA). Significance was set at $p < 0.05$.

Obtained Results.

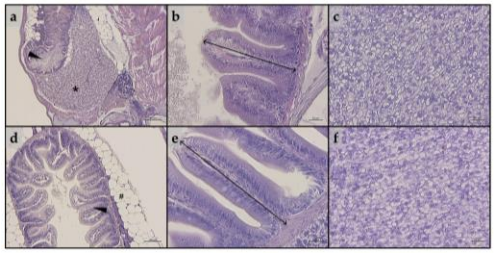

Figure 1 – Example of histomorphology of intestine and liver parenchyma of zebrafish (a-c) larvae and (d-f) juveniles.

Histological analysis. No morphological alterations or signs of inflammation were evident in liver and intestine, as also demonstrated in the histopathological indexes analyzed in the intestine, at both developmental stages. As regards liver, both larvae and juveniles belonging to all the experimental groups showed a modestly fat liver parenchyma, with a diffuse presence of hepatocytes with cytoplasm filled of fat.

For the histopathological indexes, scores were assigned as

	CTRL	PG	A1^{+}	A2^{+}	ROT	A^{-}
Larvae						
Mucosal folds height	80.7 ± 0.6	93.0 ± 7.2	84.5 ± 5.5	95.4 ± 7.5	92.5 ± 7.2	75.0 ± 4.5
Inflammatory influx	+	+	+	+	+	+
Mucosal folds fusion	+	+	+	+	+	+
Juveniles						
Mucosal folds height	166.2 ± 23.1	168.0 ± 23.0	160.0 ± 12.0	175.3 ± 26.2	162.4 ± 12.7	164.7 ± 25.5
Inflammatory influx	+	+	+	+	+	+
Mucosal folds fusion	+	+	+	+	+	+

follows: (i) inflammatory influx: + = scarce lymphocytes infiltration, ++ = moderate lymphocytes infiltration, +++ = diffused lymphocytes infiltration; (ii) mucosal folds fusion: + = 0-3 observations per section, ++ = 3-10 observations per section, +++ = > 10 observations per section.

Molecular analyses – immune and stress responses. Considering the gene expression of immune-related and of stress-related markers, no significant differences were detected in both larvae and juveniles, supporting histological analysis result (Figures not showed). As regards the larval stage, the remaining molecular analyses performed did not show significant differences, suggesting that the diets exposure time was probably too short.

In light of this, the overall welfare of the fish was not affected by the flavors' dietary administration.

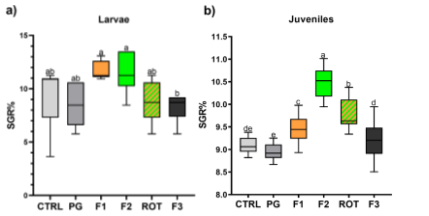

Figure – Specific growth rate (% day -1) of zebrafish larvae and juveniles fed the experimental diets.

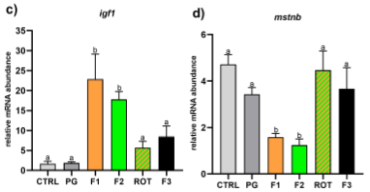

Figure 3 – Relative mRNA abundance of genes involved in growth (*igf1* and *mstnb*) analysed in juveniles' liver samples.

Biometry and growth markers. Larvae fed positive diets showed a significantly higher SGR% compared to the repulsive group, underlining that the preliminary behavioral test properly selected the flavors. Considering juveniles, SGR% mostly confirmed the results obtained in the larval stage, with all the groups fed positive diets characterized by a higher value compared to CTRL group. This result was fully supported by the expression of growth markers.

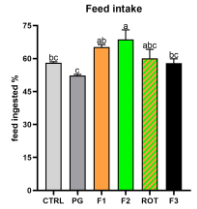

Figure 4 – % of feed ingested in zebrafish juveniles.

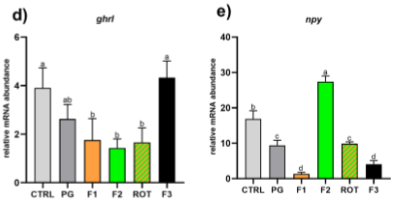

Figure 5 – Relative mRNA abundance of genes involved in appetite (*ghrl* and *npy*) analysed in juveniles' intestine and brain samples.

Feed intake and appetite markers. The attractive role of the flavors was confirmed also by the feed intake experiment. In fact, the highest feed intake was observed in both zebrafish juveniles fed positive diets, after 15 minutes of test. This result was confirmed by appetite markers gene expression, in which a significant *ghrl* downregulation in positive groups suggested that fish were in a more satiated state compared to those from the other groups. Differently, non-obvious results were obtained considering *npy*. However, NPY besides being expressed also in other tissues, interacts with different signals suggesting that its response is not exclusively dependent from the ghrelin signal [11].

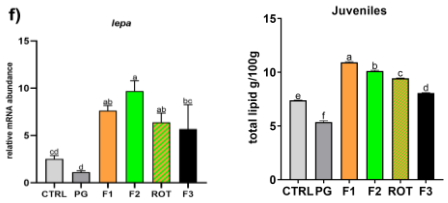

Figure 6 – Relative mRNA abundance of genes involved in appetite (*lepa* - left) and total lipid content (right) of zebrafish juveniles.

Total lipid content and appetite markers. Considering anorexigenic signals, a significant leptin upregulation, in accord with the *ghrl* expression, was observed in positive groups, further supporting their more satiated state. Since leptin expression is directly related to the amount of adipose tissue, positive groups showed a higher lipid content respect to control group, as confirmed by the total lipid analysis.

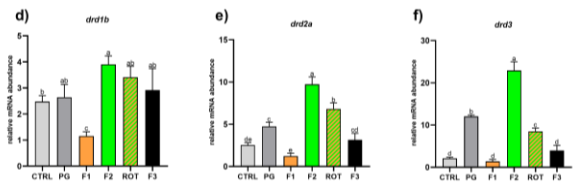

Figure 7 – Relative mRNA abundance of genes involved in reward system (*drd1b, drda,* and *drd3*) analysed in juveniles' brain samples.

Brain reward system. The F2 flavor was characterized by a longer-lasting attractive effect, evidenced in the preliminary behavioral test. Fish fed F2 diet resulted in a significant upregulation of the three dopamine receptors analyzed, compared to the CTRL group, suggesting a possible higher dopaminergic activity, possibly linked to a feeding pleasure.

To summarize, the provision of diets containing attractive flavors resulted in an increased growth rate in both larvae and juveniles. Fish fed positive diets showed higher appetite stimulus, feed ingestion and growth. However, the whole analyses results suggested the selection of the F2 flavor as the most valuable solution for maintaining its positive effect over the trial. In conclusion, these results provided important information on the possible applications of synthetic flavors for the aquaculture sector.

WP 2. Objective.

The above-described experimental design was prolonged to the zebrafish adult stage (6 months), in order to evaluate the long-term dietary effects of synthetic flavors administration, on fish physiological responses.

Methods.

The same procedures and laboratory techniques described in the *WP1 section* were performed in this phase.

Obtained/Expected Results.

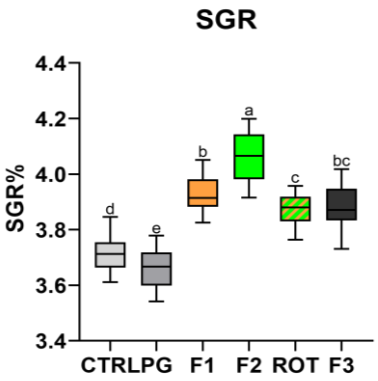

Figure 8 – Specific growth rate (% day -1) of zebrafish adults fed the experimental

Biometry. Zebrafish adults fed positive diets showed a significantly higher SGR% compared to the CTRL and PG groups, mostly confirmed the results obtained in both zebrafish larvae and juveniles (*WP1 section*).

Other analyses. Remaining analyses are still ongoing. Histological analysis and molecular expression of immune- and stress-related markers will allow to compare/confirm the absence of side effects on fish welfare, due to the flavors administration. The remaining analyses will provide an overview of fish growth, appetite, and feeding motivation considering dopaminergic activity. Finally, the feed intake will provide important results for the aquaculture sector to avoid feed wastage and economic losses by using synthetic flavors as feed attractants.

WP 3. Objective.

A two-month dietary treatment, with the same flavors previously tested, was applied on European seabass (*Dicentrarchus labrax*), a commercially relevant species for the Mediterranean aquaculture. Starting from a specific diet for seabass, the same above-described five experimental diets were produced. To date, analyses are still ongoing, however this comparative study, considering the results of the zebrafish phase, allowed to obtain important information for aquafeed sector to improve fish feeding practices.

WP 4. Under evaluation

We are currently discussing with the Hellenic Centre for Marine Research of Crete an externship during which the same flavors will be further tested on seabream (*Sparus aurata*) improving the administration technology of the flavors. Thanks to this new collaboration between the companies To Be Pharma and Aquatrade, the selected flavors will be administered through the *+Pop* technology, which uses a specific microencapsulation process able to retain the flavor on feed pellet.

- REFERENCES

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Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Course: Analisi di regressione mediante Microsoft Excel – Prof. Francesca Beolchini
2. Course: Introduzione all'ambiente LaTeX per la redazione di documenti scientifici – Prof. Francesco Spinozzi
3. Course: FTIR and Raman Imaging – Prof. Elisabetta Giorgini and Prof. Giorgia Gioacchini
4. Seminar: "Il futuro della fertilità umana sarà davvero in provetta?" – Dr. Nina Montik, 29/11/2022
5. EAS Aquaculture Europe 2023 – Vienna
6. SHARPER – European Researchers' Night 2023
7. Tutor activity for didactic laboratory activity within the Cytology and Histology course (BIO/06) included in the BSc in Biological Sciences.
8. Tutor activity for didactic laboratory activity within the Biological Basis of Nutrition course (BIO/16) included in the BSc in Biological Sciences.

List of periods spent abroad

None.

List of conferences/workshops attended and of contributions eventually presented

1. EAS Aquaculture Europe 2023 – Vienna (Austria) – Oral presentation entitled: A new set of feed additives to promote fish feed intake and welfare in aquaculture: a comparative study on zebrafish (*Danio rerio*) larval and juvenile stage. **F. Conti**, M. Zarantoniello, M. Antonucci, N. Cattaneo and I. Olivotto.

Part 3. PhD student information on publications

If not yet published, please indicate the publication status (submitted, accepted, in preparation...)

1. Cattaneo, N., Zarantoniello M., **Conti F.**, Frontini A., Chemello G., Dimichino B., Marongiu F., Cardinaletti G., Gioacchini G., Olivotto I. (2023). Dietary Microplastic Administration during Zebrafish (*Danio rerio*) Development: A Comprehensive and Comparative Study between Larval and Juvenile Stages. Animals, 13, 2256. https://doi.org/10.3390/ani13142256
2. **Conti F.**, Zarantoniello M., Antonucci M., Cattaneo N., Rattin M., De Russi G., Secci G., Lucon-Xiccato T., Lira de Medeiros A. C., Olivotto I (2023). Application of synthetic flavors in zebrafish (Danio rerio) rearing: effects on fish development, welfare, and appetite. Animals (**submitted**).

List of publications on international journals

None.

List of publications on conference proceedings

None.

List of other publications (books, book chapters, patents)

None.

[Date]

08/10/2023

Student signature

Federico Conti

Supervisor signature

Prof. Ike Olivotto