PhD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

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

Name of PhD student: Federico Conti

Title of PhD research: Use of flavourings to improve the palatability of aquafeeds used in aquaculture: 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:
[ ] XXXVI
[**X**] XXXVII

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 ingredients in aquafeed formulations may affect digestibility and reduce feed palatability in cultured fish. Vegetable ingredients are the most widely used in aquaculture and are subjected to specific production processes that aim to make them more digestible by carnivorous fish, but palatability issues still remain unsolved. The use of natural feed attractants is no more sustainable and also scarcely effective, as current data indicate that less than 50% of the feed administered is consumed by fish in reality. In addition, the uneaten feed causes an environmental imbalance and impacts the economic aspect of the aquaculture industry as feed represents 50-70% of the sectors' costs. In the present project, the effects of synthetic feed attractants, more sustainable and obtained through standardized techniques will be tested through a multidisciplinary approach. Specifically, the effects of feed attractants will be assessed within specific feed formulations on zebrafish, considering the whole life cycle, and on commercially relevant species such as seabass.

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

- BACKGROUND

Aquaculture is currently a growing sector in terms of food production. According to FAO (2018), aquaculture production grew by an average of 5.8% each year from 2001-2016. Similarly, the feed production sector is also increasing, due to high feed demands. However, the use of ingredients derived from marine sources for fish feed production, such as fishmeal and fish oils, is no longer sustainable, and in recent years efforts have been made to replace these ingredients with sustainable alternatives ones (Kader et al., 2012; Sarker et al., 2018). Vegetable meals (e.g., soy meal) are currently the most widely used, since they are an appropriate alternative protein source to fishmeal, possess a balanced amino acid profile, and are cheaper in terms of production (Zlaugotne et al., 2022). However, the adverse effects of using vegetable ingredients for carnivorous species are well known, because of the presence of anti-nutritional factors that affect the digestibility, palatability and general welfare of the cultured fish (Kader et al., 2012). To overcome the digestibility issues, vegetable meals are subjected to specific production processes that aim to make them more bioavailable and assimilable even for omnivorous and carnivorous species. One example is the use of protein hydrolysates or by adding substances such as butyric acid to minimize undesirable effects such as enteritis which are typical of carnivorous fish (Zlaugotne et al., 2022; Estensoro et al., 2016). To avoid palatability problems, on the contrary, natural feed attractants are regularly used, which are currently represented by fishing by-products such as fish meal or squid meal (Nagel et al., 2014). However, these additives are not only unsustainable, but they are also scarcely effective, as current data indicate that less than 50% of the feed administered is consumed by the fish in reality (Kong et al., 2020; Ballester-Moltó et al., 2017). This also causes an environmental imbalance as the release of organic-rich wastewater into the aquatic environment leads to eutrophication. Finally, since it is estimated that about 50-70% of the aquaculture costs derive from fish feed, reducing the wastage of uneaten feed is mandatory (Llagostera et al., 2019).

- SCIENTIFIC AIMS

The proposal of this PhD project wants to explore the application of synthetic and environmental friendly feed attractants, already used for human food consumption and produced through standardized processes to fish dietary formulations. This will allow increase attractiveness and palatability of commercial feed formulations, reducing at the same time the above-mentioned issues. By increasing feed intake, fish welfare and growth will be positively affected, 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 analyses, in order to provide robust recommendations to the feed and aquaculture industry.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective.

A set of different feed attractants have been tested during a behavioural preliminary test performed on zebrafish (*Danio rerio*) larvae to identify those feed attractants that positively attracted fish. This test allowed the identification of the three most effective feed attractants to be tested in the following phases, by adding them to a specific feed. As a negative control one feed attractant that resulted repulsive to fish was also included in the experimental design.

Methods.

Fish husbandry. Eight hundred zebrafish larvae aged 25 days post fertilization (*dpf*) were divided into forty groups of 20 individuals each and fish were singularly exposed to a different water-soluble feed attractant (named from P01-P40). Tests were performed in rectangular white plastic tanks, using a Canon LEGRIA HF R38 camera to record the behaviour of the fish during the testing time (15 mins each). During each test, two cellulose sponges (side 1 cm) were inserted into the tanks: one contained the feed attractant (1 %) while the second water and served as control for object exploration.

Statistical analysis. All video recordings were visualized and analyzed using BORIS software. A two-step analysis was performed: one-way ANOVA was firstly used considering the total time of analysis (min 0-15) to evaluate whether the larvae responded differently to several feed attractants; then, the data of the first 5 minutes (min 0-5) and the total time recorded (min 0-15) were analyzed separately by computing the overall proportion of time spent close to the novel feed attractant in these two periods of time. One-sample t-tests against the preference expected by chance (0.5) were used to test whether the preference for the novel feed attractant was different from random choice.

Obtained Results.

The average percentage of time spent in the sector with the feed attractant are showed in Figure 1; the feed attractants that showed a significant effect (one-sample t-test) are highlighted in the Figure 1 in green or red.

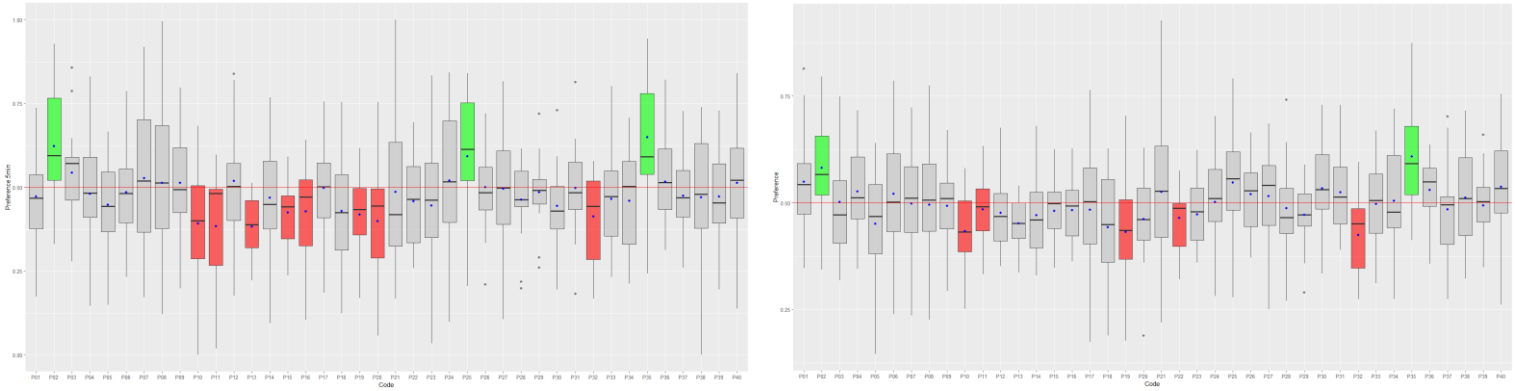

Figure 1 *– Boxplot of minutes 0-5 and 0-15 respectively, showing the average percentage of time spent by fish in the sector with the specific feed attractant. Feed attractants that showed a significant (positive, green; negative, red) effect are highlighted in the figure. ANOVA,* $F_{39,724} = 2.42$, $p = <0.0001$, $\eta^2_g = 0.12$.

The two temporal analyses revealed that in some cases, the same feed attractants showed different effects on zebrafish larvae behaviour. Analyzing different time slots is essential because the effects of each feed attractant can vary during the time in relation to its chemical nature and dilution factor. For example, novel or too much-concentrated feed attractants can initially act as alarm signals evoking avoidance or defensive behaviours. However, no one of the tested feed attractants showed this behaviour. As regards the time effect on fish behaviour, the present study evidenced that: i) P02 and P35 feed attractants showed a constant attractive effect; ii) P25 feed attractant instead, showed an attractive effect only during the first five minutes of the test; iii) the remaining feed attractants displayed a negative or neutral effect on fish. To reduce feed losses in aquaculture, the feed has to be ingested by fish as quickly as possible and thus the search for new feed additives should be focused on the feed attractants that have a quick and positive effect on fish during the first minutes of feed administration (Alves et al., 2019).

WP 2. Objective.

The results of the preliminary phase allowed the selection of three feed attractants with an attractive effect (P02, P25, and P35), and one feed attractant with a repulsive effect (P32 - negative control). These feed attractants were then added to a specific feed for zebrafish (SPAROS Lda, Área Empresarial de Marim, Lote C, 8700-221, Olhão, Portugal) in order to evaluate their attractive effect and the fish physiological responses. Through a multidisciplinary approach, growth performances and animal welfare status are under evaluation applying biometric analysis, histopathological analyses of the liver and intestine, and molecular analyses of genes involved in the regulation of growth, appetite, and stress. At this phase, analyses were performed on the larval stage of zebrafish (21 days post-fertilization).

Methods.

Fish husbandry. Zebrafish larvae (21 *dpf*) were reared in twenty-one 20L tanks to set up seven experimental dietary treatments (500 individuals per group, n = 3). From 5 *dpf* fish were fed as follows: (i) control group – fish fed with feed only (control diet); (ii) fish fed a diet with the solvent used for feed attractants production (polyethylene glycol - PG) at 1% w/w; (iii) fish fed a diet with P02 at 1% w/w; (iv) fish fed a diet with P25 at 1% w/w; (v) fish fed a diet with P35 at 1% w/w; (vi) fish fed a diet with P32 at 1% w/w (negative control).
Since several studies have shown that fish develop sensory adaptability to the same odour substance, a rotational feeding pattern is also evaluated in the present study in order to prevent the fish to get used to a certain feed attractant (Zufall et al., 2000). Therefore, a further experimental group was administered the 3 feed attractants, based on a weekly rotation (vii).

Biometry. Ten zebrafish larvae (30 per dietary group) were randomly collected from each tank at hatching (3 *dpf*) and at 21 *dpf*, and wet weight was determined by an analytical balance (precision: 0.1 mg). For each experimental group, specific growth rate (SGR) was calculated as follows: $SGR\% = ((\ln Wf - \ln Wi)/t) \times 100$, where Wf is the final wet weight, Wi, is the initial wet weight, and t, is the number of days (17). Boxplot of final weights at 21 *dpf* is showed in Figure 2.

Histology. Four zebrafish larvae (12 per dietary group) were randomly collected from each tank at 21 *dpf*. After the paraffin embedding steps, sections of 5µm cutted with a microtome were stained with Mayer hematoxylin and eosin Y, in order to study hepatic parenchyma and intestinal morphology and to measure the perivisceral tissue area. Sections were observed using a Zeiss Axio Imager.A2 microscope and images were acquired by a digital camera Axiocam 503 (Zeiss).

Molecular analysis. Total RNA extraction from four zebrafish larvae collected from each tank at 21 *dpf* (12 per dietary group) was performed followed by the cDNA synthesis using 1 µg of total RNA.
PCRs were performed allowing the relative quantification of the expression of genes involved in fish growth (insulin-like growth factor 1, *igf1*; insulin-like growth factor 2a, *igf2a*; myostatin, *mstnb*), stress response (glucocorticoid receptor, *nr3c1*; heat shock protein 70, *hsp70.1*), and appetite response (ghrelin, *ghrl*; neuropeptide y, *npy*; cannabinoid receptor 1, *cnr1*; leptin a, *lepa*).

Statistical Analyses. All data were analyzed by one-way ANOVA, with diet as the explanatory variable. All ANOVA tests were followed by Tukey's post-hoc test and significance was set at $p<0.05$.

Obtained/Expected Results.

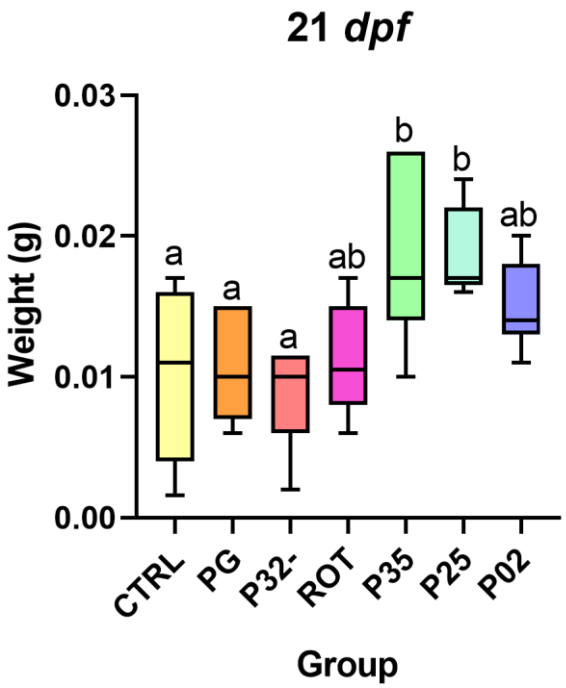

Figure 2 *– Considering the final weights of zebrafish larvae at 21 dpf, significant differences were detected among P25 and P35 groups compared with control group and negative – P32- – control group.*

Considering the final weights of zebrafish larvae at 21 *dpf*, a significantly higher mean weights distribution can be observed for the groups that received positive feed attractants (P25, P35) compared to those that received the repulsive feed attractant (P32-) and the two control groups (CTRL, PG).
Remaining analyses are still ongoing; however, histological analyses will allow to detect possible alterations in the intestinal tract, signs of inflammations, and lipid accumulation or steatosis in the liver. This will provide important information on the fish health status.
Molecular analyses will provide an overview of both the welfare status and the effects of feed attractants on fish growth and appetite, possibly improving feed intake for faster growth.

- 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: Innovazione e trasferimento tecnologico, Prof. Donato Iacobucci
2. Course: Progettare la ricerca: i progetti europei, Prof. Nicola Paone
3. Course: Environmental sustainability: the life cycle assessment, LCA, prof. Alessia Amato
4. Seminar: "Caratteristiche biofisiche dei sistemi viventi (e come guardarli)" – Prof. Ranieri Bizzarri, 23/03/2022
5. Seminar: "La Sperimentazione Animale" – Prof. Fiorenzo Conti, 09/05/2022
6. Seminar: "Le donne nella pesca"
7. PhD defense of student Matteo Zarantoniello, 08/06/2022
8. EAS Aquaculture Europe 2022 – Rimini
9. SHARPER – European Researchers' Night - 2022

List of periods spent abroad

1. 29/11/2021 – 04/12/2022 – University of Ferrara
2. 14/03/2022 – 09/04/2022 – To Be Pharma S.r.l., S.Egidio alla Vibrata (TE) - Italy

List of conferences/workshops attended and of contributions eventually presented

1. EAS Aquaculture Europe 2022 - Rimini (Italia) – Poster presentation entitled: Behavioral responses to different feed additives in zebrafish (*Danio rerio*) larvae: a preliminary study. F. Conti, T. Lucon-Xiccato, G. De Russi, M. Antonucci, N. Cattaneo, M. Zarantoniello, C. Bertolucci And I. Olivotto.

Part 3. PhD student information on publications

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

List of publications on international journals

None.

List of publications on conference proceedings

None.

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

1. Informative article published on "Il Pesce" magazine, title: "Percorsi di dottorato di ricerca: opportunità di innovazione e crescita professionale per studenti ed aziende". F. Conti, M. Antonucci, G. De Russi, M. Zarantoniello, B. Randazzo, T. Lucon-Xiccato, C. Bertolucci, I Olivotto.

[Date]

09/10/2022

Student signature	Supervisor signature
Federico Conti	Prof. Ike Olivotto