Sustainable Marine Ornamental Fish Trade: Innovative Aquaculture Practices for Public Aquariums

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Supervisors:
Prof. Daniel Montero
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PhD programme in Sustainable Aquaculture and Marine Ecosystems
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PhD thesis

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El Director, El Codirector, El Doctorando,

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List of abbreviations

ALA, alpha-linoleic acid

ANOVA, analysis of variance

ARA, arachidonic acid

ARPAL, Regional Agency for Environmental Protection–Liguria

Bc, blastocele

Bd, blastodisc

Bm, blastomere

Br, brain

°C, Celsius degrees

C, chorion

Cf, caudal fin

Ct, connective tissue

CV, Coefficient of variation

DHA, docosahexaenoic acid

DPA, docosapentaenoic acid

dpf, days post fertilization

dph, days post hatch

DW, dry weight

EAZA, European Association Zoo and Aquariums

EFA, essential fatty acid(s)

El, eye lens

EPA, eicosapentaenoic acid

EU, European Union

EUAC, European association of Aquariums Curators

Er, eye retina

eVg, early vitellogenic oocyte

FAMES, fatty acid methyl esters

FAs, fatty acid(s)

FC, filial cannibalism

g, grams

Gb, gall bladder

Gc, goblet cell

Gi, gills arches

h, hours

H, height

He, hepatocyte

Hd, **h**ead

H&E, haematoxylin and eosin

HPF, hours post fertilization

In, intestine

I-R, *Isochrysis galbana* and *Rhodomonas salina* diet

K, kelvins degrees

Kg, kilograms

L, litres

LA, linoleic acid

Lc, Leydig cells

LC-PUFA, long-chain polyunsaturated fatty acid

List of abbreviations

L/D, light/dark

Li, liver

LSS, Life Support System

Lu, lumen

m^2, square meters

m^3, cubic meters

mg, milligrams

mL, millilitres

MoRS, **M**odular Rearing System

MUFAs, monounsaturated fatty acid(s)

Mx, maxilla

N, notochord

nC, neurocranium

Nd, number of deaths

Nf, final number

Ni, initial number

Ns, number of samples

Nu, nucleus

OA, oleic acid

P, pigment

PAM, palmitic acid

PUFA, polyunsaturated fatty acid

PVC, polyvinyl chloride

pVg, previtellogenic oocyte

R, *Rhodomonas salina* diet

RAS, Recirculating Aquaculture System

SA, stearidonic acid

SAFAs, saturated fatty acid(s)

Sc, spermatocyte

SD, standard deviation

SGR, specific growth rate

St, Spermatid

STA, stearic acid

T, tail

TL, total length(s)

TLi, initial total length

TLf, final total length

T-R, *Tetraselmis suecica* and *Rhodomonas salina* diet

Tu, seminiferous tubules

ULPGC, Universidad de Las Palmas de Gran Canaria

USD, United States Dollar

UV, ultraviolet

Vc, vertebral column

Vg, vitellogenic oocyte

Vi, intestinal *villi*

Yld, yolk lipid droplet

Ys, yolk sac

Zr, zona radiata

W, watt

WAZA, World association Zoo and Aquariums

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Chapter 1. General Introduction

1.1 The marine ornamental trade

The ornamental marine fishkeeping industry, commonly known as the marine ornamental trade, is a global sector supplying wild-caught and aquaculture marine species to hobbyists and public Aquaria worldwide. Marine ornamental livestock are delivered to consumers through an international network of fishermen, aquaculture professionals, and logistic companies (Wabnitz *et al.*, 2003; Tissot *et al.*, 2010; Palmtag, 2017; Watson *et al.*, 2023). This business, which predominantly operates in tropical and subtropical regions, generates annual profit exceeding $300 million (Fig. 1.1) (Tissot *et al.*, 2010; Rhyne *et al.*, 2017; Palmtag, 2017; Biondo and Burki, 2020; Watson *et al.*, 2023).

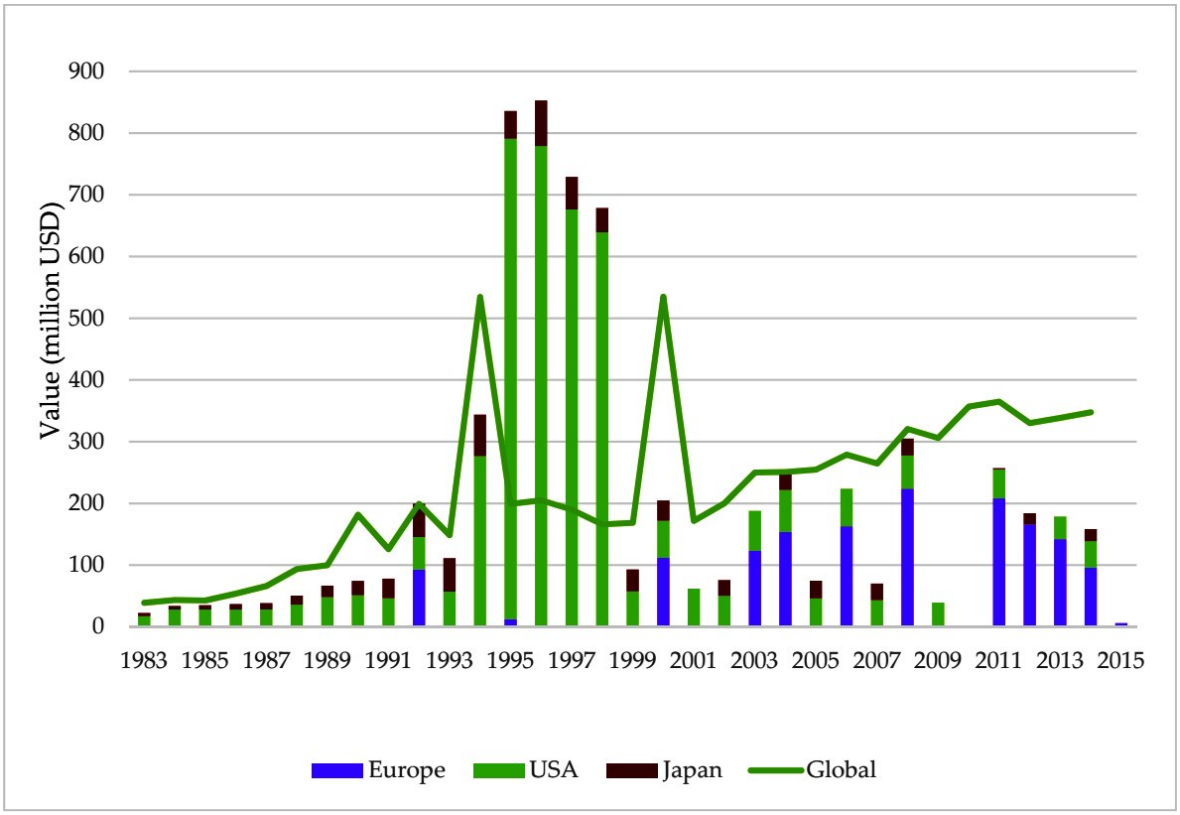

Figure 1.1: Average value in million USD of ornamentals fishes for the global export compared with import to Europe, the United States, and Japan from 1983 to 2014 (modified from Biondo and Burki, 2020).

The marine ornamental trade encompasses over 1,800 species of fish and more than 700 invertebrate species, including cnidarians, molluscs, arthropods, echinoderms, annelids, and poriferans (Rhyne *et al.*, 2012, 2017; Palmtag, 2017; Pouil *et al.*, 2019). Unlike the ornamental freshwater trade, where over 90% of species are bred in captivity, only about 10% of marine ornamental species are cultured in controlled conditions (Raghavan *et al.*, 2013; Rhyne *et al.*, 2017; Chen *et al.*, 2019; King, 2019; Pouil *et al.*, 2019).

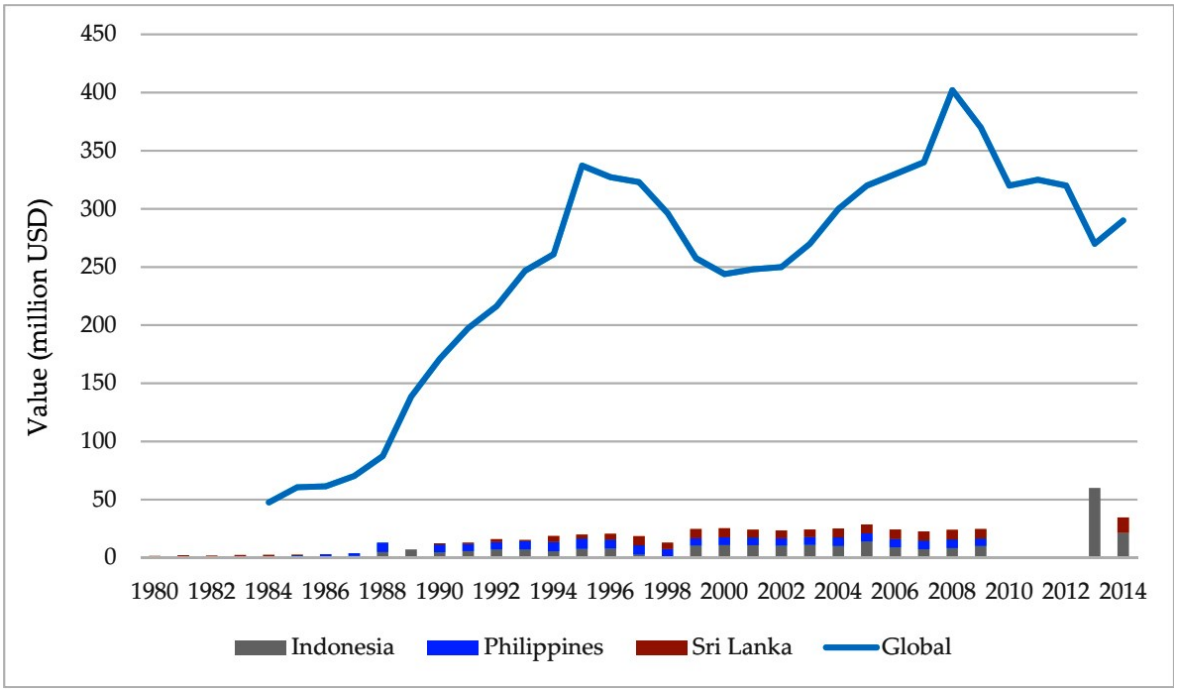

Figure 1.2: Average value in million USD of ornamentals fishes for global import compared with exports from Indonesia, the Philippines, and Sri Lanka from 1983 to 2014 (modified from Biondo and Burki, 2020).

As a result, most of marine ornamental fish and invertebrates available in the market are caught in the wild on developing countries especially in tropical and subtropical latitudes (Wabnitz *et al.*, 2003; Tissot *et al.*, 2010; Raghavan *et al.*, 2013; King, 2019). Key exporters include the Philippines, Indonesia, Solomon Islands, Sri Lanka, Australia, Fiji, the Maldives, and Palau (Fig. 1.2) (Palmtag, 2017; King, 2019; Biondo and Burki, 2020; Watson *et al.*, 2023).

The United States is the largest importer and consumer of marine ornamental species, followed by other major importers such as the United Kingdom, the Netherlands, France, Germany, Taiwan, Japan, China, Spain, and Italy (Palmtag, 2017; King, 2019; Biondo and Burki, 2020; Watson *et al.*, 2023). The collection of marine ornamentals from the wild often involves the use of unregulated methods including manual harvesting, underwater guns, nets, traps, hooks, and lines; as well as illegal methods such as sedatives (cyanide) and explosives (Wabnitz *et al*., 2003; Tissot *et al*., 2010; Rhyne *et al.*, 2014; Olivotto *et al*., 2017; Palmtag, 2017). These capture practices frequently lead to high mortality rates throughout the supply chain, from collection to maintenance, transport, and final destination (Rubec *et al*., 2001; Schmitt and Kunzman, 2005). In addition, mortality rates during transport can reach as high as 100%, largely due to improper storage and packaging techniques (Rubec *et al*., 2001; Wabnitz *et al*., 2003; Schmitt and Kunzman, 2005). Factors such as the small volume of plastic bags, overcrowding of organisms, and lack of oxygen are significant contributors to the death of specimens during transit (Schmitt and Kunzman, 2005; Rhyne *et al.*, 2017). However, even with improvements in the transportation and final survival of captured animals, the sustainability of the trade and the welfare of marine ornamental organisms remain a significant concern and are subject to ongoing debate.

1.2 Conservation strategies

In recent decades, various suppliers have made serious efforts to address sustainability challenges within the marine aquarium trade by adopting responsible collection practices and expanding aquaculture operations (Dalabajan, 2005; Tissot *et al*., 2010; Pouil *et al*., 2019; Watson *et al.*, 2023). A primary concern is the sustainability of harvesting target species from the wild, often conflicting with ecosystems preservation efforts (Sadovy and Vincent, 2002; Wabnitz *et al*., 2003; Rhyne *et al.*, 2014, 2017). This concern is heightened by the potential overexploitation of millions of fish and invertebrates traded annually, intensified by the use of destructive and illegal fishing

methods (Rubec *et al.*, 2001; Sadovy and Vincent, 2002; Wabnitz *et al*., 2003). In response, governments, stakeholders and environmental organizations undertook progressive actions to mitigate these impacts, educating fishermen on the negative consequences of unsustainable practices, providing training on environmentally friendly techniques for capturing and caring marine life (Rubec *et al.*, 2001; Wabnitz *et al*., 2003; Palmtag, 2017). Several countries have implemented management plans with varying degrees of success, including strategies such as designated collection zones, marine protected areas, no-catch zones, rotational harvesting, bans, quotas, size and sex restrictions, equipment regulations, and export limits (Rubec *et al.*, 2001; Wabnitz *et al*., 2003; Dalabajan, 2005). Additionally, efforts have been made to educate consumers on the environmental impacts of destructive fishing methods, encouraging more sustainable choices. Organizations like WAZA (World Association of Zoos and Aquariums), EAZA (European Association of Zoos and Aquaria), EUAC (European Union of Aquarium Curators), have supported this initiative by offering to the public, hobbyist and consumers the means to promote and endorse sustainable practices (Gusset and Dick, 2010, 2011; Correia *et al*., 2024).

1.3 Role of public Aquariums

Public and private Aquariums are pivotal players in the marine aquarium industry, both relying on the trade of wild-caught and aquaculture-produced marine ornamental organisms to establish their exhibits and replicate natural ecosystems. These institutions significantly contribute to global efforts in environmental conservation, as outlined in the "World Zoo Conservation Strategy" (Barongi *et al*., 2015). In recent years, many Aquariums have adopted stringent ethical guidelines for animal acquisition, while a growing number are advancing breeding programs for marine ornamental species (Tlusty *et al*., 2013; Craggs *et al*., 2017; Correia *et al*., 2024; Martino *et al*., 2023, 2024). These initiatives are driving the development, testing, and application of novel

breeding practices on a commercial scale, raising a more sustainable marine ornamental industry (Tlusty *et al*., 2013; Olivotto *et al*., 2017).

Research in marine ornamentals aquaculture plays a crucial role in increasing the diversity of species available and enhancing the efficiency and sustainability of the industry. In this context, public Aquariums serve as essential hubs for promoting sustainability and biodiversity conservation through the integration of educational and research activities (Hutchins and Thompson, 2008; Conde *et al*., 2011; Mann *et al*., 2014). Additionally, Aquariums often collaborate with universities, research institutions, and specialized companies by providing marine ornamental eggs collected from tanks (Craggs *et al*., 2017; Martino *et al*., 2024). While individual institutions initially implemented these practices, they are now incorporated into a broader global strategy through national and international networks such as EAZA, WAZA and EUAC (Correia *et al*., 2024). Thus, beyond their roles in entertainment, education, and conservation, Aquariums hold immense potential for both fundamental and applied research in areas such as breeding, nutrition, physiology, and reproduction of cultured species (Hutchins and Thompson, 2008; Gusset and Dick, 2010, 2011; Conde *et al*., 2011; Correia *et al*., 2024; Martino *et al*., 2023, 2024).

1.4 Aquaculture of marine ornamental species

The limited success of the marine ornamental aquaculture industry can be attributed to several factors, mostly the difficulty in obtaining high-quality eggs and larvae, along with the lack of knowledge regarding their nutritional requirements and specific husbandry needs, which are all essential for successful production (Tlusty *et al*., 2002; Olivotto *et al*., 2011; Pouil *et al*., 2019). In response to these challenges, there is growing interest in developing sustainable production methods for the most commercially traded species (Olivotto *et al*., 2017). This approach aims to enhance the breeding of marine ornamental species both at their sources and in countries with high demand (Wabnitz *et al*., 2003). Therefore, aquaculture of marine fish and invertebrates has the

potential to reduce environmental impacts by decreasing collection of wild specimens from natural habitats (Chen *et al.*, 2019; Watson *et al.*, 2023). One of the main benefits of research in ornamental aquaculture is the deepening of species biology.

The experimentation of new breeding techniques, particularly concerning the culture and feeding of larvae, often results in methodologies that can be adapted from related species (Olivotto *et al*., 2017; Chen *et al.*, 2019; Watson *et al.*, 2023). Research focuses on the biology of commercially important species, innovative rearing techniques, alternative live feeds, and the welfare of cultured organisms can lead to the production of specimens under controlled conditions that, when compared to those harvest from the wild, are more efficient to cope with the husbandry conditions and environmental adjustments associated with the Aquarium industry (Wittenrich, 2007; Olivotto *et al*., 2011, 2017). Moreover, aquaculture plays a key role in preserving the marine genetic diversity, contributing to the restock of endangered populations (Tlusty *et al*., 2013; Chen *et al.*, 2019; Pouil *et al*., 2019; Watson *et al.*, 2023). Ultimately, the marine ornamental aquaculture sector not only addresses current market demands but also promotes a more sustainable relationship between the marine ornamental trade and marine conservation efforts.

1.5 Broodstock and spawning management

The selection of broodstock is critical in the aquaculture process, as it significantly influences production outcomes (Chen *et al*., 2019). Depending on the species, husbandry procedures must be adapted to the broostock needs, which require a comprehensive understanding of the biological, physiological, and ecological characteristics of the target species (Olivotto *et al*., 2011). Once optimal practices for maintaining organisms in controlled conditions and completing their life cycles are established, production techniques can be refined, leading to the selection of superior breeders and the initiation of selective breeding programs aimed at producing organisms better adapted to aquaculture conditions (Chen *et al*., 2019).

Additionally, selective breeding programs may be employed to enhance specific traits that increase the desirability of animals within the marine ornamental trade (Olivotto *et al*., 2017). Generally, fish present different reproductive strategies, including gonochorism, hermaphroditism, and parthenogenesis (Munday *et al*., 2006; De Mitcheson and Liu, 2008; Kuwamura *et al*., 2020). The most common strategy among marine fishes is gonochorism, where individuals are distinctly male or female, and this differentiation remains fixed throughout their lifespan (Munday *et al*., 2006). Other strategies are also present in fish, such as hermaphroditism where fish change sex during their lifetime, being (i) sequential with protogyny (female to male) or protandry (male to female) as the most common forms (i.e. dottybacks and clownfish, including *Pseudochromis* and *Amphiprion* genus among others) (De Mitcheson and Liu, 2008; Kuwamura *et al*., 2020), or (ii) simultaneous hermaphroditism, where individuals possess both male and female reproductive organs simultaneously (i.e. hamlets, including genus *Hypoplectrus*) (De Mitcheson and Liu, 2008; Kuwamura *et al*., 2020). Finally, parthenogenesis, rare among marine fishes (i.e. zebra shark, *Stegostoma tigrinum*) and more frequent in freshwater fish such (i.e. livebearers, including genus *Poecilia*), where individuals can reproduce without males, (Kuwamura *et al*., 2020; Feldheim *et al*., 2022).

On the other hand, the spawning behaviour in terms of egg dispersal, categorize fish in (i) demersal or (ii) pelagic spawners. Thus, demersal spawners produce eggs that remain in direct contact with the substrate, either adhering to solid surfaces or being deposited in small caves as gelatinous masses. This category includes also the mouthbrooders that incubate their eggs orally until hatching (Wittenrich, 2007). Parental care is a characteristic feature of demersal spawners, such as clownfish, gobies, damselfish, blennies dottybacks and jawfishes (Shei *et al*., 2017). In contrast, pelagic spawners release eggs into the water column during brief spawning events, resulting in a greater number of eggs that disperse rapidly, precluding parental care. Notable pelagic spawners include snappers, angelfishes, and groupers, which release their eggs as part of

the plankton (Holt *et al*., 2017). The life cycle of pelagic spawners commences when males and females swim in proximity, broadcasting their eggs and sperm into the water. This reproductive strategy is the most prevalent in marine environments (Wittenrich, 2007).

The collection of pelagic eggs in aquaculture facilities and public Aquariums represents a significant challenge, being embryos susceptible to mechanical damage, particularly when traditional collecting methods are employed. In addition, the difficulty in predicting the precise timing, location of gamete release, and the duration of their permanence in the water column, coupled with the continuous movement of fish during spawning events, makes it extremely challenging to determine the exact point for collection. This issue is particularly pronounced in public Aquariums, where significant quantities of viable eggs are inadvertently lost through filtration systems and underutilized, representing a missed opportunity for breeding programs or educational initiatives. Developing innovative systems for fish eggs collection is essential to enhance reproduction success and sustainable practices, maximizing the management of pelagic-spawning species in public Aquariums.

1.5.1 Case of study: Eggs collection and development of new systems

Efficient egg collection is a critical factor in the productivity of aquaculture and Aquarium facilities. The dynamics of water flow in the broodstock tanks are crucial and should be established prior to isolating the target species (Chen *et al*., 2019). These dynamics not only influence water renewal and the removal of organic matter but can also impact egg collection efficiency (Olivotto *et al*., 2017). For ornamental species that produce adhesive eggs on solid substrates (demersal), the spawning can be easily removed and transferred to hatchery tanks (Olivotto *et al*., 2017). In contrast, collecting eggs released into the water column requires the use of an egg collector (Ohs *et al*., 2019). Egg collectors can be positioned either within the broodstock tank or externally at the outlet pipe (Ohs *et al*., 2019). When the volume or surface

area of the broodstock tank (such tanks > 100.000 L) does not permit the use of an external collector, an internal collector becomes necessary (Ohs *et al*., 2019). The most employed collectors are air-lift egg collectors, which are installed directly within the broodstock tanks. These systems consist of a screened receptacle (such as a bucket or box) equipped with floats and surrounded by small air-lifts. The air-lifts, which consist of vertical PVC pipes connected to an air supply, facilitate the transfer of surface water from the broodstock tank into the collector through an airflow, while a mesh screen retains the eggs (Ohs *et al*., 2019). However, these systems typically exhibit slow water flow, which limits their efficiency in collecting pelagic eggs from larger tanks or pools. Additionally, the high airflow generated by the bubbling within the air-lifts can stress and damage the collected eggs. Therefore, there is an increasing need to enhance egg collection techniques to maximize the number of eggs harvested during spawning events while ensuring their viability.

In this doctoral thesis, one chapter focuses on the development of an innovative device specifically designed for the efficient collection of fish eggs in large tanks, commonly used in aquaculture facilities and public Aquarium. The findings aim to enhance harvesting efficiency, thereby supporting the acquisition of high-quality eggs for fish production industry.

1.6 The importance of live feeds

Zooplankton is the primary food source for many fish and their larvae as well as other organisms in the wild. Consequently, live prey remains essential in the larval rearing process in most hatcheries (Hamre *et al.,* 2013, 2018; Hansen and Möller, 2021). Generally, early-stage fish larvae do not respond well to formulated diets or frozen feeds as their first food source. Instead, they require live prey, which stimulates their natural predatory behaviour and fulfils their nutritional needs (Olivotto *et al*., 2017; Samat *et al*., 2020). However, the production of live feed organisms is

resource-intensive, requiring significant expertise in nutrition, zoology, and microbiology (Hansen and Möller, 2021). Microalgae play a critical role in marine aquaculture as the foundation of the food web, particularly for the cultivation of live prey organisms such as rotifers, *Artemia sp.*, *Daphnia sp*., and copepods, which are in turn fed to fish larvae. Microalgae provide essential nutrients, including fatty acids, amino acids, vitamins and pigments that contribute to the health and development of these live feed organisms. Commonly used microalgae species include *Rhodomonas sp.*, *Isochrysis sp.*, *Nannochloropsis sp*., *Tetraselmis sp.*, which have multiple applications in pharmaceutics, bioremediation, biofuels and nutrition industry. They are particularly valuable in marine hatcheries due to their high content of polyunsaturated fatty acids (PUFAs) and other essential nutrients (Drillet *et al.*, 2006a; Ismar *et al.*, 2008; Guevara *et al.,* 2010; Zhang *et al*., 2013; Sirakov *et al*., 2015). Advances in microalgae culture techniques have allowed for higher productivity and more consistent prey quality, making them a cornerstone in the larviculture of marine ornamental species (Rasdi and Qin, 2014). The nutritional value of microalgae can be optimized through culture conditions, ensuring that prey organisms, such as rotifers and copepods, fulfil the dietary needs of fish larvae.

Most marine ornamentals are reared using rotifers (*Brachionus plicatilis* and *Brachionus rotundiformis*) and *Artemia sp.* nauplii due to their ease of culture at high densities (Eryalçin, 2018, 2019; Eryalçın and Tınkır, 2024). However, these organisms do not constitute the natural prey of fish larvae, may be too large for certain smaller larvae, lack essential nutrients, and often cause digestibility issues, leading to nutrient uptake deficiencies in certain species (Otero-Ferrer *et al.*, 2010; Eryalçin, 2018, 2019; Olivotto *et al*., 2017; Radhakrishnan *et al.*, 2019).

Copepods, the natural prey of many marine fish species during their larval stages, have garnered significant attention for their potential in aquaculture and hatcheries (Ajiboye *et al.*, 2011; Hamre *et al*., 2013, 2018; Radhakrishnan *et al.*, 2019; Samat *et al*., 2020). Numerous copepod species have been employed in the intensive culture of fish larvae, particularly from the

order Calanoida, including *Acartia spp*. (Schipp *et al*., 1999), *Eurytemora spp*. (Shields *et al*., 1999), *Parvocalanus spp*. (Olivotto *et al*., 2006a), and *Centropages typicus* (Olivotto *et al*., 2008b). Additionally, species from the order Harpacticoida, such as *Euterpina acutifrons* (Kraul *et al*., 1992), *Tisbe* spp. (Olivotto *et al*., 2008a), and *Tigriopus japonicus* (Fukusho, 1980), have also been investigated. Calanoid copepods, in particular, have demonstrated better results due to different factors: (i) their high content of long chain polyunsaturated fatty acids (LC-PUFAs), (ii) their pelagic nature, making them more accessible to larval fish, and (iii) their small nauplius stages, which are easier for larvae with a narrow mouth gape to capture (Payne and Rippingale, 2001;Olivotto *et al.*, 2006b; Ajiboye *et al.*, 2011; Rasdi and Qin, 2014; Radhakrishnan *et al.*, 2019). Studies have shown that providing calanoid copepods as live feed increases survival and growth rates in species such striped fangblenny (*Meiacanthus grammistes*) (Olivotto *et al.*, 2010), *Hippocampus* spp. (Job *et al*., 2006; Olivotto *et al.*, 2008c), flame angelfish (*Centropyge loriculus*) (Laidley *et al*., 2008), lemonpeel angelfish (*Centropyge flavissimus*) (Olivotto *et al.*, 2006a*),* yellow tang *(Zebrasoma flavescens*) (Pereira-Davison and Callan, 2017), palette surgeonfish (*Paracanthurus hepatus*) (DiMaggio *et al.*, 2017), mandarinfish (*Synchiropus splendidus*) (Zeng *et al.*, 2018). Therefore, the use of copepods, in particular calanoids, could significantly improve fish larvae survival and growth, reducing the need for wild specimen collection, contributing to a more sustainable and resilient ornamental fish trade (Tlusty, 2002). Despite their benefits, the continuous production of calanoid copepods remains challenging due to the need for large culture volumes, low-density cultivation, and their selective feeding behaviour, which requires mixed live algae (Holt, 2003).

1.6.1 Case of study: The Calanoid copepod *Acartia tonsa*

The use of copepods, particularly the mass production of a cosmopolitan estuarine species, the calanoid *Acartia tonsa* via cold-stored subitaneous eggs, has therefore gained interest in

aquaculture hatcheries as a promising alternative to traditional live feeds, supported by advances in technology (Drillet *et al*., 2006a, b, 2007, 2011; Ajiboye *et al*., 2011; Camus, 2012; Zeng *et al*., 2018). *Acartia tonsa*, a cosmopolitan estuarine species, is widely used in biological and ecotoxicology studies (Gorbi *et al*., 2012) and as live prey in aquaculture (Støttrup, 2000, 2006; Drillet *et al*., 2011). However, these cold-stored copepod embryos maintain some physiological activity during cold storing, which can result in changes to their nutritional quality over time (Støttrup *et al*., 1999; Drillet *et al*., 2006a, b, 2011). The most significant biochemical alterations observed during storage are related to a depletion of the content of fatty acids (FAs) of the embryos, particularly LC-PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Drillet *et al*., 2006b). Without proper enrichment, the depletion of those important FAs can negatively affect the survival and development of fish larvae (Drillet *et al*., 2006b). Besides, calanoid copepods, as many other natural preys for marine fish larvae (Støttrup, 2000), lack the ability to elongate and desaturate 18:3n-3 fatty acids into the essential LC-PUFAs, such as EPA and DHA. Their high content in n-3 LC-PUFAs is incorporated directly from their phytoplankton diet, exhibiting reduced growth and egg production when fed diets deficient in EPA and DHA (Støttrup and Jensen, 1990; Jonasdottir *et al*., 1995; Monroig *et al.,* 2022). Despite the importance of those fatty acids, there is limited information on effective protocols to enrich copepods with essential and trace elements to meet the dietary needs of marine fish larvae (Payne *et al*., 2001; Sun *et al*., 2013).

In this doctoral thesis, one chapter focus on addressing key challenges in the culture and enrichment of *A. tonsa*, specifically using live microalgae binary diets, in comparison to conventional live preys as rotifers (*B. plicatilis*). The findings contribute to advancing production of high-quality live feeds for larviculture industry, providing a sustainable alternative.

1.7 Marine ornamentals larviculture

Fish larvae are particularly delicate during the early stages of their life cycle, requiring optimal culture conditions to survive and develop. A persistent challenge in marine ornamental fish species aquaculture is the feeding of larvae during early developmental stages, particularly the transition from endogenous to exogenous feeding (Holt, 2003; Olivotto *et al*., 2017; Callan *et al.*, 2018; Hansen and Möller, 2021). Traditional live prey, such as rotifers and *Artemia salina*, often fail to support optimal survival and growth due to inadequate nutritional profiles and inappropriate prey size (Faulk and Holt, 2005; Otero-Ferrer *et al.*, 2010; Eryalçin, 2018, 2019; Olivotto *et al*., 2017; Radhakrishnan *et al.*, 2019). As previously mentioned, copepods are emerging as a promising alternative to conventional live preys, offering superior nutrition and size compatibility for larval culture across various species (Ajiboye *et al.*, 2011; Hamre *et al*., 2018; Radhakrishnan *et al.*, 2019; Samat *et al*., 2020). To ensure successful rearing in aquaculture, larval culture systems must closely replicate the open ocean environment where food is abundant, and water quality is high (Olivotto *et al*., 2017; Samat *et al*., 2020). The practice to include microalgae within the rearing environment of larval fish, well known as “green waters”, is a common method employed in larvae culture which offer several benefits such as (i) improved water quality, maintaining a positive dissolved oxygen balance, reducing nitrogen compounds and pathogenic bacteria; (ii) improved larval predatory capacity, promoting low light intensity and digestive enzyme activity; (iii) enhanced live feeds survival, increasing their nutritional value before being consumed by larvae (Setu *et al.*, 2010; Neori, 2011; Basford *et al*., 2021; Eryalçın and Tınkır, 2024). Typically, larval rearing tanks feature darkened sides, to reduce light reflection and facilitate prey recognition. Lighting is crucial and should only come from above to prevent disorientation in the larvae. Round tanks, devoid of corners, are preferable as they promote even distribution of both larvae and prey. The size and shape of the tank significantly affect the success of larval rearing (Cañedo-Orihuela *et al*., 2023). Small tanks, often referred to as microcosms, are ideal for

concentrating larvae and their live prey. Microcosms are suitable for rearing one or a few species per tank but require careful attention to system management and water parameters (Cañedo-Orihuela *et al*., 2023). The primary concern with smaller tanks is maintaining the water quality, particularly in managing nitrogen compounds like ammonia, which can quickly rise leading to high mortality rates (Olivotto *et al*., 2011). In contrast, larger tanks, or mesocosms, are more stable and offer additional benefits due to their greater water volume. Mesocosms are characterized by lower animal densities, leading to better water quality conditions and improved control of environmental variables (Shields, 2001). While their biomass output per unit volume may be lower than microcosms, mesocosms offer greater stability and realism, particularly in laboratory or field-based experiments. Maintaining water quality in closed or semi-closed systems can be challenging but is increasingly feasible with the adoption of efficient recirculating aquaculture systems (RAS). The use of RAS involve centralized filtration systems that connect multiple small tanks to a single filtration unit, thereby stabilizing water quality, reducing maintenance time, and minimizing stress on the larvae.

1.7.1 Case of study: The orchid dottyback *Pseudochromis fridmani*

The *Pseudochromidae* family, commonly known as dottybacks, features among the most popular cultured marine ornamental fish along with damselfish (*Pomacentridae*) (Thresher, 1984; Wittenrich, 2007). Their popularity in the ornamental aquaculture industry stems from their beauty, bright liveries, resilience and relatively small size, making them highly suitable for ornamental fish farming (Wittenrich, 2007; Palmtag, 2017). Dottybacks are distributed across the tropical regions of the Pacific and Indian Oceans (Lubbock, 1975; Gill, 2004) and divided in more than 150 species grouped into 24 genera, while new species are still being discovered (Gill, 2004; Gill and Allen 2011; Gill *et al*. 2012). Many dottybacks species exhibit protogynous hermaphroditism, with the ability to change sex bidirectionally observed

in some species (Wittenrich and Munday, 2005; Kuwamura *et al*., 2014). The orchid dottyback, *Pseudochromis fridmani* (Klausewitz, 1968), native to the Red Sea, is one of the most commercially valuable species in the marine ornamental trade (Wittenrich, 2007). Thus, a breeding protocol for this species is of extreme interest, as beside to reducing pressure on wild stocks, it could be an alternative income source for the trade. However, to date limited scientific data exist regarding its culture under controlled conditions (Brons, 1996; Moe, 1997; Wittenrich, 2007; Mies *et al*., 2014; Chen and Zeng, 2021a b; Chen *et al*., 2023). In the literature, past studies on reproductive biology of *P. fridmani* lacks in detailed description as well as in-depth analysis (Brons, 1996; Moe, 1997). The most critical challenges are related to the broodstock management, and to the handling of early developmental stages (Mies *et al*., 2014; Chen and Zeng, 2021a b; Chen *et al*., 2023). Broodstock formation is often a difficult task considering that *P. fridmani* usually present extreme intraspecific aggressive behaviour that may result in death of the weaker specimens (Wittenrich, 2007; Mies *et al*., 2014). In addition, when spawning activity begins, the female lays a demersal egg mass that may be protected or cannibalized by the male (filial cannibalism) before hatching occurs (Wittenrich, 2007; Mies *et al*., 2014, Chen *et al*., 2023). Although various studies have documented egg cannibalism in *P. fridmani*, the specific mechanism behind this behaviour remains unknown, posing a significant bottleneck for aquaculture production of this and many other dottyback species. (Sargent, 1992; Mies *et al*., 2014; Shei *et al*., 2017; Chen *et al.*, 2023). Similarly, although its larviculture has been successfully achieved under controlled conditions using calanoid copepods and "green water" techniques (Chen and Zeng, 2021a, b), high mortality rates linked to physiological changes and nutritional deficiencies continue to hinder its mass production (Chen and Zeng, 2021a, b).

In this doctoral thesis, two chapters focus on addressing key challenges in the aquaculture of *P. fridmani*, specifically related to broodstock management, and feeding of early

developmental stage up to 15 days post-hatch (dph). The findings contribute to advancing mass production techniques, providing a sustainable alternative to the capture of wild specimens.

1.8 Objectives

The present doctoral thesis aims to strengthen the marine ornamental industry through innovative practices in public Aquariums developing sustainable culture techniques for marine ornamental fish species, including eggs collection management, and the development of high-quality live preys to improve larval quality. Besides, it is also aimed at improving the production of fish species in a public Aquarium, using the marine ornamental fish orchid dottyback (*Pseudochromis fridmani*) as an industrial case study.

To achieve the general objective; the following specific objectives were addressed:

- To develop an advanced fish egg collection system that addresses the need to effectively utilize the potential of large-volume tanks exhibited in public Aquariums, which host a diverse range of regularly spawning species, while optimizing the management and increasing the number of collected eggs available for conservation and research projects.
- To improve the broodstock husbandry of the orchid dottyback, *Pseudochromis fridmani* (Klausewitz, 1968), through the fish conditioning, pair formation and new insights on reproductive biology of the species.
- To develop an enrichment protocol for live feeds based on microalgae, assessing its effects on the survival and biochemical composition of the calanoid copepod *Acartia tonsa* (Dana, 1849) *versus* the rotifer *Brachionus plicatilis* (Müller, 1786).
- To improve the orchid dottyback *Pseudochromis fridmani* larval production through the use of high-quality live prey.

Chapter 2. General Materials and Methods

2.1 General design

In alignment with the outlined objectives, the research conducted within this Doctoral Thesis is structured around four key studies that aim to advance sustainable aquaculture techniques at industrial scale for high demanded marine ornamental fish species, leveraging natural spawning events in the exhibit and curatorial large-volume tanks of public Aquariums. A schematic diagram of the thesis is presented in Fig. 2.1.

- **The first study** (Chapter 3) details the development of a novel patented fish egg collection device designed for large-scale tanks, comparing its performance with traditional air-lift egg collectors in terms of efficiency, flow dynamics, and yield of collected eggs.
- **The second study** (Chapter 4) focuses on optimizing the broodstock management of the orchid dottyback *Pseudochromis fridmani* under controlled conditions. This research establishes comprehensive husbandry guidelines for this (Fig 2.2).
- **The third study** (Chapter 5) assesses the effects of nutritional enrichment using live microalgae mixtures on both conventional and novel live prey species. It evaluates the biochemical differences between the rotifer *Brachionus plicatilis* and the calanoid copepod *Acartia tonsa* for their application as first feed in the larviculture of marine ornamental fish. Furthermore, it offers a detailed comparison of the fatty acid profiles of the tested live preys (Fig 2.3).
- **The fourth study** (Chapter 6) applies the findings from the previous research to improve the production of *P. fridmani* larvae, through the implementation of enriched live preys in the larval culture of this species. It evaluates larval performance, tracks the nutritional composition throughout the early developmental stages, and describes the morphological changes in skeletal ossification, intestinal, and liver structures up to 15 days post-hatch

(dph). This study contribute to identify the minimum nutritional requirements necessary to optimize larval performance, allowing comparison between *P. fridmani* larvae development with that of other marine ornamental teleost, offering key insights to enhance early diet protocols and support sustainable production (Fig 2.4).

The whole experimentation was conducted in the quarantine areas belonging to the Tropical Department of the Aquarium of Genoa (Genoa, Liguria, Italy). All procedures involving animals were performed according to the Guide for Care and Use of Laboratory Animals of the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The collected samples were preserved at the Regional Agency for Environmental Protection–Liguria (ARPAL), Department of Organic Chemistry and Physical Analysis (Genoa, Liguria, Italy). Sample analysis was conducted at the laboratories of the Highly Specialized Aquaculture and Biotechnology Service (SABE) located at the Marine Scientific and Technological Park of ECOAQUA University Institute of the University of Las Palmas de Gran Canaria (Canary Islands, Spain).

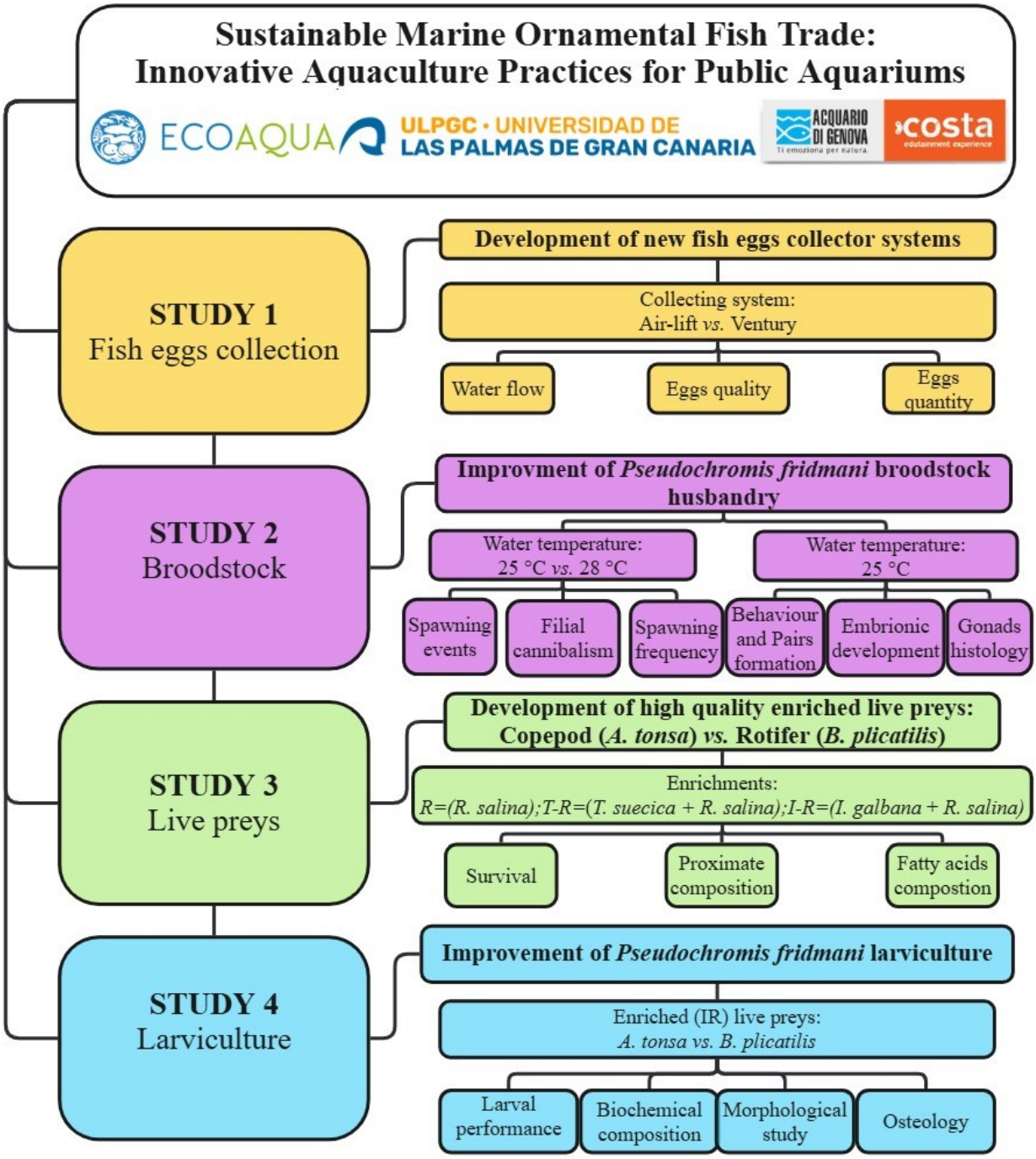

Figure 2.1: Thesis general design flow chart. R= *Rhodomonas salina*; T-R= *Tetraselmis suecica* + *Rhodomonas salina*; I-R= *Isochrysis galbana* + *Rhodomonas salina*

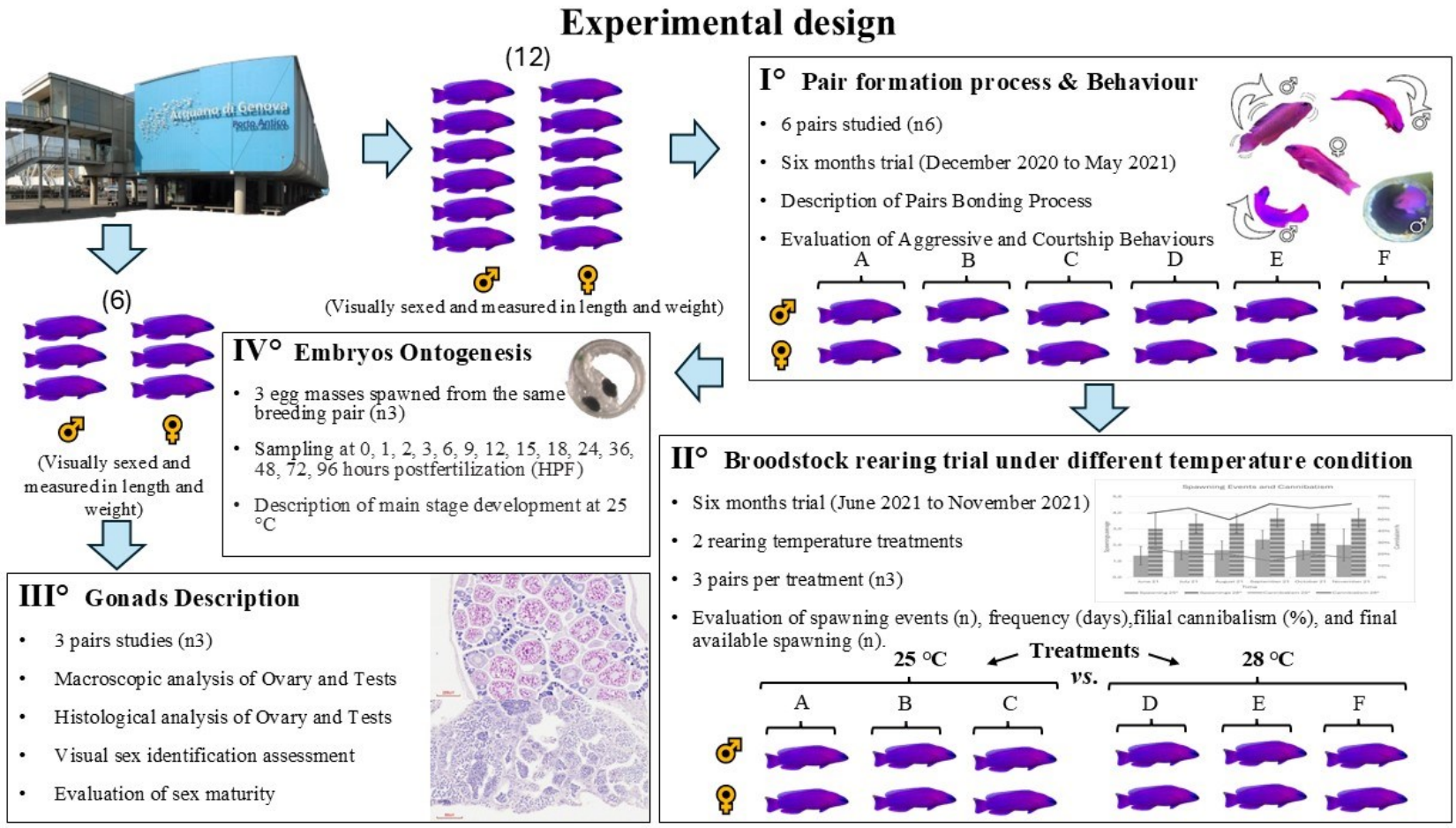

Figure 2.2: Study 2 experimental design: 6 pairs of *P. fridmani* were obtained and divided in two treatments, 25 °C and 28 °C, for six months studying the effects on spawning production, spawning frequency and filial cannibalism. In addition, a detailed description of pair formation behaviour, gonads histology and embryos ontogeny was performed.

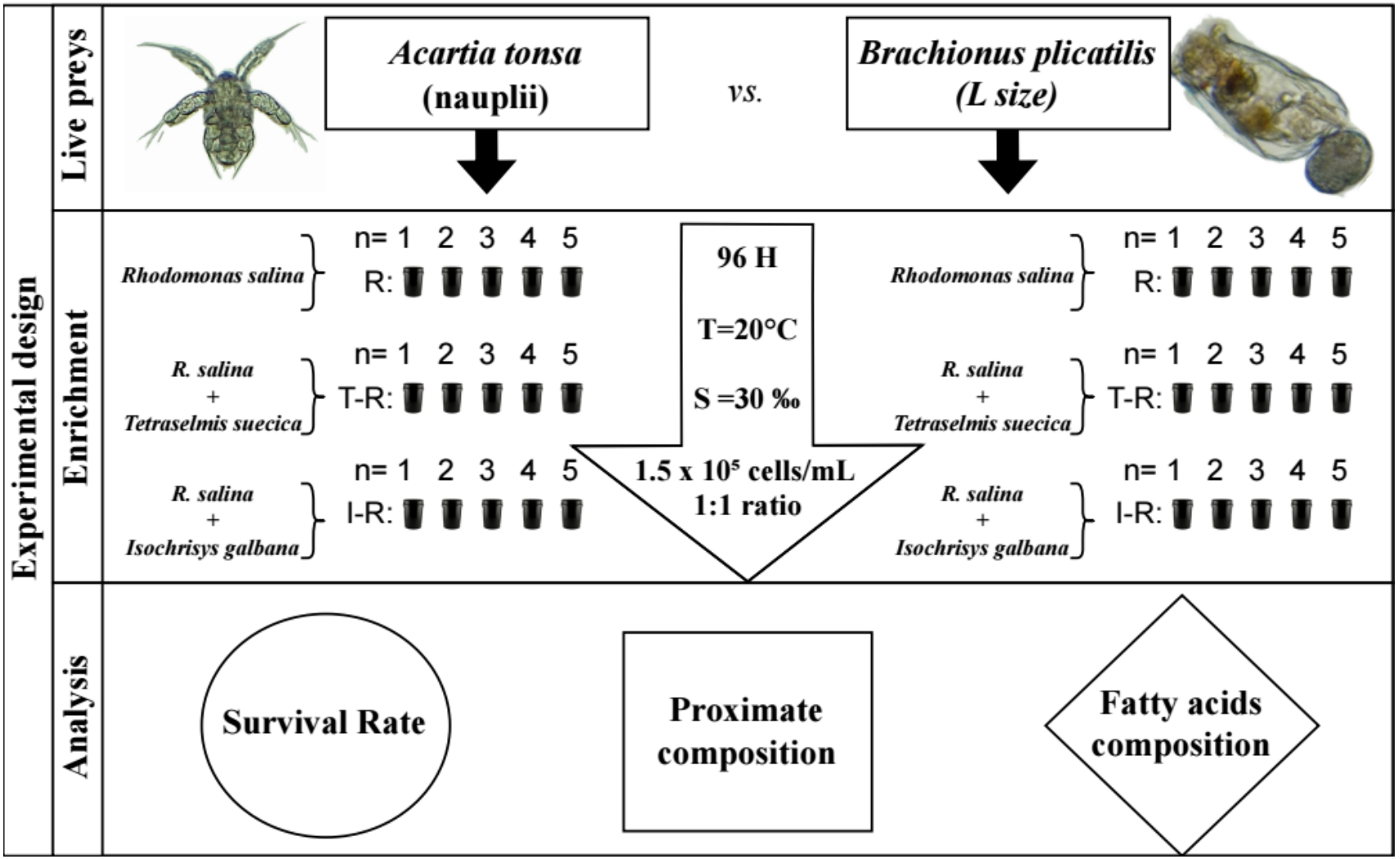

Figure 2.3: Study 3 experimental design: The copepod *A. tonsa* and the rotifer *B. plicatilis* were divided into three experimental feeding treatments (n = 5) and fed with the designated diets during 96 h. Effects on survival rate, proximate and fatty acids composition were studied. Abreviaturas: R= *Rhodomonas salina*; T-R= *Tetraselmis suecica* + *Rhodomonas salina*; I-R= *Isochrysis galbana* + *Rhodomonas salina*

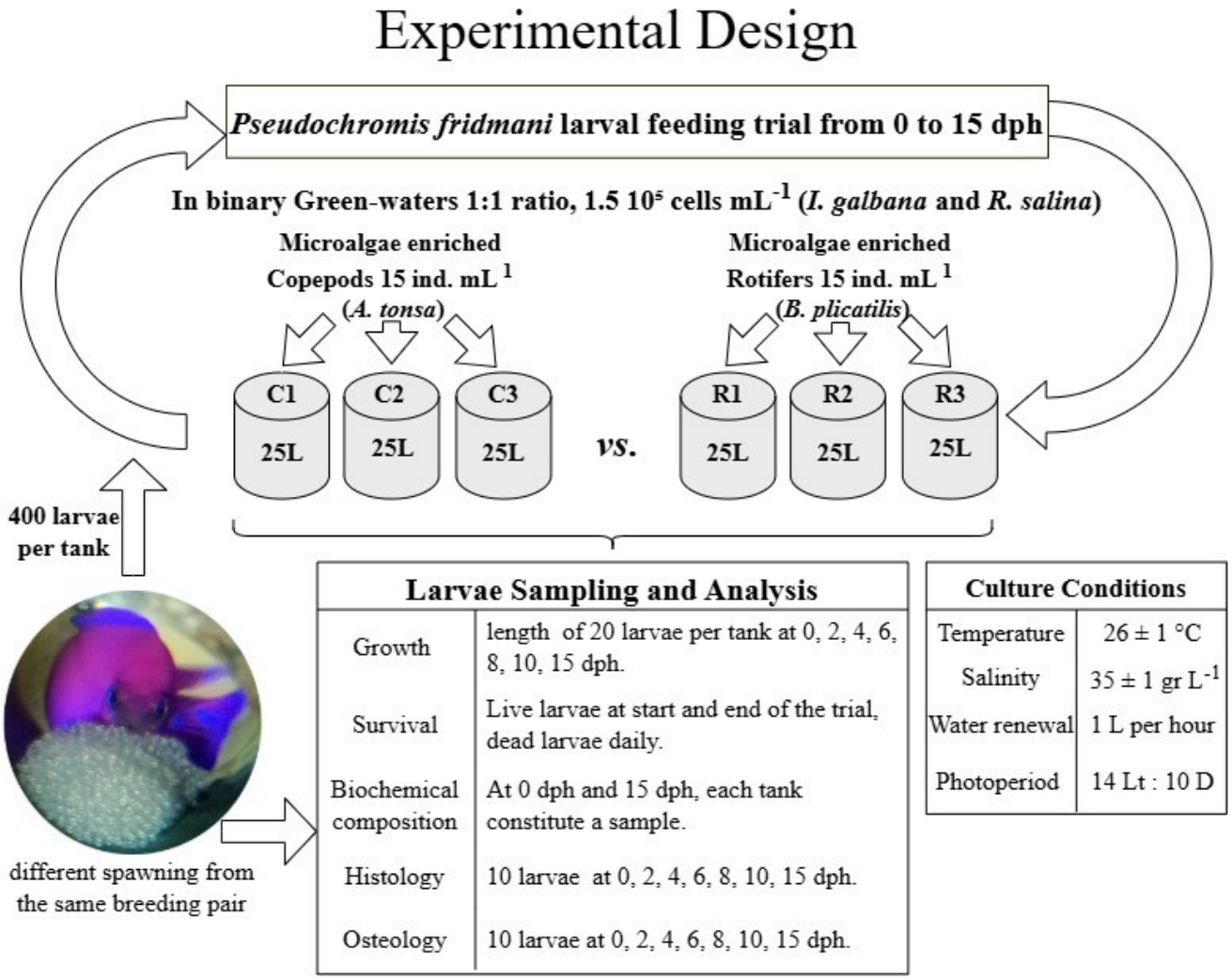

Figure 2.4: Study 4 experimental design: *Pseudochromis fridmani* larvae were divided in two experimental feeding treatments (n = 3) and fed with the designated diets for 15 days. Effects on growth, biochemical composition, histological modification of liver and intestine, and skeletal mineralization were studied. C= Copepods; R= Rotifers; L= Liters; Lt= Light; D= Dark.

2.2 Fish eggs collector devices

To assess the performance of the eggs collectors systems, Venturi *versus* Air-lift (Experiment 1, Chapter 3), a comprehensive study was conducted over a six-month period (September 2022 – February 2023). The invention presented in this document emerges from the challenge to optimize suction strength to effectively collect fish eggs without damaging them. Both the Venturi-based system and the traditional air-lift system were tested using same methodologies. The experimental setup was established in the "Tropical lagoon" tank (Aquarium of Genoa, Italy), a large exhibition space (*ca.* 200 m^3) that houses a diverse range of marine fish species (see section 2.2.3). This setting provided an ideal environment for testing the efficiency of the egg collection system under realistic aquaculture conditions. Both egg collectors were positioned daily at 4:00 PM and operated concurrently until the following day at 10:00 AM, for a total of 18 hours of collection throughout the night. Each day, the contents of the devices were harvested and concentrated into a one-litre beaker for subsequent abundance calculations. The eggs collected were counted using a stereomicroscope (EZ4, Leica, Germany), and their viability was assessed. The suction capacity (L/h) of each system was determined through pre-collection tests measuring the volume of water processed per hour. Data were recorded and compared to evaluate collection performance. Water quality parameters, including pH, salinity, and temperature, were closely monitored using manual probes to ensure precise measurements and maintain optimal conditions for egg spawning and collection.

2.2.1 Air-lift system

Collection of pelagic eggs with air-lift systems have been widely implemented during hatchery operations in aquaculture and Aquariums industry. These systems leverage air pressure to create turbulent water flows, allowing the transport of eggs from the broodstock rearing environment to collection devices. While air-lift systems have been effective in their

primary function, they are not without limitations. The turbulence generated during its operation can result in significant mechanical stress on the eggs, leading to damage and reduced viability. The air-lift egg collection system used in this study was designed with same structure and size of the Venturi eggs collector, changing only the form of water propulsion (Fig. 2.5). This system operated via a continuous flow of low-pressure air, which created an upward current in the collection tube. The airflow was carefully calibrated to prevent water overflowing and minimize eggs damage. Collected eggs were transferred into dedicated beaker for subsequent analysis.

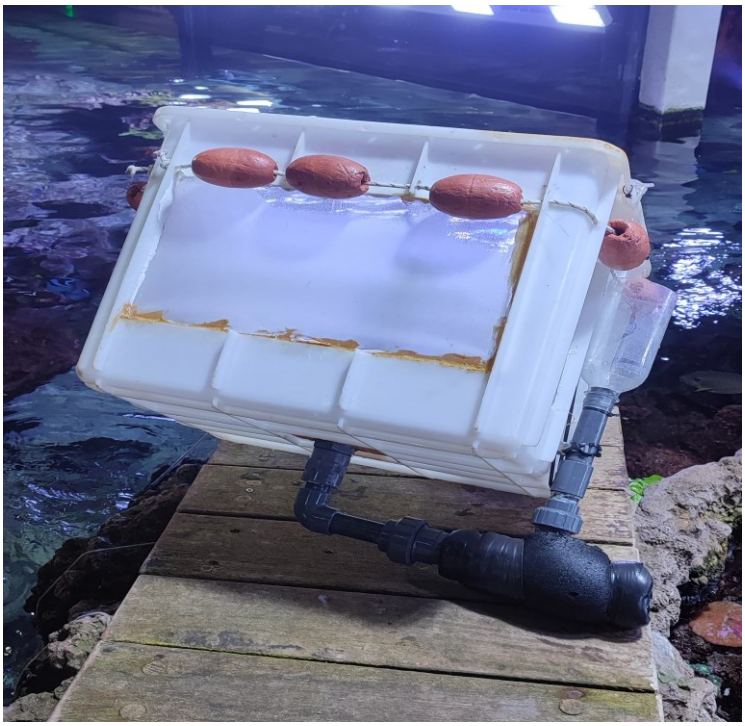

Figure 2.5: The Air-lift eggs collecting device employed during experimentation.

2.2.2 Development of new collecting systems

The new egg collection system utilizes a Venturi tube to enhance the collection process while minimizing mechanical stress on the eggs. This system involved the use of a Venturi collection tube connected to a low-pressure pump. By integrating the principles of Venturi dynamics into the egg collection process, the new system offers a significant advancement

over traditional air-lift methods. The Venturi tube design allows for the acceleration of water flow without inducing turbulence, ensuring that eggs are collected efficiently while maintaining their integrity. The system incorporates a water pump to drive the suction process eliminates the need for air compression, thereby addressing the issues associated with foam generation and eggs damage (Fig. 2.6). This innovation is poised to transform egg collection methodologies, offering a solution that enhances both efficiency and egg viability.

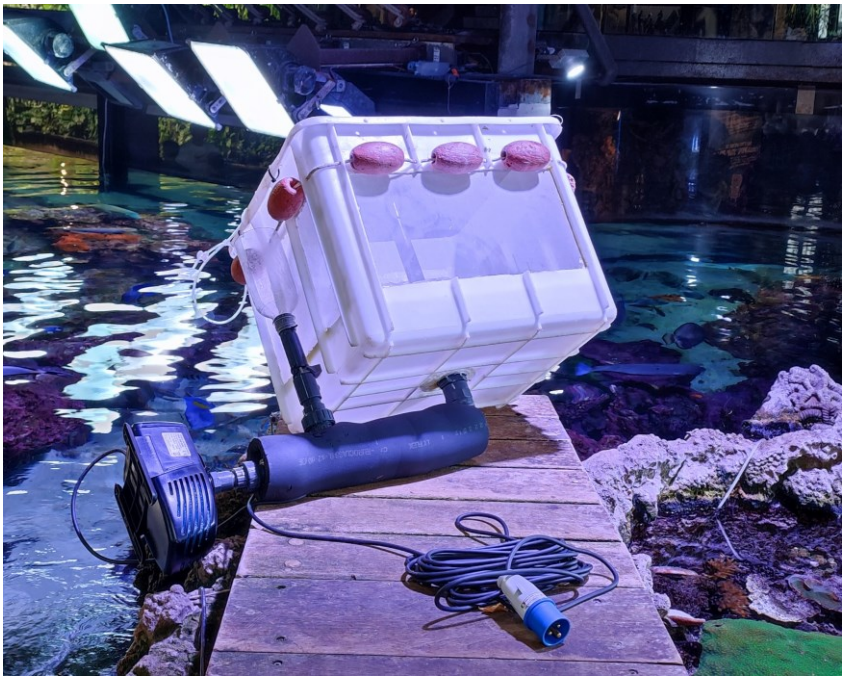

Figure 2.6: The new patented Venturi eggs collecting device configured with a dedicated submersible pump, positioned on the edge of the "Tropical lagoon" tank (NA9) at the Aquarium of Genoa (Italy).

2.2.3 Experimental designed tank in the Public Aquarium of Genoa (NA9)

The two collectors described in Experiment 1 were simultaneously tested from September 2022 to February 2023 in one of the main exhibit tanks of the Aquarium of Genoa (Italy), known as the “Tropical lagoon” (tank code NA9) (Fig. 2.7). This large-volume tank simulated the natural habitat of Indo-Pacific regions and, with a total volume of 190,000 litres, provided environmental conditions closely resembling those encountered in the wild. Spanning an area

of 220 m^2, it housed more than 90 species of tropical marine fishes and over 30 species of invertebrates.

The tank was equipped with advanced filtration systems powered by two 8 m^3/h pumps, including two biological filters, two sand filters, two ozone-supplied protein skimmers, and an ultraviolet sterilization system (Fig. 2.9). Aquarium maintenance adhered to strict protocols, including regular water changes and constant monitoring of water quality. Physicochemical parameters were measured three times per week, maintaining a pH of 8, salinity at 35 ‰, temperature at 25°C, NO_2 levels below 0.02 mg/L, and NH_4 levels below 0.02 mg/L throughout the testing period. Lighting was programmed to replicate natural day-night cycles, with varying light intensity to simulate daily fluctuations. Feeding procedures were meticulously managed by Aquarium staff, who provided fresh food (chopped fish, molluscs, and crustaceans) enriched with a vitamin mix powder to ensure optimal nutritional quality.

The collectors were activated in the afternoon from 16:00 to 9:00 the following morning, during which the presence of eggs was regularly checked. Cleaning and maintenance of the devices were conducted consistently throughout the testing period to ensure proper functionality and reliable data collection. When eggs were found in the morning, they were carefully concentrated in a beaker for subsequent analysis. Egg identification and evaluation of abundance and quality were performed by averaging three 1 mL^{-1} samples using a stereomicroscope (EZ4, LEICA, Germany), allowing for detailed viability examination (live/death).

During the testing period, both collectors were evaluated based on the quantity and viability of the eggs collected, as well as the volume of water processed (L/h) within the NA9 tank. Data from this evaluation, recorded over six months, excluded days with no spawning events. To simplify and minimize the margin of error during the comparison, 10 days were selected in which the target species, the batfish *Platax orbicularis*, spawned (Fig. 2.8). This selection

ensured a focused and consistent basis for evaluating the performance of the two collection systems. Furthermore, this approach offered a robust assessment of their efficiency and impact on egg quality under controlled yet realistic conditions.

Figure 2.7: The tropical marine exhibit tank "Tropical lagoon" (NA9), Aquarium of Genoa (Italy).

Figure 2.8: Group of batfish *Platax orbicularis* swimming in the "Tropical lagoon" (NA9), Aquarium of Genoa (Italy).

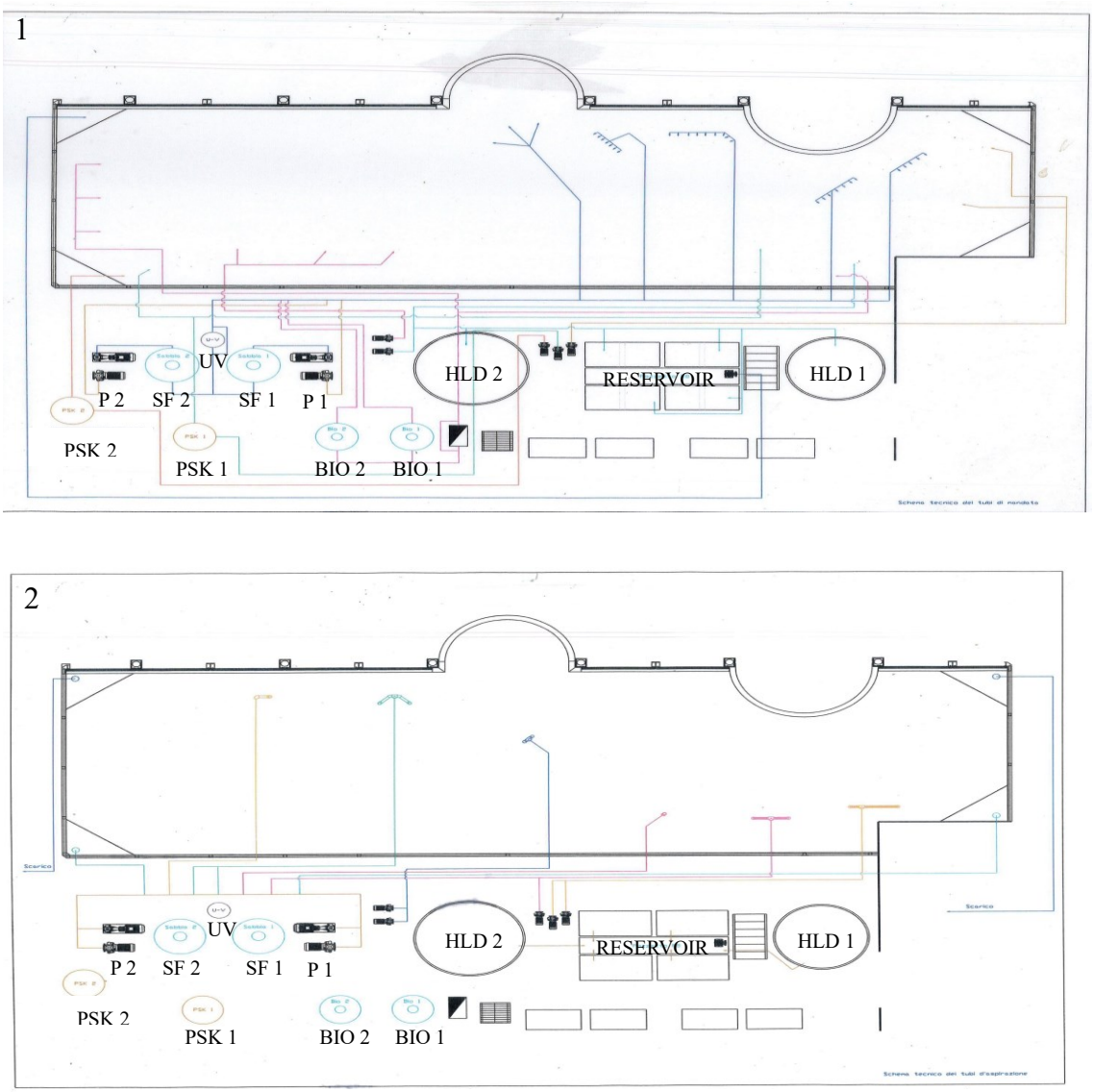

Figure 2.9: Filtration diagram of the "Tropical lagoon" (NA9) with detailed water inlet (1) and outlet (2). P= Main pump; PSK= Protein skimmer; SF= Sand filter; BIO= Biological filter; UV= Ultraviolet sterilization; HLD= Holding tank.

2.3 Broodstock rearing

The orchid dottybacks of Experiment 2 and 4 (Chapters 4 and 6) were obtained from a stock belonging to the Aquarium of Genoa (Genova, Liguria, Italy). The broodstock population consisted of 12 adult *P. fridmani*, which were measured (total length, weight) and acclimated in a customized recirculating system named "T1" (see section 2.3.1; Fig. 2.10). The selected broodstock, with total length (mean ± SD) 10.2 ± 1.2 cm and weight 8.4 ±1.7 g were originated from the same brood older than 1 year. The sorted specimens were split in 6 pairs and released

simultaneously in the assigned partitions of two glass aquaria. Each tank was divided in three partitions of approximately 133 L without any decoration or gravel. Each partition housed one *P. fridmani* pair and two polyvinyl chloride (PVC) pipe (30 cm long and 3.2 cm in diameter) to provide hiding places and breeding substrate where fish could spawn. Since *P. fridmani* do not present any sexual dichromatism or dimorphism, specimens were visually sexed, trying to identify females as smaller with a remarkably swollen belly, and males as larger and slender.

2.3.1 Recirculating system T1

System T1 was composed by 6 glass aquaria of 400 L, connected to a 500 L sump (Fig. 2.10). The experiment area was isolated to avoid visual interference in the behaviour and spawning activity by Aquarium staff. Water quality was ensured by a biological filtration, sand filter, UV sterilizer (PAN UV EASY 116, Panaque, Italy) and heat exchanger, all powered by the main pump (Sena, Astralpool, Italy). Temperature was maintained at 25 ± 1 °C, salinity at 35 ± 1 g L^{-1} and photoperiod at 12L:12D provided by two 30 W fluorescent lights tube (Master TL-D, Philips, Nederland) suspended 20 cm above each broodstock tank. Fishes were fed twice a day *ad libitum* using mixed diets based on marine chopped fish (cod, sardines), mussels, shrimps and vitamin premix (1 g*kg) combined with enriched live cultured mysids and frozen adult *Artemia* administrated once a day. A daily maintenance, which involved cleaning of the drain mesh and siphoning of feed waste and debris was carried out. Water samples from the tanks were collected three times a week to check the main parameters and keep Nitrogen compounds, NH_4 and NO_2, below 0.01 mg L^{-1}.

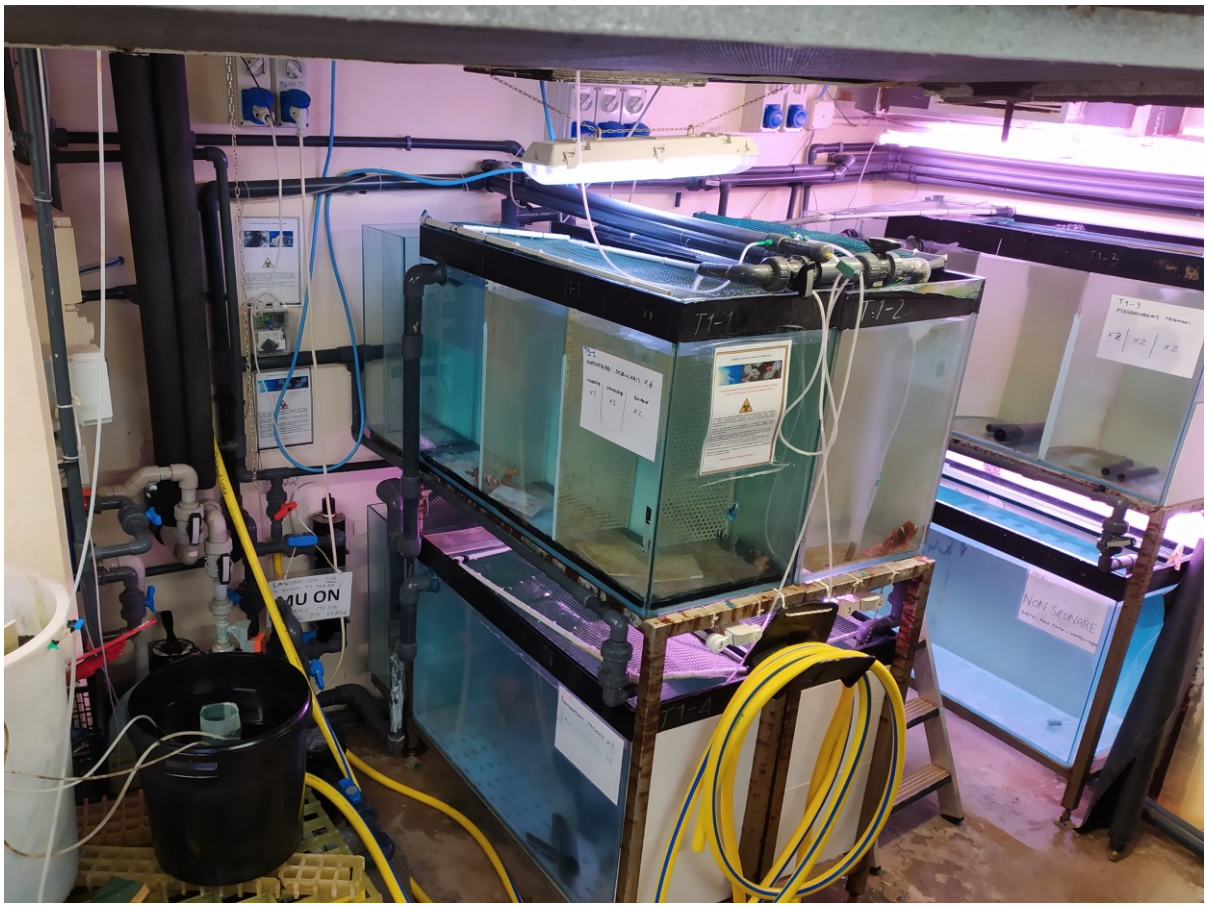

Figure 2.10: The broodstock system "T1" located in the tropical department quarantine area of the Aquarium of Genoa (Italy).

2.3.2 Behaviour

In Experiment 2 (Chapter 4) the reproductive and behavioural aspects of *P. fridmani* under controlled conditions were studied. Daily observations on the pair formation process of 6 candidate pairs were made during six months (from December 2020 to May 2021), in daytime (from 8:00 am to 6:00 pm), every 2 h (n= 6) over a 10 min period recording aggressive or courtship behaviour. A semi-quantitative score was defined ranging from 0 to 3, where 0, 1, 2 and 3 represented respectively absent, low, mild and high behavioural level. Animal observations of behaviour in general were made following recommendations published by Altmann (1974). When high level of aggressive behaviour in a pair was observed, the largest individuals were temporally separated in a transparent cage maintaining visual contact between specimens. Aggression was determined according to the level of stress and damage

inflicted by the dominant fish: (i) frayed fins; (ii) bite marks; (iii) constriction in a corner. Reproductive behaviour level was determined according to the courtship activity: (i) male swimming in out nest; (ii) male shaking open fins in front of the female; (iii) pair sharing same PVC pipe. A pair was considered formed when accepted food simultaneously, no aggressions were observed and started courtship producing first spawning.

2.3.3 Spawning activity

The effects of increased temperatures on spawning dynamic and filial cannibalism of orchid dottybacks was also tested in Experiment 2 (Chapter 4), during six month (from June 2021 to November 2021) the six pairs obtained (see section 2.1; Fig. 2.2) were divided in two groups (n= 3), exposed respectively to 25 ± 1 °C and to 28 ± 1 °C water temperature, and visually monitored. The aquarium tank selected for the 28 °C condition was equipped with a heater which progressively increased (3 days, 1° per day) and then maintained stable temperature during the trial. Pair spawning activity was monitored at least five times a day, and the PVC pipe chose by the male to create the nest was also visually inspected with a flashlight, to verify the presence of egg masses. The total number of spawning per month, the spawning frequency (days) and the total number of available spawning per month were measured. When spawning occurred, the eggs were left to the care of the male until hatch checking for possible filial cannibalism. The rate of filial cannibalism (FC) represented the monthly frequency of egg mass that was completely cannibalized by the brood fish during the incubation period. The FC was calculated as follow:

$$FC = \frac{\text{Number o f egg batches cannibalized in a month}}{\text{Total number of monthy spawnings}} \times 100\%$$

Since the 4th day post-fertilization (dpf), when eyes of the embryos became visible as black spot with silver reflections, selected egg mass were shifted from the broodstock tank to the larval culture system Modular Rearing System (MoRS) for the Experiment 4 (Chapter 6) (see

section 2.5). Hatching events were considered successful only when more than 90 % of the larvae hatched from the egg mass.

2.3.4 Embryonic development

The main embryonic development stages (morula, blastula, gastrula, etc.) of *P. fridmani* were identified and described in Experiment 2 (chapter 4). Three different spawning from the same breeding pair kept at 25 °C were selected for the embryonic development study (n=3) (see section 2.6.1). During the experiment morphological data were recorded, photographing embryos at 5x and 10x magnification with stereomicroscope (EZ4, Leica, Germany) connected to a digital camera (Motic, Xiamen, China). The diameters mean of 20 eggs was measured with a micrometre scale ocular.

2.4 Live preys rearing

2.4.1 Microalgae culture

The marine microalgae *Tetraselmis suecica* (Kylin) Butcher 1959 and *Isochrisys galbana* (Parke 1949) were already present in Aquarium of Genoa, while *Rhodomonas salina* (Wislouch) D.R.A. Hill & R. Wetherbee 1989 strain was obtained from the Mediterranean Culture Collection of Villefranche (MCCV118, EMBRC, France). These algae were used to fed and enrich experimental live preys and implement larval culture with the “green waters” technique (Experiment 3 and 4, Chapters 5 and 6, respectively). Algae were grown on a F/2 medium (Guillard and Ryther, 1962), with starter cultures scaled from 1 L to 5 L flasks before being inoculated into 100 L polycarbonate cylinders (Fig. 2.11) filed with 1 µm filtered seawater (cartridge filter, Swan®, Taiwan), salinity 30 ± 1 g L^{-1}, UV sterilized (PAN UV EASY 116, Panaque®, Italy) and mixed with air filtered with cotton. Cylinders were cleaned weekly to prevent cross-contamination and ensure that cultures remained in optimal

conditions. Cultures were kept at 20 ± 1 °C with 24 h light regime illumination based on 10.000 ºK led light (Fig. 2.11). Concentrations of cultures was determined every 3 days using a haemocytometer (Neubauer chamber) (Marienfeld Superior, Germany) to count by triplicate the number of cells mL^{-1} under a microscope (DME, Leica, Germany).

Figure 2.11: Microalgae cultured in the facilities of the Aquarium of Genoa, (Italy): a) *Tetraslmis suecica*; b) *Isochrisys galbana;* c) *Rhodomonas salina*; d) Culture room. The scale bar present in the down left corner of each microalgae species corresponds to 5µm.

2.4.2 Rotifers culture: *Brachionus plicatilis*

The main rotifer cultures of *B. plicatilis* (L strain), with a size range of 180-250 µm, employed in Experiment 3 and 4 (Chapters 5 and 6, respectively) was fed on *R. salina, I. galbana* and S.parkle® (INVE Aquaculture, Belgium) at constant salinity 30 ± 1 g L^{-1} and temperature 20 ± 1 °C in two transparent cylindrical tanks of 80 L each (Fig. 2.12). Cultures were maintained under gentle airflow and kept at an inversely proportional volume, with daily dilution or harvesting depending on the tank. Additionally, a 100% water change was performed weekly as part of periodic system cleaning to ensure a healthy environment. Cultures were 12 L/12 D (Light/Dark) illuminated by two 30 W fluorescent tubes (Fig. 2.12). Density estimations of rotifers were done daily by averaging three 1 mL^{-1} samples trough a stereomicroscope (EZ4, LEICA, Germany) to maintain optimal population levels, preventing overcrowding, which could degrade water quality.

Figure 2.12: Rotifers (*Brachionus plicatilis*) culture facilities.

2.4.3 Copepods culture: *Acartia tonsa*

The calanoid copepod *A. tonsa,* with a range size of 70 µm (newly hatched nauplii) till 1.5 mm (adult females) employed in Experiment 3 and 4 (Chapters 5 and 6 respectively), was obtained from the commercial product "Copepod Eggs" distributed by Algova® (Germany), cists were hatched following the company's instructions. The nauplii hatched were cultured in 100 L polycarbonate cylinders, maintained at a temperature of 25 ± 1 °C, salinity 35 ± 1 g L^{-}, and fed equally on *R. salina* and *I. galbana.* Cultures were moved by a gentle airflow and renewed weekly with a 100% water change. The copepod population was monitored daily by averaging three 1 mL samples under a stereomicroscope (EZ4, Leica, Germany), ensuring that densities were sufficient to meet the nutritional needs and there was not contamination.

2.4.4 Live preys enrichment

In Experiment 3 (Chapter 5) the relationship between the changes of proximal and fatty acids compositions depending on food source was studied, to produce high-quality live feeds. Copepod *A. tonsa* used in the Experiment 3 (Chapter 5) were obtained from cold stored eggs (Algova®, Germany) and hatched following the instructions provided by the producer, while rotifers *B. plicatilis* were collected from the culture tanks (see section 2.4.2). Copepods eggs were washed from the buffer solution with 50 µm mesh sieve, inoculated in marine water and incubated during 24 hours at 24 ± 1 °C in a glass flask at the density of 40.000 eggs L^{-1} with air bubbling. The newly hatched nauplii of *A. tonsa* at N I stage and the rotifer *B. plicatilis* were immediately concentrated using a 50 µm mesh sieve and counted under a stereomicroscope in triplicate (EZ4, Leica, Germany). The experiment was launched in the MoRS facility (see section 2.5.1), after splitting live preys into three experimental feeding treatments (n = 5) as follows: Treatment R (control): mono algal diet based on *R. salina*; Treatment T-R: binary diet (1:1 ratio) based on *T. suecica* and *R. salina*; Treatment I-R:

binary diet (1:1 ratio) based on *I. galbana* and *R. salina*. Tanks were kept at 20 ± 1 °C, 30 ‰ salinity and 14L/10D photoperiod, checking water quality parameters daily. All microalgae treatments were formulated with a ratio of 1:1 based on cell count, chosen to facilitate the application of the enrichment and the reintegration of the consumed algae. Starting culture density was established in 10 ind. mL^{-1} for *A. tonsa* and 280 ind. mL^{-1} for *B. plicatilis* by counting individuals as described for zooplankton cultures (see section 2.4.2). To estimate the survival rate of each experimental treatment a daily counting of copepods nauplii and rotifers was carried out by triplicate in each tank. Microalgae concentration was daily checked and kept at $1.5x10^5$ cells mL^{-1} (1:1) in each tank following identical protocol applied for phytoplankton cultures (see section 2.4.1). During 96 h, rotifers and copepods were fed with the designated diets after 10 % daily water exchange supplied by MoRS connected to the culture tanks. The feeding trials were stopped on day 4 (96 h) when nauplii of *A. tonsa* were moulting from the fourth, preventing metamorphosis to the copepodite stage. The duration of the experiment (96 h) ensured to avoid any influence, which could come from the endogenous feeding of the first developmental stage (N I), to the final biochemical composition of *A. tonsa* nauplii.

2.5 Larval rearing

Larvae of Experiment 4 (Chapter 6) were reared per triplicate under controlled conditions, after checking a good spawning quality (enough oocyte yield, a good fertilization and viability rates and a period with high hatching and larvae survival rates). Following the methodology of the section 2.3, eggs mass were transferred to 25 L hatching tank. Larvae were concentrated to the surface of the hatching tank using a light torch and scooped in a 500 mL beaker. Six groups of 400 larvae were selected and moved in six rearing tanks of 25 L belonging to the MoRS, reaching a density of 16 larvae L^{-1} for each tank. Larval culture tanks were provided with a water renewal of

1 L per hour and with 1 bubble air per second, which ensured slow water circulation. Two feeding diets based on enriched copepods and rotifers were tested to determine their effect on survival, growth, biochemical composition, first bones calcification, liver and intestinal morphology of *P. fridmani* larvae from 1 to 15 dph. The effects of the following diets were tested by triplicate (n=3): **Diet I:** The calanoid copepod *A, tonsa* (15 ind. mL^{-1}), enriched with the microalgae *R. salina* and *I. galbana;* **Diet II:** The rotifer *B. plicatilis* (15 ind. mL^{-1}) enriched with the microalgae *R. salina* and *I. galbana.* The "green waters" techniques using binary live microalgae *R. salina* and *I. galbana,* 1:1 ratio at 1.5 10^5cells mL^{-1}, was established in all experimental tanks. The cleaning of the drain mesh, siphoning of debris, counting of died larvae and water analysis were carried out daily in each experimental tank. After cleaning maintenance, the density of microalgae and live preys was estimated (see section 2.4) and reset to schedule trial concentration values. Temperatures and dissolved oxygen concentrations were measured daily, while water quality was monitored 3 times week to prevent ammonia and nitrite build-up.

2.5.1 Modular rearing system (MoRS)

The Experiment 3 and 4 (Chapters 5 and 6, respectively) were conducted inside the Life Support System (LSS) named Modular Rearing System (MoRS), designed and built for this PhD project, to optimize space and water usage while maintaining a stable and controlled environment (Fig. 2.13). The main feature of MoRS was to change the number of tank depending on the experiment design. Cylindrical culture tanks of 25 L each with black walls, were equipped with air line and a central drain mesh. Each tank was maintained at bain-marie condition in 600 L glass aquarium, with the possibility to be independent or with a water renewal from MoRS depending on the needs. This modular configuration provided increased flexibility in experimental management, enabling the manipulation of multiple experimental groups simultaneously without cross-interference. Mors filtration system includes a 200 L

sump supported by a biological tower, 5µ mechanical filtration, protein skimmer (Aqua Medic, Germany), UV sterilization (PAN UV EASYMS 130, Panaque, Italy). Water temperature inside MoRS tanks was controlled by heating device coupled with automatic thermostat (Panaque, Italy). All the MoRS was subjected to a photoperiod, adapted to the experimental needs, using two 40 W fluorescent tubes with a colour temperature of 10.000 ºK. Analysis of the main water parameters such as temperature, salinity, pH, and ammonia levels was carried out three times a week, in order to keep the same chemical and physical water parameters of the broodstock. Continuous aeration maintained optimal oxygen levels, preventing hypoxic zones within the tanks.

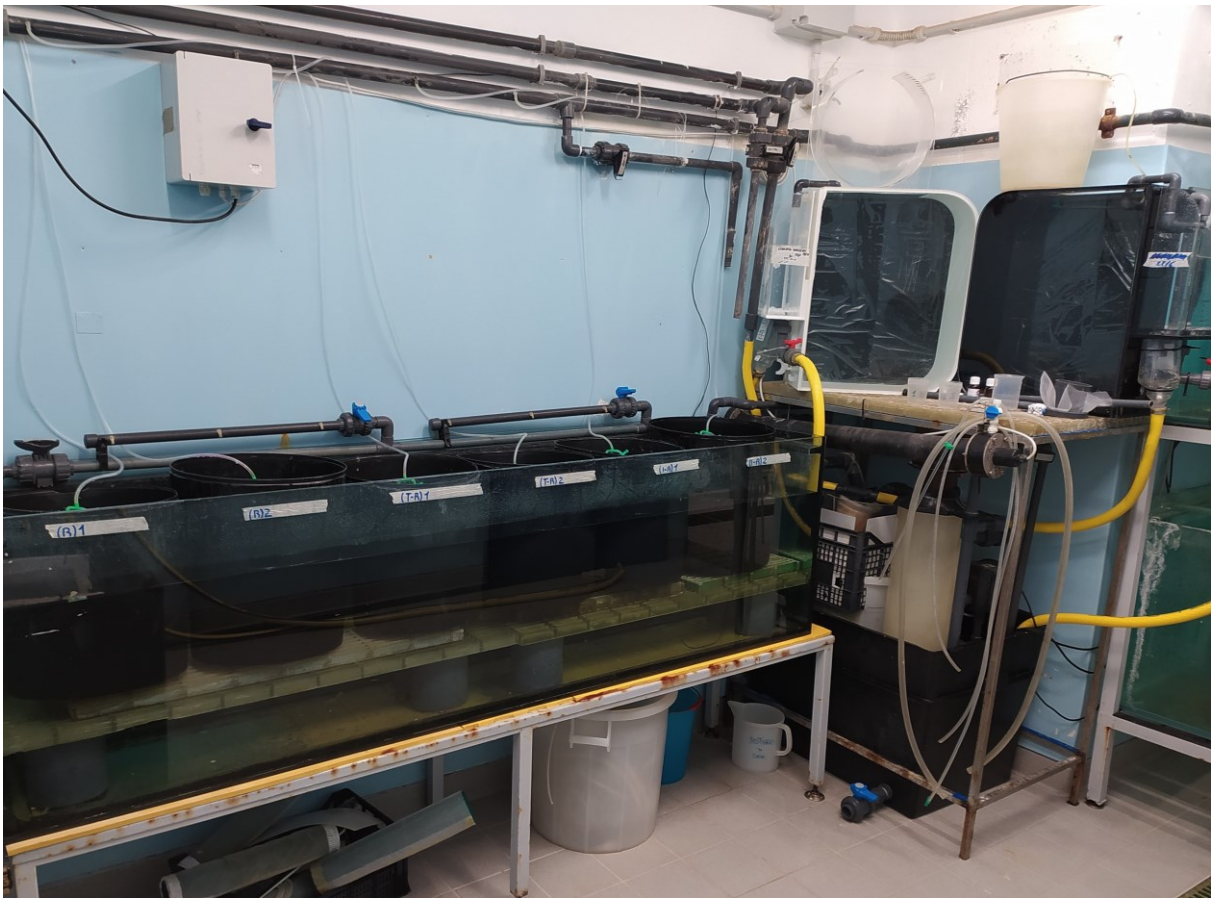

Figure 2.13: The larval culture system "MoRS" (Modular Rearing System) developed as part of the research project undertaken for this doctoral thesis and located at the Tropical Department of the Aquarium of Genoa. Designed to optimize the rearing conditions for marine ornamental species, providing a modular and adaptable platform for larval culture studies.

2.6 Sampling procedures

2.6.1 Broodstock and embryos

Three orchid dottybacks (*P. fridmani)* breeding pairs were sampled from the stock of Aquarium of Genoa (F1) for subsequent histological analysis of the gonads (Fig. 2.2), to investigate sexual patterns and functional sex (Experiment 2, Chapter 4). All efforts were made to minimize suffering, and humane endpoint was quickly applied with cold marine water (0 ± 1 °C) as described by Chen *et al.* (2014) and then included in 50 mL Falcon polyethylene tubes and preserved in buffered formalin (4%). Twenty eggs were respectively collected three different spawning at 0, 1, 2, 3, 6, 9, 12, 15, 18, 24, 36, 48, 72, 96 hours post-fertilization (HPF) from each selected spawning. Samples were collected directly from the aquarium tanks with the support of blade and pipet, leaving the rest of egg mass to the care of the male, and samples fixed in buffered formalin (4%) for subsequent analysis.

2.6.2 Live feeds

In order to characterize live feeds nutritional profile (Experiment 3, Chapter 5), samples of each microalgae species (20 L) used for live prey were collected at the beginning of the experiment from their respective phytoplankton culture tanks (Fig. 2.3). At the end of the feeding Experiment 3, each experimental culture tank of copepods and rotifers (n = 5) generated a sample. The resulting samples were centrifuged (Rotofix 32°, Hettich, Germany), split in vials per triplicate, lyophilized (Alpha 1-2 LD plus, Christ; Germany) and stored at -20 °C for subsequent analysis. Each tank was fully filtered through a 50 μm mesh sieve, the content rinsed with deionized water to remove residual salts, lyophilized (Alpha 1-2 LD plus, Christ; Germany) and stored in vial at -20 °C for subsequent analysis. The collected samples were preserved at the Regional Agency for Environmental Protection–Liguria (ARPAL), Department of Organic Chemistry and Physical Analysis (Genoa, Liguria, Italy).

2.6.3 Larvae

In Experiment 4 (Chapter 6), 20 larvae per tank were collected at the beginning (0 dph), during (2, 4, 6, 8, 10 dph) and at the end of the feeding trial (15 dph) and individually measured for total length (TL) in order to assess growth. Besides, those samples were kept for histological and osteological studies (Fig. 2.4). Thus, larvae were previously euthanized with cold water (0 ± 1 °C) as described by Chen *et al.* (2014) and then included in 5 mL Eppendorf polyethylene tubes and preserved in buffered formalin (4%) for subsequent histological and osteological analysis. Additional larvae were collected at the beginning (0 dph) and at the end (15 dph) of the feeding trial for proximate composition and FA analysis. In order to reach a sufficient sample size for the 0 dph sample, due to the tinny size of the newly hatched larvae, a different spawning was collected from the same breeding pair and hatched as described previously (see section 2.5) and larvae sampled for biochemical analysis. Additionally, at the end of the trial all larvae (15 dph) present in each experimental tank constituted a sample (n = 3 per treatment). Larvae were washed with deionized water to remove sea salts, included in 5 mL Eppendorf polyethylene tubes, lyophilised (Alpha 1-2 LD plus, Christ; Germany) and then stored at -80 °C until analysis.

2.7 Growth performance

Density estimation of live feeds used in Experiment 3 (Chapter 5), as well as the growth performance of fish larvae in Experiment 4 (Chapter 6) were estimated using the following formulae:

- Larval growth was assessed by estimating total length (TL) under a stereomicroscope (EZ4, Leica, Germany).

$$\text{TL (mm)} = \text{Mean of replicates (n)}$$

- Specific growth rate (SGR) was calculated in relation to total length registered during the feeding trial, where TLf was the final larval total length (mm), TLi was the initial larval total length (mm) and Δt was the time between sampling days (Hopkins, 1992; Lugert *et al.*, 2014).

$$\text{SGR (\%)} = \frac{[\ln(\text{TLf}) - \ln(\text{TLi})]}{\Delta t} \text{ x } 100$$

- The coefficient of variation (CV) of total length was calculated as the ratio between the standard deviations and the mean lengths (Bowker 1995).

$$\text{CV (\%)} = \frac{\text{TL Standard Deviation}}{\text{TL Mean}} \text{ x } 100$$

- The density estimation of live preys in Experiment 3 and the counting of live larvae in Experiment 4, allowed to calculate the survival rates per tank. Where Nf was the number of living specimens at the end of the experimental period, Ni was the initial numbers stocked in each tank and Ns was the number of samples taken during the trial (Cortay *et al.*, 2019).

$$\text{Survival (\%)} = \frac{\text{Nf}}{(\text{Ni} - \text{Ns})} \text{ x } 100$$

- In Experiment 4 larvae cumulative mortality for each treatment was also determined, where Nd was the number of total dead larvae, Ni was the initial larvae numbers stocked in each tank and Ns was the number of larvae sampled during the trial.

$$\text{Cumulative mortality (\%)} = \frac{\text{Nd}}{(\text{Ni} - \text{Ns})} \text{ x } 100$$

2.8 Samples analyses

The different analysis was carried out at the laboratories of the Highly Specialized Aquaculture and Biotechnology Service (SABE) located at the University Institute of Sustainable Aquaculture and Marine Ecosystems (IU ECOAQUA), belonging to the University of Las Palmas de Gran Canaria (Canary Islands, Spain).

2.8.1 Biochemical composition

Proximate composition of microalgae, live preys and larvae was analysed following standard procedures (Association of Official Analytical Chemists [AOAC], 2019), samples were previously split in vials per triplicate, frozen, lyophilized (Alpha 1-2 LD plus, Christ; Germany), homogenized and stored at -80 °C for subsequent analysis. Moisture was determined by thermal dehydration until constant mass at 105 °C. Ash content was determined by combustion at 600 °C for 12 h. Crude protein content was determined by the Kjeldahl method, and crude lipid was extracted following the Folch method (Folch *et al*., 1957). Carbohydrate was estimated by difference [Percentage carbohydrate content = 100 − (% moisture + % ash + protein + % fat)]. The analysis of FAs was carried out by transmethylation of the total lipids in fatty acid methyl ester (FAMES) as described by Christie and Han (2010), separated, and identified by gas chromatography (7820A GC System, Agilent Technologies, United States) following the conditions described by Izquierdo *et al*., (1990). All gross composition data were expressed as percentages of dry weight (DW ± SD), while the fatty acids content was expressed as percentage of the total identified fatty acids (DW ± SD). All analyses were conducted in triplicate and results used to compare the different experimental groups.

2.8.2 Histological analysis

To describe histological modifications in the broodstock and larvae tissues (Experiment 2 and 4), samples were fixed in buffered formalin 4% (see section 2.6), processed in histology cassettes and dehydrated using a Thermo Scientific STP 120-2 (Thermo Shandon Limited, United Kingdom). Paraffin-embedded samples were cut at 3 µ on a Leica Jung Autocut 2055 microtome (Leica, Nussloch, Germany) (Fig. 2.14). Slides were stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970). A semi-quantitative score of the tissue

to define and evaluate the structural and cellular conditions was employed, ranging from 0 to 3, where 0, 1, 2 and 3 represented absent, low, mild and severe conditions (Betancor *et al.*, 2012).

The histological assessment was completed with the quantitative image analysis, determining the length, width, diameter, surface area of cells and/or specialized structures. All mounted sections were scanned with Motic Easy Scan Pro digital scanner (Motic, Xiamen, China) operated using the Motic DS Assistant software (Motic VM V1 Viewer 2.0) and examined using Image Pro Software (Media Cybernetics, USA). Three different trained independent blind observers evaluated the slides to estimate visual differences among treatments.

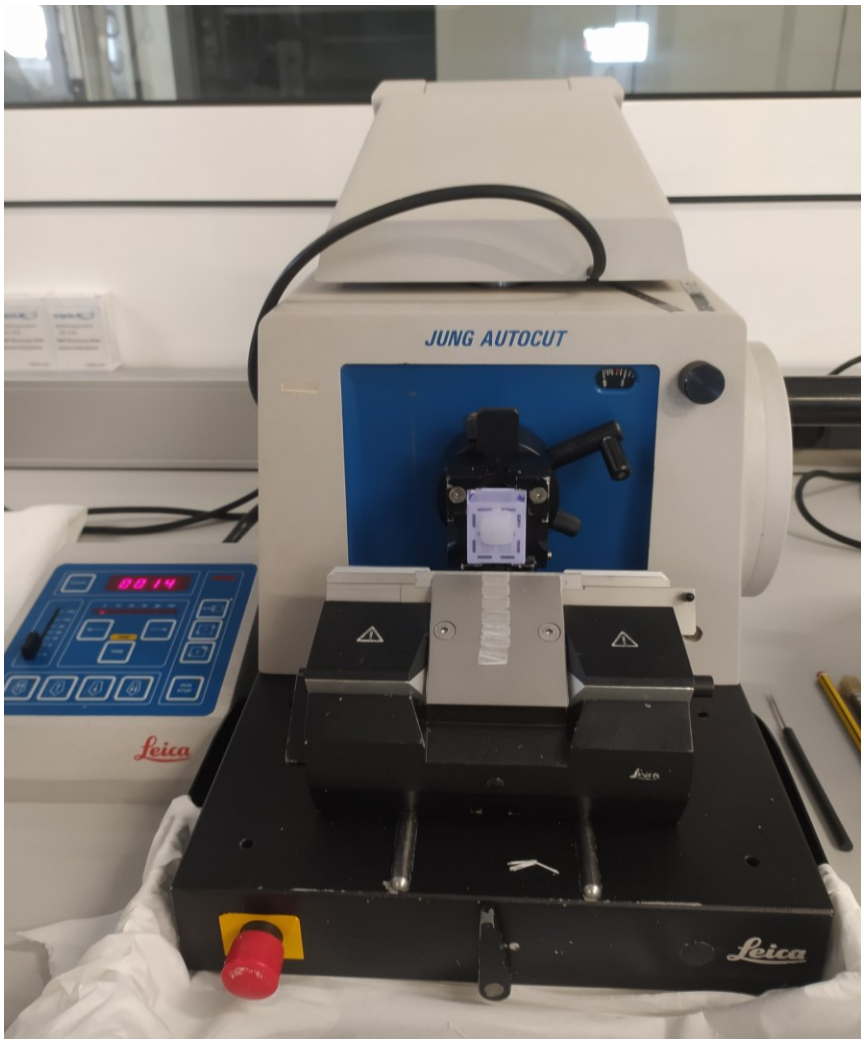

Figure 2.14: Microtome used to cut samples and generate sections mounted in the slides**.**

2.8.3 Osteological analysis

Osteological analysis was conducted in Experiment 4 to evaluate larvae first ossification and skeletal development, comparing the effect of different feeding treatment. For those analyses, 10 larvae fixed in buffered formalin 4% from each sampling day were stained with Alizarin Red and Alcian Blue following the acid-free double staining protocol of Walker and Kimmel (2007) modified (Fig. 2.15). This involved clearing soft tissues with an enzymatic digestion solution and staining cartilage with Alcian Blue and bone with Alizarin Red. Different millimolar concentrations of magnesium chloride ($MgCl_2$) were used to prepare alcian blue solution depending on the larvae age respectively: 100 mM from 0 to 2 dph, 140 mM from 4 to 6 dph, 180 mM from 8 to 10 dph and 200 mM at 15 dph. Stained larvae were examined using an Olympus CX41 microscope (Olympus, Hamburg, Germany) connected to an Olympus XC30 camera (Olympus), which was linked to a computer using Image Pro Software (Media Cybernetics, USA), to observe the development and ossification of key skeletal structures, such as the vertebral column, cranial bones, and fin rays.

Figure 2.15: Larvae of *P. fridmani* 15 days post hatch stained with modified Walker and Kimmel (2007) method.

The degree of ossification and any skeletal deformities (e.g., scoliosis, lordosis, or kyphosis) were recorded for each specimen. Three trained independent blind observers evaluated the samples to define visual differences among treatments. A semi-quantitative score ranging from 0 to 3 of skeletal mineralization was defined for cranial region (maxilla, gill arches, neurocranium), vertebral column, and caudal fin complex. Score 0 was assigned as absence of mineralized matrix; score 1 was defined as low bones mineralization; score 2 as moderate bones mineralization; and score 3 as completely mineralized bones. skeleton mineralization was evaluated for each dietary group.

2.9 Statistical analysis

All data resulting from the experiments were analysed using appropriate statistical methods to ensure robust and reliable interpretations. Levene's test was used to assess homogeneity of variances and the Shapiro Wilk test to assess normality, applying log-transformed percent data when assumptions were not accomplished. Confidence levels were established at 95 % ($P < 0.05$) for the analysis and the results were presented as mean with the standard deviation (SD) unless otherwise mentioned. To compare the two eggs collecting systems (Experiment 1), focusing on egg abundance and viability, the *T*-Student test (*t*-test) was performed. To determine the specific effect of different rearing temperature on broodstock spawning activity and filial cannibalism (Experiment 2) and compare the use of high-quality live preys during larval first feeding (Experiment 4), a *T*-Student test (*t*-test) was also applied to the data. To independently compare the differences in live feed proximate and FAs composition (Experiment 3) a One-way analysis of Variance (ANOVA) was applied to data and means were compared. Moreover, comparison between groups was calculated by Tukey post-hoc test for pairwise comparisons and Games-Howell to determine specific differences between treatments. To obtain a more integrated interpretation of diet on copepods and rotifers survival, a Two-way repeated measures ANOVA

with two fixed factors, time and diet, was applied (Experiment 3). Statistical reprocessing of the data was carried out using SPSS statistical software package (IBM SPSS V27.0 for Windows; SPSS Inc., Chicago, IL, USA), and graphical representations of the data were generated using 365 MSO Excel (Microsoft Corporation, v. 2410, USA) for clarity in presenting results. All conclusions were drawn based on statistically significant outcomes, ensuring that the interpretations of the data were valid and reliable.

Chapter 3. Development of new fish eggs collecting system

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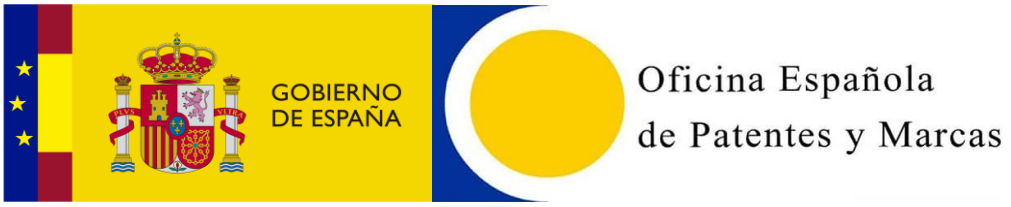
This work was submitted to the **Spanish Patent and Trademark Office** (OEPM, January 2024) Patent number P202430037 titled "SYSTEM FOR COLLECTING EGGS OF AQUATIC ANIMALS"



3.1 Brief Summary of the Invention

One of the significant limitations in developing aquaculture protocols for marine fish species lies in broodstock management and the collection of fertilized embryos (commonly referred to as eggs) for examination and further development. Efficient egg collection is crucial for successful aquaculture operations. Marine fish employ diverse spawning strategies; for example, pelagic fish often release sperm and eggs simultaneously into the water, where fertilization and embryo development occur. Once fertilized, pelagic eggs float and are carried by water currents. In a tank environment, these eggs must be promptly removed before entering filtration system components, where they would be destroyed.

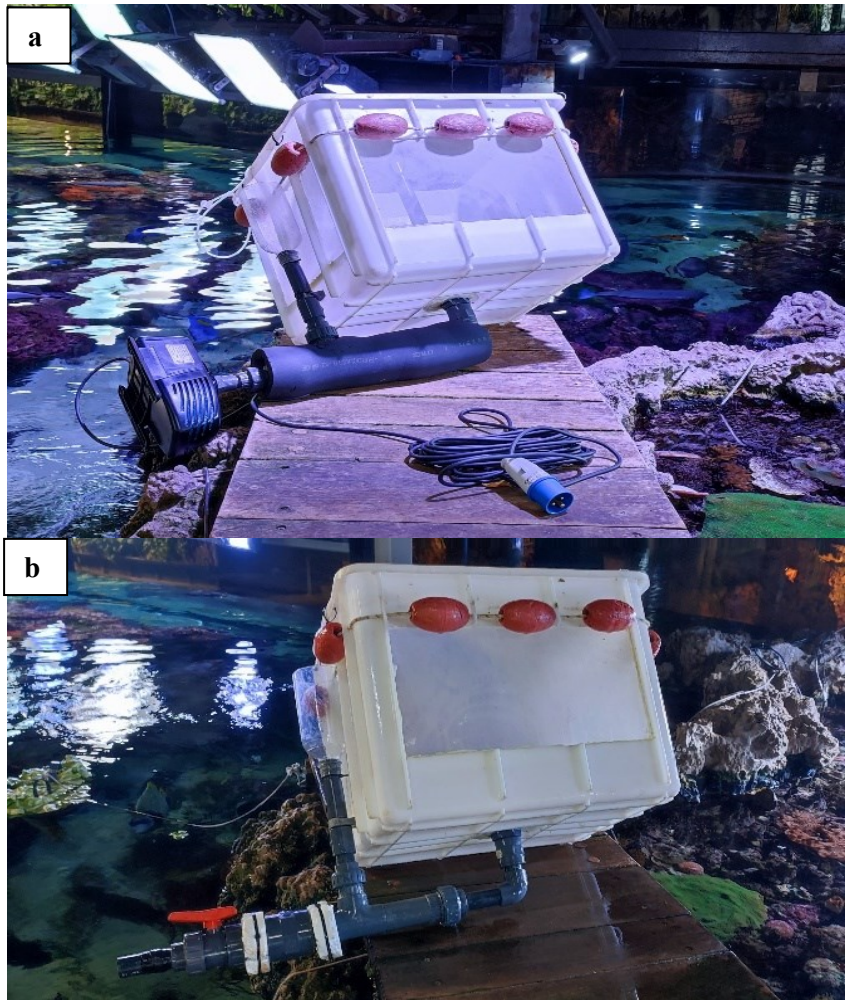


Figure 3.1: Different Venturi egg collectors powered by a dedicated pump (a) and designed to be connected to the tank's main pump (b).

Thus, there is a need to implement egg collectors and collection techniques tailored to the target tanks and reproductive strategies of the species in question, ensuring maximum harvest while preventing egg damage. The invention, named the “Venturi Egg Collector,” facilitates the collection of spawn with minimal supervision and handling by a single technician during aquaculture operations, particularly in the ornamental species sector. This device can be customized in size and shape based on specific needs (tank dimensions, number and size of broodstock, egg size, etc.). Moreover, it can either be powered by a dedicated pump (Fig. 3.1a) or alternatively connected directly to the main pump of the tank housing the collector itself (Fig. 3.1b).

3.2 Novelty and Inventive Activity

The development of a new patent stems from the need to improve upon the existing invention, the Air-lift Collector (US Patent No. 2006/0130771 A1). The current collector, while functional, has several limitations: its suction flow rate is insufficient for large-volume tanks, and the turbulent and irregular airflow generated by air bubbles can damage or trap a portion of the collected eggs. The primary objectives of this improvement were to enhance the device's collection capacity and eliminate the foam-generating effect inherent in the previous air-lift system. To achieve this, the new collector model incorporates a Venturi tube (Fig. 3.2) powered by a water pump, which fully replaces the air-lift system. The Venturi tube features a significant narrowing in its interior, which notably increases the water flow velocity. This increase in velocity creates a vacuum in a side-mounted pipe connected to the suction collector (Fig. 3.2). By integrating the Venturi tube, the new collector achieves a significant improvement in suction flow rate and consequently in egg abundance (Fig. 3.S1 and S2), and substantially reducing turbulence generated by the previous air-lift system increasing eggs viability (Fig. 3.S3 and S4). This modification also eliminates air bubbles, thereby removing the foam effect entirely from the collector's operation.

Figure 3.2: Cross-sectional view of a Venturi tube.

3.3 Background

The currently existing device, the Air-lift Collector (US Patent No. 2006/0130771 A1), designed for the collection of aquatic animal eggs, operates using an air-lift pump (Fig. 3.3). Its functionality relies on an airline entering the proximal end of the egg transport tube, generating a water flow into the concentrator vessel. Consequently, the eggs flow toward the concentrator along with water and air, while mesh screens retain the eggs and allow excess water to return to the tank.

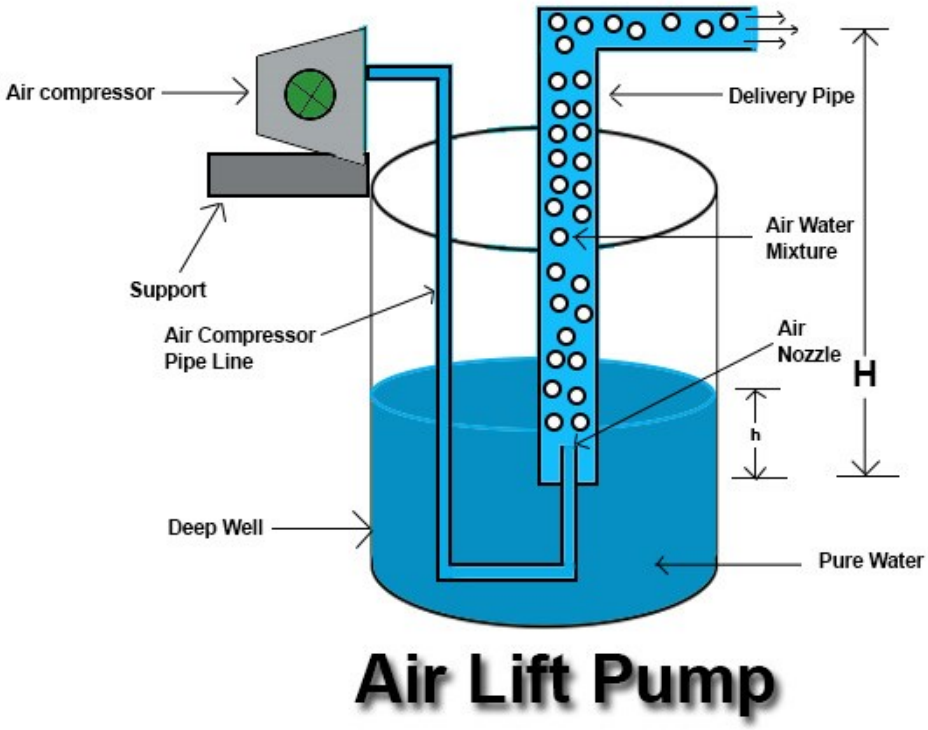


Figure 3.3: Functional description of the air-lift pump. H= Height.

During tests conducted with both types of egg collectors, the air-lift and the Venturi, significant differences were observed regarding the volume of water processed (L/h), the abundance of eggs harvested and their viability (Fig. 3.S1, S2, S3 and S4). The Venturi Collector proved to be user-friendly, requiring minimal attention and handling during installation by a single technician. The main advantage of this new collector was its superior suction and water-processing capacity compared to the air-lift collector. Specifically, the suction force in the Venturi Collector depended on the water flow rate provided (2,400 L/h), generating a suction force of 1,800 L/h—four times greater than that of the Air-lift Collector, which processed only 450 L/h. It was observed that the relationship between the volume of water processed and the number of eggs collected was directly proportional, although the quantity and diversity of eggs depended on the condition of the broodstock and dispersal factors such as surface area (m^2) and water dynamics in the tank. Unlike the air-lift Collector, the Venturi Collector showed no signs of damaged eggs or eggs trapped dry along the edge of the concentration vessel.

These updates could therefore be described in a new patent or utility model, with broad applications in the breeding and reproduction of aquatic species, both in the aquaculture sector and in public Aquariums.

Supplementary Files

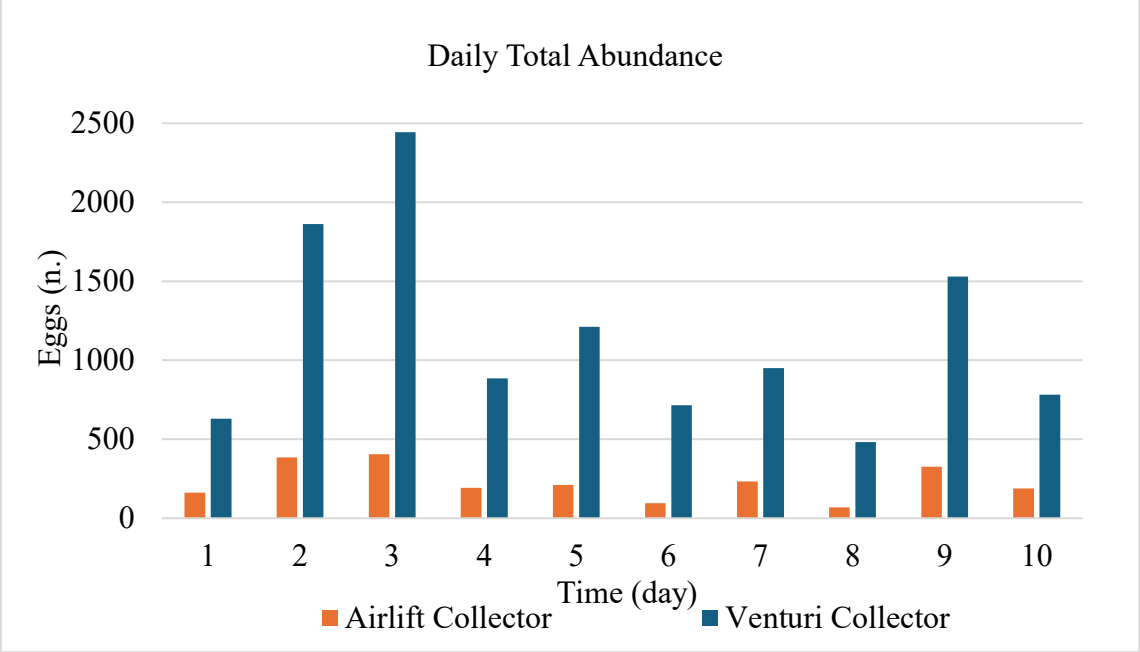


Figure 3.S1: Daily average of batfish (*P. orbicularis*) eggs abundance harvested with Air-lift and Venturi Collectors in the Tropical lagoon tank (NA9), Aquarium of Genoa (Italy).

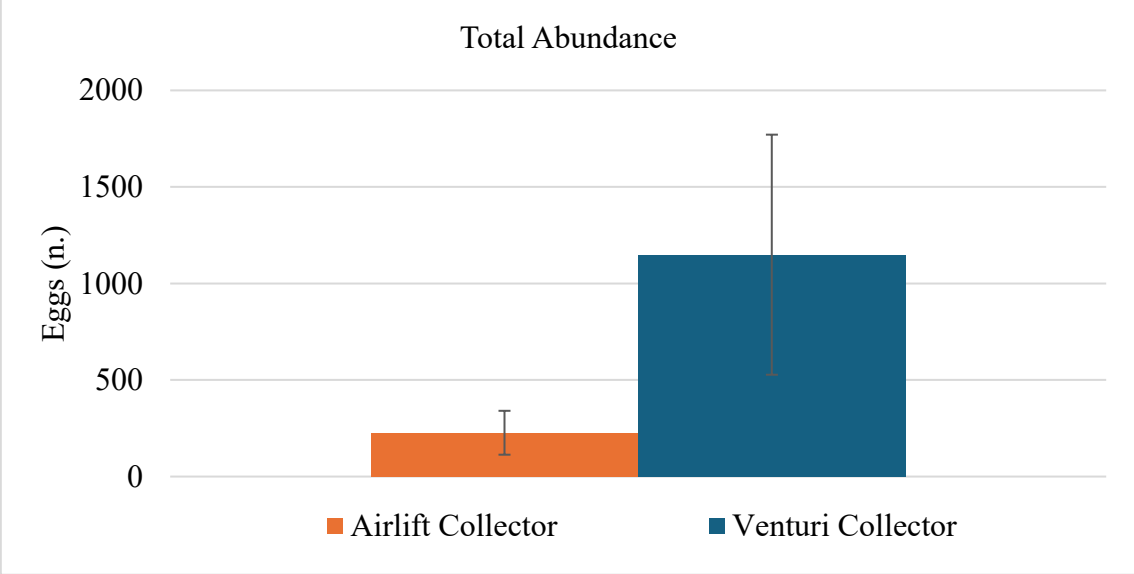


Figure 3.S2: Total average of batfish (*P. orbicularis*) eggs abundance harvested with Air-lift and Venturi Collectors in the Tropical lagoon tank (NA9), Aquarium of Genoa (Italy).

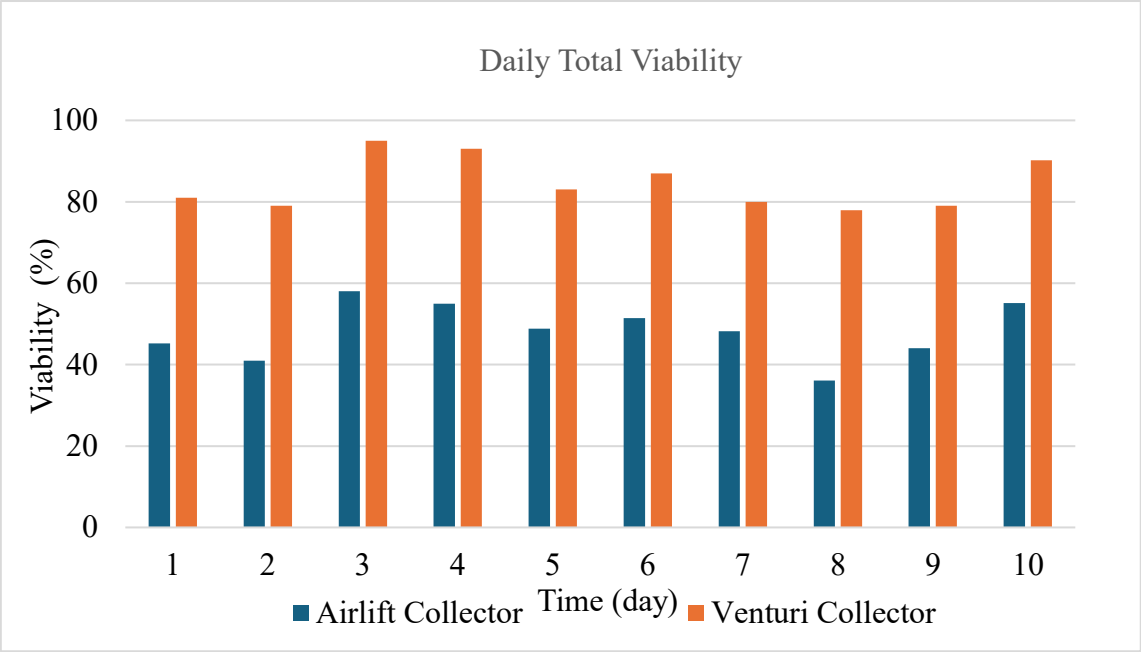


Figure 3.S3: Daily average of batfish (*P. orbicularis*) eggs viability harvested with Air-lift and Venturi Collectors in the Tropical lagoon tank (NA9), Aquarium of Genoa (Italy).

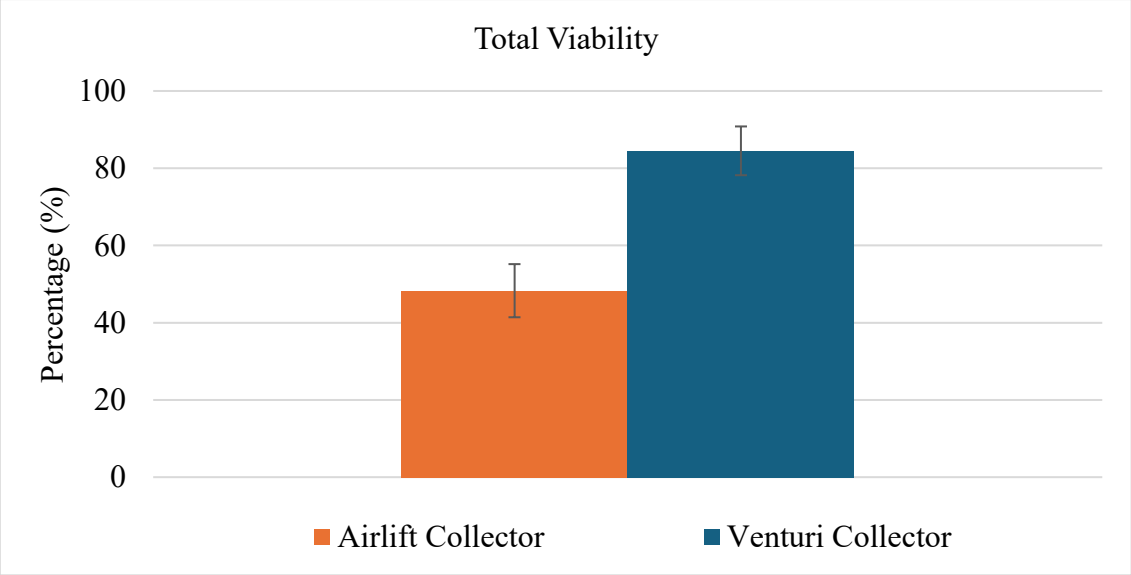


Figure 3.S4: Total average of batfish (*P. orbicularis*) eggs viability harvested with Air-lift and Venturi Collectors in the Tropical lagoon tank (NA9), Aquarium of Genoa (Italy).

Chapter 4. Broodstock spawning activity and filial cannibalism in orchid dottyback *Pseudochromis fridmani* (Klausewitz, 1968) under different rearing temperatures

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4.1 Abstract

The orchid dottyback *Pseudochromis fridmani* is considered one of the most attractive species in the marine ornamental fish trade because of its bright colour, resilience, and relatively small size. Culturing orchid dottybacks presents bottlenecks related to broodstock and spawning conditioning, which impede large-scale production. The present study addresses the knowledge gaps with regard to the management of *P. fridmani* under controlled conditions, describing the pair formation process, the associated behaviours, and their gonad histology at 25 °C. In addition, the effects of low (25 °C) and high (28 °C) temperatures on spawning activity and filial cannibalism were studied. Twelve specimens were divided into six pairs and monitored for 12 months. Water temperature strongly influenced broodstock spawning activity and filial cannibalism. At 28 °C, a significant increase in spawning rate was observed, but concurrent filial cannibalism also increased, leading to spawn loss. Notably, the total monthly number of spawnings that successfully developed until hatching did not differ significantly between pairs maintained at 28 and 25 °C. Histological analysis of the gonads (n=6) suggested that the evaluation of total length and body shape may be a reliable and rapid tool for visual sex differentiation in this species. In summary, maintaining the broodstock temperature around 25 °C facilitates the management and improves the performance of this highly demanded species, enhancing its production under controlled rearing conditions. This study represents an improvement in the husbandry of this scarcely known species, contributing to its large-scale production and the conservation of natural stocks.

4.2 Introduction

4.2.1 Marine ornamentals aquaculture

Aquaculture of marine ornamental species plays a key role in the marine aquarium fish trade and is considered a sustainable alternative to wild-capture fisheries (Holt, 2003; Olivotto *et*

al., 2003; Olivotto *et al*., 2011). Over the past decades, this trade has expanded worldwide, becoming a multi-million-dollar industry exceeding 360 million US$ per year in value (Palamatag, 2017; Pouil *et al*., 2019). In addition, approximately 90% of the marine fish sold are still captured in the wild, and in some countries, destructive capturing methods are still employed, resulting in negative repercussions on coral reefs and the surrounding marine ecosystems (Olivotto *et al*., 2003; Palmtag, 2017). Consequently, concerns have been raised regarding the overexploitation of natural stocks, leading to an increasing interest in the development of aquaculture techniques (Pouil *et al*., 2019). Aquaculture is a highly effective strategy for advancing sustainability as it has the potential to significantly alleviate fishing pressure on coral reef ecosystems (Tlusty, 2002; Olivotto *et al*., 2017; Palmtag, 2017). Moreover, hatchery-produced juvenile fish are hardier, less susceptible to disease, and survive better than wild specimens maintained under controlled conditions (Tlusty, 2002; Olivotto *et al*., 2017; Chen *et al.*, 2020; Watson *et al.*, 2023). The study of the reproductive biology of marine ornamental fish is essential for achieving successful production as well as for understanding their ecological roles in the wild (Chen *et al.*, 2020; Watson *et al.*, 2023). However, the reproductive behaviour and spawning of reef fish are frequently difficult to observe in field studies, which typically focus only on describing species distribution, habitat, and morphology (Lubbock, 1975; Zander, 2017). Consequently, relevant information is scarce in the literature, and the production of new marine species under controlled conditions is often constrained by (i) knowledge gaps in broodstock spawning management and (ii) lacking knowledge of larval nutrition, which are generally the main bottlenecks in scaling up to mass production (Holt, 2003; Olivotto *et al*., 2017; Chen *et al*., 2020, 2023; Martino *et al*., 2023).

4.2.2 The orchid dottyback *Pseudochromis fridmani*

Dottybacks (Pseudochromidae) are a family of small, vibrantly coloured marine fish found in the Indo-Pacific region (Thresher, 1984; Wittenrich, 2007). They are among the most traded marine ornamental fish and are popular choices for home aquariums, along with damselfish (*Pomacentridae*), because of their esthetics, bright colour, resilience, and relatively small size (Thresher, 1984; Wittenrich, 2007; Palmtag, 2017). Dottybacks comprise more than 150 species in 24 genera, and new species are still being discovered (Gill, 2004; Gill and Allen, 2011; Gill *et al*., 2012). The orchid dottyback *Pseudochromis fridmani* (Klausewitz, 1968) is a small, captivating reef species endemic to the Red Sea (Lubbock, 1975; Moe, 1997; Gill, 2004) and is considered one of the most commercially attractive dottybacks in the marine aquarium trade (Wittenrich, 2007). Orchid dottybacks are characterised by a bright purple, elongated body with a black stripe across the eyes, in addition to their hardiness and resilience to being kept in an aquarium (Moe, 1997). Optimising the breeding management for this species is of significant interest to the aquarium industry, as it not only provides a viable and sustainable aquaculture alternative for trade but also contributes to the protection of wild populations from overexploitation. In this context, it is important to understand the reproductive and behavioural aspects of this fish under controlled conditions.

4.2.3 Breeding the orchid dottybacks

Although orchid dottybacks have been bred under controlled conditions, they display several characteristics that hinder large-scale production. Similar to most dottybacks, *P. fridmani* was described as a protogynous hermaphrodite, developing first as a female and becoming a male later, depending on age and social dynamics (Wittenrich, 2007). There is limited scientific literature supporting hermaphroditism and bidirectional sex change in this species, although these reproductive strategies are probably natural features of dottyback biology (Wittenrich

and Munday, 2005; Wittenrich, 2007; Kuwamura *et al*., 2014). To date, some of the most critical challenges are related to broodstock and spawning management (Chen *et al*., 2023), as comprehensive studies on pair formation, sexual behaviour, spawning dynamics, gonadal histology, and filial cannibalism (FC) in orchid dottybacks are limited or completely lacking (Wittenrich, 2007; Mies *et al*., 2014; Chen *et al*., 2023). Recently, Chen *et al.* (2023) described the early embryonic and larval ontogeny of *P. fridmani* under controlled conditions, providing valuable insights into the life cycle of this species. These findings documented year-round spawning and high FC, encouraging future studies to understand and manage cannibalism by broodstock and optimise larval dietary nutrition during the first 14 days post-hatching (DPH). Indeed, previous studies on *P. fridmani* breeding and larval culture lack detailed descriptions and in-depth analyses (Brons, 1996; Moe, 1997). However, recent studies focused on the production of high-quality live feeds (Martino *et al*., 2023), which are considered to result in the best culture performance, e.g., when *P. fridmani* larvae were fed enriched calanoid copepods during the first 15 DPH (Martino *et al*., 2024). Generally, pairing dottybacks may be difficult, considering that within the confines of a small tank, co-housing of two or more similarly sized individuals typically results in fighting (Lubbock, 1975). It was recommended that *P. fridmani* be kept in groups of individuals with different sizes for reducing intraspecific aggression, which can be mitigated through providing hiding structures and refuges (Lubbock, 1975; Wittenrich, 2007; Mies *et al*., 2014). In addition, individuals that spend their entire life cycle together, including the larval stages, should be less predisposed to exhibit aggressive intragroup behaviour, naturally forming heterosexual pairs as they mature (Mies *et al*., 2014). When spawning begins, the female lays a demersal egg mass that may be protected or consumed by the male before hatching can occur (Wittenrich, 2007; Mies *et al*., 2014, Chen *et al*., 2023). Although different studies have documented FC in *P. fridmani,* the specific mechanisms eliciting this behaviour are unknown, rendering it an aquaculture

bottleneck for this and many other dottyback species (Sargent, 1992; Mies *et al*., 2014; Shei *et al*., 2017; Chen *et al.*, 2023). Temperature severely affects metabolism and behaviour, especially in aquatic ectotherms, which can have important implications for trophic interactions and population dynamics (Pankhurst and Munday 2011; Visser, 2008; Sunday *et al*., 2010). Indeed, FC may be influenced by temperature and altered via several mechanisms, such as decreases in dissolved oxygen, embryo developmental issues, courtship behaviour, and energy costs of parental care (Payne *et al*., 2002, 2004; Manica, 2004; Klug, 2009; Vallon *et al*., 2016; Matsumoto *et al*., 2018). Elevated temperatures give rise to higher metabolic rates, which typically result in altered behaviour, higher activity levels, and increased energy demands (Manica, 2004; Klug, 2009; Biro *et al*., 2009, Sunday *et al*., 2010).

In the present study, we tested the effects of high (28 °C) and low (25 °C) temperatures on the broodstock management and reproductive performance of the orchid dottyback *P. fridmani* to determine whether continuous exposure to these conditions would induce changes in spawning activity and FC. The behaviours involved in the bonding process of orchid dottybacks under controlled conditions were investigated for 6 months, and the histology of mature gonads was characterized. Overall, the present study contributes to enhancing our knowledge of *P. fridmani* reproductive biology as well as improving broodstock management of this highly demanded species.

4.3 Materials and methods

All experiments described below (Fig. 2.2) were conducted in quarantine areas belonging to the Tropical Department of the Aquarium of Genoa (Genoa, Liguria, Italy). Histological analysis was performed in the laboratories of the Highly Specialized Aquaculture and Biotechnology Service (SABE), located at the Marine Scientific and Technological Park of the ECOAQUA Institute of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain).

All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the European Union Directive (2010/63/EU).

4.3.1 Study species and broodstock conditioning

A total of 18 adult *P. fridmani* kept at 25 ± 1 °C were procured from the Aquarium of Genoa (Genoa, Liguria, Italy) and were allowed to acclimate in a dedicated recirculating system (Fig. 2.2) measuring the main biometric index (total length, weight) (Table 4.1). All fish used in this experiment, with total length and weight expressed as mean ± standard deviation (SD), i.e., 10.2 ± 1.2 cm and 8.4 ±1.7 g, were older than 1 year and originated from the same pair of parents (Table 4.1). Because *P. fridmani* does not present any obvious sexual dimorphism, specimens were visually sexed assuming females to be smaller with a swollen belly and males to be larger and more slender. The sorted specimens were split into nine pairs (Table 4.1), three of which were killed through cooling to 0 ± 1 °C in marine water to minimise suffering (Chen *et al.,* 2014) and were then placed in 50 mL Falcon polyethylene tubes and preserved in buffered formalin (4 %) for subsequent histological analyses (Fig. 2.2). The remaining six pairs (Table 4.1) were simultaneously placed in assigned partitions of two glass aquaria connected to the same recirculating system (Fig. 2.2). Each aquarium was divided into three partitions of approximately 133 L (Fig. 4.S1), without any decoration or gravel. Partition housed one *P. fridmani* pair and two uncapped polyvinyl chloride (PVC) pipes (30 cm long and 3.2 cm in diameter) as shelter and spawning substrate. The low number of individuals used in this study was because of limited available space. Access to the experimental area was restricted to a few people to avoid interference with behaviour and spawning activity.

Table 4.1: Total length and weight of 18 *P. fridmani* individuals selected for the experimental broodstock trial (1 year) and histological analysis of gonads.

***P. fridmani* Biometry**	**Specimens**	**Pair**	**Sex**	**TL (cm)**	**Weight (g)**
Broodstock Study	1	A	Male	12.1	10.9
	2		Female	9.8	6.6
	3	B	Male	10.3	7.3
	4		Female	8.4	8.6
	5	C	Male	11.3	10.4
	6		Female	9.5	8.1
	7	D	Male	11.2	9.6
	8		Female	10.1	10.3
	9	E	Male	11.9	9.8
	10		Female	8.6	5.4
	11	F	Male	10.4	6.6
	12		Female	8.2	5.23
Total mean			Male	11.2 ± 0.7	9.1 ± 1.7
			Female	9.1 ± 0.8	7.8 ± 1.9
Gonads Histology	13	G	Male	11.5	9.6
	14		Female	8.9	6.6
	15	H	Male	11.3	9.8
	16		Female	9.6	8.6
	17	I	Male	10.8	7.9
	18		Female	88	6.2
Total mean			Male	11.2 ± 0.4	9.1 ± 1.0
			Female	9.1 ± 0.4	7.1 ± 1.3
Total				10.2 ± 1.2	8.4 ±1.7

Water quality was maintained using biological filtration, a sand filter, a UV steriliser (Panaque, Italy), and a heat exchanger, all powered by a main pump (Astralpool, Italy). Temperature was maintained at 25 ± 1 °C, salinity at 35 ± 1 g L^{-1}, and the photoperiod at 12:12 h light:dark using two 30 W fluorescent lights tube (Philips, Nederland) suspended 20 cm above each broodstock tank. The fish were fed twice per day *ad libitum* using mixed diets based on marine chopped fish (cod and sardines), mussels, shrimp, and vitamin premix (1 g/kg). In addition, the broodstock was fed enriched live-cultured *Mysis* and frozen adult *Artemia* once a day. Daily maintenance included cleaning the drain mesh and siphoning the

feed waste and debris. Water samples were collected from the recirculating system three times per week to check the main parameters and maintain nitrogen compounds, ammonia (NH_4^+), and nitrite (NO_2) below 0.01 mg L^{-1}.

4.3.2 Behaviour and pair formation

After isolation (see section 2.1), daily monitoring of the behaviour and pair formation process of the six pairs under controlled conditions was performed for 6 months (from December 2020 to May 2021). A semi-quantitative score was defined to describe aggressive and courtship behaviours, ranging from 0 to 3, where 0, 1, 2, and 3 represent absent, low, mild, and high behavioural levels, respectively. This scoring system was assigned through observations made during daytime (from 8:00 am to 6:00 pm), every 2 h (n= 6) over a 10 min period recording aggressive and courtship behaviour during the 6 months of the experiment. Aggression was determined based on the level of stress and damage inflicted by the dominant fish: (i) constriction in a corner, (ii) frayed fins, and (iii) bite marks. The reproductive behaviour level was determined according to courtship activity: (i) male swimming in and out of the PVC pipe, (ii) male quivering with open fins in front of the female, and (iii) pair sharing the same PVC pipe (Fig. 4.2). The monthly aggression and courtship scores for each pair were obtained by averaging the daily observation scores. Animal behavioural observations in general were made following the recommendations of Altmann (1974). When a high level of aggressive behaviour was observed in a pair, the larger individual was temporarily placed in a separated, transparent floating cage, maintaining visual contact between the specimens. The physical isolation of the aggressive individual lasted for a maximum of 1 week, with the decision to release it earlier, as determined by the nature of the interactions observed between the specimens. A pair was considered bonded when they

simultaneously accepted food, displayed no signs of aggression, and initiated courtship, ultimately resulting in the first spawning event.

4.3.3 Spawning activity and filial cannibalism

The effects of different rearing temperatures on the spawning dynamics and FC of orchid dottybacks were investigated for 6 months (from June 2021 to November 2021). The six pairs obtained (see section 2.2) were divided into two groups (n= 3), kept at 25 ± 1 °C and 28 ± 1 °C, respectively. The aquarium tank selected for the 28 °C treatment was equipped with a heater, which progressively increased the temperature from 25 to 28 °C over 3 days (1 °C per day) and then maintained it stable at 28 °C during the 6-month trial. Using a flashlight, all pairs were checked at least five times per day to verify the presence of egg masses in the PVC pipe. The total number of eggs spawned per month and the spawning frequency (days) were calculated. When spawning occurred, the eggs were left to the care of the male until hatching to test the occurrence of FC. The rate of FC represented the monthly frequency of egg masses that were completely cannibalised by broodfish during the incubation period. FC was calculated as follows (Chen *et al*., 2023):

$$FC = \frac{\text{Number o f egg masses cannibalized in a month}}{\text{Total number of monthly spawnings}} \times 100\%$$

The total number of egg masses available for hatching per month was calculated by subtracting the number of egg masses cannibalised during the month from the total monthly spawning number. All data resulting from spawning activity and FC were tested for homogeneity of variances using the Levene test and for normal distribution using the Shapiro-Wilk test; log-transformation of percent data was applied when these assumptions were not met. Student's *t*-test was used to assess differences between treatments. Analyses were conducted using the SPSS statistical software package (IBM SPSS V27.0 for Windows; SPSS Inc., Chicago, IL, USA), and statistical significance is reported at $p < 0.05$.

4.3.4 Gonadal histology

Histological analysis of the gonads was conducted to investigate the sexual patterns and maturity of three *P. fridmani* pairs, which were not used for the observation experiment described in section 2.2 (see section 2.1). Gonads from each sampled fish were removed, macroscopically analysed, and fixed in 4% paraformaldehyde at 4 °C for 48 h. Thereafter, the samples were dehydrated, embedded in paraffin (Thermo Fisher Scientific STP 120-2; Thermo Shandon Limited, United Kingdom), sectioned at 3 μm thickness using a Leica Jung Autocut 2055 microtome (Leica, Nussloch, Germany), and stained with haematoxylin and eosin (Martoja and Martoja-Pierson, 1970). Histological sections were used to confirm whether orchid dottyback gonads contained exclusively testicular or ovarian tissue or had both types of tissue. The mounted sections were scanned using a Motic Easy Scan Pro digital scanner (Motic, Xiamen, China) operated using Motic DS Assistant software (Motic VM V1 Viewer 2.0), and examined using Image Pro Software (Media Cybernetics, USA). Three trained independent blind observers evaluated the slides to estimate visual differences among the samples.

4.3.5 Embryos development

To achieve greater sample representativeness and minimize errors in development timing, three different egg masses from the same breeding pair kept at 25 °C were selected for the embryo ontogeny study. Twenty eggs were respectively collected at 0, 1, 2, 3, 6, 9, 12, 15, 18, 24, 36, 48, 72, 96 hours postfertilization (HPF) from each selected spawning. Samples were collected directly from the aquarium tanks with the support of blade and pipet, leaving the rest of egg mass to the care of the male. During the experiment morphological data were recorded, photographing embryos at 5x and 10x magnification with stereomicroscope (EZ4, Leica, Germany) connected to a digital camera (Motic, Xiamen, China) and samples fixed in

buffered formalin (4%). Eggs diameter was measured with a micrometre scale ocular. The main embryonic development stages were identified and described (morula, blastula, gastrula) from the moment that most of the samples reached that stage according to Gilbert, (2014) for zebrafish.

4.4 Results

4.4.1 Pair formation and Reproductive behaviour

The average time required for pair formation was 7.5 ± 2.8 weeks. During this period, distinct levels of aggressive behaviour were observed among the pairs, exhibiting a consistent and gradual decline. This reduction ultimately ceased, coinciding with the first spawning and formation of pair bonds (Fig. 4.1; Table 4.2). Only two pairs, A and F, exhibited high levels of intraspecific aggression which negatively affected food intake of all individuals in the tanks, as fighting continued during feeding. Different types of bite-related injuries were documented in these pairs, including haemorrhagic lesions, desquamation, and frayed fins. Therefore, physical isolation (see section 2.2) was necessary for pairs A and F. After 2 months of experimentation, all pairs exhibited courtship behaviour (Fig. 4.1). Additionally, four pairs (B, C, D, and E) spawned for the first time, thereby completing the bonding process (Table 4.2). The males measured 11.2 ± 0.7 cm in length and weighed 9.1 ± 1.7 g (Table 4.1), and they spent most of the time inside the PVC pipe. The females were 9.1 ± 0.8 cm in length and 7.8 ± 1.9 g in weight (Table 4.1), swimming in open areas and using pipes to take shelter. A few days before spawning (3 days), while females typically presented with a swollen abdomen, the males showed increased courtship behaviour, swimming in and out of their preferred pipe (Fig. 4.2).

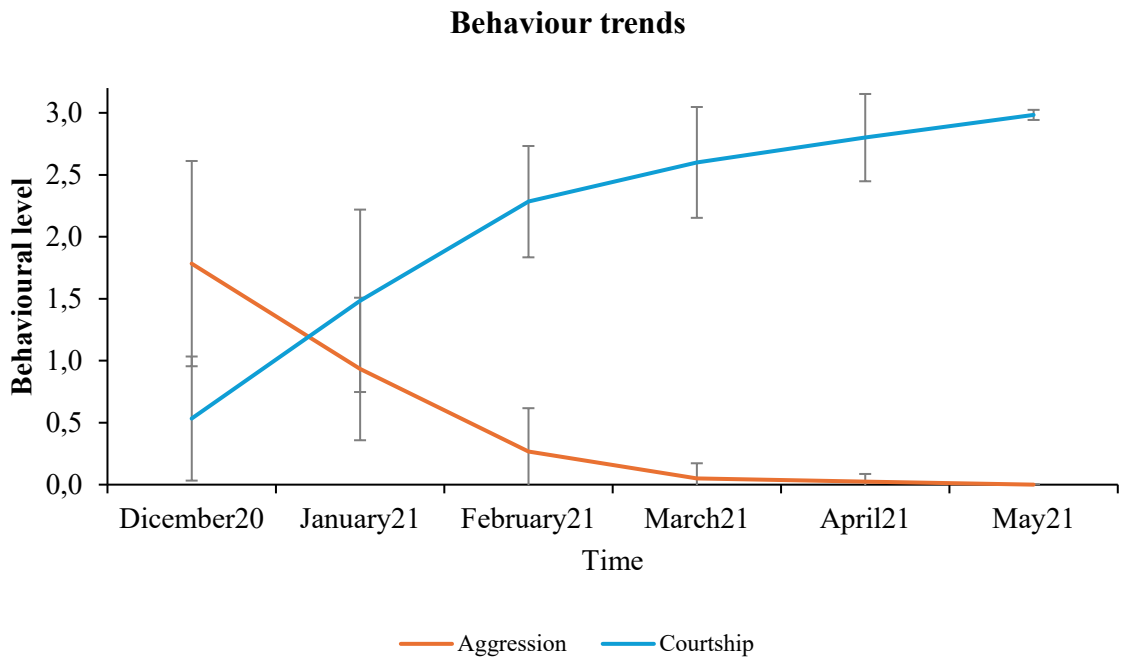

Figure 4.1: Behavioural level trends (aggression and courtship) of six *P. fridmani* pairs during six months pair formation trial (December 2020 to May 2021).

At this stage, the male was constantly courting the female, chasing and turning in front of her, quivering with open fins, and then swimming back into the chosen PVC pipe (Fig. 4.2). This courtship process of swimming in and out lasted until the female followed the male and shared the same PVC pipe (Fig. 4.2). The use of a glass tank and an open-ended pipe in the present study provides a great opportunity to describe this behaviour. The male then slowly encircled the female inside the pipe, quivering, and inducing the female to spawn the egg mass. At this time, the female started to lay egg masses slowly, while the male fertilised them with sperm. This process generally took 2 h, after which the female left the PVC pipe and returned to swim in the open areas, while males started taking care of the egg mass inside the PVC pipe, swimming out for a few seconds only for feeding.

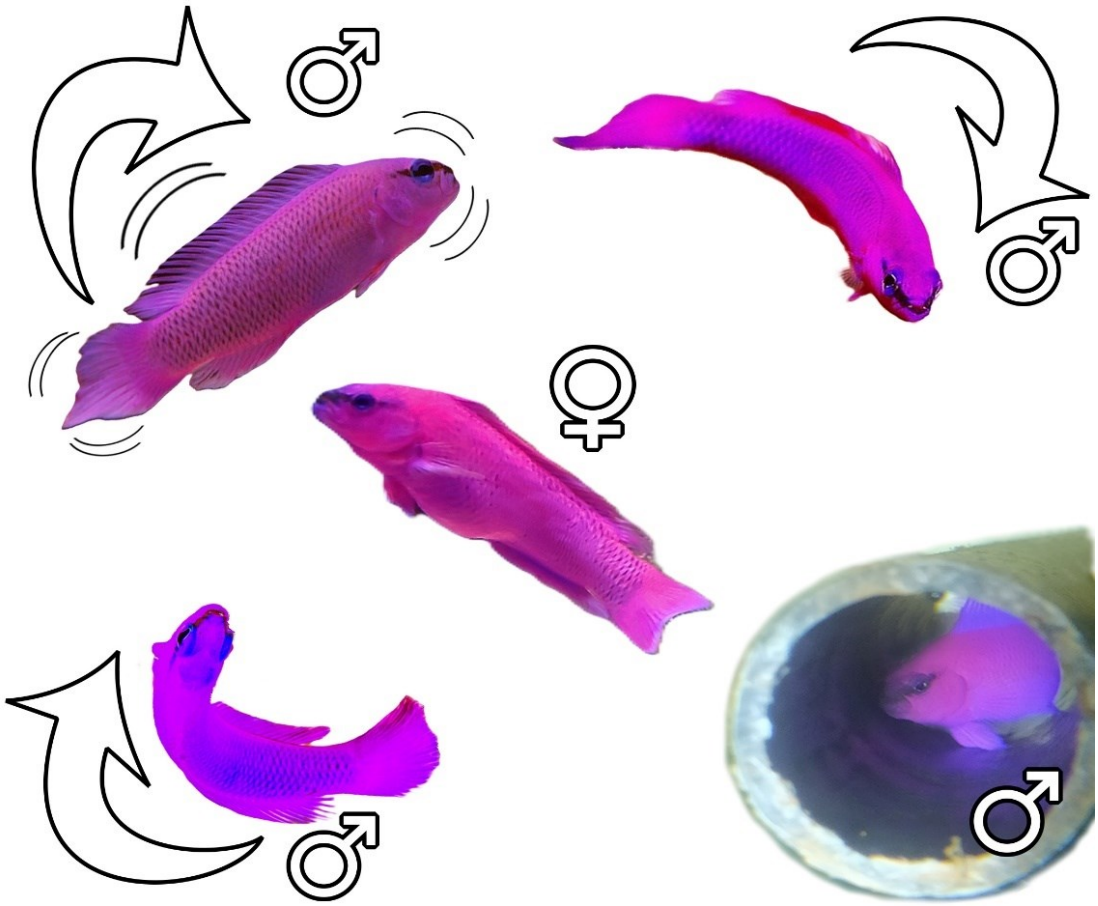

Figure 4.2: Schematization of the orchid dottyback *P. fridmani* courting behaviour: (i) Female swims in open area; (ii) Male prepares and defends the nest inside the PVC pipe; (iii) Male swims out of the pipe looking for the female; (iv) Male swims in front of the female shaking vigorously the body; (v) Male swims back into the PVC pipe, guiding the female by indicating the way.

Table 4.2: Pairs formation and behavioural analysis (aggression and courtship) of six *P. fridmani* pairs during six months (December 2020 to May 2021). Semiquantitative scoring of behaviour (Mean ± SD): score 0: no; score 1: low; score 2: medium score 3: high.

***P. fridmani* pairs**	**Dicember20**		**January21**		**February21**		**March21**		**April21**		**May21**		**Time to bond (weeks)**
	Aggression	**Courtship**	**Aggression**	**Courtship**	**Aggression**	**Courtship**	**Aggression**	**Courtship**	**Aggression**	**Courtship**	**Aggression**	**Courtship**	
A	2.5 ± 0.6	-	1.3 ± 0.4	0.9 ± 0.2	0.6 ± 0.4	1.9 ± 0.3	-	2.3 ± 0.3	-	2.8 ± 0.5	-	3.0 ± 0.0	9
B	0.8 ± 0.2	1.2 ± 0.5	-	2.2 ± 0.7	-	2.8 ± 0.2	-	3.0 ± 0.0	-	3.0 ± 0.0	-	3.0 ± 0.0	4
C	0.9 ± 0.3	1.0 ± 0.3	0.7 ± 0.4	1.6 ± 0.2	0.2 ± 0.1	2.0 ± 0.3	-	2.5 ± 0.4	-	2.9 ± 0.5	-	3.0 ± 0.0	8
D	1.6 ± 0.3	0.6 ± 0.3	0.9 ± 0.3	2.0 ± 0.3	-	2.4 ± 0.4	-	2.9 ± 0.5	-	3.0 ± 0.0	-	3.0 ± 0.0	6
E	2.1 ± 0.5	0.4 ± 0.2	1.0 ± 0.2	1.9 ± 0.4	-	2.8 ± 0.5	-	3.0 ± 0.0	-	3.0 ± 0.0	-	3.0 ± 0.0	6
F	2.8 ± 0.7	-	1.7 ± 0.6	0.3 ± 0.2	0.8 ± 0.5	1.8 ± 0.3	0.3 ± 0.3	1.9 ± 0.6	0.2 ± 0.2	2.1 ± 0.5	-	2.9 ± 0.6	12
Tot. mean + SD	1.8 ± 0.8	0.5 ± 0.5	0.9 ± 0.6	1.5 ± 0.7	0.3 ± 0.4	2.3 ± 0.4	0.1 ± 0.1	2.6 ± 0.4	0.0 ± 0.1	2.8 ± 0.4	-	3.0 ± 0.0	7.5 ± 2.8

4.4.2 Spawning activity and Filial cannibalism

TheThe six pairs of *P. fridmani* spawned constantly throughout the experiment, regardless of the temperature (Table 4.3; Fig. 4.3). Spawning was observed only in the afternoon, between 2 pm and 7 pm. The *P. fridmani* pairs exhibited similar monthly spawning event numbers when maintained at the same water temperature (Table 4.3). Nevertheless, at 28 °C the total of monthly spawning events was significantly higher (3.4 ± 0.1; t = -19.6; $p < 0.0001$) compared with that of pairs kept at 25 °C (1.8 ± 0.1) (Table 4.3). Moreover, the frequency of spawning events was similar between the pairs maintained at the same water temperature, whereas it was significantly increased at higher temperatures (Table 4.3). Indeed, pairs kept at 25 °C presented significantly lower total spawning frequency (19.8 ± 2.2 days), compared with those kept at 28 °C (9.3 ± 0.4 days) (t = 7.36; p = 0.0018) (Table 4.3). FC was affected by temperature and varied greatly between the two treatments (Table 4.3; Fig. 4.3). At 25 °C, the pairs presented significantly lower levels of total monthly FC (18.5% ± 8.6%) compared to that at 28 °C (59.1% ± 6.4%; t = -9.33, $p < 0.0022$) (Table 4.3). There was no significant difference in the total available egg masses between the treatments (Table 4.3; t = 0.16, p = 0.888).

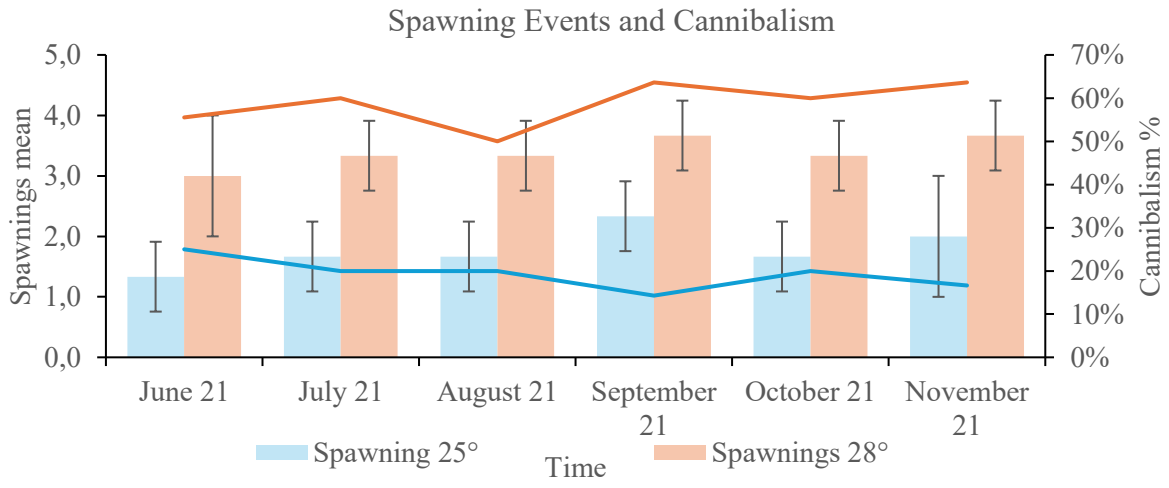

Figure 4.3: Total mean spawning and filial cannibalism rate (%) of six *P. fridmani* pairs exposed to different water temperature conditions (n= 3) over 6 months period.

Table 4.3: Monthly spawning events, spawning frequency, filial cannibalism rates and available spawning of six *P. fridmani* pairs reared under different water temperature over six months (25 °C; 28 °C). Values (Mean ± SD) followed by different superscript letters in the same row are significantly different ($p < 0.05$).

Monthly spawning events (n.)				**Spawning frequency (days)**				**Monthly Filial cannibalism (%)**				**Available spawnings (n.)**			
Pair	**25°C**	**Pair**	**28°C**	**Pair**	**25°C**	**Pair**	**28°C**	**Pair**	**25°C**	**Pair**	**28°C**	**Pair**	**25°C**	**Pair**	**28°C**
A	1.8 ± 0.4	D	3.5 ± 0.5	A	17.8 ± 6.0	D	8.9 ± 1.5	A	27.3 ± 27.4	D	52.4 ± 12.5	A	1.7 ± 0.5	D	1.7 ± 0.5
B	1.7 ± 0.8	E	3.3 ± 0.5	B	22.1 ±9.6	E	9.3 ± 1.3	B	10.0 ± 20.4	E	60.0 ± 15.3	B	1.5 ± 0.8	E	1.7 ± 0.5
C	1.8 ± 0.8	F	3.3 ± 0.8	C	19.6 ± 9.1	F	9.8 ± 3.1	C	18.2 ± 22.1	F	65.0 ± 11.4	C	1.7 ± 0.8	F	1.2 ± 0.4
Total	1.8 ± 0.1^{a}	Total	3.4 ± 0.1^{b}	Total	19.8 ± 2.2^{b}	Total	9.3 ± 0.4^{a}	Total	18.5 ± 8.6^{a}	Total	59.1 ± 6.4^{b}	Total	1.6 ± 0.1	Total	1.5 ± 0.3

4.4.3 Gonadal histology

Macroscopic analysis confirmed the correct classification of the six specimens, comprising three males and three females (Fig. 4.4 and 4.5; Table 4.1). The ovaries were lobular paired structures that occupied a substantial portion of the abdominal cavity and were larger than the male testes. Their colour ranged from yellowish to orange, depending on the maturation stage of the oocytes, reflecting their advanced reproductive development. Enlarged size of the ovaries was correlated with the presence of vitellogenic oocytes, which is a sign of imminent spawning readiness. Histological sections of the ovaries (Fig. 4.4 a and b) displayed a range of oocyte developmental stages, typical of asynchronously spawning species. Smaller, less-developed previtellogenic oocytes were present, characterised by large nuclei and a small basophilic cytoplasm. In contrast, larger vitellogenic oocytes were abundant and filled with prominent yolk granules and lipid droplets (Fig. 4.4 a). These features are characteristic of the later stages of oocyte maturation and preparation of eggs for fertilisation. The zona radiata, a specialised extracellular membrane protein, was also visible surrounding the more mature oocytes, indicating their functional readiness (Fig. 4.4 b).

The testes were elongated, symmetrical, and paired organs located along the ventral side of the body cavity. They appeared whitish to pale cream in colour, with a smooth and firm texture, indicative of sexual maturity. Histological sections of the testes revealed well-defined seminiferous tubules, each containing multiple stages of spermatogenesis (Fig. 4.5 a and b). The tubules were lined with spermatocytes that progressed to spermatids (Fig. 4.5 b). The interstitial tissue between the tubules showed the presence of Leydig cells, which are responsible for androgen production and are critical for regulating spermatogenesis (Fig. 4.5 b).

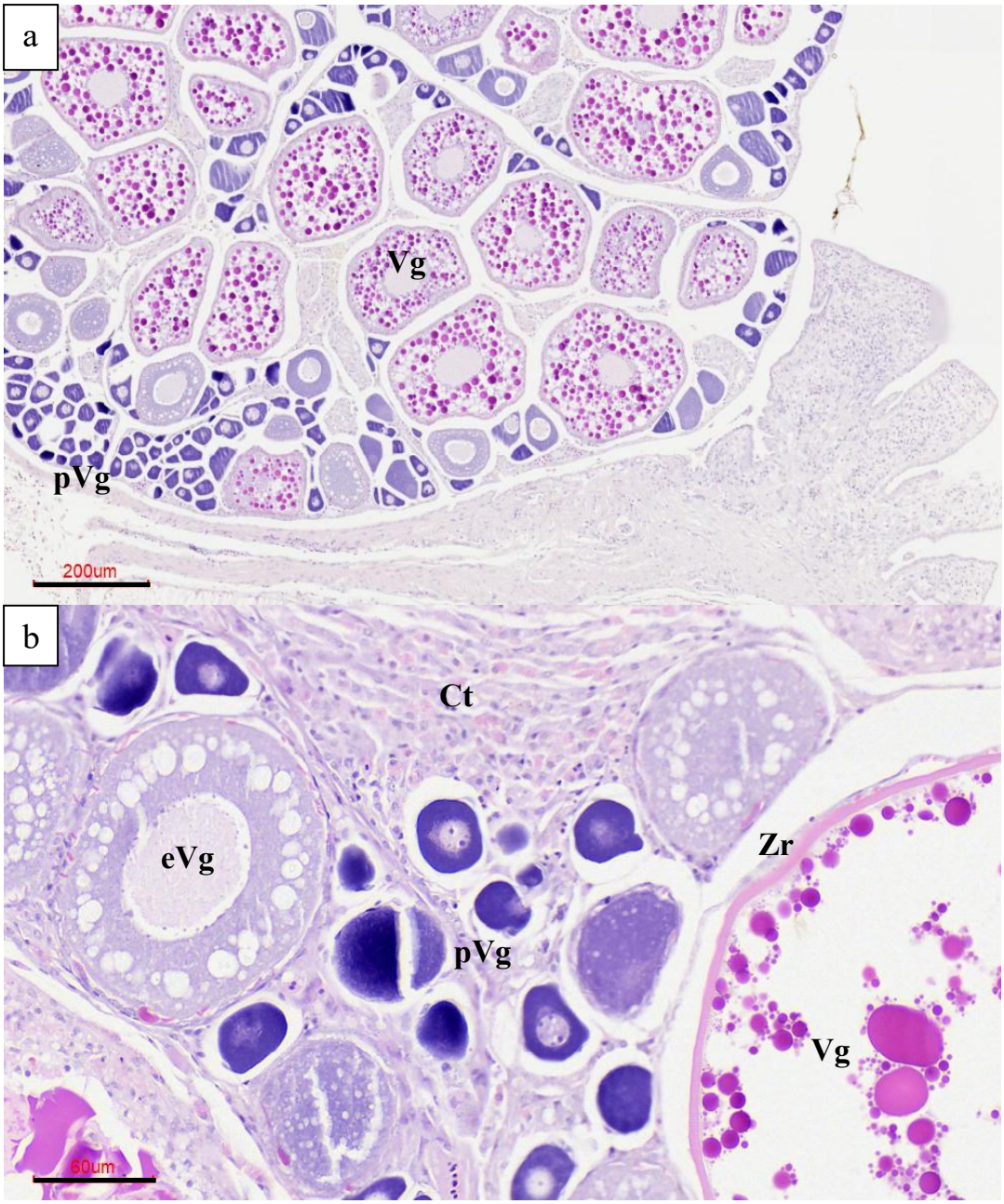

Figure 4.4: Histological examination of *P. fridmani* ovary: (a) Expanded view of mature ovary with different follicular development stages, scale bar 200µm; (b) Detailed view of the various stages of oocyte maturation., scale bar 60µm; eVg= early Vitellogenic oocyte; pVg= Previtellogenic oocyte; Vg= Vitellogenic oocyte; Ct= Connective tissue; Zr= Zona radiate.

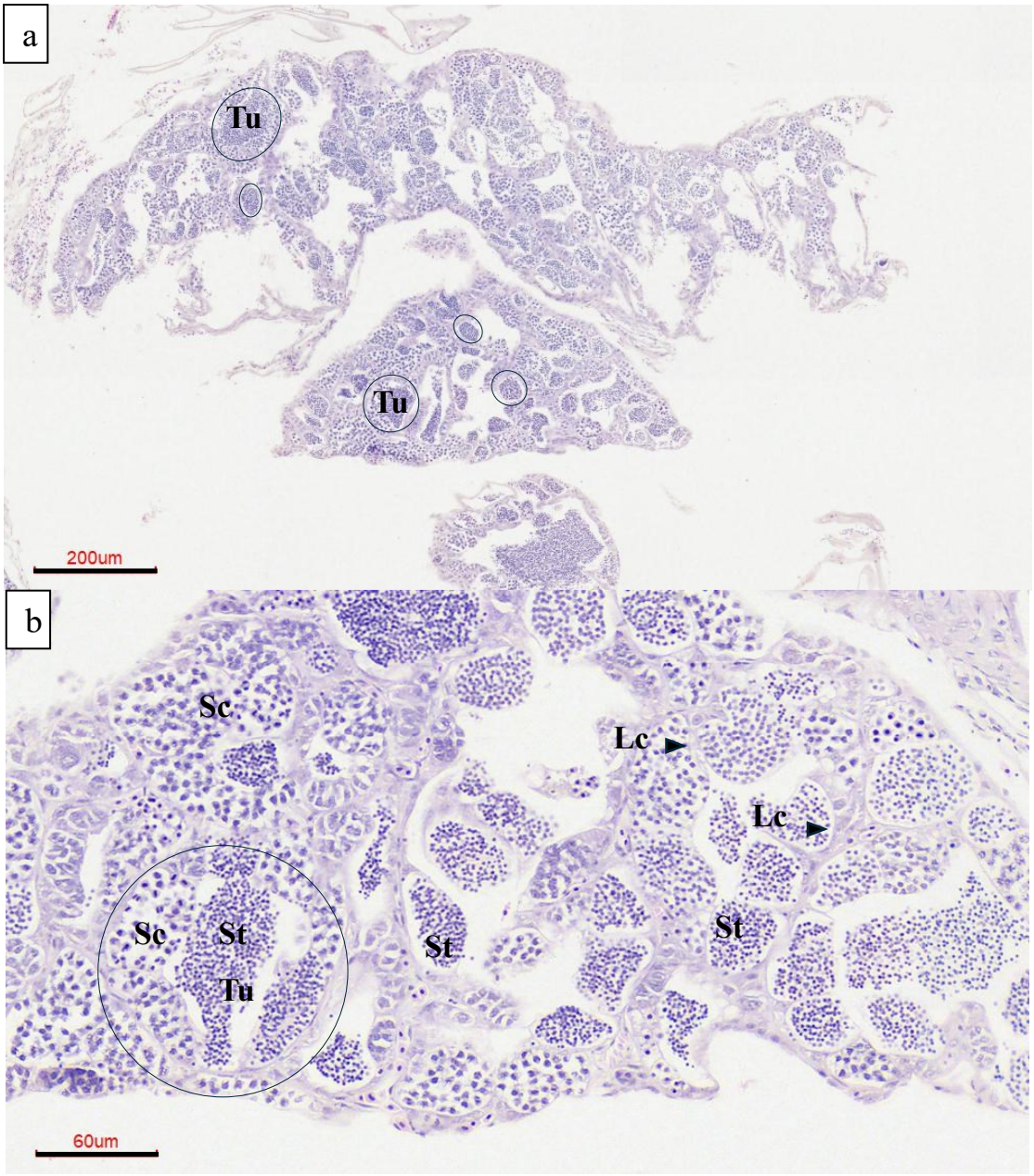

Figure 4.5: Histological examination of *P. fridmani* testicular tissue: (a) Expanded view of mature test at various stages of spermatogenesis scattered in the gonad, scale bar 200µm; (b) Detailed view of the various maturation stages, scale bar 60µm; Sc= Spermatocyte; St= Spermatid; Tu= Seminiferous tubules (highlighted by a black circle); Lc=Leydig cells (highlighted by a black arrow).

4.4.4 Embryos development

The embryonic development of orchid dottybacks at 25 °C occurred within approximately 96 HPF (Table 4.S1). After spawning occurred, size of individual egg varied between 1.0 ± 0.1 mm, and all were aggregate by fine sticky threads which give stability to the egg mass without attach to the substrate. Each egg was spherical in shape and transparent containing spherical yolk with 0.5 ± 0.1 mm diameter. After spawning, 0 HPF (Fig. 4.S2 a), discoidal meroblastic cleavage was going to start while chorion was completely expanded. After 1 HPF the beginning of the cleavage was observed with the formation of the blastodisc (4 cells) (Fig. 4.S2 b), while at 2 HPF the blastodisc become several cell layers (16 cells stage) reaching morula stage (Fig. 4.S2 c). At 3 HPF the early blastula formation was observed with very small blastomeres (512 cells) (Fig. 4.S2 d). After 6 HPF followed the flattening of blastodisc with the blastomeres migrating downward over the yolk (Fig. 4.S2 e). After 9 HPF the completion of blastula stage was observed with the beginning of epiboly (Fig. 4.S2 f). After 12 HPF, gastrulation began with the migration of blastoderm equidistantly around the yolk, elevated with a thin layer of blastomeres on the surface (Fig. 4.S2 g). Blastoderm cells spread over the yolk mass and reached the blastopore, indicating that late gastrula stage was ready at 15 HPF (Fig. 4.S2 h). The organogenesis started after 18 HPF with primitive formations of head and neuroepithelial (Fig. 4.S2 i), while at 24 HPF the brain and the eyes differentiation were clearly visible, the neural groove extends along embryonic axis, and the notochord appeared over the yolk mass (Fig. 4.S2 j). At 36 HPF, the embryo increased in size and formation of the eye lens were evident, tail extended beyond yolk and its movements were clearly visible, as well as the absorption of yolk was visible (Fig. 4.S2 k). At 48 HPF embryo increased in size, gill arches primordia, mussels and heart were noticed with recognizable upper and lower jaw (Fig. 4.S2 l). At 72 HPF the embryo increased in size, yolk absorption became increasingly clear, gill arches were recognizable, and the heart become prominent and

exhibited a rhythmic beat with blood circulation. Some melanophores along the posterolateral part of the embryo were evident and the retina appeared heavily pigmented, muscles become prominent, as well as pectoral fin formation (Fig. 4.S2 m). At 96 HPF the maxilla, gill arches and muscles were well developed, black pigments appeared on the body. Embryos were particularly active with the dorsal and ventral fin fold, twisting within the egg. Occupying the entire space of egg, and the tail completely wrapped around the head, they were ready to hatch (Fig. 4.S2 n). Hatching took place in the evening on completion of 96 HPF at water temperature of 25°C and dark conditions.

4.5 Discussion

The present study analysed the current challenges concerning the reproductive biology and broodstock management of the orchid dottyback, *P. fridmani*, highlighting the importance of controlling rearing conditions to improve broodstock performance. The most critical aspect of the reproductive biology of orchid dottybacks were assessed, showing how rearing temperature plays a key role in spawning activity and FC.

4.5.1 Reproductive behaviour and pair formation

The timing of pair formation varied among pairs and was closely influenced by aggression and courtship levels. Therefore, considering that aggression is more intense among individuals of the same sex (Brons, 1996; Moe, 1997; Wittenrich, 2007), pairs A and F may have undergone sex reversal to establish a reproductive pair, as suggested by the higher aggression observed in these fish during the bonding period (Wittenrich and Munday, 2005). Nevertheless, it cannot be excluded that *P. fridmani* is either incapable of bidirectional sex change or is not truly hermaphroditic. In this scenario, the increased aggression and resulting delay in pair formation may be attributed to issues related to gonadal maturation or

behavioural incompatibility between individuals, thus requiring more time to establish a bond. In contrast, pairs B, C, D, and E probably already had an established hierarchy, presenting low aggressiveness and earlier bonding (Wittenrich and Munday, 2005). Previous studies have reported that bonding between *P. fridmani* pairs occurs rapidly (Mies *et al*., 2014; Chen *et al*., 2023). This discrepancy with regard to the time required for pair formation may be influenced by factors such as the origin of the individuals used in the study, their age, size, sexual maturity, and pre-existing social relationships (Mies *et al*., 2014; Chen *et al*., 2023). Moreover, temporary isolation of males proved to be effective in achieving successful pairing among aggressive individuals. Indeed, continuous visual contact between specimens probably facilitated the establishment of a hierarchy and mutual acceptance, while preserving physical integrity by minimising the risk of negative interactions. The observations on reproductive behaviour of orchid dottybacks helped better understand the pair bonding process under controlled conditions, allowing us to identify the timing of pair formation (7.5 ± 2.8 weeks, n=6) when no aggressions were observed and both fish started courtship and finally spawned.

4.5.2 Spawning activity and filial cannibalism

Orchid dottybacks are demersal spawners, with spherical eggs surrounded by a sticky, fibrillar, and elastic extracellular matrix which gave stability to the egg mass until hatch. In the present study the eggs morphology and embryos development sequence were in accordance with previous studies conducted at higher temperature on *P. fridmani* (27 ± 1 °C) (Chen *et al*., 2023), and similar dottyback species as *P. flavivertex* (27 ± 0.5 °C) (Olivotto *et al*., 2006) and *P. dilectus* (29 ± 1 °C) (Madhu *et al*., 2016). Broodstock pairs were highly influenced by water temperature, which played a key role in optimising the breeding and spawning management of *P. fridmani* under controlled conditions. At 28 °C, the total number of monthly spawning (3.4 ± 0.1) was significantly higher compared to that at 25 °C; however,

egg cannibalism also increased (59.1% ± 6.4%) resulting in a reduced number of available egg masses for hatching (1.5 ± 0.3). Similar results were also reported in the beaugregory damselfish (*Stegastes leucostictus,* Castelnau, 1855), which showed increased FC at high temperatures and low oxygen levels (Payne *et al*., 2002; 2004). In addition, our findings align with those recently reported by Chen *et al.* (2023), who also observed a high occurrence of FC, predominantly during the later stages of the incubation period (72–96 HPF). In our study, the higher number of total spawning and FC observed in pairs kept at 28 °C can be explained by the increased temperature which may directly affect reproductive processes by promoting hormone synthesis and action within the hypothalamus–pituitary–gonadal axis, generating a stress condition that led males to consume eggs (Donelson *et al*., 2010; Pankhurst and Munday, 2011). Generally, FC is attributed to a multitude of factors, such as water parameters (Payne *et al*., 2002; 2004), egg developmental issues (Mies *et al*., 2014; Chen *et al*., 2023), meeting the energy cost of parental care (Manica, 2004; Klug, 2009), and/or restarting courtship with a partner (Matsumoto *et al*., 2018). Therefore, the causes of FC remain unclear, but they may be multifactorial and influenced by the specific behavioural traits of individuals as well as their rearing conditions. Adopting appropriate rearing conditions adjusted to the needs of each species, particularly with regard to water temperature, is essential for the successful breeding of marine fish (Wittenrich, 2007; Chen *et al.*, 2020). Previous studies reported high FC from several dottyback species kept under controlled rearing conditions at high temperatures, including sunrise dottyback *P. flavivertex* (27 ± 0.5 °C, Olivotto *et al*., 2006), orchid dottyback *P. fridmani* (27 °C, Mies *et al*., 2014; 27.5–29 °C, Chen *et al.*, 2023), and redhead dottyback *P. dilectus* (29 ± 1 °C, Madhu *et al*., 2016). The results of the present study showed that pairs kept at a lower temperature (25 °C) also presented continuous spawning over time, with significantly less FC compared to pairs kept at 28 °C, thus mitigating the risk of egg loss during aquaculture operations. Furthermore, also the spawning

frequency of orchid dottybacks significantly increased with temperature (28 °C; 9.3 ± 0.4 days), which is in line with previous studies reporting similar frequencies at similar temperatures (Mies *et al.,* 2014; Chen *et al.*, 2023).

The continuous spawning observed throughout the experiment was not correlated with water temperature but rather seemed to be a behaviour associated with bonding strength of the pair, likely influenced by isolation, food availability, and other rearing conditions (Chen *et al.*, 2023). Maintaining the broodstock at 25° C appears to be a viable strategy for mitigating FC while ensuring continuous spawning over time. This approach may improve overall larval production efficiency by reducing egg loss during aquaculture operations. These findings have significant implications for the aquaculture of *P. fridmani*, highlighting the importance of optimising rearing conditions to enhance broodstock management, reproductive success, and aquaculture outcomes.

4.5.3 Gonads histology and embryo ontogeny

The lack of clear sexual dimorphism in *P. fridmani* may lead to pairing challenges, potentially resulting in unintentional pairing of individuals of the same sex, which frequently results in aggressive behaviour (Mies *et al*., 2014). In our study, macroscopic analysis of the gonads suggested that the evaluation of total length and body shape of individuals may be a relatively reliable and fast proxy for visually identifying the sex of orchid dottybacks. Indeed, histological analysis highlighted the presence of both previtellogenic and vitellogenic oocytes in ovary tissue, while testes were in active spermatogenesis. This confirmed that these individuals were sexually mature, with gonads fully developed for reproductive purposes and potentially undergoing multiple spawning events. However, limitations are present as our histological analysis was based on six sexually mature fish (> 1 year old) originating from the

same brood. Therefore, in the case of younger specimens, this approach may be less effective compared to its application on well-developed and sexually mature specimens.

Wittenrich and Munday (2005) confirmed bidirectional sex changes in three dottyback species, suggesting that other dottyback species can exhibit the same. Our results showed that the gonads of males contained only spermatocytes and spermatozoa, and no ovarian tissues were observed, hindering hypothetical sex reversals. However, despite the fact that individuals were clearly male and unable to change sex under specific environmental conditions, they may start sex reversal (male to female), as demonstrated in other dottyback species (Wittenrich and Munday, 2005; Wittenrich, 2007; Kuwamura *et al*., 2020). Similar results were obtained by Wittenrich and Munday (2005), who histologically examined the gonads of *P. flavivertex* specimens that had undergone sex change and described the presence of only testes or ovary tissues in males and females, respectively. Bidirectional sex change can be advantageous when mate choice is limited, and mating occurs within a restricted home range (Munday, 2002). Therefore, to improve the breeding and broodstock management of orchid dottybacks under controlled conditions, further in-depth studies are needed to verify the presence of hermaphroditism and bidirectional sex changes in this species by manipulating the population structure of isolated pairs (male-male) or harems.

The embryonic development analysis helped to better understand the early ontogeny of *P. fridmani*, confirming previous study conducted at different temperature (Chen *et al*., 2023) and providing guidelines for the improvement of its management. Orchid dottybacks are egg mass layers, with spherical eggs surrounded by a sticky, fibrillar, and elastic extracellular matrix which gave stability to the egg mass until hatch. In the present study the eggs morphology and embryos development sequence were in accordance with previous studies conducted at higher temperature on *P. fridmani* (27 ± 1 °C) (Chen *et al*., 2023), and similar dottyback species as *P. flavivertex* (27 ± 0.5 °C) (Olivotto *et al*., 2006b) and *P. dilectus* (29 ±

1 °C) (Madhu *et al*., 2016). In comparison with the findings of Chen *et al*., (2023), our results revealed similar timing during embryonic ontogeny, despite differences in sampling methodologies and rearing temperatures. This observation suggests that minor variations in rearing temperature may not significantly impact the overall timing of embryonic development in this species. Moreover, this resilience to temperature variation could indicate that the species has evolved mechanisms to buffer developmental processes against minor thermal changes, possibly as an adaptation to fluctuating environmental conditions in its natural habitat. Further research should explore the limits of this thermal tolerance, as well as the potential associated mechanisms.

4.6 Conclusions

The present study contributes to the knowledge of the reproductive biology of *P. fridmani* by providing in-depth descriptions of the effects of temperature on the spawning and FC of this highly demanded and charming species, improving its breeding under controlled conditions and enhancing large-scale production in the marine ornamental fish trade. Broodstock formation may be critical over the first 2 months, with possible episodes of aggression, whereas courtship and spawning behaviour start shortly afterwards. The behaviour was clearly pair-specific and influenced by sexual and social incompatibility, which may be quickly resolved through temporary isolation of the aggressive specimens. The importance of the rearing temperature to improve broodstocks performance was highlighted, suggesting that temperatures higher than 25 °C increase monthly spawning as well as FC, thus reducing the final number of available egg masses for hatching. Therefore, maintaining the rearing temperature around 25 °C may be optimal for enhancing broodstock management and performance of *P. fridmani* under controlled conditions. Although the histological analysis of *P. fridmani* gonads did not definitively confirm or rule out

the possibility of bidirectional sex changes in this species, total length and body shape emerged as relatively reliable indicators for sex determination.

In conclusion, this study represents not only an improvement in the culture techniques of this scarcely known species but also constitutes a reference for studying dottyback ecology and population dynamics in the wild. To improve the breeding and broodstock management of orchid dottybacks under controlled conditions, further in-depth studies are needed to completely understand the factors promoting FC and investigate the presence of hermaphroditism and bidirectional sex change in this species.

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Supplementary Files

Figure 4.S1: The *P. fridmani* broodstock tank set in three partitions and housing three spawning pairs during experimentation.

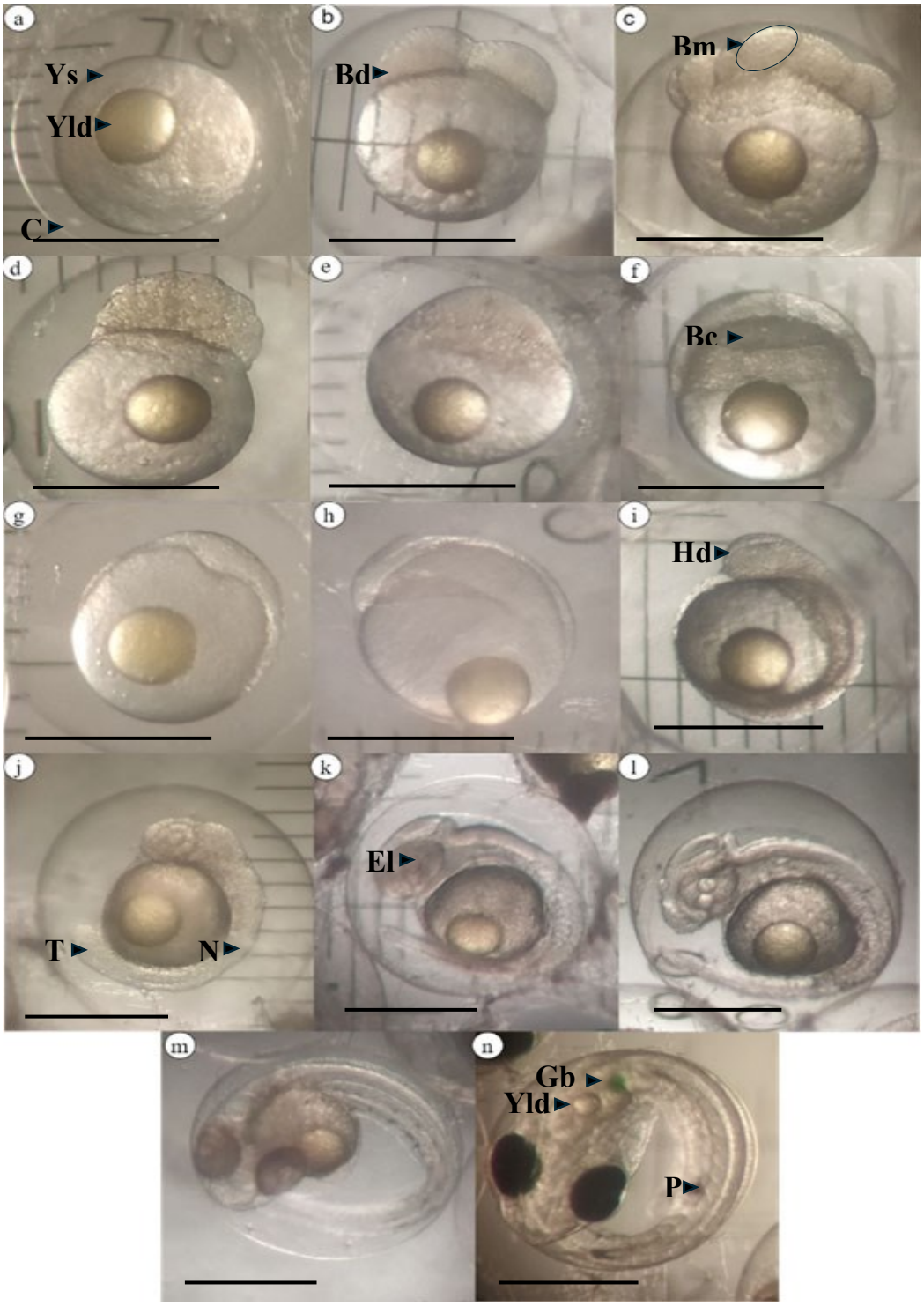

Figure 4.S2: Embryonic development of *P. fridmani* (25 ± 1 °C): (a) 0 HPF, chorion is completely expand; (b) 1 HPF, 4 cells stage; (c) 2 HPF, 16 cell stages; (d) 3 HPF, early blastula , 516 cell stage; (e) 6 HPF, blastodisc flattening stage (f) 9 HPF, late blastula and epiboly stage; (g) 12 HPF, early gastrula stage; (h) 15 HPF, late gastrula stage; (i) 18 HPF, begin organogenesis and primitive formations; (j) 24 HPF, notochord appeared with primitive head and tail over the yolk; (k) 36 HPF, eye lens and embryo movements were clearly visible; (l) 48 HPF, gill arches, mussels and heart primordia with evident absorption of yolk sac, (m) 72 HPF, gill arches, upper and lower jaw with eyes highly pigmented; (n) 96 HPF, embryo completely developed bent double, black pigments and complete absorption of yolk sac. The scale bar present in the down left corner of each developmental stage correspond to 500µm. Ys= Yolk sac; Yld= Yolk lipid droplet; C= Chorion; Bd= Blastodisc; Bm= Blastomere; Bc= Blastocele; H= Head; T= Tail; N= Notochord; El= Eye lens; Gb= Gall bladder; P= Pigment.

Table 4.S 1: Embryonic developmental stage of *P. fridmani* at 25 C° water temperature (n=3).

***Pseudochromis fridmani* embryos development (25 °C)**		
Time (h)	**Developmental stage**	**Figure**
0	Dilated chorion wall	a
1	Begin cleavage 4 blastomeres	b
2	Morula 16 blastomeres	c
3	512 blastomeres early blastula	d
6	Flattened blastodisc	e
9	Late blastula, epiboly	f
12	Early gastrula	g
15	Late gastrula	h
18	Begin organogenesis, primordium	i
24	Notochord and head	j
36	Eye lens	k
48	Gill arch, mussels, heart	l
72	Jaws, eyes pigmentation	m
96	Body pigmentation, development complete	n

Chapter 5. Live microalgae-based diets as enrichment to improve the nutritional profile of the calanoid copepod *Acartia tonsa* (Dana, 1849) nauplii

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5.1 Abstract

Most emerging species in marine aquaculture and the Aquarium industry have a sensitive and small-mouthed larval stage, and their culture is very challenging due to a lack of appropriate early feeding protocols. The use of copepods has been widely recognized as a good alternative to traditional fish live feeds (Rotifers and *Artemia spp*.), especially for new species targeted for the multibillion-dollar marine ornamental trade. However, most copepod culture trials are focused on biomass production, while their nutritional requirements and enrichment protocols remain scarcely known. The main objective of this study was to determine the dietary contribution of three microalgae-based diets, provided as a mono R (*Rhodomonas salina*) or binary I-R (*Isochrysis galbana* + *R. salina*) and T-R (*Tetraselmis suecica* + *R. salina*), on the biochemical composition (proximate and fatty acids) and survival of the calanoid copepod *Acartia tonsa* (nauplii) *versus* the conventional rotifer *Brachionus plicatilis* throughout 96 h exposure. Both live preys were characterized by the highest protein and total n-3 long-chain poly unsaturated fatty acids (PUFAs) content when fed with the binary diet I-R compared to other treatments, although the nutritional value of *A. tonsa* nauplii was always much higher compared to *B. plicatilis*. In general, there was a wide difference in proximate and fatty acid (FAs) composition between the two preys, denoting *A. tonsa* as better live feed in terms of capacity to retain n-3 LC-PUFAs, especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). The present study highlights the nutritional profile of enriched *A. tonsa* nauplii and contributes to the production of high-quality live feeds, which may be able to reach the nutritional needs of new marine fish species during their larval development, as well as contributing to the improvement of *A. tonsa* culture

5.2 Introduction

5.2.1 Marine fish larvae production and conventional live feeds

In the last decades, the production of high-quality marine fish species and its diversification were constrained by a bottleneck of non-optimized nutritional food sources for larval stages, inducing low growth and survival (Hamre *et al.,* 2013; Hansen and Möller, 2021). The success in the larval breeding of new marine species is determined mainly during the early life stages of development, during the transition from endogenous to exogenous nutrition (Olivotto *et al.,* 2011, 2017). At this stage, the live feed should be adapted to the morphological characteristics of the larvae and their needs in terms of nutritional requirements. In contrast to their natural environment, where larvae have access to a large variety of feeding regimes, the main live preys employed in hatcheries consists of rotifers (*Brachionus* spp.) and brine shrimps (*Artemia* spp.). Rotifers have small size, suitable as first feeding for the earliest stages of several fish and shrimp larvae species (Dhont *et al*., 2013). Meanwhile, *Artemia* shows larger size variability compared to rotifers and can be hatched from dormant cysts, which can be easily distributed and stored for prolonged periods of time (Dhont *et al*., 2013). However, both preys have a limited ability to synthesize important long-chain polyunsaturated fatty acids (LC-PUFAs) from shorter fatty acids (Lubzens *et al*., 1985; Birkou *et al*., 2012; Dey *et al.,* 2022). The rate of synthesis of these fatty acids (FAs) is rather low, especially for docosahexaenoic acid (DHA: 22: 6n-3), and to supply large amounts of polyunsaturated fatty acids (PUFAs) to marine fish larvae, live preys must be enriched with PUFA-rich food (Birkou *et al*., 2012). Several techniques have been developed to improve the biochemical composition of rotifers and *Artemia* (e.g., oil emulsions, phytoplankton, etc.), or even to seek new types of live preys whose composition may fulfil nutritional requirements of certain fish species (Rasdi and Qin, 2014).

5.2.2 Copepods as live feed

The use of copepods has been widely recognized as a good alternative to traditional live feeds (Ajiboye *et al*., 2011; Camus, 2012; Zeng *et al*., 2018). Copepods can improve the survival and growth for a variety of fish larvae due to their small developmental stages, swimming behaviour and FAs profile which includes high content of LC-PUFAs (Rasdi and Qin, 2014; Zeng *et al.,* 2018). Therefore, there is a considerable interest in studying the mechanisms responsible for its production (Shields *et al.,* 1999; Drillet *et al.,* 2011). New techniques allow producing large quantities of calanoid copepods using cold stored subitaneous eggs (Støttrup, *et al*., 1999; Drillet *et al*., 2006a; Drillet *et al*., 2006b; Peck and Holste, 2006; Drillet *et al*., 2007; Peck *et al*., 2008; Drillet *et al*., 2011). However, cold-stored copepod embryos still retain a physiological activity during this period and their nutritional quality may change over this time (Støttrup *et al*., 1999; Drillet *et al*., 2006a, b, 2011). In particular, the major biochemical differences that occur during the storage period are related to the embryo FAs content (Drillet *et al*., 2006b). Previous studies demonstrated that after 6 months of cold storage, the quality of copepods obtained from those eggs was suboptimal for the Saddleback clownfish (*Amphiprion polymnus*) larval rearing (Olivotto *et al.,* 2012). Therefore, unless proper enrichment can be assured, these changes in FAs may affect larval survival and development. Most calanoid copepods, which constitute the natural food source for many marine fish larvae (Støttrup, 2000), are unable to elongate and desaturate the 18:3n-3 fatty acid to produce significant amounts of the LC-PUFAs, eicosapentaenoic (EPA: 20:5n-3) and DHA fatty acids (Monroig *et al*., 2022*)*. Their high content in n-3 LC-PUFAs is incorporated directly through the phytoplankton, exhibiting reduced growth and egg production when, in opposition, fed diets deficient in EPA and DHA (Støttrup and Jensen, 1990; Jonasdottir *et al*., 1995). Moreover, food quantity and quality affect copepod ingestion, survival, growth, and fecundity (Siuda and Dam, 2010; Drillet *et al.,* 2011; Chen *et al*., 2012; Rasdi and Qin, 2014;

Besiktepe and Dam, 2020). The use of a mixed diet has been proven to sustain large copepod cultures for many generations (Alajmi and Zeng, 2013). Otherwise, monospecific diets may cause nutritional deficiencies, because of the inadequate content of one or more essential nutrients (Puello-Cruz *et al*., 2009).

5.2.3 *Acartia tonsa* enrichment study

Since most copepod culture trials are focussed on the quantity of mass production, the nutritional requirement and enrichment potential of copepod remains scarcely known (Drillet *et al*., 2011; Rasdi and Qin, 2014). The nutritional value of copepods is less predictable due to the variety of algae species available as feed (Rasdi and Qin, 2014; Malzahn *et al*., 2015). Little information to date is available on adequate protocols to enrich copepods with nutritional and trace elements required for marine fish larvae (Payne and Rippingale, 2001; Sun *et al*., 2013). The copepod *Acartia tonsa* (Dana, 1848) is a ubiquitous cosmopolitan estuarine species that is often used in biology and ecotoxicology studies among others (Gorbi *et al*., 2012), but also as live prey for aquaculture (Støttrup 2000, 2006; Drillet *et al*., 2011). Indeed, many studies have already highlighted the relevance of *A. tonsa* as live feed favouring fish larval survival and growth in comparison to rotifers and *Artemia* nauplii (Shields *et al*., 1999; Malzahn *et al.,* 2015). In this study we examined the dietary contribution of three microalgae species (*Rhodomonas salina, Isochrysis galbana* and *Tetraselmis suecica*), provided as a mono- or binary-diets, on the biochemical composition (proximate and fatty acids) and survival of *A. tonsa* nauplii *versus* the conventional live feed *Brachionus plicatilis*. Specifically, we focus on the relationship between the changes of fatty acids compositions depending on food source, in order to produce high-quality live feeds (Hansen and Möller, 2021), able to reach the nutritional needs of new marine fish larvae species. Therefore, the

identification of adequate microalgal enrichment for *A. tonsa* may enhance the production of high-quality marine fish as also contributes to the culture of this copepod species.

5.3 Materials and methods

The experiment was accomplished in accordance with the EU Directive 2010/63/EU for animal experiments, or the National Research Council's Guide for the Care and Use of Laboratory Animals.

5.3.1 Facilities

The entire experiment was held in the quarantine areas of the Aquarium of Genoa (Genoa, Liguria, Italy), using the facilities described below:

5.3.1.1 Phytoplankton cultures

The marine microalgae *T. suecica* (Kylin) Butcher 1959*, I. galbana* (Parke 1949) and *R. salina* (strain number MCCV118) (Wislouch) D.R.A. Hill & R. Wetherbee 1989, were used in the present study as food source for copepods and rotifers. They were grown in 80 L polycarbonate cylinders, inoculated into 1 µm filtered seawater (salinity 30‰), UV sterilized, and mixed with filtered air. All algae species were grown on a F/2 medium (Guillard and Ryther, 1962). Microalgae cultures were maintained at 20 ± 1 °C with rear illumination based on 10.000 K fluorescent tubes with a 24 h light regime. The algae were harvested during the log phase. Concentration of algal cultures (cells mL^{-1}) (Table 5.1) was determined daily, using Neubauer chamber (Marienfeld Superior, Germany) under a microscope (DME, Leica, Germany).

Table 5.1: Concentration of microalgae cultures expressed as mean value with standard deviation (n . 10^6 cells mL-1 ± SD), during the 4-day experiment.

Microalgae	Day 1	Day 2	Day 3	Day 4
Tetraselmis suecica	2.1 ± 0.1	2.5 ± 0.0	2.8 ± 0.1	3.1 ± 0.1
Isochrysis galbana	5.2 ± 0.1	5.9 ± 0.1	6.4 ± 0.2	6.8 ± 0.2
Rhodomonas salina	2.4 ± 0.1	2.6 ± 0.1	2.9 ± 0.0	3.2 ± 0.0

5.3.1.2 Zooplankton cultures

Rotifers (*B. plicatilis*), with a size range of 180-250 μm (L size), were cultured on S.parkle® (INVE Aquaculture, Belgium) at 30 ± 1 ‰ salinity and 20 °C in two transparent cylindrical tanks of 80 L each.

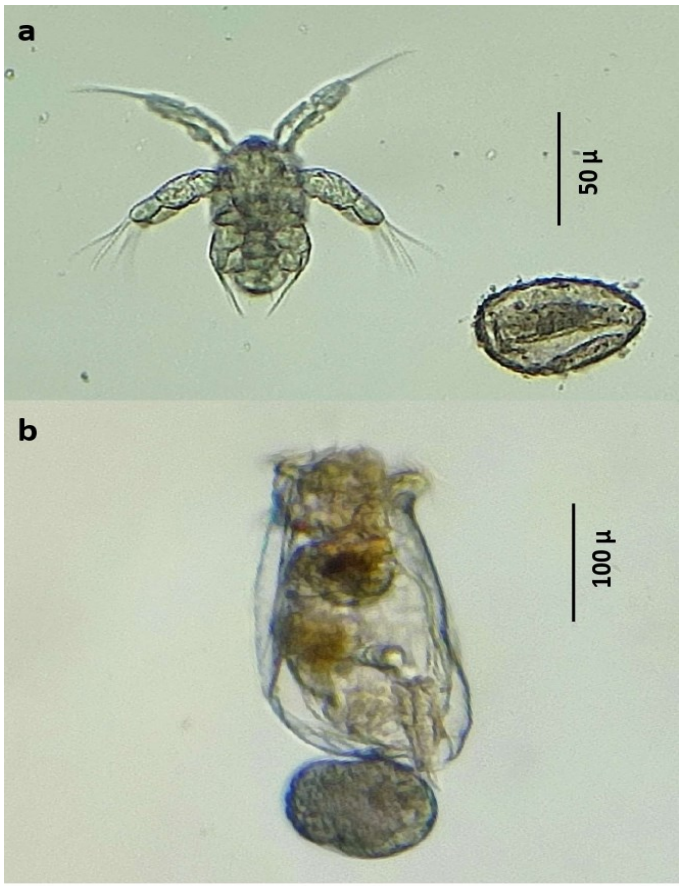

Figure 5.1: Nauplius stage of *Acartia tonsa* 24 h post hatch (a), and female of the rotifer Brachionus plicatilis (b).

Cultures were renewed weekly with a 100% water change keeping a mean culture density of 200 individuals mL^{-1} moved by a gentle airflow. The culture was 12L/12D (Light/Dark) illuminated by two 30 W fluorescent tubes. Density estimations of rotifer, with a mean size of 220 μm (Fig. 5.1 b), were done daily by averaging three 1 mL samples, and each of the samples was checked under a stereomicroscope (EZ4, Leica, Germany). Nauplii of the calanoid copepod (*A. tonsa*) with a mean size of 70 μm (Fig. 5.1 a) were obtained by cold stored eggs distributed by Algova® (Germany).

5.3.1.3 Modular Rearing System (MoRS)

The experiment was conducted inside the Life Support System (LSS) named MoRS (Modular Rearing System). This was connected to six experimental cylindrical tanks, with volume of 20 L, and equipped with air line. MoRS filtration system includes a 200 L sump supported by a biological tower, 5 μ mechanical filtration, protein skimmer (Aqua Medic, Germany), UV sterilization (PAN UV EASYMS 130, Panaque, Italy). Water temperature inside MoRS tanks was in average 20 ± 1°C (mean ± SD) with minimum variation and controlled by heating device coupled with automatic thermostat (Panaque, Italy). The whole system was illuminated 12L/12D by two 40 W fluorescent tubes, oxygen level range from 6 to 7 mg/L and salinity was established at 30 ± 1‰.

5.3.2 Experimental design

Cold stored eggs of *A. tonsa* preserved at 4 °C distributed by Algova® (Germany) were hatched following the instructions provided by the producer, while the rotifers *B. plicatilis* were collected from the culture tanks (see section 2.1; Fig. 2.3). Copepods eggs were washed from the buffer solution with 50 μm mesh sieve, inoculated in marine water and incubated during 24 hours at 24 ± 1 °C in a glass flask at the density of 40.000 eggs L^{-1} with air

bubbling. The newly hatched nauplii of *A. tonsa* at N I stage and the rotifer *B. plicatilis* were immediately concentrated using a 50 μm mesh sieve and counted under a stereomicroscope in triplicate (EZ4, Leica, Germany). The experiment was launched after splitting live preys into three experimental feeding treatments (n = 5) as follows (Fig. 2.3): Treatment R (control): mono algal diet based on *R. salina*; Treatment T-R: binary diet (1:1 ratio) based on *T. suecica* and *R. salina*; Treatment I-R: binary diet (1:1 ratio) based on *I. galbana* and *R. salina*. The three species of algae used in this study were chosen because of their significant differences in LC-PUFAs composition, which allowed a comparison of the effects of the diets on copepods and rotifers FAs composition. *R. salina* was selected as the control diet due to its good nutritional profile and suitable cell size for *A. tonsa* and *B. plicatilis* culture (Drillet *et al.*, 2006a; Ismar *et al*., 2008; Guevara *et al.,* 2010; Zhang *et al*., 2013). While *T. suecica* and *I. galbana* were selected to generate the binary diets, combined with the control, since they are among the most frequently used species in commercial mariculture industry (Sirakov *et al*., 2015). All microalgae treatments were formulated with a ratio of 1:1 based on cell count, chosen to facilitate the application of the enrichment and the reintegration of the consumed algae. Each treatment was split into five replicates, incorporated in five tanks of 20 L (n = 5), configured without water exchange with the MoRS facility to standardise the culture conditions. Starting culture density was established in 10 ind. mL^{-1} for *A. tonsa* and 280 ind. mL^{-1} for *B. plicatilis* by counting individuals as described for zooplankton cultures. Respectively were estimated 200.000 specimens in each copepod tank and 5.600.000 specimens in each rotifer tank ensuring a sufficient final sample size for the subsequent biochemical analysis, avoiding overpopulation. During 96 h, rotifers and copepods were fed with the designated diets after 10 % daily water exchange supplied by MoRS connected to the culture tanks. Tanks were kept at 20 ± 1 °C, 30 ‰ salinity and 14L/10D photoperiod (Gorbi *et al.,* 2012), checking water quality parameters daily. Microalgae concentration was daily

checked and kept at $1.5 \cdot 10^5$ cells mL^{-1} (1:1) in each tank following identical protocol applied for phytoplankton cultures (see section 2.4.1), ensuring enough feed for live preys and avoiding starvation. High microalgae concentration was chosen as it has been described to support high survival rates and increase culture performance of *A. tonsa* and *B. plicatilis* (Zhang *et al*., 2013; Rahman *et al.,* 2018; Sew *et al*., 2018; Besiktepe and Dam, 2020). To estimate the survival rate of each experimental treatment a daily counting of copepods nauplii and rotifers (Fig. 5.1) was carried out by triplicate in each tank following identical protocol applied for zooplankton cultures (see section 2.4.2) (Table 5.2).

Table 5.2: Concentration of rotifers (*Brachionus plicatilis*) and number of females, as well as nauplii of calanoid copepods (*Acartia tonsa*) measured in culture tanks during the feeding experimentation using three microalgae treatments (R= *Rhodomonas salina;* I-R= *Isochrysis galbana* + *Rhodomonas salina*; T-R= *Tetraselmis suecica* + *Rhodomonas salina*). All values are expressed as mean value with standard deviation (mean value mL-1 ± SD).

Live feed	Treatment	Day 1		Day 2		Day 3		Day 4	
		Individuals	Females	Individuals	Females	Individuals	Females	Individuals	Females
B. plicatilis	R	280 ± 0.1	13.3 ± 0.8	278.2 ± 0.8	10.2 ± 0.5	277 ± 0.7	15.6 ± 1.1	276.6 ± 0.9	12.3 ± 1.6
	I-R	280 ± 0.1	14 ± 0.2	277.8 ± 1.6	12.6 ± 0.8	277.4 ± 1.5	14.8 ± 0.8	276.6 ± 1.1	18.5 ± 0.8
	T-R	280 ± 0.1	13.8 ± 0.6	274.6 ± 4.7	8.4 ± 0.3	272.2 ± 2.7	8.1 ± 0.6	267.8 ± 1.6	10.4 ± 1.1
		Nauplii		Nauplii		Nauplii		Nauplii	
A. tonsa	R	10 ± 0.1	-	8.8 ± 0.1	-	8.4 ± 0.1	-	8.0 ± 0.1	
	I-R	10 ± 0.1	-	9.6 ± 0.1	-	9.2 ± 0.1	-	8.8 ± 0.1	
	T-R	10 ± 0.1	-	9.6 ± 0.1	-	9.0 ± 0.1	-	8.7 ± 0.1	

5.3.3 Samples collection

Samples of each microalgae species (20 L) employed for live prey diets were collected at the beginning of the experiment from their respective phytoplankton culture tanks. The resulting samples were centrifuged (Rotofix 32°, Hettich, Germany), split in vials per triplicate, lyophilized (Alpha 1-2 LD plus, Christ; Germany) and stored at -20 °C for subsequent analysis. The feeding trials were stopped on day 4 (96 h) when nauplii of *A. tonsa* were

moulting from the fourth (N IV) to the fifth (N V) developmental stage, preventing metamorphosis to the copepodite stage (Leandro *et al*., 2006). The exogenous feeding was observed starting from 24 hours post hatch at N II stage. The duration of the experiment (96 h) ensured to avoid any influence, which could come from the endogenous feeding of the first developmental stage (N I), to the final biochemical composition of *A. tonsa* nauplii. At the end of the feeding trials, each experimental culture tank of copepods and rotifers (n=5) generated a sample (Table 5.2). Each tank was fully filtered through a 50 μm mesh sieve, the content rinsed with deionized water to remove residual salts, lyophilized (Alpha 1-2 LD plus, Christ; Germany) and stored in vial at -20 °C for subsequent analysis. The collected samples were preserved at the Regional Agency for Environmental Protection–Liguria (ARPAL), Department of Organic Chemistry and Physical Analysis (Genoa, Liguria, Italy).

5.3.4 Samples Analysis

Samples analysis was carried out at the laboratories of the Highly Specialized Aquaculture and Biotechnology Service (SABE) located at the University Institute of Sustainable Aquaculture and Marine Ecosystems (IU ECOAQUA), belonging to the University of Las Palmas de Gran Canaria (Canary Islands, Spain). Proximate composition of microalgae diets, *A. tonsa* nauplii and *B. plicatilis* was conducted following standard procedures (Association of Official Analytical Chemists [AOAC], 2019). Moisture was determined by thermal dehydration until constant mass at 105 °C. Ash content was determined by combustion at 600 °C for 12 h. Crude protein content (N · 6.25) was determined by the Kjeldahl method, and crude lipid was extracted following the Folch method (Folch *et al*., 1957). Carbohydrate was estimated by difference [Percentage carbohydrate content = 100 − (% moisture + % ash + protein + % fat)]. The analysis of FAs was carried out by transmethylation of the total lipids in FAMES (Fatty acid methyl ester) as described by Christie and Han (2010), separated, and

identified by gas chromatography (7820A GC System, Agilent Technologies, United States) following the conditions described by Izquierdo and co-authors (1990). All gross composition data were expressed as percentages of dry weight (DW ± SD), while the fatty acids content was expressed as percentage of the total lipid (DW ± SD). All analyses were conducted in triplicate.

5.3.5 Data analysis

All data were analysed with Levene's test to assess homogeneity of variances and a Shapiro Wilk test to assess normality, applying log-transformed percent data when assumptions were not accomplished. Differences in proximate and FAs composition were assessed by One-way analysis of Variance (ANOVA). Comparison between groups was calculated by Tukey post-hoc test for pairwise comparisons, and Games-Howell. To study the effect of diet on survival of the copepods and the rotifers, a Two-way repeated measures ANOVA with two fixed factors, time and diet, was used. The significance level of 0.05 (*P* value) was tested using SPSS statistical software package (IBM SPSS for Windows V27.0; SPSS Inc., Chicago, IL, USA). Unless stated otherwise, measurements are presented as mean ± standard deviation (SD).

5.4 Results

5.4.1 Analysis of biochemical composition of microalgae

- **Proximal composition**

All data of microalgae gross composition were expressed as percentages of dry weight (DW ± SD, Table 5.3). *T. suecica* showed significantly higher protein (29.0 ± 0.7 %) and lipid (25.1 ± 0.5 %) content in comparison to the others two microalgae tested (ANOVA, $P < 0.05$, Table 5.3). While *R. salina* showed the highest percentages of carbohydrates (16.1 ± 0.5 %) and ash

(52.5 ± 0.5 %) (ANOVA, $P < 0.05$, Table 5.3). Moisture content was significantly higher in *I. galbana* (10.6 ± 0.7 %) (ANOVA, $P < 0.05$, Table 5.3), meanwhile there were no differences (ANOVA, $P \geq 0.05$) between *R. salina* (8.12 ± 0.50 %) and *T. suecica* (8.63 ± 0.55 %) species.

- **Fatty acid composition**

The fatty acid composition of microalgae was expressed as percentage of the total lipid DW ± SD (Table 5.3). In comparison to other algae, *I. galbana* showed the highest levels of total n-3 LC-PUFAs, total monounsaturated (MUFAs), total n-9 FAs, oleic acid (OA; 18:1n-9), and DHA (ANOVA, $P < 0.05$, Table 5.3), while EPA and total saturated fatty acids (SAFAs) were significantly lower (ANOVA, $P < 0.05$, Table 5.3). *T. suecica* showed the highest percentage of myristoleic acid (14:1n-5), palmitic acid (16:0), vaccenic acid (18:1n-7), linoleic acid (LA; 18:2n-6), gamma-Linoleic acid (18:3n-6), alpha-Linolenic acid (ALA; 18:3n-3), stearidonic acid (SA; 18:4n-3), arachidonic (ARA; 20:4n-6), Eicosatetraenoic acid (20:4n-3), total n-3 and total n-6 FAs (ANOVA, $P < 0.05$, Table 5.3). In the same way, high contents in myristic acid (14:0) were detected in *R. salina* (ANOVA, $P < 0.05$, Table 5.3). Finally, all the studied FAs ratios were significantly higher in *R. salina* (ANOVA, $P < 0.05$, Table 5.3), except for DHA/EPA and DHA/ARA ratio, which levels were significantly higher in *I. galbana* (ANOVA, $P < 0.05$, Table 5.3).

Table 5.3: Proximal composition (lipid, protein, carbohydrate, ash and moisture content; g/100g dry weight) and fatty acid composition (mg/g dry weight) of microalgae used as food for live preys, n=3.

Microalgae	***Tetraselmis suecica***	***Isochrysis galbana***	***Rhodomonas salina***
Lipids	25.1 ± 0.5^{c}	18.8 ± 0.2^{b}	14.2 ± 0.6^{a}
Proteins	29.0 ± 0.7^{c}	22.6 ± 0.4^{b}	17.3 ± 0.5^{a}
Carbohydrates	9.4 ± 0.6^{a}	11.3 ± 0.7^{b}	16.1 ± 0.5^{c}
Ash	36.5 ± 0.7^{a}	47.3 ± 0.5^{b}	52.5 ± 0.5^{c}
Moisture	8.6 ± 0.6^{a}	10.6 ± 0.7^{b}	8.1 ± 0.5^{a}
14:0	0.1 ± 0.0^{a}	1.4 ± 0.1^{b}	1.8 ± 0.2^{c}
14:1n-5	0.3 ± 0.1^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
16:0	3.2 ± 0.2^{b}	0.7 ± 0.0^{a}	0.8 ± 0.2^{a}
16:1n-7	0.2 ± 0.1^{a}	0.7 ± 0.1^{b}	0.9 ± 0.2^{b}
16:3n-1	3.4 ± 0.1	-	-
18:0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.5
18:1n-9	1.9 ± 0.5^{b}	3.4 ± 0.2^{c}	0.5 ± 0.0^{a}
18:1n-7	0.8 ± 0.1^{b}	0.5 ± 0.1^{a}	0.3 ± 0.2^{a}
18:2n-6	1.8 ± 0.1^{c}	1.0 ± 0.0^{b}	0.7 ± 0.1^{a}
18:3n-6	0.2 ± 0.0^{b}	0.1 ± 0.0^{a}	-
18:3n-3	4.8 ± 0.0^{c}	1.7 ± 0.2^{a}	3.7 ± 0.4^{b}
18:4n-3	3.7 ± 0.2^{c}	2.8 ± 0.3^{b}	0.7 ± 0.1^{a}
20:1n-5	0.4 ± 0.1	-	-
20:2n-6	0.1 ± 0.0	-	-
20:4n-6 ARA	0.3 ± 0.0^{b}	0.1 ± 0.0^{a}	-
20:3n-3	0.1 ± 0.0	-	-
20:4n-3	0.2 ± 0.0^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
20:5n-3 EPA	2.2 ± 0.2^{b}	0.6 ± 0.1^{a}	2.1 ± 0.2^{b}
22:1n-11	-	0.1 ± 0.0	-
22:5n-3	-	0.1 ± 0.0	-
22:6n-3 DHA	0.1 ± 0.1^{a}	4.5 ± 0.2^{c}	1.3 ± 0.1^{b}
Σn-3[1]	11.3 ± 0.3^{c}	9.8 ± 0.2^{b}	8.0 ± 0.7^{a}

Σn-6[2]	2.4 ± 0.1[c]	1.2 ± 0.1[b]	0.7 ± 0.1[a]
Σn-9[3]	2.3 ± 0.2[b]	3.9 ± 0.2[c]	0.5 ± 0.0[a]
ΣSAFAs[4]	3.5 ± 0.2[b]	2.3 ± 0.1[a]	3.5 ± 0.4[b]
ΣMUFAs[5]	4.2 ± 0.2[b]	5.4 ± 0.2[c]	1.9 ± 0.3[a]
Σn-3 LC-PUFAs[6]	2.6 ± 0.2[a]	5.3 ± 0.3[c]	3.6 ± 0.3[b]
ratio n-3/n-6	4.7 ± 0.4[a]	8.2 ± 0.5[b]	10.8 ± 1.5[c]
ratio n-3/n-9	4.9 ± 0.4[b]	2.5 ± 0.2[a]	15.7 ± 0.8[c]
ratio n-6/n-9	1.1 ± 0.1[b]	0.3 ± 0.0[a]	1.5 ± 0.3[b]
ratio DHA/EPA	0.1 ± 0.1[a]	7.6 ± 0.5[c]	0.6 ± 0.0[b]
ratio DHA/ARA	0.3 ± 0.4[a]	71.1 ± 1.2[c]	32.6 ± 2.0[b]
ratio EPA/ARA	6.6 ± 0.0[a]	9.4 ± 0.6[b]	52.6 ± 2.5[c]

Values (mean ± SD) followed by different superscripts within a row were ($P < 0.05$) significantly different among the three treatments. n= number of replicates; SAFA= saturated fatty acid; MUFA= monounsaturated fatty acid; LC-PUFA= long chain polyunsaturated fatty acid; DHA =docosahexaenoic acid; EPA =eicosapentaenoic acid; ARA =arachidonic acid. [1]Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. [2]Includes 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. [3]Includes 18:1n-9, 18:2n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9. [4] Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. [5]Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. [6]Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

5.4.2 Analysis of biochemical composition of rotifers

- **Proximal composition**

The gross composition of rotifers was expressed as percentage of DW ± SD (Table 5.4). The group fed with treatment R showed the highest lipid content (17.3 ± 1.1 %), followed by I-R (15.4 ± 0.8%) and T-R (13.1 ± 1.0%) groups (ANOVA, $P < 0.05$, Table 5.4). Protein was the major constituent in all groups, but I-R treatment showed the highest content (58.7 ± 1.6%) (ANOVA, $P < 0.05$, Table 5.4). Carbohydrate did not show any significant difference among all treatments (ANOVA, $P \geq 0.05$, Table 5.4). While the group T-R showed the higher ash and moisture content (ANOVA, $P < 0.05$, Table 5.4), no difference was observed between I-R and R treatments (ANOVA, $P \geq 0.05$, Table 5.4).

- **Fatty acid composition**

The FA composition of the rotifer *B. plicatilis* fed with the three experimental diets was expressed as percentage of the total lipid DW ± SD (Table 5.4). Rotifers fed on diet I-R showed significantly higher content in total n-3 LC-PUFAs, total n-6 FAs, OA, LA, and DHA fatty acids, in comparison to other groups (ANOVA, $P < 0.05$, Table 5.4). Group fed on diet R showed higher percentage in 14:0, 16:0, palmitoleic acid (16:1n-7), stearic acid (18:0), vaccenic acid, ALA, as also total SAFAs and MUFAs series than other groups (ANOVA, $P < 0.05$, Table 5.4). FAs ratios n-6/n-9, DHA/EPA and DHA/ARA were significantly higher in rotifers fed on I-R treatment, while n-3/n-6 ratio was higher with R treatment. (ANOVA, $P < 0.05$, Table 5.4).

Table 5.4: Proximal composition (lipid, protein, carbohydrate, ash and moisture content; g/100 g dry weight) and fatty acid composition (mg/g dry weight) of rotifers (Brachionus plicatilis), fed with three microalgae diets (R= Rhodomonas salina; I-R= Isochrysis galbana + Rhodomonas salina; T-R= Tetraselmis suecica + Rhodomonas salina), n=5.

Brachionus plicatilis	**T-R**	**I-R**	**R**
Lipids	13.1 ± 1.0^{a}	15.4 ± 0.8^{b}	17.3 ± 1.1^{c}
Proteins	57.4 ± 0.8^{ab}	58.7 ± 1.6^{b}	56.2 ± 1.7^{a}
Carbohydrates	7.8 ± 0.6	8.7 ± 0.9	7.2 ± 1.0
Ash	21.7 ± 0.4^{b}	17.2 ± 1.7^{a}	19.4 ± 0.6^{a}
Moisture	18.6 ± 2.1^{b}	14.6 ± 1.5^{a}	13.6 ± 2.1^{a}
14:0	0.2 ± 0.1^{a}	0.2 ± 0.1^{a}	0.9 ± 0.1^{b}
14:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
16:0	1.6 ± 0.4^{a}	1.6 ± 0.2^{a}	2.6 ± 0.3^{b}
16:1n-7	0.3 ± 0.1^{a}	0.5 ± 0.1^{ab}	0.7 ± 0.1^{b}
16:3n-1	0.2 ± 0.0	-	-
18:0	0.4 ± 0.1^{a}	0.5 ± 0.0^{a}	0.8 ± 0.1^{b}
18:1n-9	1.3 ± 0.4^{ab}	1.7 ± 0.2^{b}	1.0 ± 0.1^{a}
18:1n-7	0.6 ± 0.1^{a}	0.5 ± 0.1^{a}	0.9 ± 0.1^{b}
18:2n-6	0.8 ± 0.2^{ab}	1.2 ± 0.3^{b}	0.6 ± 0.0^{a}
18:3n-6	0.1 ± 0.0	-	-
18:3n-3	1.7 ± 0.5^{a}	1.4 ± 0.2^{a}	2.7 ± 0.3^{b}
18:4n-3	0.7 ± 0.3	0.5 ± 0.1	0.7 ± 0.1
20:1n-5	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.1
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-6 ARA	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:3n-3	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
20:4n-3	1.0 ± 0.3	0.9 ± 0.1	0.9 ± 0.0
20:5n-3 EPA	1.1 ± 0.3	1.4 ± 0.1	1.2 ± 0.1
22:1n-11	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
22:5n-3	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
22:6n-3 DHA	0.5 ± 0.1^{a}	1.4 ± 0.1^{c}	0.8 ± 0.1^{b}

Σn-3[1]	5.7 ± 1.3	6.1 ± 0.6	7.0 ± 0.5
Σn-6[2]	1.3 ± 0.2^{ab}	1.7 ± 0.3^{b}	1.2 ± 0.1^{a}
Σn-9[3]	2.0 ± 0.4	2.0 ± 0.2	2.0 ± 0.3
ΣSAFAs[4]	2.4 ± 0.5^{a}	2.5 ± 0.3^{a}	4.4 ± 0.5^{b}
ΣMUFAs[5]	4.0 ± 0.8^{ab}	3.9 ± 0.4^{a}	4.9 ± 0.3^{b}
Σn-3 LC-PUFAs[6]	3.0 ± 0.6^{a}	4.1 ± 0.3^{b}	3.4 ± 0.2^{a}
ratio n-3/n-6	4.4 ± 1.1^{ab}	3.7 ± 0.4^{a}	6.1 ± 0.8^{b}
ratio n-3/n-9	2.9 ± 0.8	3.0 ± 0.2	3.6 ± 0.7
ratio n-6/n-9	0.7 ± 0.2^{ab}	0.8 ± 0.1^{b}	0.6 ± 0.0^{a}
ratio DHA/EPA	0.5 ± 0.1^{a}	1.0 ± 0.1^{c}	0.7 ± 0.0^{b}
ratio DHA/ARA	2.8 ± 0.5^{a}	7.4 ± 2.2^{b}	3.7 ± 0.6^{a}
ratio EPA/ARA	5.7 ± 2.0	7.2 ± 1.7	5.5 ± 1.1

Values (mean ± SD) followed by different superscripts within a row were ($P < 0.05$) significantly different among the three treatments. n= number of replicates; SAFA= saturated fatty acid; MUFA= monounsaturated fatty acid; LC-PUFA= long chain polyunsaturated fatty acid; DHA =docosahexaenoic acid; EPA =eicosapentaenoic acid; ARA =arachidonic acid. [1]Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.[2]Includes 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6.[3]Includes 18:1n-9, 18:2n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9. [4] Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. [5]Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. [6]Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

5.4.3 Analysis of biochemical composition of copepods

- **Proximal composition**

Data of copepods gross composition were expressed as percentage of DW ± SD in Table 5.5, where protein represented the major constituent in all groups. The highest value for protein was found in copepods fed with binary diets T-R (71.8 ± 0.7 %) and I-R (70.7 ± 0.7 %), which showed significantly higher content compared to the group fed on R diet (ANOVA, $P < 0.05$, Table 5.5). Copepod fed with T-R showed also significantly higher lipid content compared to other diets (18.0 ± 0.3 %) (ANOVA, $P < 0.05$, Table 5.5), meanwhile no

differences were observed in groups fed on I-R and R treatments (ANOVA, $P \geq 0.05$, Table 5.5). Carbohydrate constituted the smallest portion of the nauplii proximate composition, with values ranged between 4 ± 0.4 % in T-R to 9.1 ± 0.6 % for treatment R. Data of ash and moisture content were showing no statistical difference among all treatments (ANOVA, $P \geq 0.05$, Table 5.5).

- **Fatty acids composition**

The FA composition of *A. tonsa* nauplii fed with the three experimental diets was expressed as percentage of the total lipid DW ± SD (Table 5.5). Total n-3 FAs and total n-3 LC-PUFAs, as well as EPA, DHA, were significantly higher in copepods fed on I-R compared with other two diets (ANOVA, $P < 0.05$, Table 5.5). Group fed on diet T-R gave copepod higher percentage of 16:0, OA, LA, ALA, vaccenic acid, ARA, as also MUFAs, total n-6 and n-9 FAs series (ANOVA, $P < 0.05$, Table 5.5). FAs ratios n-3/n-6 and n-3/n-9 were significantly lower (ANOVA, $P < 0.05$, Table 5.5) in group fed on T-R, while DHA/EPA and DHA/ARA ratio levels were significantly higher with I-R treatment (ANOVA, $P < 0.05$, Table 5.5). Finally, copepods fed on R and I-R did not show any significant differences in EPA/ARA ratio (ANOVA, $P \geq 0.05$, Table 5.5), meanwhile treatment T-R was significantly lower (ANOVA, $P < 0.05$, Table 5.5) compared to the other diets.

Table 5.5: Proximal composition (lipid, protein, carbohydrate, ash and moisture content; g/100 g dry weight) and fatty acid composition (mg/g dry weight) of copepods (*A. tonsa*) nauplii, fed with three microalgae diets (R= *Rhodomonas salina*; I-R= *Isochrysis galbana* + *Rhodomonas salina*; T-R= *Tetraselmis suecica* + *Rhodomonas salina*), n=5.

Acartia tonsa	**T-R**	**I-R**	**R**
Lipids	18.0 ± 0.3^{b}	16.4 ± 0.5^{a}	15.9 ± 0.3^{a}
Proteins	71.8 ± 0.7^{b}	70.7 ± 0.7^{b}	68.1 ± 0.6^{a}
Carbohydrates	4.0 ± 0.4^{a}	6.4 ± 0.6^{b}	9.1 ± 0.6^{c}
Ash	6.3 ± 0.4	6.6 ± 0.6	6.9 ± 0.2
Moisture	7.2 ± 0.7	7.6 ± 0.6	8.4 ± 0.7
14:0	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.2
14:1n-5	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
16:0	3.8 ± 0.4^{c}	1.2 ± 0.2^{a}	2.5 ± 0.3^{b}
16:1n-7	0.6 ± 0.5	0.2 ± 0.0	0.2 ± 0.0
16:3n-1	0.2 ± 0.0	-	-
18:0	1.3 ± 0.1^{b}	0.7 ± 0.1^{a}	1.4 ± 0.2^{b}
18:1n-9	1.3 ± 0.3^{b}	0.7 ± 0.1^{a}	0.6 ± 0.2^{a}
18:1n-7	0.6 ± 0.1^{b}	0.3 ± 0.0^{a}	0.4 ± 0.1^{a}
18:2n-6	0.7 ± 0.1^{b}	0.4 ± 0.1^{a}	0.2 ± 0.1^{a}
18:3n-6	0.1 ± 0.0	-	-
18:3n-3	1.8 ± 0.2^{b}	1.0 ± 0.1^{a}	1.2 ± 0.1^{a}
18:4n-3	0.7 ± 0.2	0.5 ± 0.1	0.4 ± 0.1
20:1n-5	0.2 ± 0.1	0.1 ± 0.0	-
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	-
20:4n-6 ARA	0.2 ± 0.0^{b}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
20:5n-3 EPA	2.9 ± 0.3^{ab}	2.9 ± 0.1^{b}	2.5 ± 0.1^{a}
22:1n-11	0.1 ± 0.0	-	-
22:5n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1

22:6n-3 DHA	2.1 ± 0.3[a]	6.7 ± 0.2[c]	4.6 ± 0.2[b]
Σn-3[1]	7.8 ± 0.8[a]	11.5 ± 0.3[c]	9.2 ± 0.4[b]
Σn-6[2]	1.1 ± 0.2[c]	0.7 ± 0.2[b]	0.4 ± 0.1[a]
Σn-9[3]	1.5 ± 0.3[b]	0.8 ± 0.1[a]	0.8 ± 0.1[a]
ΣSAFAs[4]	5.6 ± 0.5[b]	2.5 ± 0.1[a]	4.6 ± 0.4[b]
ΣMUFAs[5]	3.2 ± 0.6[b]	1.5 ± 0.1[a]	1.7 ± 0.2[a]
Σn-3 LC-PUFAs[6]	5.3 ± 0.5[a]	9.9 ± 0.2[c]	7.6 ± 0.4[b]
ratio n-3/n-6	7.2 ± 1.7[a]	17.2 ± 3.7[b]	24.5 ± 5.0[b]
ratio n-3/n-9	5.5 ± 0.9[a]	14.7 ± 2.0[b]	12.2 ± 2.1[b]
ratio n-6/n-9	0.8 ± 0.2	0.9 ± 0.4	0.5 ± 0.1
ratio DHA/EPA	0.7 ± 0.1[a]	2.3 ± 0.1[c]	1.9 ± 0.1[b]
ratio DHA/ARA	12.2 ± 3.2[a]	96.8 ± 5.0[c]	80.9 ± 6.7[b]
ratio EPA/ARA	16.7 ± 3.6[a]	42.0 ± 2.7[b]	43.6 ± 3.2[b]

Values (mean ± SD) followed by different superscripts within a row were ($P < 0.05$) significantly different among the three treatments. n= number of replicates; SAFA= saturated fatty acid; MUFA= monounsaturated fatty acid; LC-PUFA= long chain polyunsaturated fatty acid; DHA =docosahexaenoic acid; EPA =eicosapentaenoic acid; ARA =arachidonic acid. [1]Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. [2]Includes 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6.[3]Includes 18:1n-9, 18:2n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9. [4] Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. [5]Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. [6]Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

5.4.4 Survival

During the feeding trials, survival rates were different among preys. While *B. plicatilis* survival did not change significantly after exposure to each treatment, *A. tonsa* showed different survival peaks over time depending on the type of diet administered (Fig. 5.2, "diet · day", $F_{6.54} = 117.28$, $P < 0.05$). Thus, from day 2, survival of nauplii fed with binary diets I-R (96.2 ± 0.8 %) and T-R (95.8 ± 0.9 %) was significantly higher than nauplii fed on R treatment (87.9 ± 0.7 %) (ANOVA, $P < 0.05$, Fig. 5.2). At the end of the experiment, survival

was significantly higher in binary diets, especially with I-R treatment. (88.1 ± 0.7 %) (ANOVA, $P < 0.05$).

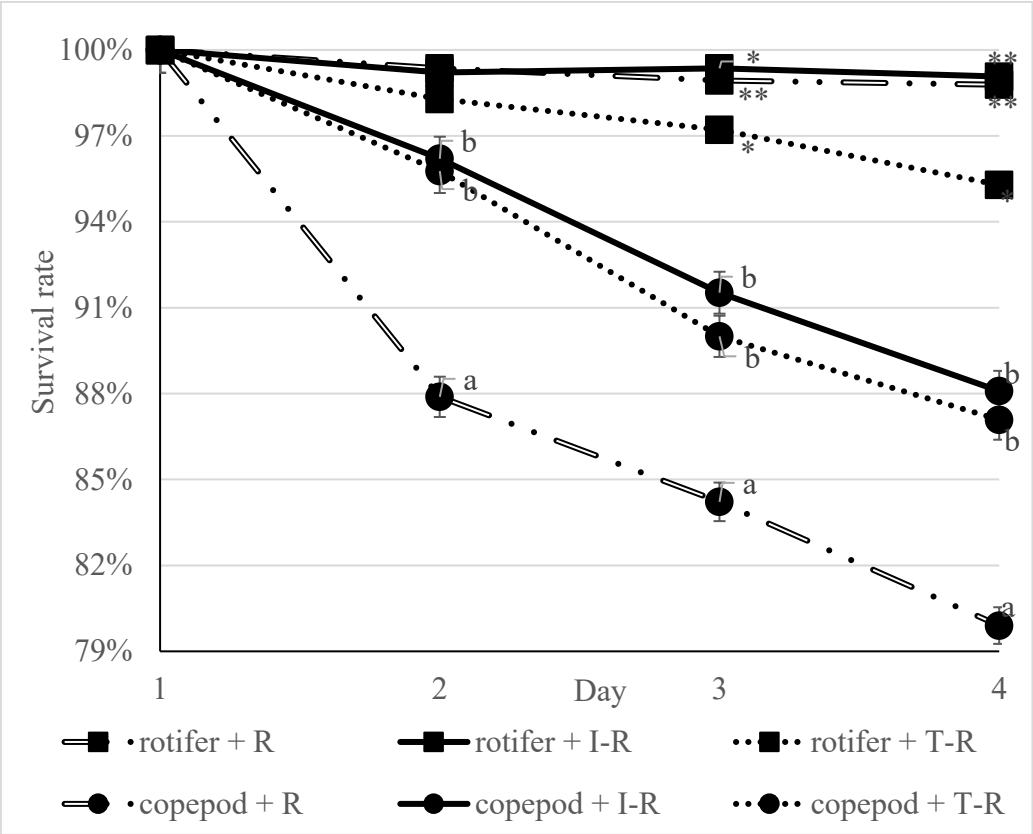

Figure 5.2: Survival (% ± SD) of *Brachionus plicatilis* (rotifer) and *Acartia tonsa* (copepod) nauplii fed with three experimental diets. Values (mean ± SD) followed by different superscripts were ($P < 0.05$) significantly different among the three treatments. R= *Rhodomonas salina*; I-R= *Isochrysis galbana* + *Rhodomonas salina*; T-R= *Tetraselmis suecica* + *Rhodomonas salina*

5.5 Discussion

5.5.1 Biochemical composition of live preys

The present study showed how different mono and binary diets based on microalgae (T-R, I-R and R) affect the survival and biochemical composition of *A. tonsa* nauplii and the rotifer *B. plicatilis*. In both preys, the use of binary diets based on the combination of *I. galbana* and *R. salina* (I-R treatment) improved their protein and LC-PUFAs content as well as the survival of *A. tonsa* nauplii, compared to control and T-R treatment. Indeed, among all microalgae employed in this study the treatment I-R showed the highest nutritional value, especially in

LC-PUFAs. It is plausible that this binary formula took advantage of the content in EPA of *R. salina* and the DHA content of *I. galbana*, generating potentially a LC-PUFAs boosted diet. Although these microalgae strains naturally produce high amounts of PUFAs, their FA content and composition can vary with environmental parameters such as the composition of the growth medium, nutrient availability, or illumination (Fernandes *et al*., 2016). Therefore, it was important to standardize the microalgae culture conditions, in order to ensure the same FA enrichment day by day. In general, the biochemical composition of *A. tonsa* nauplii fed on I-R treatment was the richest in terms of protein, lipids, total LC-PUFAs, EPA and DHA, also compared to *B. plicatilis* fed with the same treatment. Despite being a monoalgal diet, *R. salina* (control) has shown to better improve the nutritional value of *A. tonsa*, especially in EPA and DHA compared to the binary diet T-R. Indeed, this was probably due to the composition of *T. suecica* which showed the lowest content in LC-PUFAs, especially in DHA, compared to the other microalgae employed to generate treatments (Table 5.3). These results agreed with previous studies reporting the excellence of *Rhodomonas spp.* as diet for calanoid copepods (Buttino *et al*., 2009; Dahl *et al*., 2009; Ismar *et al.*, 2008; Zhang *et al.,* 2013), suggesting that *R. salina* is an outstanding candidate to generate binary diet for the calanoid copepod *A. tonsa*. On the contrary, the rotifer *B. plicatilis* was characterized by a lower content in proximate and FAs composition, even after enrichment with I-R treatment, compared to *A. tonsa* nauplii. Retention ability of LC-PUFAs was clearly species specific (Dey *et al.,* 2022). Although *B. plicatilis* is able to synthetize long chain PUFAs, it showed a limited ability to retain EPA and DHA compared to *A. tonsa* (Birkou *et al.*, 2012). On the contrary, *A. tonsa* nauplii were able to retain high quantity of n-3 LC-PUFAs acquired exclusively from their phytoplankton diet, especially EPA and DHA. These results are consistent with those obtained by Støttrup *et al.* (1999), where *A. tonsa* nauplii fed with *Rhodomonas baltica* until 12 days post-hatch reached the highest levels of EPA and DHA

after 3 days of culture. The high percentage of DHA and EPA observed in copepods nauplii after 4 days fed with I-R, suggested that the combination between *R. salina* and *I. galbana* could be an optimal food source for *A. tonsa,* as diet or enrichment. Besides, results also suggest that *A. tonsa* could retain the portion of saturated fatty acids (14:0, 16:0 and 18:0), and polyunsaturated fatty acids (18:3n-3, 18:4n-3, 20:4 n-6, 20:5 n-3 and 22:6n-3), particularly EPA and DHA, which may be catabolized by the copepod as important growth factors (Veloza *et al*., 2005; Besiktepe and Dam, 2020). The ability to retain LC PUFAS could be related to the growth and reproduction of calanoid copepods, providing them with a considerable energy store and enabling their growth also during the food-scarce seasons (Saba *et al*., 2009; Chen *et al.*, 2012; Besiktepe and Dam, 2020). This high LC-PUFAs retention capacity makes *A. tonsa* an optimal live feed for marine larval cultures, which may replace totally or partially both rotifers and even *Artemia* throughout all marine fish larvae development. Compared to conventional live preys, *A. tonsa* present not only a high content in protein, DHA, and EPA, but also an attractive swimming behaviour and wide size range (*ca.* 70 µm of newly hatched nauplii till 1.5 mm of adult females) (Drillet *et al.,* 2006b, 2007, 2011; Dhont *et al*., 2013; Rasdi and Qin, 2014; Hansen*,* 2017). On the other hand, the latest cold storing techniques of *A. tonsa* eggs, resisting at 4° C for several months (Støttrup, 2006), represent to date the easiest way to manage high quantities of copepods nauplii, although it implies the inevitable decrease in viability and nutritional content (Drillet *et al*., 2011). In this sense, the enrichment exposure of *A. tonsa* nauplii to microalgae rich in LC-PUFAs as *I. galbana* and *R. salina*, arrange this decrease in nutritional content improving their nutritional value before being administrated as live feed to marine fish larvae. Indeed, the increased importance of LC-PUFAs (e.g., EPA and DHA) for marine fish larvae has been reviewed for many fish species (Olivotto *et al*., 2003; Faulk and Holt, 2005; Olivotto *et al.,* 2006ab; Dey *et al.,* 2022).

5.5.2 Survival

Finally, none of the diets fed to *B. plicatilis* produced significant mortality, only the group fed on T-R treatment showed reduced survival. That difference could be related to the size and motility of the microalgae employed as enrichment. Indeed, *T. suecica* presented larger cell size and higher motility, around 17 µm in cell length and four flagella, compared to *R. salina* and *I. galbana* which showed respectively 11 µm and 5 µm cell length, both with two flagella. In contrast, the calanoid copepod *A. tonsa* showed significant differences in survival depending on the treatment supplied, with the highest survival rate when fed on I-R treatment. The mortality observed in all culture trials of *A. tonsa* nauplii could be explained since they pass through the delicate phase of moulting where mortality is due not only to the food source but also to many other multifactorial causes such as energy reserves, parents nutrition, eggs storage conditions, storage time, etc. (Drillet *et al*., 2006b, 2011). Despite all treatment showing mortality, these results highlight the importance of using live microalgae binary diets as food source for *A. tonsa* nauplii stages.

5.6 Conclusions

The present study provides evidence of the importance of enriching *A. tonsa* nauplii with binary microalgal diet based on the combination of *I. galbana* and *R. salina* (I-R) in order to supply an adequate content of n-3 LC-PUFAs. Moreover, it showed how the biochemical composition of *A. tonsa* and *B. plicatilis* depended on their FAs retention ability and the diet supplied. The wide differences in nutritional composition observed between both preys revealed *A. tonsa* as superior live feed, with higher protein content and improved ability to retain LC-PUFAs (EPA, DHA), compared to *B. plicatilis.* In conclusion, this study highlights the biochemical profile of enriched *A. tonsa* nauplii and contributes to the production of high-quality live feeds, which may be able to

reach the nutritional needs and enhance the production of new marine fish species, as well as contributing to the improvement of *A. tonsa* culture.

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Chapter 6. Enriched calanoid copepods *Acartia tonsa* (Dana, 1849) enhances growth, survival, biochemical composition and morphological development during larval first feeding of the orchid dottyback fish *Pseudochromis fridmani* (Klausewitz, 1968)

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6.1 Abstract

In a time of unprecedented coral reef decline, improved aquaculture protocols and high-quality live feeds may contribute to the sustainability of the ornamentals industry producing new valuable and stronger specimens bred under controlled conditions, mitigating the pressure on wild natural stocks. In the present study, the effect of microalgae enriched live feed diets (15 ind. mL^{-1}), the copepod *Acartia tonsa* and the rotifer *Brachionus plicatilis,* were tested on early larvae stages (0 to 15 days post hatch) of the orchid dottyback *Pseudochromis fridmani*. Enriched *A. tonsa* significantly improved *P. fridmani* larval growth, survival, biochemical composition, as well as the morphological development of liver, intestine and skeletal system, compared to rotifers treatment. The results obtained showed that is feasible to totally replace the enriched rotifer *B. plicatilis* with the enriched copepod *A. tonsa* during first larval feeding of orchid dottybacks, resulting in a more robust larvae with better culture performance. Indeed, feeding larvae with enriched *A. tonsa* avoid the different negative effects when used enriched rotifers diets, which led to liver steatosis and nuclear pyknosis, delays in intestine and skeletal development, altered proximate composition and FA content. This study provided valuable information about rearing and breeding under controlled conditions of *P. fridmani*, contributing to its mass scale production and to the implementation of new hatchery protocols employing high quality live feeds.

6.2 Introduction

6.2.1 The marine ornamental trade

In recent decades, the marine ornamental fish trade has exponentially increased its value becoming a multibillion-dollar industry, expanding worldwide and targeting thousands of coral reef species (Rhyne *et al.*, 2012, 2017; Palmtag, 2017; Pouil *et al.*, 2019; Watson *et al.*, 2023). This rapid growth generated many controversies about its sustainability, since more than 90% of the marine ornamental fish and invertebrates traded species are from wild origin,

caught in developing countries especially in tropical and subtropical latitudes (Raghavan *et al.*, 2013; Chen *et al.*, 2019; King, 2019; Watson *et al.*, 2023). As a result, uncontrolled fishing and bad practices may lead to the overexploitation of natural stocks and high mortality rates along the whole trade process (Pouil *et al.*, 2019). In this context, there is a growing interest in developing new larval culture methods and feeding protocols for the most traded marine ornamental species. Aquaculture of fish and invertebrates can therefore support the marine ornamental trade to mitigate the impact on the environment, reducing the need to collect from the wild and offering specimens bred under controlled conditions which are more resistant and sustainable (Tlusty, 2002; Olivotto *et al.*, 2017; Chen *et al.*, 2019; Watson *et al.*, 2023).

6.2.2 Marine ornamental larval husbandry

The main bottleneck in the culture of many marine ornamentals happens during their early developmental stages, specifically during the larval transition from endogenous to exogenous feeding (Holt, 2003; Olivotto *et al*., 2017; Callan *et al.*, 2018; Hansen and Möller, 2021). Indeed, successful rearing of marine fish larvae during first feeding period depends on the proper availability, quantity and quality of food supplied (Olivotto *et al*., 2017; Samat *et al*., 2020). Furthermore, live feeds should match the mouth size of the larvae and their nutritional requirements, while delays in adaptation to the live feed may lead to starvation and nutrient deficiency, resulting in slow growth and low survival (Chen *et al*., 2019; Hamre *et al*., 2018; Hansen and Möller, 2021). The practice to include microalgae within the rearing environment of larval fish, well known as “green waters”, is a common method employed in larvae culture which offer several benefits such as (i) improved water quality, maintaining a positive dissolved oxygen balance, reducing nitrogen compounds and pathogenic bacteria; (ii) improved larval predatory capacity, promoting low light intensity and digestive enzyme

activity; (iii) enhanced live feeds survival, increasing their nutritional value before being consumed by larvae (Setu *et al.*, 2010; Neori, 2011; Basford *et al*., 2021; Eryalçın and Tınkır, 2024). The use of live preys such as rotifers and *Artemia spp*. for ornamental species does not always sustain survival and optimal larval growth as they may have an inadequate nutritional profile and/or unsuitable size (Otero-Ferrer *et al.*, 2010; Eryalçin, 2018, 2019; Olivotto *et al*., 2017; Radhakrishnan *et al.*, 2019). In opposition, copepods are considered a suitable alternative as live preys, improving the larval cultures of several fish species (Ajiboye *et al.*, 2011; Hamre *et al*., 2018; Radhakrishnan *et al.*, 2019; Samat *et al*., 2020). The better larval performance using calanoid copepods has been attributed to several factors, including (i) the small size of these preys (generally ranging from 40 μ to 2 mm), (ii) their pelagic swimming behaviour and (iii) the high content in long-chain polyunsaturated fatty acids (LC-PUFAs) that may meet requirements of larvae, also with small mouth gape (Olivotto *et al.*, 2006a, b; Ajiboye *et al.*, 2011; Rasdi and Qin, 2014; Radhakrishnan *et al.*, 2019). Hence, calanoid copepods increased survival and growth rate in Clark's anemonefish (*Amphiprion clarkii*, Olivotto *et al.*, 2008a), striped fangblenny (*Meiacanthus grammistes,* Olivotto *et al.*, 2010), longsnout seahorse (*Hippocampus reidi,* Olivotto *et al.*, 2008b), spotted pipefish (*Stigmatopora argus*, Payne *et al.*, 1998), flame angelfish (*Centropyge loriculus*, Laidley *et al*., 2008), lemonpeel angelfish (*Centropyge flavissimus*, Olivotto *et al.*, 2006a*),* yellow tang *(Zebrasoma flavescens,* Pereira-Davison and Callan, 2017), palette surgeonfish (*Paracanthurus hepatus*, DiMaggio *et al.*, 2017), mandarinfish (*Synchiropus splendidus*, Zeng *et al.*, 2018) and barramundi (*Lates calcarifer,* Rasdi and Qin, 2018). New techniques allow producing massive quantities of copepods using cold stored subitaneous eggs of the calanoid copepod *A. tonsa,* hatched similarly as *Artemia* cyst (Støttrup *et al.*, 1999; Payne and Rippingale, 2001; Drillet *et al.*, 2006a, b, 2007, 2011; Peck and Holste, 2006; Peck *et al.*, 2008). A recent study described how enrichment exposure to microalgae rich in LC-PUFAs

such as *Isochrysis galbana* and *Rhodomonas salina* improves the nutritional value of *A. tonsa* nauplii, especially in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Martino *et al.*, 2023). Therefore, enriched copepods could act as natural high-quality live feed with higher LC-PUFAs content compared to rotifers, which may be replaced totally or partially throughout all fish larvae development of certain marine species (Rasdi and Qin, 2014; Hamre *et al.*, 2018; Radhakrishnan *et al.*, 2019; Samat *et al*., 2020; Martino *et al.*, 2023).

6.2.3 *Pseudochromis fridmani* larval feeding

The orchid dottyback *Pseudochromis fridmani,* Klausewith, 1968 (Pseudochromidae*)* is an ornamental fish species endemic of the Red Sea, widely commercialized in the marine ornamental trade (Wittenrich, 2007). Recently, Chen *et al.* (2023) described its embryonic and larval development stages, targeting future research to reduce filial cannibalism through broodstock management and to improve larval culture through the early development. Indeed, scientific information on its fatty acid (FA) requirements and on the effects of first feeding on liver, intestine and skeletal development is scarce, although it has been reared under controlled conditions with rotifers as larval diet (Brons, 1996; Moe, 1997), observing best survival rates when larvae were fed combining rotifer and copepods using “green waters” (Chen and Zeng, 2021a, b). Therefore, the present study aimed to evaluate the effect of copepods compared to more conventional live preys, on early larvae of *P. fridmani* cultured from 0 to 15 days post hatch (dph). Thus, *A. tonsa* and *B. plicatilis,* were enriched and compared as monospecific live feed diets on *P. fridmani* larvae growth rate, survival, biochemical composition, first bones calcification and histological modification of intestine and liver. The results provide valuable information on the breeding of *P. fridmani* contributing

to its mass scale production and to the implementation of new hatchery protocols employing high quality live feeds.

6.3 Material and Methods

All the experimentation was conducted in the quarantine areas belonging to the Tropical Department of the Aquarium of Genoa (Genoa, Liguria, Italy). All procedures involving animals were performed according to the Guide for Care and Use of Laboratory Animals of the European Union Directive (2010/63/EU). All efforts were made to minimize larvae suffering and a humane endpoint was quickly applied with 0 ± 1 °C freezing marine water (Chen *et al.,* 2014). The collected samples were preserved at the Regional Agency for Environmental Protection–Liguria (ARPAL), Department of Organic Chemistry and Physical Analysis (Genoa, Liguria, Italy). Sample analysis was conducted at the laboratories of the Highly Specialized Aquaculture and Biotechnology Service (SABE) located at the Marine Scientific and Technological Park of ECOAQUA University Institute of the University of Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain).

6.3.1 Broodstock conditioning

A total of three breeding pairs of *Pseudochromis fridmani* (total length between 9.8 ± 1.5 cm) were kept in a 400 L glass aquarium split in three partitions of approximately 133 L each. Numerous PVC pipe segments (*ca.* 30 cm length and 32 mm diameter) were used to provide hiding places and breeding substrate where fish could create the nests (Fig. 6.S1). Since this specie does not present any sexual dichromatism or dimorphism (Mies *et al.,* 2014), pairs were composed identifying females as smaller with a remarkably swollen belly, and male as larger and slender preparing and defending the nest. Each breeding pair was fed twice a day *ad libitum* following the feeding protocol of the Aquarium facilities, which were based on

fresh chopped fish, mussels, and shrimps, live cultured mysis and frozen adult *Artemia.* Daily maintenance, which involved cleaning of the drain mesh and siphoning of feed waste and debris was carried out. Temperature was maintained at 26 ± 1 °C, salinity at 35 ± 1 g L^{-1} and photoperiod at 10L:14D provided by two 30 W fluorescent lights tube suspended 20 cm above the water surface. Water samples were collected three times a week to check the main parameters and keep nitrogen compounds below 0.01 mg L^{-1}, NH_4^+ as well NO_2.

6.3.2 Spawn management and larval culture system

Pair spawning activity was monitored twice a day, and the PVC pipe chose by the male to create the nest was also visually inspected to verify the presence of egg masses (Fig. 6.S1). When spawning occurred, the egg masses were left to the care of the male. Since the 4th dpf, when eyes of the embryos became visible as black spot with silver reflections, the egg mass was shifted from the broodstock tank to the larval culture system MoRS (Modular Rearing System) (Fig. 6.S2; Martino *et al.,* 2023). Hatching events were considered successful only when more than 90 % of the larvae hatched from the egg mass. The MoRS housed six cylindrical culture tanks with black walls of 25 L each, with a central 300 µ drain mesh that kept the larvae separated from the filtration. Each tank was maintained at bain-marie condition in 600 L glass aquarium and supplied with a 1 L per hour water flow (Fig. 6.S2). Water salinity was kept at 35 ± 1 g L^{-1}, while temperature at 26 ± 1 °C within minimum variation controlled by a heating device coupled with automatic thermostat (Panaque, Italy). All the MoRS was subjected to a photoperiod 14 L:10 D using two 40 W with a colour temperature of 10.000 K, fluorescent tubes. Daily analysis of the main water parameters was carried out three times a week, in order to keep the same chemical and physical water parameters of the broodstock (see section 2.1).

6.3.3 Live feeds culture techniques

The marine microalgae *Rhodomonas salina* and *Isochrysis galbana* were used to fed and enrich both experimental live preys and to implement the larval culture with the "green waters" technique. Algae were grown on a F/2 medium (Guillard and Ryther, 1962), inoculated into 100 L polycarbonate cylinders filed with 1 µm filtered seawater (cartridge filter, Swan®, Taiwan), salinity 30 ± 1 g L^{-1}, UV sterilized (PAN UV EASY 116, Panaque®, Italy) and mixed with air filtered with cotton. Cultures were kept at 20 ± 1 °C with illumination on a 24 h light regime and log phase during the whole experimentation harvesting and refilling cultures with new medium (Martino *et al.,* 2023). Concentrations of cultures was determined daily using a haemocytometer (Neubauer chamber) to count by triplicate the number of cells mL^{-1} under a microscope (DME, Leica, Germany). Both rotifers and copepods have been cultured to feed the orchid dottybacks during the larval trial. The rotifers *B. plicatilis* (L strain), with a range size of 180-250 µ, was cultured on *R. salina* and *I. galbana* at 30 ± 1 g L^{-1} salinity and 20 ± 1 °C in two 80 L cylindrical tanks. The calanoid copepod *A. tonsa,* with a range size of 70 µm newly hatched nauplii till 1.5 mm adult females, was obtained from the commercial product "Copepod Eggs" distributed by Algova® (Germany), cists were hatched following the company's instructions. The nauplii hatched were cultured in 100 L polycarbonate cylinders and fed equally on *R. salina* and *I. galbana.* Density estimation of rotifers and copepods were done daily by averaging three 1 mL samples, and each of the samples was counted under a stereomicroscope (EZ4, Leica, Germany). Rotifers and copepods were enriched/fed with binary microalgal diet based on *I. galbana* and *R. salina* prior to be used as live preys following Martino *et al.* (2023) (Table 6.1), utilizing 1:1 ratio at 1.5 10^5cells mL^{-1} to facilitate the application of the enrichment and the reintegration of the algae consumed. The microalgae used in this study were chosen because of their high content in LC-PUFAs, suitable cell size for both *A. tonsa* and *B.*

plicatilis and as well because they are among the most popular species in commercial mariculture industry (Ismar *et al*., 2008; Guevara *et al.,* 2010; Zhang *et al*., 2013; Sirakov *et al*., 2015).

6.3.4 Experimental design

In this study, early hatched larvae from the same breeding pair were concentrated to the surface of the hatching tank using a light torch and scooped in a 500 mL beaker. Six groups of 400 larvae were placed in six rearing tanks of 25 L belonging to the MoRS, reaching a density of 16 larvae L^{-1} for each tank (see section 2.1; Fig, 2.4). Larval culture tanks were provided with a water renewal of 1 L per hour and with 1 bubble air per second, which ensured slow water circulation. Two feeding diets based on enriched copepods and rotifers were tested to determine their effect on survival, growth, biochemical composition, first bones calcification, liver and intestinal morphology of *P. fridmani* larvae from 1 to 15 dph. The effects of the following diets were tested by triplicate (n=3):

Diet I: The calanoid copepod *A, tonsa* (15 ind. mL^{-1}), enriched with the microalgae *R. salina* and *I. galbana.*

Diet II: The rotifer *B. plicatilis* (15 ind. mL^{-1}) enriched with the microalgae *R. salina* and *I. galbana.*

The "green waters" techniques using binary live microalgae *R. salina* and *I. galbana,* 1:1 ratio at 1.5 10^5cells mL^{-1}, was established in all experimental tanks. The cleaning of the drain mesh, siphoning of debris, counting of died larvae and water analysis were carried out daily in each experimental tank. After cleaning maintenance, the density of microalgae and live preys was estimated (see section 2.4.1) and reset to scheduled trial concentration values.

Table 6.1: Summary of the live preys proximal composition (protein and lipid; g/100g dry weight) and fatty acid composition (mg/g dry weight), *A. tonsa* and *B. plicatilis*, both enriched with microalgae (*Isochrysis galbana* + *Rhodomonas salina*), n= 5, extract from Martino *et al.* (2023).

Live preys	*A. tonsa*	*B. plicatilis*
Crude lipids	16.4 ± 0.5	15.4 ± 0.8
Crude proteins	70.7 ± 0.7^{b}	58.7 ± 1.6^{a}
14:0	0.5 ± 0.1	0.2 ± 0.1
16:0	1.2 ± 0.2	1.6 ± 0.2
18:0	0.7 ± 0.1	0.5 ± 0.0
18:1n-9 OA	0.7 ± 0.1^{a}	1.7 ± 0.2^{b}
20:4n-6 ARA	0.1 ± 0.0	0.2 ± 0.0
20:5n-3 EPA	2.9 ± 0.1^{b}	1.4 ± 0.1^{a}
22:6n-3 DHA	6.7 ± 0.2^{b}	1.4 ± 0.1^{a}
ΣSAFAs[1]	2.5 ± 0.1	2.5 ± 0.3
ΣMUFAs[2]	1.5 ± 0.1^{a}	3.9 ± 0.4^{b}
Σn-3 LC-PUFAs[3]	9.9 ± 0.2^{b}	4.1 ± 0.3^{a}
n-3/n-6	17.2 ± 3.7^{b}	3.7 ± 0.4^{a}
n-3/n-9	14.7 ± 2.0^{b}	3.0 ± 0.2^{a}
n-6/n-9	0.9 ± 0.4	0.8 ± 0.1
DHA/EPA	2.3 ± 0.1^{b}	1.0 ± 0.1^{a}
DHA/ARA	96.8 ± 5.0^{b}	7.4 ± 2.2^{a}
EPA/ARA	42.0 ± 2.7^{b}	7.2 ± 1.7^{a}

Values (mean ± SD) followed by different superscript letters within a row were ($p < 0.05$) significantly different among treatments. n= number of replicates; SAFA= saturated fatty acid; MUFA= monounsaturated fatty acid; LC-PUFA= long chain polyunsaturated fatty acid; DHA =docosahexaenoic acid; EPA =eicosapentaenoic acid; ARA =arachidonic acid; OA= oleic acid. [1] Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. [2]Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. [3]Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

6.3.4.1 Samplings

Samples of 20 larvae per tank were collected and individually measured in total length (TL) at the beginning (0 dph), during (2, 4, 6, 8, 10 dph) and at the end of the feeding trial (15 dph) in order to assess growth, histological and osteological modifications. Thus, larvae were previously euthanized with cold water 0 ± 1 °C (Chen *et al.,* 2014) and then included in 5 mL Eppendorf polyethylene tubes and preserved in buffered formalin (4%)

until analysis. Additional larvae were collected at the beginning (0 dph) and at the end (15 dph) of the feeding trial for proximate and FA composition analysis. In order to reach a sufficient sample size for the 0 dph sample, due to the tinny size of the recently hatched larvae, a different spawning was collected from the same breeding pair and hatched as described previously (see section 2.5) and larvae sampled (*ca*. 2000) for biochemical analysis. Additionally, at the end of the trial all larvae (15 dph) present in each experimental tank constituted a sample (n=3). Larvae were washed with deionized water to remove sea salts, included in 5 mL Eppendorf polyethylene tubes, lyophilised (Alpha 1-2 LD plus, Christ; Germany) and then conserved at -80 °C until analysis.

6.3.4.2 Larval Growth and Survival

Larval growth was assessed by estimating TL under a stereomicroscope (EZ4, Leica, Germany).

$$\text{TL (mm)} = \text{Mean}$$

Specific growth rate (SGR) was calculated in relation to total length registered during the feeding trial using the following formula:

$$\text{SGR (\%)} = \frac{[\ln(\text{TLf}) - \ln(\text{TLi})]}{\Delta t} \text{ x } 100$$

Where TLf was the final larval total length (mm), TLi was the initial larval total length (mm) and Δt was the time between sampling days (Hopkins, 1992; Lugert *et al.*, 2014). The coefficient of variation (CV) of total length was calculated as the ratio between the standard deviations and the mean lengths according to the formula (Bowker 1995):

$$\text{CV (\%)} = \frac{\text{TL Standard Deviation}}{\text{TL Mean}} \text{ x } 100$$

At the end of the trial, the dead and live larvae count allowed to calculate the final survival rates per tank with the formula:

$$\text{Final Survival (\%)} = \frac{\text{Nf}}{(\text{Ni} - \text{Ns})} \text{ x } 100$$

Where Nf was the number of living larvae at the end of the experimental period, Ni was the initial larvae number stocked in each tank and Ns was the number of larvae sampled during the trial (Cortay *et al.*, 2019). Each day cumulative mortality for each treatment replicate were also determined as follows:

$$\text{Cumulative mortality (\%)} = \frac{\text{Nd}}{(\text{Ni} - \text{Ns})} \text{ x } 100$$

Where Nd was the number of total dead larvae, Ni was the initial larvae numbers stocked in each tank and Ns was the number of larvae sampled during the trial.

6.3.4.3 Biochemical composition analysis

Analysis of proximate and fatty acid composition, of *P. fridmani* larvae was performed at 0 and 15 dph comparing the composition of larvae fed with the two experimental diets. All samples were homogenised and analysed by triplicate. Proximate composition was conducted following standard procedures (Association of Official Analytical Chemists [AOAC], 2019). Moisture was determined by thermal dehydration until constant mass at 105°C. Ash content was determined by combustion at 600°C for 12 h- Crude protein content was determined using the method described by Kjeldahl and crude lipid was extracted following the chloroform-methanol method described by Folch *et al.* (1957). Fatty acid methyl esters (FAMES) profiles were obtained by transmethylation of total lipids (Christie and Han, 2010) and separated by gas-liquid chromatography (GLC), under the conditions previously described by Izquierdo *et al.* (1990).

6.3.4.4 Histological analysis

To characterize histological changes in the liver, intestine and bones, 10 larvae fixed in buffered formalin 4% from each sampling day were processed in histology cassettes and dehydrated using a Thermo Scientific STP 120-2 (Thermo Shandon Limited, United Kingdom). Paraffin-embedded larvae were cut at 3 µ on a Leica Jung Autocut 2055 microtome (Leica, Nussloch, Germany). Sampled larvae were stained with haematoxylin and eosin (H&E) for the liver and the intestine morphological studies (Martoja and Martoja-Pierson, 1970). A semi-quantitative score of the liver conditions, steatosis and nuclear pyknosis, was defined ranging from 0 to 3, where 0, 1, 2 and 3 represented absent, low, mild and severe hepatic conditions (Betancor *et al.*, 2012), respectively. The histological assessment was completed with the quantitative image analysis of the liver, regarding 10 hepatocyte (diameter) and 10 hepatocyte nuclei (diameter and surface area), and intestine, measuring its luminal diameter, 10 villi (length and width) and 10 goblet cells surface area. The mounted sections were scanned with a Motic Easy Scan Pro digital scanner (Motic, Xiamen, China) operated using the Motic DS Assistant software (Motic VM V1 Viewer 2.0) and examined using Image Pro Software (Media Cybernetics, USA). Three different trained independent blind observers evaluated the slides to estimate visual differences among treatments.

6.3.4.5 Osteological analysis

For this analysis, 10 larvae fixed in buffered formalin 4% from each sampling day were stained with alizarin red and alcian blue following the acid-free double staining protocol of Walker and Kimmel (2007) modified. Different millimolar concentrations of magnesium chloride ($MgCl_2$) were used to prepare alcian blue solution depending on the larvae age respectively: 100 mM from 0 to 2 dph, 140 mM from 4 to 6 dph, 180 mM

from 8 to 10 dph and 200 mM at 15 dph. Stained larvae were examined using an Olympus CX41 microscope (Olympus, Hamburg, Germany) connected to an Olympus XC30 camera (Olympus), which was linked to a computer using Image Pro Software (Media Cybernetics, USA). The preparations were evaluated by three trained independent blind observers to define visual differences among treatments. A semi-quantitative score ranging from 0 to 3 of skeletal mineralization was defined for cranial region (maxilla, gill arches, neurocranium), vertebral column, and caudal fin complex. Score 0 was assigned as absence of mineralized matrix; score 1 was defined as low bones mineralization; score 2 as moderate bones mineralization; and score 3 as completely mineralized bones. The effect of different live diets on first skeleton mineralization was evaluated for each dietary group.

6.3.5 Statistical analysis

Analyses were conducted using SPSS statistical software package (IBM SPSS V27.0 for Windows; SPSS Inc., Chicago, IL, USA), setting the significance level for all analyses at < 0.05 (P value). All data resulting from comparative studies regarding growth, survival, biochemical composition, histological and osteological analysis were analysed using Students' t-test. The homogeneity of the variable and the normal distribution were tested using respectively Levene's and Shapiro Wilk's tests, while percentage data were log-transformed in order to obtain variance homogeneity. Unless stated otherwise, measurements are presented as mean ±standard deviation (SD).

6.4 Results

6.4.1 Larval growth and survival

Generally, the use of enriched *A. tonsa* as a live prey induced a significant increase of *P. fridmani* larvae length (*t*-test, $p < 0.05$; Fig 6.1) and growth (*t*-test, $p < 0.05$; Fig.6.2) since 4 dph until the end of the trial (15 dph) respect to *B. plicatilis*. Furthermore, no significant differences in CV of total length were observed among feeding protocols *(t*-test, $p \geq 0.05$; Fig. 6.3). By the end of the trial final survival of larvae reared with copepods diet was significantly higher (82 % ± 4.3 %) than larvae fed with rotifers (54 % ± 3.6 %) (*t*-test = 99.86, $p < 0.05$). The incidence of larval mortality was mainly observed during the first 3 dph in larvae fed with copepods and during the first 5 dph in larvae fed with rotifers (11 % and 34% respectively) (Fig. 6.4).

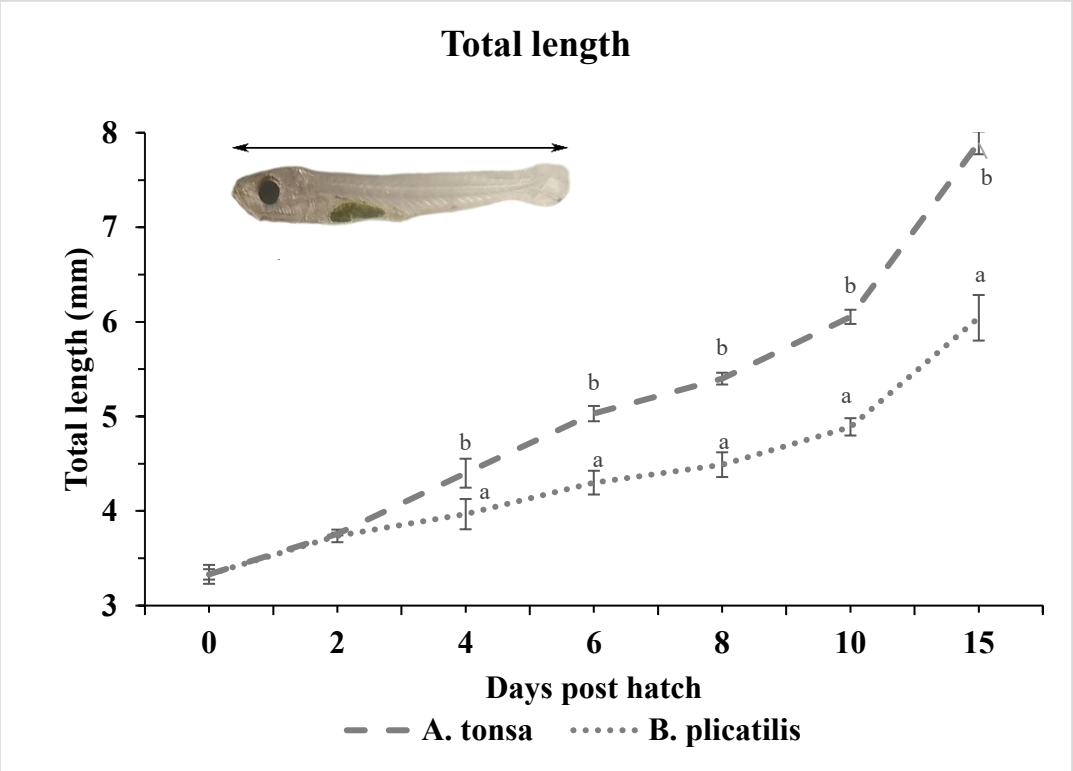

Figure 6.1: Total length parameters (mm) of *P. fridmani* larvae fed experimental diets at 0, 2, 4, 6, 8, 10 and 15 days post hatch (mean ± SD, n = 3). Different letters above the bars indicate significant differences among feeding regime ($p < 0.05$).

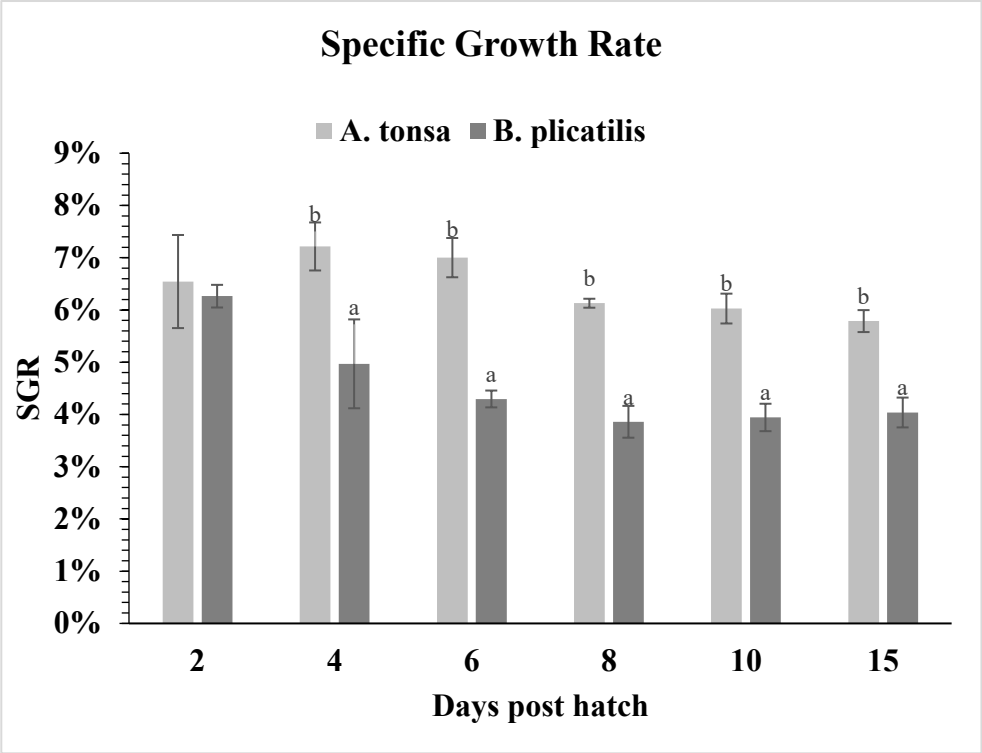

Figure 6.2: Specific growth rate (SGR, % day-1) of *P. fridmani* larvae fed experimental diets from 0 to 15 days post hatch (mean ± SD, n = 3). Different letters above the bars indicate significant differences among feeding regime ($p < 0.05$).

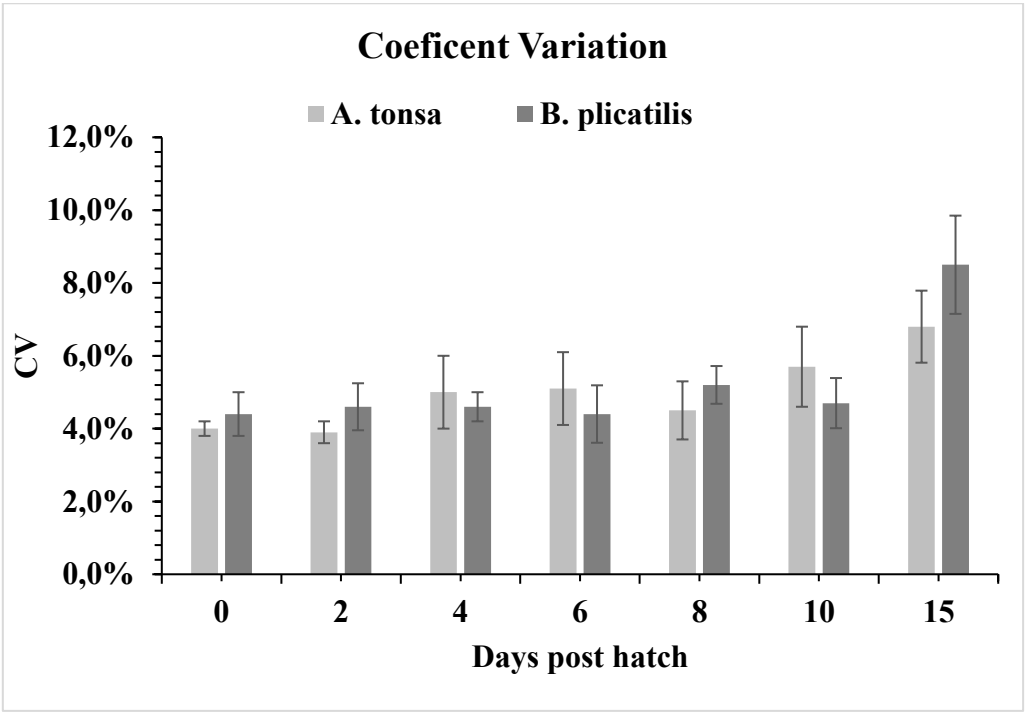

Figure 6.3: Coefficient of variation of total length in *P. fridmani* larvae fed with experimental diets from 0 to 15 days post hatch (mean ± SD, n = 3).

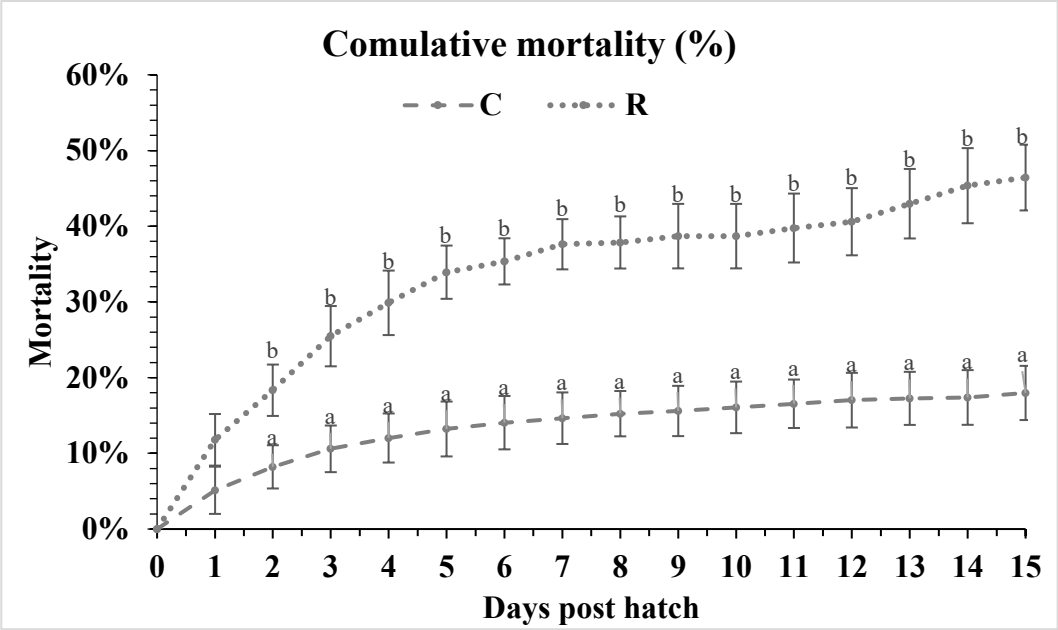

Figure 6.4: Cumulative mortality (%) through ontogeny of *P. fridmani* larvae fed with experimental diets from 0 to 15 days post hatch (mean ± SD, n = 3). Different letters above the bars indicate significant differences among feeding regime ($p < 0.05$). C= Copepods; R= Rotifers.

6.4.1 Whole larvae biochemical composition

The Proximate composition of *P. fridmani* larvae was expressed as percentage of DW ± SD, meanwhile their fatty acid composition was expressed as grams per 100g of identified fatty acids from whole larval body (Table 6.2). At the hatching time (0 dph), the main fatty acids found in *P. fridmani* newly hatched larvae were the total saturated fatty acids (SAFAs) and LC-PUFAs, such as palmitic acid (PAM, 16:0), docosapentaenoic acid (DPA, 22:5n-3), EPA, and DHA (Table 6.2). In contrast, at the end of the trial, larvae fed with copepods showed significantly higher lipid content (15.2 ± 0.1 %) compared to those fed with rotifers (*t*-test = 10.44, $p < 0.05$; Table 6.2). Protein and moisture content showed no significant difference among treatments (*t*-test, $p \geq 0.05$), while ash content was significantly higher in larvae fed with rotifers (17.4 ± 0.6 %) (*t*-test = 8.1, $p < 0.05$; Table 6.2). In term of FAs, larvae fed with copepods were characterized by significantly higher levels of LC-PUFAs, α-linolenic acid (ALA, 18:3n-3), EPA and DHA compared to the groups fed with rotifers (*t*-test, $p < 0.05$; Table 6.2). While larvae fed with rotifers significantly increased their content in SAFAs,

especially in PAM, and stearic acid (STA, 18:0) as well as in DPA compared to the group fed with copepods (*t*-test, $p < 0.05$; Table 6.2). However, orchid dottybacks larvae (15 dph) fed with copepods were characterized by a significantly higher DHA/ARA, EPA/ARA, n-3/n-6 and n-3/n-9 ratios (*t*-test, $p < 0.05$; Table 6.2), compared to the rotifer treatment. The FAs ratio DHA/EPA did not show any difference (*t*-test, $p \geq 0.05$; Table 6.2) between treatments.

Table 6.2: Proximal composition (protein. lipid, ash and moisture content; g/100g dry weight) and fatty acid composition (mg/g dry weight) of *P. fridmani* larvae at 0 dph (control) and 15 dph fed with copepods (C) and rotifers (R), n=3.

***P. fridmani* larvae**	***0 dph***	***C15 dph***	***R15 dph***
Crude protein	67.3 ± 0.2	55.6 ± 0.7	54.9 ± 0.6
Crude lipid	16.0 ± 0.3	15.2 ± 0.1[b]	14.1 ± 0.3[a]
Crude ash	12.7 ± 0.1	14.7 ± 0.8[a]	17.4 ± 0.6[b]
Crude moisture	7.6 ± 0.3	8.5 ± 0.7	7.8 ± 0.3
14:0	0.2 ± 0.0	0.6 ± 0.1[b]	0.2 ± 0.0[a]
14:1n-5	-	0.1 ± 0.0	0.1 ± 0.0
15:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
16:0	3.5 ± 0.1	2.8 ± 0.1[a]	3.1 ± 0.1[b]
16:1n-7	0.5 ± 0.1	1.0 ± 0.0[b]	0.6 ± 0.0[a]
16:3n-1	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
18:0	1.9 ± 0.2	1.2 ± 0.0[a]	1.7 ± 0.1[b]
18:1n-9	2.0 ± 0.1	2.3 ± 0.1	2.2 ± 0.1
18:1n-7	0.7 ± 0.1	0.8 ± 0.0	0.9 ± 0.1
18:2n-6	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.1
18:3n-3	0.5 ± 0.1	0.7 ± 0.0[b]	0.5 ± 0.1[a]
18:4n-3	0.1 ± 0.0	0.4 ± 0.0[b]	0.1 ± 0.0[a]
20:0	0.1 ± 0.0	-	0.1 ± 0.0
20:1n-9	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:2n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-6	0.1 ± 0.0	-	0.1 ± 0.0
20:4n-6 ARA	-	0.3 ± 0.0	0.4 ± 0.1
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.1 ± 0.0	0.4 ± 0.0[b]	0.2 ± 0.0[a]
20:5n-3 EPA	0.9 ± 0.1	0.7 ± 0.0[b]	0.5 ± 0.0[a]
22:1n-11	-	0.1 ± 0.0	0.1 ± 0.0
22:5n-6	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:5n-3	0.4 ± 0.1	0.2 ± 0.0[a]	0.3 ± 0.0[b]

22:6n-3 DHA	2.1 ± 0.1	1.3 ± 0.1^{b}	0.8 ± 0.1^{a}
Σn-3[1]	4.2 ± 0.2	3.7 ± 0.0^{b}	1.8 ± 0.2^{a}
Σn-6[2]	1.9 ± 0.1	1.5 ± 0.1^{b}	1.1 ± 0.1^{a}
Σn-9[3]	2.2 ± 0.1	2.7 ± 0.1^{b}	2.0 ± 0.1^{a}
ΣSAFAs[4]	5.8 ± 0.2	4.9 ± 0.1^{a}	5.2 ± 0.1^{b}
ΣMUFAs[5]	3.7 ± 0.1	4.8 ± 0.1	4.6 ± 0.1
Σn-3 LC-PUFAs[6]	3.6 ± 0.2	2.6 ± 0.0^{b}	1.8 ± 0.2^{a}
n-3/n-6	2.2 ± 0.1	2.5 ± 0.1^{b}	1.7 ± 0.1^{a}
n-3/n-9	1.9 ± 0.1	1.4 ± 0.1^{b}	0.9 ± 0.1^{a}
DHA/EPA	2.4 ± 0.2	1.9 ± 0.1	1.8 ± 0.2
DHA/ARA	3.0 ± 0.2	3.7 ± 0.6^{b}	2.3 ± 0.1^{a}
EPA/ARA	1.2 ± 0.1	1.9 ± 0.2^{b}	1.3 ± 0.2^{a}

Values (mean ± SD) followed by different superscript letters within a row were ($p < 0.05$) significantly different among treatments. n= number of replicates; SAFA= saturated fatty acid; MUFA= monounsaturated fatty acid; LC-PUFA= long chain polyunsaturated fatty acid; DHA =docosahexaenoic acid; EPA =eicosapentaenoic acid; ARA =arachidonic acid. [1]Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3. [2]Includes 16:2n-6, 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. [3]Includes 18:1n-9, 18:2n-9, 20:1n-9, 20:2n-9, 20:3n-9 and 22:1n-9. [4] Includes 14:0, 15:0, 16:0, 17:0, 18:0, and 20:0. [5]Includes 14:1n-5, 14:1n-7, 15:1n-5, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9 and 22:1n-11. [6]Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3.

6.4.2 Histological analysis

Liver examination in larvae fed copepods showed since 2 dph until the end of the trial better organization and a well-preserved architecture, with prominent basophilic nucleus mostly located at a central position in the cell (*t*-test, $p < 0.05$; Table 6.3; Fig. 6.5 a; 6.6 a). Although some lipid infiltration was observed in the form of micro-vacuolization, the copepods treatment denoted a lower extension compared to those observed in larvae fed rotifers (Fig. 6.5; 6.6). Indeed, the use of rotifers significantly increased liver insults in larvae (*t*-test, $p < 0.05$; Table 6.3), denoted by the presence of cellular steatosis, hypertrophy and nucleus pyknosis (Fig. 6.5 b; 6.6 b).

The intestine of larvae fed copepods presented since 4 dph until the end significantly higher diameter (*t*-test, $p < 0.05$; Table 6.4) compared to rotifers treatment. By the end of the trial, all the histological measurements including *villi* length and width, goblets cells number and surface area, were significantly higher in larvae fed copepods (*t*-test, $p < 0.05$; Table 6. 5; Fig. 6.7). The first goblet cells were observed at 6 dph in larvae fed with copepods, while groups fed with rotifers exhibited at 8 dph (Table 6.4). Significant increase in terms of goblet cells number and surface area was also registered since 10 till 15 dph in larvae fed on copepods (*t*-test, $p < 0.05$; Table 6.4).

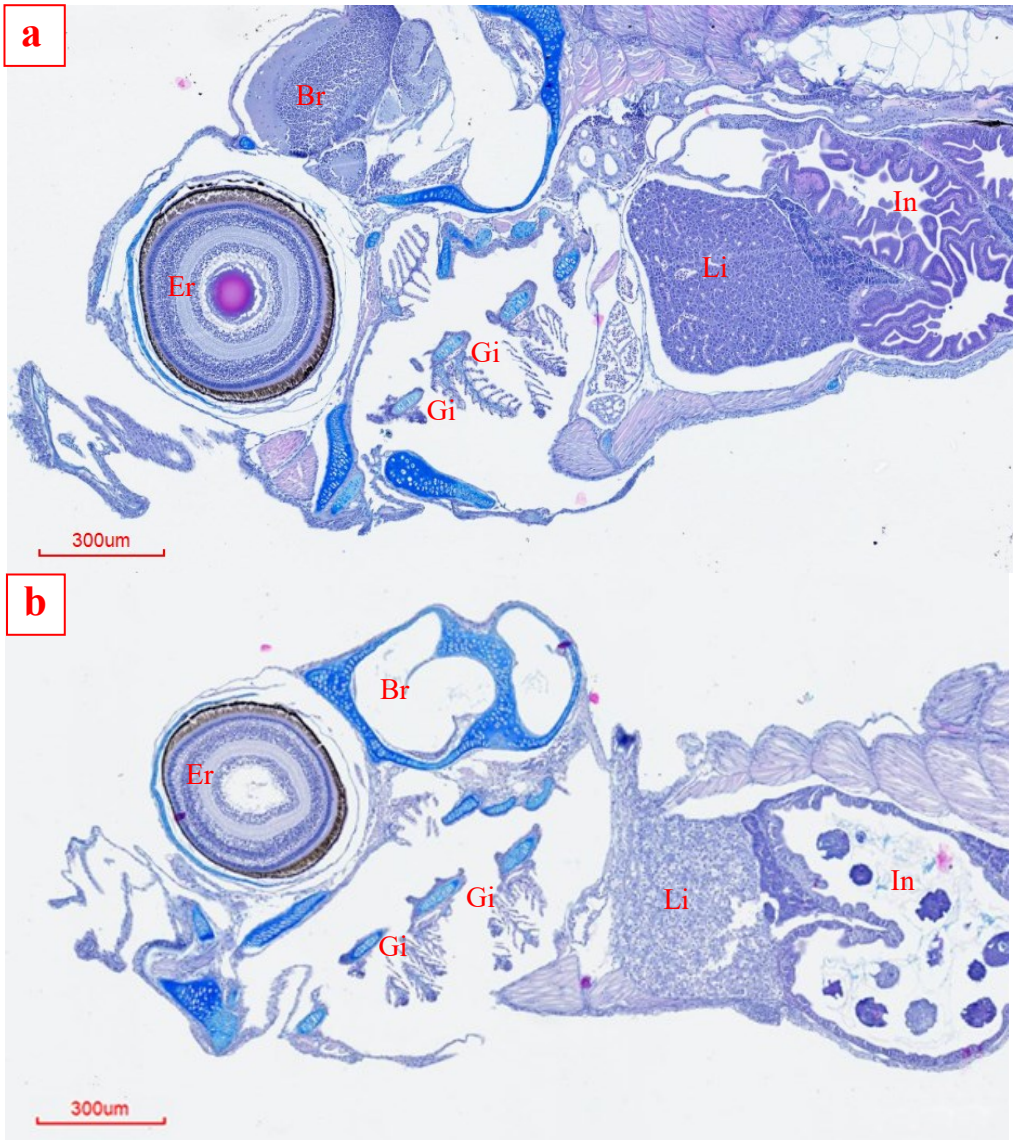

Figure 6.5: Microscopic view (20x magnification) of *Pseudochromis fridmani* larvae at 15 dph, fed with copepods (A) and rotifers (B). Er= Eye retina; Br= Brain; Gi= Gills; Li= Liver; In= Intestine.

Table 6.3: Histological analysis of hepatic tissue of *P. fridmani* larvae fed with the experimental diets for 15 days. Values (mean ± SD) followed by different superscript letters in the same row are significantly different ($p < 0.05$). Semiquantitative score value (steatosis and pyknosis; n= 5): score 0: normal liver histomorphology, score 1 and 2: low and mild hepatic conditions, and score 3: severe hepatic conditions. Quantitative analysis values were expressed in micrometres (hepatocyte diameter, nucleus diameter and nucleus area).

Liver	**0 dph**	**2 dph**		**4 dph**		**6 dph**		**8 dph**		**10 dph**		**15 dph**	
Diet	**Control**	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***
Steatosis	0.4 ± 0.5	0.8 ± 0.4	1.4 ± 0.5	1.2 ± 0.4^{a}	2.4 ± 0.5^{b}	0.6 ± 0.5^{a}	2.0 ± 0.7^{b}	0.8 ± 0.4^{a}	2.2 ± 0.8^{b}	0.4 ± 0.5^{a}	2.0 ± 0.7^{b}	0.4 ± 0.5^{a}	1.8 ± 0.4^{b}
Pyknosis	0.2 ± 0.4	0.4 ± 0.5	1.0 ± 0.7	0.6 ± 0.5^{a}	1.8 ± 0.4^{b}	0.2 ± 0.4^{a}	1.6 ± 0.5^{b}	0.4 ± 0.5^{a}	1.6 ± 0.5^{b}	0.2 ± 0.4^{a}	1.2 ± 0.4^{b}	0.2 ± 0.4^{a}	1.2 ± 0.4^{b}
Hepatocyte diameter	9.5 ± 0.9	8.8 ± 0.7^{a}	11.6 ± 1.1^{b}	11.4 ± 1.4^{a}	15.1 ± 1.1^{b}	10.8 ± 1.0^{a}	17.1 ± 1.4^{b}	9.7 ± 1.1^{a}	16.1 ± 1.5^{b}	11.1 ± 3.1^{a}	16.5 ± 1.0^{b}	14.2 ± 0.6^{a}	15.7 ± 0.6^{b}
Nucleus diameter	3.7 ± 0.4	3.6 ± 0.3^{b}	2.6 ± 0.3^{a}	4.4 ± 0.2^{b}	2.6 ± 0.1^{a}	4.1 ± 0.1^{b}	3.1 ± 0.4^{a}	4.3 ± 0.3^{b}	2.9 ± 0.2^{a}	4.7 ± 0.3^{b}	3.1 ± 0.5^{a}	4.4 ± 0.4^{b}	3.4 ± 0.4^{a}
Nucleus area	10.8 ± 1.3	10.9 ± 0.8^{b}	8.5 ± 0.8^{a}	16.1± 1.4^{b}	10.7 ± 1.5^{a}	14.4± 1.7^{b}	10.2 ± 0.7^{a}	16.5 ± 1.2^{b}	8.8 ± 0.8^{a}	17.0 ± 1.2^{b}	9.8 ± 1.8^{a}	16.9 ± 0.8^{b}	9.4 ± 0.4^{a}

Table 6.4: Histological analysis of intestine tissue of *P. fridmani* larvae fed the experimental diets for 15 days (n= 5). Values (mean ± SD) followed by different superscript letters in the same row are significantly different ($p < 0.05$). Goblet cells n. correspond to the total number observed in each liver sample

Intestine	***0 dph***	***2 dph***		***4 dph***		***6 dph***		***8 dph***		***10 dph***		***15 dph***	
Diet	**Control**	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***
Diameter	80.0 ± 7.2	121.0 ± 14.9	98.6 ± 10.4	167.3 ± 12.8^{b}	133.9 ± 15.5^{a}	178.4 ± 13.1^{b}	129.8 ± 11.4^{a}	191.5 ± 14.9^{b}	155.9 ± 16.3^{a}	201.2 ± 11.4^{b}	164.4 ± 16.2^{a}	221.7 ± 13.3^{b}	168.9 ± 7.4^{a}
Villi length	24.9 ± 3.3	22.8 ± 4.2	25.4 ± 6.0	34.4 ± 3.6	26.7 ± 6.0	35.7 ± 6.9	27.6 ± 6.3	32.0 ± 4.1	30.2 ± 6.9	39.9 ± 10.5	29.3 ± 6.1	60.0 ± 13.6^{b}	33.7 ± 2.9^{a}
Villi width	28.1 ± 6.2	21.7 ± 5.2	23.1 ± 3.3	33.3 ± 4.4	25.3 ± 3.2	32.7 ± 4.1	27.5 ± 1.3	31.1 ± 3.3	28.4 ± 5.1	33.1 ± 2.2	31.1 ± 5.6	39.0 ± 3.6^{b}	28.2 ± 2.1^{a}
Goblet cell n.	-	-	-	-	-	0.6 ± 0.5	-	2.8 ± 2.5	0.6 ± 1.3	5.2 ± 2.8^{b}	0.2 ± 0.4^{a}	12.6 ± 1.5^{b}	2.6 ± 1.1^{a}
Goblet cell area	-	-	-	-	-	25.9 ± 6.3	-	26.7 ± 3.2	19.6 ±3.6	30.1 ± 3.1^{b}	14.4 ± 0.0^{a}	46.3 ± 10.9^{b}	23.4 ± 4.4^{a}

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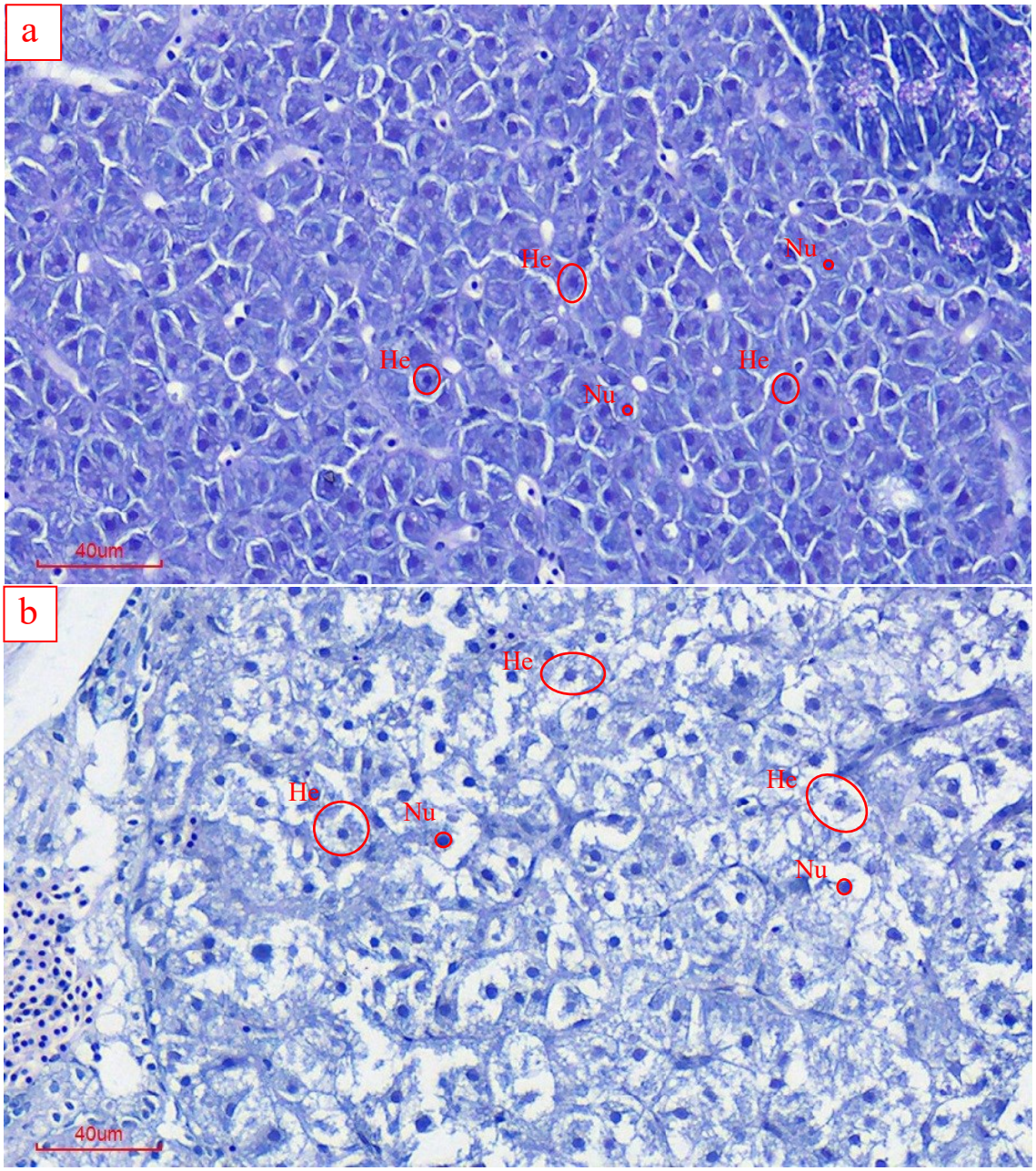

Figure 6.6: Microscopic view (40x magnification) of *Pseudochromis fridmani* liver at 15 days post hatch fed with copepods (a) and rotifers (b). He= Hepatocyte; Nu= Nucleus

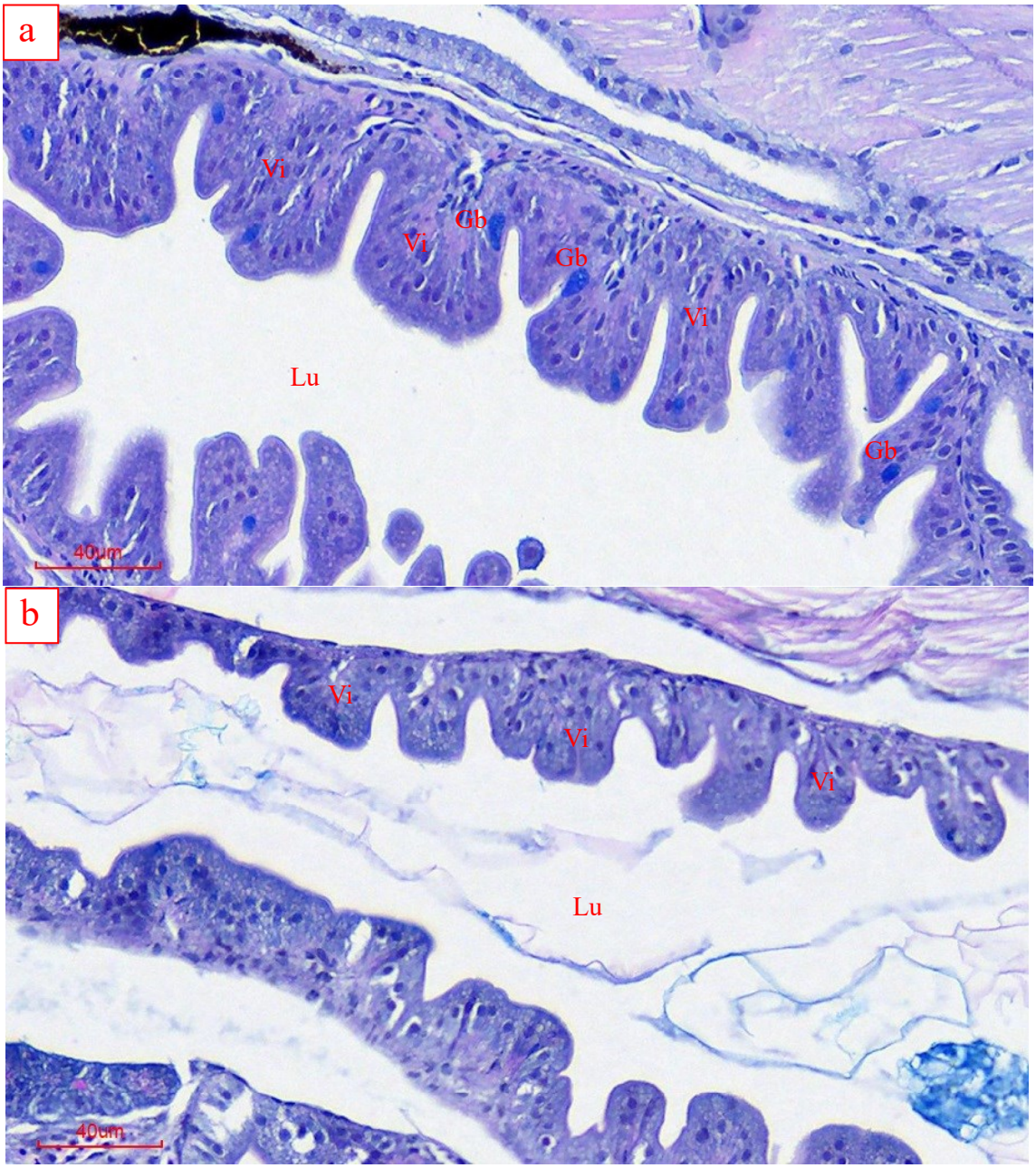

Figure 6.7: Microscopic view (40x magnification) of *Pseudochromis fridmani* intestine at 15 days post hatch fed with copepods (a) and rotifers (b). Vi= *Villi*; Lu= *Lumen*; Gb= Goblet Cell.

6.4.3 Osteological analysis

Larvae of *P. fridmani* showed differences in skeletal development depending on the diet administered (Table 6.5, Fig. 6.8, 6.9). In both treatment first bones formation was detected noticeable from 6 dph onwards, starting from the cranial region and proceeding towards the tail complex direction. The mineralization of maxilla as first ossified structures was significantly higher (*t*-test, $p < 0.05$; Table 6.5) in larvae fed copepods until 10 dph (Fig. 9 a).

Calcification was limited to the cranial region until 10 dph when gill arches, neurocranium and vertebral column started to ossify and to be appreciated, without statistical difference between treatments (*t*-test, $p \geq 0.05$; Table 6.5; Fig. 6.8). By the end of the trial the tail fin bones were observed in both treatments, however, skeletal mineralization and development of larvae fed copepods, except the maxilla, was significantly higher (*t*-test, $p < 0.05$; Table 6.5; Fig. 6.9 a), concerning the whole skeleton (cranial region, vertebral column and tail fin), compared to rotifers treatment (Fig. 6.9 b,). Remarkably, no incidence of skeletal deformities was observed during the study in both treatments.

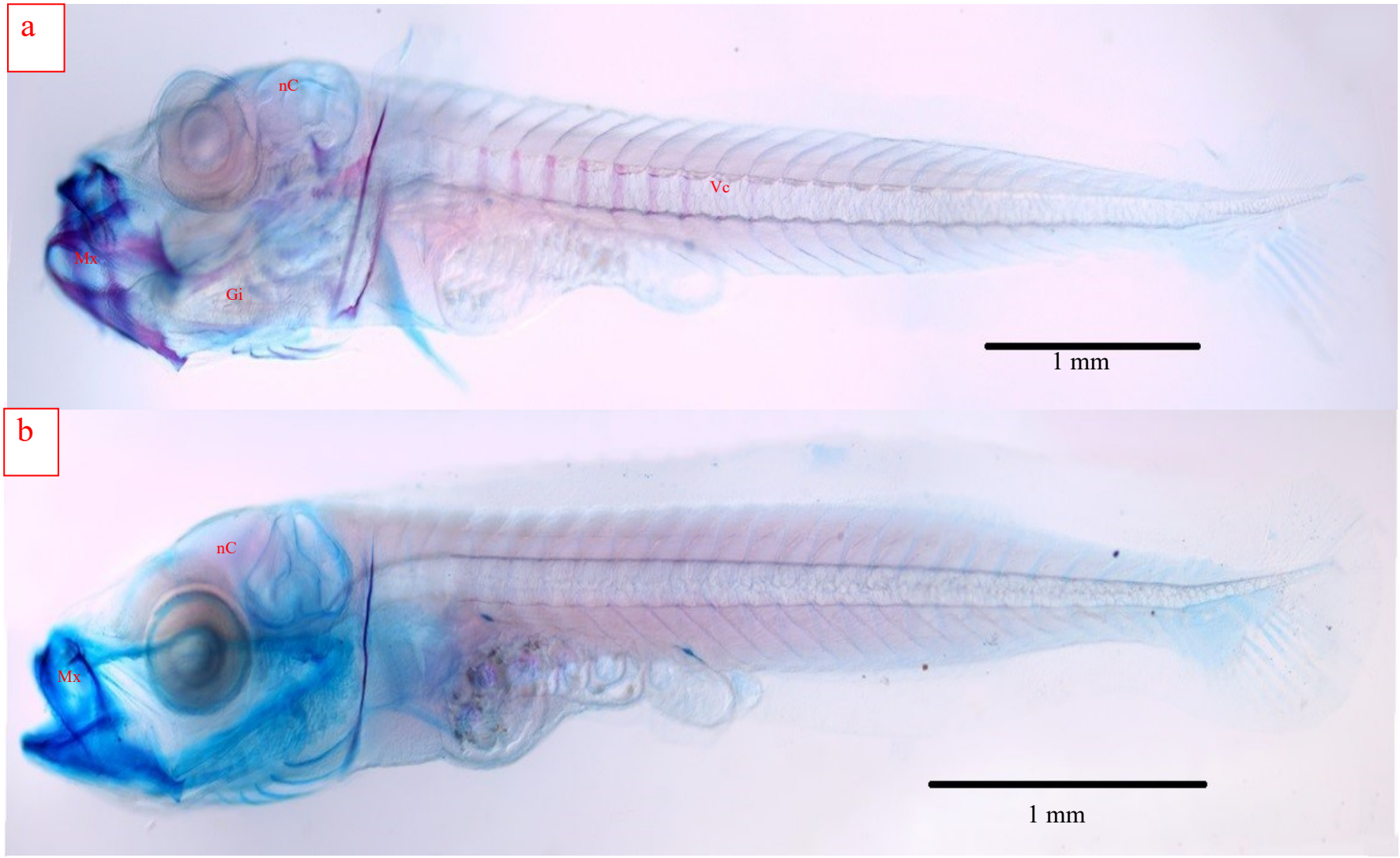

Figure 6.8: *Pseudochromis fridmani* larvae at 10 days post hatch showing different skeletal mineralization (red) depending on the diet administered, copepods (a) and rotifers (b). Maxilla= Mx; Gill arches= Gi; Neurocranium= nC; Vertebral column= Vc

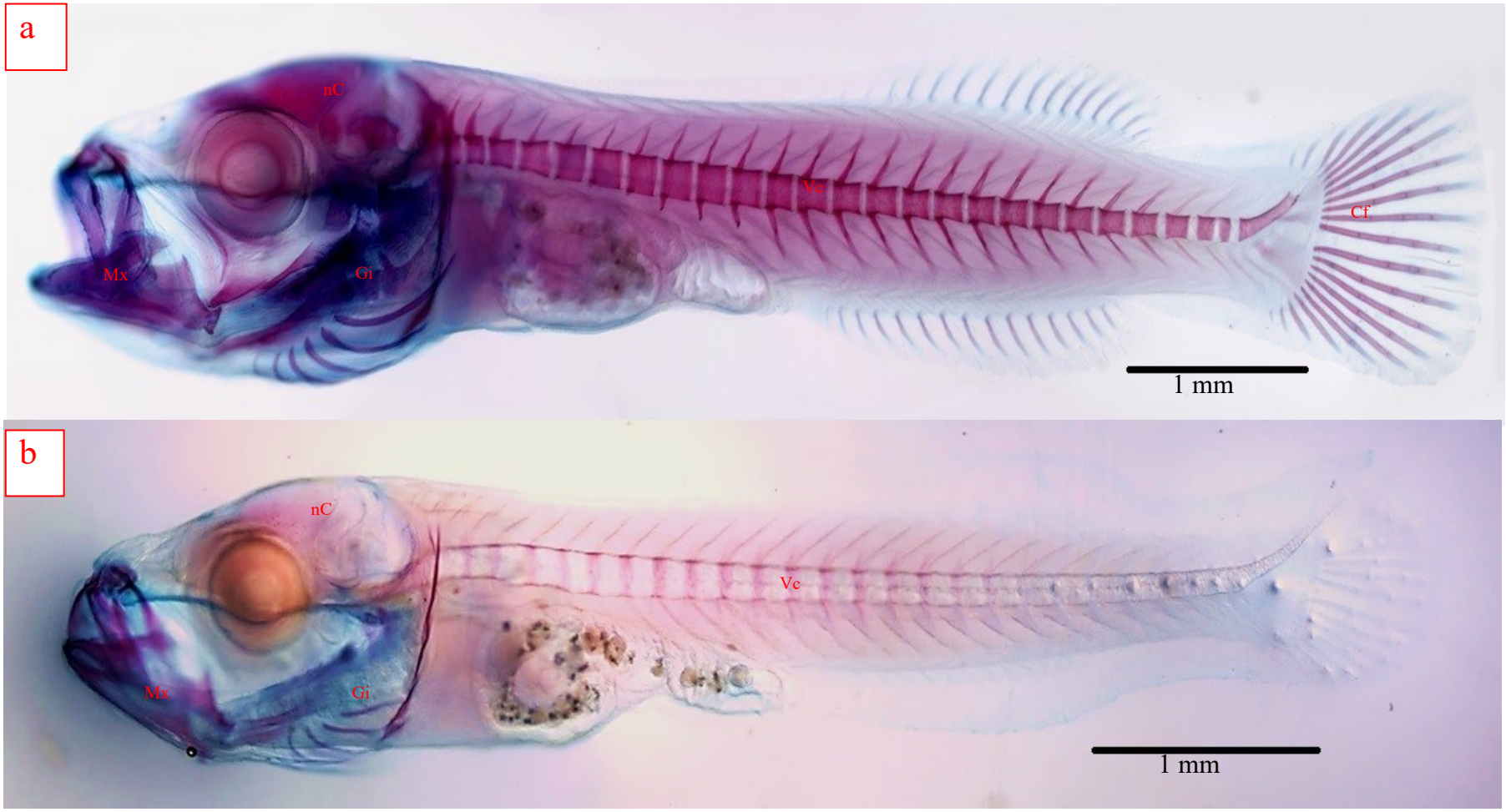

Figure 6.9: *Pseudochromis fridmani* larvae at 15 days post hatch showing different skeletal mineralization (red) depending on the diet administered, copepods (a) and rotifers (b). Maxilla= Mx; Gill arches= Gi; Neurocranium= nC; Vertebral column= Vc, Caudal fin= Cf

Table 6.5: Evaluation of bones mineralization in *P. fridmani* larvae fed with the experimental diets for 15 days. Values (mean ± SD) followed by different superscript letters in the same row are significantly different ($p < 0.05$). Semiquantitative score value (mean ± SD, n= 10): score 0: no bones mineralization; score 1: low mineralization; score 2: medium mineralization; score 3: high mineralized bones.

Skeleton	**6 dph**		**8 dph**		**10 dph**		**15 dph**	
Diet	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***	***A. tonsa***	***B. plicatilis***
Maxilla	1.1 ± 0.3^{b}	0.2 ± 0.4^{a}	1.8 ± 0.4^{b}	1.0 ± 0.3^{a}	2.5 ± 0.5^{b}	1.2 ± 0.4^{a}	2.9 ± 0.3	2.2 ± 0.5
Gill arches	-	-	-	-	1.8 ± 0.6	0.8 ± 0.4	2.7 ± 0.5^{b}	1.6 ± 0.5^{a}
Neurocranium	-	-	-	-	1.6 ± 0.5	0.7 ± 0.5	2.6 ± 0.4^{b}	1.4 ± 0.5^{a}
Vertebral column	-	-	-	-	1.2 ± 0.4	0.5 ± 0.5	2.7 ± 0.5^{b}	1.5 ± 0.5^{a}
Tail fin	-	-	-	-	-	-	2.2 ± 0.4^{b}	0.8 ± 0.4^{a}

6.5 Discussion

The present study revealed how important is the choice of the first diet to improve larvae culture of marine fish, which do not fully adapt to conventional live feed. The hitherto unknown effect of enriched copepods on growth performance, survival, nutritional and morphological conditions (histological and osteological) of cultured *P. fridmani* larvae were described, demonstrating for the first time that the calanoid copepod *A. tonsa* is better alternative to other live preys such as rotifer and can be used successfully during the early larval feeding of this species. Comparable results have been also reported in the Asian seabass larvae (*Lates calcarifer,* Bloch 1790*),* which were fed on the copepod *Cyclopina kasignete* resulting in better growth, survival, and increased the essential fatty acid contents of EPA and DHA (Rasdi and Qin, 2018).

6.5.1 Larval Growth and survival

The use of enriched copepods improved final larval survival (82 ± 3.6 % at 15 dph) in comparison with previous studies on *P. fridmani* reporting 72 ± 8 % at 8 dph (Chen and Zeng, 2021a) and 64 ± 10 % at 19 dph (Chen and Zeng, 2021b), both co-feed with rotifer *B. rotundiformis* and the calanoid copepod *Parvocalanus crassirostris* in green waters, respectively. The larvae hatched with developed jaws and gut (0 dph), consuming the rest of

the yolk reserves and starting with the first feeding between 1 and 2 dph. The lower and shorter peak of mortality (0-3 dph) observed on larvae fed copepods compared to groups fed rotifer treatment (0-5 dph), might be related to the live prey differences, as the higher nutritional composition of *A. tonsa* and its wide range of sizes which may have affected hunting success (Drillet *et al*., 2006 a, b, 2007; Hamre *et al.*, 2018; Martino *et al.*, 2023). In addition, larvae fed rotifer treatment showed peaks mortality at 13-15 dph, which could be linked to the inadequate prey size for this developmental stage (Radhakrishnan *et al.*, 2019).

6.5.2 Whole larvae biochemical composition

Hence, by the end of the trial orchid dottybacks larvae fed with copepods treatment presented higher total lipid and LC-PUFAs composition, especially in EPA and DHA compared to larvae fed rotifer treatment. Indeed, although larvae were capable to ingest enriched rotifers, these preys apparently did not fulfil completely the larval nutritional requirements. Accordingly, rotifers enriched with binary microalgal diets can be deficient in certain essential nutrients for marine larvae, such as the LC-PUFAs DHA and EPA, compared to enriched calanoid copepods (Martino *et al*., 2023). An inadequate fatty acid content, particularly LC-PUFAs, may lead to reduced appetite, growth, swimming activity, survival as well as development and skeleton anomalies in marine fish larvae (Izquierdo, 1996; Chen *et al*., 2019; Roo *et al*., 2019; Hansen and Möller, 2021), especially during the transition from endogenous to exogenous feeding. A previous study using similar species, such as the sunrise dottyback, *Pseudochromis flavivertex* (Rüppell, 1835), suggest that the use of enriched rotifers and *Artemia* with elevated levels of n-3 HUFA are essential for rearing this species, while larvae fed on not enriched live preys did not past 7dph (Olivotto *et al*., 2006b).

6.5.3 Histological studies

Fish larvae are especially sensitive to these non-optimal feeding conditions or nutritional stress factors because most tissues and organs are under progressive differentiation and development (Gisbert *et al*., 2008; Glencross, 2009). Indeed, the liver is a good biomarker for nutritional effects of feeding regimes, as the hepatocytes respond quickly to nutritional changes in fish larvae (Gisbert *et al*., 2008; Glencross, 2009). In the present study, the high hepatic lipid accumulation of the larvae fed rotifers might be related to poor diet nutritional composition, since inadequate feeding regimes are reported to cause high fat deposition in hepatocytes leading to liver steatosis (Montero *et al*., 2001; Gisbert *et al*., 2008). Comparable results were obtained by Carvalho *et al.*, (2019) with meagre larvae (*Argirosomus regius*), which showed high level of liver steatosis and granulomatosis when fed on diet with low LC-PUFAs concentration. The monounsaturated fatty acids, particularly oleic acid (OA; 18:1n-9), were reported to be the primary cause of hepatic steatosis appearance in other fish species such as gilthead seabream (*Sparus aurata*) (Caballero *et al*., 2004). Furthermore, since the excess of this FA is mainly degraded in peroxisomes and marine fish has a lower capacity to replicate this organelle, these are slowly metabolized contributing to the lipid droplet formation (Carvalho *et al.*, 2021). As confirmation of this, the composition of the enriched live preys in OA was respectively 0,7% in copepods vs 1,7% in rotifers as described in Martino *et al.* (2023). Additionally, the histological analysis of the digestive system of *P. fridmani* larvae provided a reliable index of the nutritional condition generated by treatments along the feeding trial. The increase of total goblet cells number, intestine diameter and improved *villi* morphology on copepods treatment may be explained by the fact that mostly marine fish larvae do not possess a well-developed digestive system, therefore may need exogenous digestive enzymes from live prey to help their digestion (Yúfera and Darias, 2007), which copepods are known to contain in higher levels (Zeng *et al.*, 2018; Conceição *et al*., 2010). In addition, copepods also contain higher proportion of phospholipids than rotifers,

which are more effectively digested than triacylglycerol by fish larvae (Conceição *et al*., 2010). The successful development of the digestive system is essential for both growth and survival of the larvae, as malfunction would delay nutrient intake and cause inflammatory disorders, immune dysfunction, and imbalance in nutrients (Rombout *et al.,* 2011; Zhang *et al*., 2020).

6.5.4 Osteological studies

Generally, the temporal sequence of the larvae bony structure's formation of *P. fridmani* larvae obtained with the acid free staining method (Walker and Kimmel, 2007) allowed detecting and quantifying cartilage and bone in larvae with a minimum size, confirming the suitability of this method for skeletal development studies of similar fish larvae species. Indeed, in order to detect bones mineralization in early larvae, is preferable to use the Walker and Kimmel (2007) acid free staining procedures rather than the traditional double staining protocols of Dingerkus and Uhler (1977), since the acid contained might decalcify the matrix. By the end of the trial, larvae fed with copepods treatment were characterized by an increased skeletal mineralization degree (cranial region, vertebral column and caudal fin complex), compared to groups fed with rotifers, evidencing the first bones formation in this species (e.g. maxilla mineralization between 4 - 6 dph). The difference in bones mineralization observed between treatments may be associated to the delay in growth performance of larvae fed with rotifer treatment, which at 15 dph presented same total length and mineralization degree of 10 dph larvae fed with copepods treatment. Indeed, nutrients intake was described in many studies as important factors affecting skeleton issues insurgence, in particular associated with n-3 PUFAs dietary content (Cahu *et al*., 2003; Roo *et al*., 2019). The difference observed in mineralization timing and culture performance between treatments is attributable to the enhanced nutritional composition of copepods, which behaved presumably as natural live feed for orchid dotty back larvae promoting growth and development.

6.6 Conclusions

In conclusion, the feeding trial performed in this study demonstrated the importance of copepods diet during early larval development of *P. fridmani,* providing valuable information about the breeding of this species. The copepod *A. tonsa* enriched with live microalgae (*R. salina* and *I. galbana*), improved larval growth, survival, biochemical composition, as well as liver, intestine and skeletal development, compared to enriched *B. plicatilis*. The better nutritional quality of these copepods, overall richer in LC-PUFAs, seems to be one of the main factors. Nevertheless, rotifers demonstrated to enable growth of larvae till 15 dph, despite negatively affected the culture performance reducing fish survival and biochemical composition, generating anomalies in liver, intestine, as well as delay in growth and skeleton mineralization. Therefore, the supplementation of *A. tonsa* diet during first feeding of orchid dottyback larvae would be a useful tool to enhance *P. fridmani* breeding in controlled conditions, representing an improvement in the culture techniques of this scarcely known species, as also a source of sustainable and stronger specimens for the ornamental trade, even allowing restocking activities of overexploited populations.

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Supplementary Files

Figure 6.S1: Male specimen of the orchid dottyback *Pseudochromis fridmani* taking care of its egg mass.

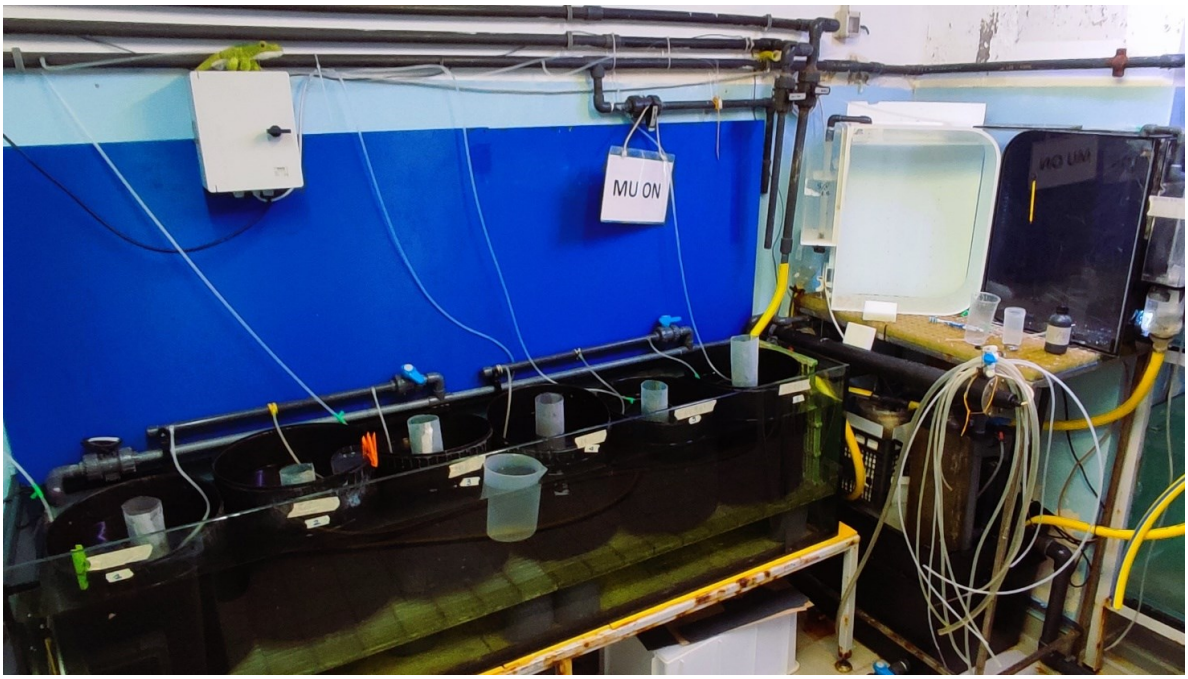

Figure 6.S2: The larval culture system "MoRS" (Modular Rearing System).

Chapter 7. General Discussion

7.1 What measures can be implemented to ensure the long-term viability of marine ornamental species in trade over the coming decades?

Ensuring the long-term viability of marine ornamental species in trade over the coming decades requires a comprehensive and multifaceted approach that integrates ecological, economic, and social considerations. Overexploitation of wild populations, habitat degradation, and climate change pose significant challenges to the sustainability of the marine ornamental trade (Wabnitz *et al*., 2003; Olivotto *et al*., 2017; Palmtag, 2017; Watson *et al*., 2023). To address these challenges and secure the future of this industry, it is imperative to implement coordinated measures that include international cooperation for adequate regulations, advancements in aquaculture, stakeholder education, socioeconomic actions promoting habitat conservation, and targeted research on the biology and ecology of species with high industrial value (Wabnitz *et al*., 2003; Palmtag, 2017; Watson *et al*., 2023).

Public Aquariums play a pivotal role in driving these efforts by acting as hubs for education, innovation, and the dissemination of sustainable practices. Through their unique ability to connect with diverse audiences, Aquariums can promote awareness of the ecological impacts of unsustainable ornamental trade practices while advocating for responsible consumer behaviours. By partnering with conservation organizations, research institutions, and industry stakeholders, public Aquariums can facilitate the sharing of knowledge and resources, including the exchange of captive-bred species, to create a more sustainable supply chain (Gusset and Dick, 2010, 2011; Mann *et al*., 2014; Correia *et al*., 2024). These initiatives contribute to reducing the environmental impact of the ornamental trade by offering alternatives to wild harvesting.

The implementation of aquaculture techniques for marine ornamental species represents a cornerstone of sustainable trade. Aquariums can contribute significantly by developing breeding

programs that close the life cycle of valuable species, thereby reducing reliance on wild populations and providing an alternative source of ornamental fish and invertebrates (Palmtag, 2017; Watson *et al*., 2023). Innovations in larviculture, live feed production, and water quality management have allowed an increasing number of species to be bred under controlled conditions, reducing pressure on natural habitats while preserving biodiversity (Pouil *et al*., 2019; Tlusty *et al*., 2013; Olivotto *et al*., 2017; Chen *et al*., 2019). Public Aquariums, as centres of expertise, can demonstrate these techniques to stakeholders, fostering wider adoption of aquaculture solutions within the industry.

Education and capacity-building are essential components of sustainability in the marine ornamental trade. Public Aquariums are uniquely positioned to lead education initiatives aimed at consumers, traders, and fishers, highlighting the importance of sustainable practices and the ecological consequences of overexploitation (Palmtag, 2017; Correia *et al*., 2024). Training programs for *in-situ* collectors and traders, focused on species handling, transport, and best practices, can improve the overall sustainability of the trade (Rubec *et al*., 2001; Wabnitz *et al*., 2003; Correia *et al*., 2024). Additionally, Aquariums can leverage their public outreach capabilities to advocate for the certification and labelling sustainably sourced products, fostering ethical consumer choices and industry accountability.

Socioeconomic factors also play a crucial role in shaping the practices of the ornamental fish trade. Many coastal communities depend on this industry for their livelihoods (Wabnitz *et al*., 2003). By collaborating with public Aquariums, these communities can gain access to training in aquaculture techniques, reducing dependence on wild harvests while creating new economic opportunities. Ensuring equitable access to resources and fostering community support for conservation initiatives are vital for achieving long-term resilience and sustainability (Watson *et al*., 2023).

Research and innovation remain indispensable for ensuring the future viability of marine ornamental species. Studies on species biology, ecology, reproduction and husbandry technologies are key to developing effective conservation and aquaculture strategies (Olivotto *et al*., 2017; Palmtag, 2017; Chen *et al*., 2019). Public Aquariums can act as incubators for such research, hosting collaborative projects that drive advancements in larval rearing, nutritional optimization, and disease management (Chen *et al*., 2019; Pouil *et al*., 2019). By integrating their findings into broader conservation efforts, Aquariums contribute to the scaling of aquaculture practices while ensuring ecological integrity.

In conclusion, the role of public Aquariums is central to advancing the sustainability of the marine ornamental trade. Through education, research, and collaboration, they can catalyse the adoption of responsible practices, reduce environmental impacts at the source, and support biodiversity conservation. As the ornamental fish trade continues to grow, a shift toward responsible practices will be essential to balance economic development with the protection of our oceans and the incredible biodiversity they sustain.

7.2 What advancements are being made in the development of sustainable aquaculture practices for marine ornamental species?

Significant advancements in sustainable aquaculture practices for marine ornamental species have been achieved, particularly in breeding technologies and larval culture. Historically, as previously mentioned, many marine ornamentals were challenging to breed under controlled conditions due to their unknown reproductive biology, complex larval culture and specific nutritional requirements difficult to fulfil with traditional feeds (Tlusty *et al*., 2002; Olivotto *et al*., 2011; Pouil *et al*., 2019).

Closing the life cycle under industrial conditions requires success in reproduction, achieved through improved broodstock management and species-specific husbandry (Chapters 3 and 4), and

larval culture, dependent on high-quality live feeds and advancements in larval care (Chapters 5 and 6). Recent innovations, such as **enriched live feeds and optimized larval culture systems**, have enabled successful breeding and rearing of high-value ornamental species (Olivotto *et al*., 2017; Chen *et al*., 2019; Watson *et al*., 2023).

Public Aquariums have played a pivotal role in fostering these innovations. As centres of biodiversity and hubs for knowledge exchange, they have also facilitated the **development and transfer of advanced egg collection systems** (Tlusty *et al*., 2013; Barongi *et al*., 2015; Ohs *et al*., 2019; Correia *et al*., 2024). These systems, particularly in large-volume tanks, streamline the **collection of naturally spawned eggs**, minimizing handling stress, maximizing hatch rates, and **ensuring a consistent supply of high-quality eggs for larviculture** (Ohs *et al*., 2019). By integrating these technologies, public Aquariums contribute not only to sustainable aquaculture but also to public awareness and education, highlighting their role in advancing marine ornamental aquaculture. Despite these advancements, challenges remain in scaling up always more improved aquaculture practices for marine ornamentals. While many species still require further research to overcome breeding and rearing bottlenecks, the role of public Aquariums as centres rich in biodiversity is always more fundamental (Tlusty *et al*., 2013; Correia *et al*., 2024).

Understanding the reproductive biology of target species is crucial for scaling up production. Factors such as gonad development, reproductive behaviour, spawning success, and environmental conditions like temperature must be carefully studied (Chapter 4).

Linked with this, and in connection with the spawning success in terms of larval survival, **the larval nutrition and feeding** constitutes an important bottleneck for the breed success**. Innovations in live feeds,** including enriched rotifers, and calanoid copepods, have significantly improved larval survival rates (Hamre *et al.,* 2013, 2018; Hansen and Möller, 2021) (Chapter 5). Calanoid copepods, with their superior nutritional profile, align closely with the dietary needs of marine ornamental larvae, enhancing growth and survival during initial feeding stages (Olivotto *et*

al., 2017; Samat *et al*., 2020). Further research is needed to diversify and enhance live feeds, ensuring they meet the specific nutritional requirements of different species (Ajiboye *et al*., 2011; Rasdi and Qin, 2014; Zeng *et al.,* 2018).

The success of fish production is also linked with the **optimization and standardization of larval husbandry** (Chapter 6) through the **advances obtained in nutrition** (Chapter 5). The utilization of the "green water" technique to start mesocosms and microcosms culture has also demonstrated substantial benefits for larviculture. These create a **more naturalistic rearing environment** simulating natural ecosystems on a small or intermediate scale, thereby reducing stress and increasing the overall health and survival rates of larvae (Setu *et al.*, 2010; Samat *et al*., 2020; Basford *et al*., 2021). Moreover, these techniques can be adapted for industrial scale larviculture, making them highly versatile tools for both research and commercial aquaculture operations (Neori, 2011; Eryalçın and Tınkır, 2024).

In conclusion, significant progress has been made in developing aquaculture practice able to source alternatives to the wild catch marine ornamental species. From advancements in breeding (Chapter 3 and 4) and larval rearing techniques (Chapters 5 and 6) to the adoption of innovative technologies (Chapters 3 and 5), all these efforts contributed to a more sustainable and resilient marine ornamental trade. However, important information on the biology of many traded species, culture techniques and their nutritional requirements are still scarce or completely unknown, requiring collaboration among scientists, public Aquariums, and consumers in order to fill these knowledge gaps and develop always more sustainable aquaculture practice.

7.3 What innovative techniques, strategies and tools can be implemented to enhance the efficiency and effectiveness of fish egg collection in public Aquariums?

Enhancing the efficiency and effectiveness of fish egg collection in public Aquariums is a crucial aspect of advancing marine ornamental aquaculture and conservation efforts. Nowadays, egg collection is particularly significant in public Aquariums, where maintaining genetic diversity, supporting endangered species, and ensuring the sustainability of captive populations are primary objectives (Barongi *et al*., 2015; Hutchins and Thompson, 2008; Gusset and Dick, 2010; Conde *et al*., 2011; Correia *et al*., 2024). Over the years, several strategies and tools have been developed to refine this process, while the construction of larger tanks designed to replicate complex ecosystems has expanded their potential to integrate cutting-edge technologies like advanced egg collection systems, further supporting biodiversity conservation and aquaculture practice (Ohs *et al*., 2019).

The **development of a newly patented Venturi-based egg collecto**r represents a major innovation in this field (Chapter 3). By leveraging the pressure differential of the Venturi effect, this system eliminates air bubbles during aspiration, minimizes turbulence, and significantly reduces mechanical stress on eggs. Testing within one of the largest exhibition tanks at the Genoa Aquarium demonstrated notable improvements in both the average quantity and quality of collected eggs during the experimental period, as well as a higher total yield (Chapter 3).

The benefits of this system extend beyond the immediate improvements in egg collection. By minimizing turbulence and mechanical stress, the Venturi-based collector enhances the abundance and quality of collected eggs, which are critical factors for successful hatching and larval development (Olivotto *et al*., 2017; Chen *et al*., 2019; Ohs *et al*., 2019). Furthermore, the Venturi-based design allows for the processing of larger volumes of water, making its integration particularly suitable for use in aquaculture and research facilities, as well as big Aquarium tanks,

ensuring that the benefits of this technology are accessible to a diverse audience. The ability to handle larger water volumes also allows for the continuous and simultaneous collection of eggs from multiple spawning sites within a tank, streamlining operations and reducing labour demands, providing a cost-effective upgrade to enhance breeding programs.

The success of industrial-scale egg collection in public Aquariums depends on understanding target species' biology and behaviour, including reproductive strategies, spawning cycles, and environmental conditions (Olivotto *et al*., 2011). Synchronizing egg collection systems with species-specific environmental cues, such as light, temperature, and water flow, can significantly enhance efficiency (Olivotto *et al*., 2006a; Ohs *et al*., 2019). Continuous innovation within aquariums drives the development and adoption of advanced technologies, advancing marine ornamental aquaculture.

Additionally, improved egg collection systems contribute to conservation by supporting breeding and stock enhancement programs for endangered species. Improved egg quality increases survival rates during hatching and larval rearing, bolstering breeding program success and safeguarding the future of threatened marine species (Tlusty *et al*., 2013; Ohs *et al*., 2019).

In conclusion, the development and implementation of advanced egg collection systems, such as the Venturi-based design (Chapter 3), represents a significant step forward in enhancing the efficiency and effectiveness of fish egg collection. These innovations not only address longstanding challenges associated with traditional methods, offering a more reliable, versatile, and sustainable solution, but also position public Aquariums at the forefront of marine conservation and ornamental aquaculture diversification. By integrating these systems with a deep understanding of species biology, eco-friendly practices, and emerging technologies, stakeholder as public Aquariums can strengthen their breeding programs, support conservation efforts, and contribute to the sustainability of marine ornamental trade.

7.4 What approaches can be applied to improve the management and productivity of *Pseudochromis fridmani* broodstock?

The management and productivity of *Pseudochromis fridmani* broodstock, commonly known as the orchid dottyback, present significant challenges due to the complex interplay of environmental, physiological, and behavioural factors that influence its reproductive success (Wittenrich, 2007; Mies *et al*., 2014; Chen and Zeng, 2021a b, Chen *et al*., 2023). This species, prioritized by the Genoa Aquarium for industrial production, was studied in the present thesis to enhance broodstock management through improved rearing conditions, particularly temperature control, to optimize spawning and minimize filial cannibalism (Chapter 4).

Temperature, a critical environmental factor, significantly affects reproductive outcomes (Pankhurst and Munday, 2011; Visser, 2008; Sunday *et al*., 2010). While higher temperatures (28 °C) increased spawning frequency, they also elevated filial cannibalism, reducing the number of viable eggs. Conversely, maintaining **broodstock at 25 °C minimized cannibalism while sustaining stable spawning, improving overall productivity** (Chapter 4). This temperature not only reduces the incidence of filial cannibalism but also maintains a stable spawning frequency over time, ensuring a consistent supply of viable eggs (Chapter 4). Moreover, the rearing temperature did not seem to significantly affect the timing of embryonic development (Chapter 4) or overall egg quality, which further supports the idea that maintaining a temperature of 25 °C could be beneficial for broodstock management.

Aggression during pair formation further influences reproductive success, as social dynamics, including courtship and dominance, can delay stable pairing affecting their overall breeding performance (Mies *et al*., 2014; Chen *et al*., 2023). Aggression is more pronounced between individuals of the same sex, often leading to temporary incompatibility (Brons, 1996; Moe, 1997; Wittenrich, 2007). Temporary **isolation of aggressive individuals while maintaining visual contact can foster hierarchy establishment and mutual acceptance**, resulting in more stable

pairs and improved spawning outcomes (Chapter 4). Addressing aggressive behaviour is critical in species like *P. fridmani*, where sexual dimorphism is absent, complicating sex determination (Wittenrich, 2007; Mies *et al*., 2014; Chen *et al*., 2023). Using macroscopic analysis of body size and shape, such as identifying larger, slender males and shorter females with swollen bellies, improves sex identification accuracy and reduces aggression linked to improper pairing (Chapter 4).This approach helps in the accurate process of pairing individuals and minimizes the occurrence of aggression due to improper sex identification. Additionally, the species' potential for bidirectional sex change, observed in related dottybacks, warrants further exploration (Wittenrich and Munday, 2005; Kuwamura *et al*., 2020). Understanding the environmental triggers for sex change, such as mate absence, could improve reproductive success in limited pairing scenarios. These findings have profound implications for optimizing broodstock productivity and management protocols, supporting the sustainable production of *P. fridmani*.

In conclusion, improving the management and productivity of *P. fridmani* broodstock requires a multifaceted approach that takes into account the critical role of environmental factors, particularly water temperature. Maintaining a temperature of 25 °C appears to be the most effective strategy for enhancing spawning activity while minimizing filial cannibalism. Additionally, managing aggression during pair formation, ensuring proper food and water quality, and accurately identifying sexes for pairing are essential components of an effective broodstock management plan. By implementing these approaches, it is possible to optimize the breeding and reproductive performance of *P. fridmani*, ultimately contributing to the sustainable industrial production of this high-demand species in the marine ornamental trade. Further research into the factors influencing filial cannibalism, as well as the potential for sex change in this species, would provide additional insights into the management of *P. fridmani* broodstock and the improvement of breeding protocols for this species.

7.5 Once the spawning success has been reached, what methods can be employed to enhance the nutritional quality of feed for larvae production success?

Enhancing the nutritional quality of feed provided to larvae is a key aspect of successful larviculture, particularly in the cultivation of marine ornamental species. The early developmental stages of these species are highly sensitive to dietary inputs, necessitating feeds that are not only nutritionally adequate but also adapted to their specific physiological needs (Hamre *et al.,* 2013; Olivotto *et al.,* 2011, 2017; Hansen and Möller, 2021). *Brachionus plicatilis* is widely used in aquaculture for its ease of cultivation, but alternative live feeds like **calanoid copepods offer superior nutrition**, improving larval survival and growth (Ajiboye *et al*., 2011; Zeng *et al*., 2018) (Chapter 5).

Enrichment of live feeds enhances their nutritional content by exposing them to nutrient-rich diets, increasing levels of essential components such as EPA, DHA, proteins, vitamins, and minerals (Birkou *et al*., 2012; Rasdi and Qin, 2014; Eryalçin, 2018, 2019; Eryalçın and Tınkır, 2024). However, rotifers may have limited capacity to retain these essential components, potentially compromising their nutritional value for some species (Faulk and Holt, 2005; Olivotto *et al*., 2017; Radhakrishnan *et al*., 2019).

In contrast, the calanoid copepod *Acartia tonsa* naturally possesses a more favourable biochemical composition, characterized by higher levels of proteins and lipids such as EPA and DHA. Indeed, in the present study **larvae_fed with enriched *A. tonsa* exhibited enhanced growth performance, survival rates, and morphological development** compared to those fed with enriched rotifers (Chapter 6). This superiority is attributed not only to the copepod's intrinsic nutritional profile but also to its efficient assimilation and retention of enriched nutrients, making it a more reliable vector for delivering essential dietary components to larvae (Saba *et al*., 2009; Chen *et al.*, 2012; Besiktepe and Dam, 2020) (Chapter 5).

Another aspect to consider is the selection of appropriate microalgae species used in the enrichment process of live feeds. For example, **microalgae such as *Rhodomonas salina*, *Isochrysis galbana* can positively influence *A. tonsa* nutritional quality** (Chapter 5). These microalgae differ in their biochemical compositions, particularly in fatty acid profiles, which in turn affect the nutritional quality of the copepods that consume them (Rasdi and Qin, 2014; Malzahn *et al.,* 2015). Therefore, copepods enriched with a combination of these microalgae species exhibit a more balanced and enhanced nutrient profile, thereby providing a superior feed option for marine larvae (Siuda and Dam, 2010; Drillet *et al.,* 2011; Chen *et al*., 2012; Rasdi and Qin, 2014; Besiktepe and Dam, 2020), including *P. fridmani* (Chapter 6).

In conclusion, high nutrient microalgae can be effectively used to enrich live feed provided to marine larvae, with *Acartia tonsa* emerging as a particularly advantageous option over traditional rotifers like *Brachionus plicatilis*. The superior biochemical composition of *A. tonsa*, combined with its natural prey-like characteristics, makes it a highly suitable feed that meets the complex nutritional requirements of newly hatched larvae. While the resultant improvements in larval growth, survival, and overall health underscore the value of integrating *A. tonsa* into larviculture practices. Future research aimed to refine enrichment methodologies, copepod culture techniques and diversification in species, will ensure further enhancements in the feeding and nutritional strategies employed in marine ornamental aquaculture.

7.6 Can the use of enriched calanoid copepods as live feed improve the efficiency of *Pseudochromis fridmani* larviculture?

As shown in Chapter 6, the use of enriched calanoid copepods, particularly *Acartia tonsa*, as live feed showed efficacy for improving the efficiency of *Pseudochromis fridmani* larviculture. Calanoid copepods provide critical biological and **nutritional benefits during early larval development**, significantly enhancing survival, growth, and health (Holt, 2003; Olivotto *et al*.,

2006a; Hamre *et al*., 2018). In *Pseudochromis fridmani* larviculture, where high mortality often results from suboptimal nutrition and inappropriate feed sizes, **enriched copepods improve survival, growth, and morphogenesis compared to conventional feeds like rotifers** (Cahu *et al*., 2003; Olivotto *et al*., 2008b; Chen *et al*., 2019, 2023; Chen and Zeng, 2021a, b) (Chapter 6).

The **bioavailability of critical nutrients in copepods and their superior digestibility** reduce energy expenditure during larvae digestion and facilitate nutrient assimilation (Zeng *et al.*, 2018; Conceição *et al*., 2010). This is particularly beneficial for *P. fridmani* larvae, which require high-quality feed to support their rapid development (Chen and Zeng, 2021a b).

The **incorporation of enriched *Acartia tonsa* into the larviculture of *Pseudochromis fridmani*** addresses key challenges in marine ornamental aquaculture, significantly enhancing larval development and survival. The nutritional profile of copepods provide a **balanced diet that supports the rapid development of vital systems**, including the digestive and skeletal structures essential for metamorphosis and post-larval viability (Chapter 6). Histological analyses revealed **improved liver function** in larvae fed enriched copepods, with better-organized hepatocytes, reduced lipid infiltration, and fewer instances of liver steatosis, reflecting enhanced metabolic efficiency. Additionally, **intestinal development**, characterized by increased goblet cell density, villi length, and luminal diameter, demonstrated superior nutrient absorption and digestive efficiency (Chapter 6).

Enriched *A. tonsa* also **enhance skeletal mineralization**, particularly in critical structures such as the maxilla, gill arches, and vertebral column, resulting in more robust larvae (Chapter 6). These combined benefits underscore the key role of copepods in improving larviculture outcomes for *P. fridmani*, providing a sustainable and effective solution to enhance marine fish production.

In conclusion, the use of enriched calanoid copepods, particularly *Acartia tonsa*, represents a highly effective strategy for improving the efficiency of *Pseudochromis fridmani* larviculture. By **addressing critical nutritional needs during early development**, enriched copepods enhance

larval survival, growth, and development while contributing to the sustainability and economic viability of marine ornamental fish aquaculture. Future research should focus on optimizing enrichment protocols for copepods and assessing their long-term impacts on larval health and performance. Such efforts will undoubtedly establish enriched copepods as the gold standard in live feed solutions, setting new benchmarks for success in the industry. Additionally, the experimentation of new breeding techniques, particularly regarding the culture and feeding of marine ornamental larvae, will be essential to develop methodologies that can be adapted to related species, ensuring broader applicability across aquaculture practices.

7.7 Can those improvements contribute to the cost-effective and sustainable development of marine ornamental aquaculture?

The advancements in marine ornamental aquaculture discussed herein provide significant contributions to both cost-effectiveness and sustainability in the sector. The innovative techniques applied in the present studies **address critical bottlenecks in breeding, larval rearing, and nutritional management, offering economic advantages while reducing environmental impacts.**

- **The introduction of the Venturi egg collector represents a breakthrough in egg harvesting technology**. By minimizing turbulence and mechanical stress during egg collection, this system increases the yield of the harvest and enhances eggs quality and, consequently, hatch rate and larval quality. The ability to operate in large-scale tank environments allows facilities to optimize resource use, reducing labour costs and operational inefficiencies. From an environmental perspective, this technology supports more sustainable broodstock management and sourcing of ornamental species, ensuring a consistent supply of high-quality eggs.

- **The improvement of broodstock management, further supports sustainable aquaculture practices**. For our practical case (*P. fridmani*), temperature control emerges as a critical factor, with findings indicating that maintaining broodstock at 25°C minimized filial cannibalism while ensuring consistent spawning activity. Effective management strategies, including social behaviour monitoring and pair bonding optimization, reduce aggression and improve reproductive success. These measures not only enhance productivity but also reduce resource waste associated with failed spawning attempts, contributing to long-term economic and environmental sustainability. In addition, these refinements in broodstock management create a cascading effect, improving every subsequent stage of the aquaculture process.
- Nutritional innovations, particularly **the use of enriched live feeds such as calanoid copepods (e.g., *Acartia tonsa*)**, **address critical bottlenecks in larval rearing** by offering superior nutritional profiles tailored to the dietary needs of species like *Pseudochromis fridmani*. These benefits reduce mortality rates and production costs, making copepods a highly promising live feed for aquaculture. Despite their advantages, the widespread adoption of copepod cultivation in large-scale hatcheries remains limited due to the logistical and economic challenges. Consequently, many hatcheries continue to rely on traditional live feeds, such as *Brachionus plicatilis*, due to their ease of cultivation and established production protocols. Public Aquariums, with their focus on developing and refining methods for breeding a wide variety of species, represent ideal environments for advancing the use of copepods. By leveraging their unique expertise and resources, public Aquariums can lead the way in transferring high-quality live feeds like *Acartia tonsa* into aquaculture practices, ultimately supporting the broader industry in transitioning toward more sustainable and efficient production methods.
- **The integration of these nutritional advancements into larviculture protocols has demonstrated significant benefits for *P. fridmani* production**. These outcomes result in

more robust juvenile fish, increasing their market value and reducing the costs associated with rearing fragile larvae. This approach not only enhances survival rates but also improves the overall efficiency of aquaculture operations for marine ornamental species.

- Furthermore, as experienced, **collaboration between public Aquariums, private aquaculture enterprises and academic institutions can lead to technological innovations that benefit the industrial sector**. Joint research projects, shared resources, and coordinated breeding programs enable Aquariums to optimize their production processes while contributing valuable insights to commercial aquaculture operations.

Collectively, **the innovations presented in the present studies form a cohesive framework for cost-effective and sustainable marine ornamental aquaculture**. By addressing key challenges in egg collection, broodstock management, larval nutrition and culture, **they reduce operational costs, enhance productivity, improve fish welfare and mitigate environmental impacts**. Furthermore, these technologies and practices can serve as models for other species, promoting scalability and broader adoption across the aquaculture industry.

In conclusion, the integration of innovative technologies showed by this thesis, optimized broodstock management protocols, and advanced nutritional strategies represent a transformative step towards sustainable and economically viable marine ornamental aquaculture. With public Aquariums acting as hubs for research, education, and species conservation, the marine ornamental aquaculture industry is well-positioned to continue its trajectory towards a more sustainable and resilient future.

Chapter 8. General Conclusions

1. The implementation of advanced egg collection systems, such as the Venturi collector, has significantly **improved the efficiency of pelagic egg harvesting in large tanks** by minimizing mechanical damage and ensuring higher egg viability compared to traditional air-lift systems. This advancement represents a substantial improvement in the management of spawning events and resource utilization.

2. Maintaining broodstock at a stable temperature of 25°C has been conclusively shown to **reduce filial cannibalism, enhance spawning activity, and improve overall reproductive efficiency** in *P. fridmani*. This temperature also functioned as a critical factor in broodstock management.

3. **Total length (TL) and body shape have proven to be a relatively reliable and rapid set of measurements for sexing** *P. fridmani*. Those non-invasive parameters offer an efficient method for broodstock identification, contributing to better pair formation and reproductive success.

4. The combined use of live microalgae, specifically *I. galbana* and *R. salina* (I-R), has demonstrated **exceptional effectiveness as a natural enrichment strategy for live prey**, significantly boosting their content of long-chain polyunsaturated fatty acids (LC-PUFAS), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

5. Enriching calanoid copepods (*Acartia tonsa*) and rotifers (*Brachionus plicatilis*) with combined microalgae (I-R) **enhances survival rates and nutritional value in both live prey** types.

6. Enriched copepods exhibited **higher protein and lipid content, along with superior retention of essential fatty acids** as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential for optimal larval health and development.

7. Larviculture of *P. fridmani* showed marked **improvements during the first 15 days post-hatch (dph)** when enriched copepods were used instead of traditional live feeds. This resulted in enhanced larval performance, better growth rates, and improved biochemical composition.

8. The use of enriched copepods in larviculture **significantly promoted proper digestive, hepatic, and skeletal development in *P. fridmani* larvae**. Larvae fed with enriched copepods displayed **well-organized hepatic and intestinal structures, reduced cellular damage, and improved overall physiological health**, underscoring the importance of proper nutrition.

9. This research provide a **robust foundation for long-term advancements in ornamental fish aquaculture, delivering validated methodologies for broodstock, live feed, and larval management** with industrial applications in conservation, scientific research, and sustainable commercial breeding.

Chapter 9. Resumen en español

9.1 El comercio de ornamentales marinos

El comercio de ornamentales marinos es una industria global dedicada a suministrar tanto especies silvestres capturadas como cultivadas en acuicultura a los aficionados de acuarios domésticos como a Acuarios públicos y centros de investigación. Este comercio opera a través de una red internacional de pescadores, profesionales de la acuicultura y empresas de logística (Wabnitz *et al*., 2003; Tissot *et al*., 2010; Palmtag, 2017; Watson *et al*., 2023). Principalmente activo en regiones tropicales y subtropicales, genera ganancias anuales superiores a los 300 millones de dólares (Tissot *et al*., 2010; Rhyne *et al*., 2017; Palmtag, 2017; Watson *et al*., 2023). El comercio abarca más de 1,800 especies de peces y 700 especies de invertebrados, incluyendo cnidarios, moluscos, artrópodos, equinodermos, anélidos y poríferos (Rhyne *et al*., 2012, 2017; Palmtag, 2017; Pouil *et al*., 2019). A diferencia del comercio ornamental de agua dulce, donde más del 90% de las especies son criadas en cautiverio, solo alrededor del 10% de las especies marinas ornamentales presentes en el mercado se cultivan en condiciones controladas (Raghavan *et al*., 2013; Rhyne *et al*., 2017; Chen *et al*., 2019; King, 2019; Pouil *et al*., 2019). Como consecuencia, la mayoría de los peces e invertebrados ornamentales marinos en el mercado son capturados en países en desarrollo, principalmente en zonas tropicales y subtropicales (Wabnitz *et al*., 2003; Tissot *et al*., 2010; Raghavan *et al*., 2013; King, 2019).

La recolección de ornamentales marinos se realiza mediante diversos métodos, incluyendo la extracción manual de rocas vivas e invertebrados utilizando equipos de buceo (Wabnitz *et al*., 2003; Tissot *et al*., 2010; Rhyne *et al*., 2014), o empleando técnicas a menudo no reguladas como redes, anzuelos, palangres y trampas, e incluso ilegales como armas submarinas, sedantes y explosivos (Wabnitz *et al*., 2003; Rhyne *et al*., 2014; Olivotto *et al*., 2017; Palmtag, 2017). Estas prácticas contribuyen a elevadas tasas de mortalidad a lo largo de la cadena de suministro, desde la

recolección hasta el transporte y la entrega final (Rubec *et al*., 2001; Schmitt and Kunzman, 2005). Además, durante el transporte, las tasas de mortalidad pueden alcanzar el 100%, principalmente debido a técnicas inadecuadas de almacenamiento y embalaje (Rubec *et al*., 2001; Wabnitz *et al*., 2003; Schmitt & Kunzman, 2005). Sin embargo, incluso con las mejoras en el transporte y la supervivencia final de los animales capturados, la sostenibilidad del comercio y el bienestar de los organismos marinos ornamentales siguen siendo una preocupación importante y son objeto de un debate continuo.

9.2 Estrategias de conservación y el papel de los Acuarios publicos

En las últimas décadas, diversos proveedores han realizado esfuerzos significativos para abordar los desafíos de sostenibilidad en el comercio de acuarios marinos mediante la adopción de prácticas responsables de recolección y la expansión de operaciones de acuicultura (Dalabajan, 2005; Tissot *et al*., 2010; Pouil *et al*., 2019; Watson *et al*., 2023). Una de las principales preocupaciones es la sostenibilidad de la extracción de especies silvestres, un problema que frecuentemente entra en conflicto con los esfuerzos de preservación de los ecosistemas (Sadovy and Vincent, 2002; Wabnitz *et al*., 2003; Rhyne *et al*., 2014, 2017). Este problema se agrava por la sobreexplotación potencial de millones de peces e invertebrados comercializados anualmente, intensificada por métodos de pesca destructivos e ilegales (Rubec *et al*., 2001; Sadovy & Vincent, 2002; Wabnitz *et al*., 2003).

En respuesta a estos desafíos, gobiernos, organizaciones ambientales y otros sectores clave han implementado medidas progresivas, como la educación a pescadores sobre las consecuencias negativas de las prácticas insostenibles y la capacitación en técnicas responsables para la captura y el cuidado de la vida marina (Rubec *et al*., 2001; Wabnitz *et al*., 2003; Palmtag, 2017). Diversos países han adoptado planes de gestión que incluyen estrategias como zonas designadas de recolección, áreas marinas protegidas, restricciones de captura, recolección rotativa, cuotas,

limitaciones de tamaño y sexo, regulaciones de equipos y límites de exportación (Rubec *et al*., 2001; Wabnitz *et al*., 2003; Dalabajan, 2005). Organizaciones internacionales como WAZA, EAZA y EUAC han respaldado estas iniciativas, proporcionando herramientas para fomentar prácticas responsables entre consumidores y aficionados (Gusset & Dick, 2010, 2011; Correia *et al*., 2024).

Los Acuarios públicos y privados tienen un rol central en el comercio de ornamentales marinos, dependiendo de organismos capturados en la naturaleza y de aquellos producidos en acuicultura para sus exhibiciones y la recreación de ecosistemas naturales. Estas estructuras contribuyen a los esfuerzos globales de conservación, según lo planteado en la "World Zoo Conservation Strategy" (Barongi *et al*., 2015). En los últimos años, los Acuarios han adoptado pautas éticas más estrictas para la adquisición de animales y han incrementado los programas *ex-situ* de cría de especies ornamentales marinas (Tlusty *et al*., 2013; Craggs *et al*., 2017; Correia *et al*., 2024; Martino *et al*., 2023, 2024). Estos programas han impulsado el desarrollo y la aplicación de prácticas innovadoras de cría a escala comercial, fortaleciendo una industria ornamental marina más sostenible (Tlusty *et al*., 2013; Olivotto *et al*., 2017). En este contexto, los Acuarios no solo actúan como centros de conservación y educación, sino también como nodos para actividades de investigación fundamentales y aplicadas en acuicultura ornamental marina, esencial para diversificar las especies disponibles y mejorar la eficiencia y sostenibilidad de la industria (Hutchins & Thompson, 2008; Gusset & Dick, 2010, 2011; Conde *et al*., 2011; Martino *et al*., 2023, 2024).

9.3 Acuicultura de especies marinas ornamentales

El éxito limitado de la industria de la acuicultura marina ornamental puede atribuirse a varios factores, principalmente a la dificultad para obtener huevos y larvas de alta calidad, así como al desconocimiento de sus requisitos nutricionales y necesidades específicas de manejo, todos ellos esenciales para la producción exitosa de especies marinas (Tlusty *et al*., 2002; Olivotto *et al*.,

2011; Pouil *et al*., 2019). En este contexto, el interés por la investigación en cultivos de ornamentales ha crecido, permitiendo desarrollar métodos de producción sostenibles, contribuyendo a una mejor comprensión de la biología y a mejorar la cría de las especies más comercializadas (Wabnitz *et al*., 2003; Olivotto *et al*., 2017). Por lo tanto, la acuicultura de peces e invertebrados tiene el potencial de reducir los impactos ambientales al disminuir la recolección de ejemplares silvestres de sus hábitats naturales (Chen *et al*., 2019; Watson *et al*., 2023). La experimentación con nuevas técnicas de cría, especialmente en lo que respecta al cultivo y alimentación de larvas, frecuentemente genera metodologías adaptables a especies relacionadas (Olivotto *et al*., 2017; Chen *et al*., 2019; Watson *et al*., 2023). La investigación centrada en la biología de especies de interés comercial, el desarrollo de técnicas innovadoras de cultivo, la identificación de alimentos vivos alternativos y el bienestar de los organismos cultivados puede dar lugar a ejemplares mejor aclimatados al cautiverio en comparación con aquellos recolectados en estado salvaje (Wittenrich, 2007; Olivotto *et al*., 2011, 2017). Además, la acuicultura desempeña un papel clave en la preservación de la diversidad genética marina, contribuyendo a la repoblación de especies en peligro de extinción (Tlusty *et al*., 2013; Chen *et al*., 2019; Pouil *et al*., 2019; Watson *et al*., 2023).

9.4 Manejo de reproductores y desoves

La selección de reproductores es un aspecto crucial en acuicultura, ya que influye significativamente en los resultados de producción (Chen *et al*., 2019). Dependiendo de la especie, los procedimientos zootécnicos deben adaptarse a sus características biológicas, fisiológicas y ecológicas (Olivotto *et al*., 2011). Una vez establecidas prácticas óptimas para mantener a los organismos en condiciones controladas, es posible perfeccionar las técnicas de producción y seleccionar reproductores superiores, iniciando programas de cría selectiva para producir organismos mejor adaptados a la acuicultura (Chen *et al*., 2019). También puede emplearse

manipulación genética para mejorar rasgos deseables en el comercio de ornamentales marinos (Olivotto *et al*., 2017). Los peces presentan estrategias reproductivas como gonocorismo, hermafroditismo y partenogénesis. El gonocorismo, donde los individuos son exclusivamente machos o hembras, es la estrategia más común (Munday *et al*., 2006). El hermafroditismo puede ser secuencial como en los dottybacks o peces doncellas (*Pseudochromis spp.)* (proterándrico, de macho a hembra) y peces payasos (*Amphiprion spp.)* (proterogínico, de hembra a macho), o simultáneo, como en los hamlets o méritos de arrecife (*Hypoplectrus spp.*) (individuos con ambos sexos) (De Mitcheson & Liu, 2008; Kuwamura *et al*., 2020). La partenogénesis es extremadamente rara entre las especies marinas (tiburón zebra, *Stegostoma tigrinum*), observada más comúnmente en especies de agua dulce (*Poecilia* spp.) (reproducción sin machos) (Kuwamura *et al*., 2020). Los peces pueden ser desovadores demersales, que producen huevos adheridos al sustrato aportando cuidado parental, o pelágicos, que liberan huevos en la columna de agua a la merced de las corrientes. Esta última estrategia reproductiva es la más prevalente en el medio ambiente marino (Wittenrich, 2007).

En especies con huevos adhesivos, los desoves pueden transferirse fácilmente a tanques de incubación, mientras que para huevos pelágicos se utilizan sistemas de recolección (Ohs *et al*., 2019). La recolección de huevos pelágicos en instalaciones de acuicultura y Acuarios públicos representa un desafío significativo, ya que los embriones son susceptibles a daños mecánicos, especialmente cuando se emplean métodos tradicionales de recolección. Este problema es particularmente evidente en los Acuarios públicos, donde grandes cantidades de huevos viables se pierden frecuentemente a través de los sistemas de filtración, lo que representa una oportunidad desaprovechada para los programas de reproducción o las iniciativas educativas. Desarrollar sistemas innovadores para la recolección de huevos de peces es esencial para mejorar el éxito reproductivo y fomentar prácticas sostenibles, maximizando la gestión de especies de desove pelágico en los Acuarios públicos.

9.5 Importancia de los alimentos vivos

El plancton es la principal fuente de alimento para muchos peces y sus larvas, así como para otros organismos marinos. Por ello, las presas vivas siguen siendo esenciales en el proceso de cría larval en la mayoría de los criaderos (Hamre *et al*., 2013, 2018; Hansen y Möller, 2021). Las larvas de peces en etapas tempranas no responden adecuadamente a dietas formuladas o alimentos congelados, necesitando presas vivas que estimulen su comportamiento natural de depredación (Olivotto *et al*., 2017; Samat *et al*., 2020). Las microalgas desempeñan un papel fundamental en la acuicultura marina, al ser la base de la red trófica, especialmente en el cultivo de organismos como rotíferos, *Artemia sp*. y copépodos, que posteriormente se alimentan a las larvas de peces. Estas microalgas aportan nutrientes esenciales, incluidos ácidos grasos, aminoácidos y vitaminas, que contribuyen al desarrollo de las presas vivas. Especies comunes como *Rhodomonas sp.*, *Isochrysis sp.* y *Tetraselmis sp.* son ampliamente utilizadas en acuicultura debido a su alto contenido en ácidos grasos poliinsaturados (PUFAs) y otros nutrientes esenciales (Drillet *et al*., 2006a; Ismar *et al*., 2008; Guevara *et al*., 2010; Zhang *et al*., 2013; Sirakov *et al*., 2015). Los rotíferos (*Brachionus plicatilis* y *Brachionus rotundiformis*) y los nauplios de *Artemia* sp. son los organismos más utilizados en la cría larval debido a su facilidad de cultivo en altas densidades. Sin embargo, estos no constituyen las presas naturales de muchas larvas marinas, pueden ser demasiado grandes para algunas especies y a menudo provocan problemas digestivos y deficiencias en la absorción de nutrientes (Otero-Ferrer *et al*., 2010; Eryalçin, 2018, 2019; Olivotto *et al*., 2017; Radhakrishnan *et al*., 2019). En contraste, los copépodos, presas naturales de muchas especies marinas durante sus etapas larvales, han demostrado un alto potencial en acuicultura (Ajiboye *et al*., 2011; Hamre *et al*., 2013, 2018; Radhakrishnan *et al*., 2019; Samat *et al*., 2020). En particular, los copépodos calanoides, como *Acartia spp.*, destacan por su alto contenido de ácidos grasos poliinsaturados de cadena larga (LC-PUFAs), su naturaleza pelágica que facilita su acceso a las larvas y su pequeño tamaño en estadios naupliares (Payne y

Rippingale, 2001; Olivotto *et al*., 2006b; Ajiboye *et al*., 2011; Rasdi y Qin, 2014; Radhakrishnan *et al*., 2019). A pesar de sus beneficios, la producción continua de copépodos calanoides sigue siendo un desafío debido a los grandes volúmenes de cultivo requeridos y su alimentación selectiva, que exige mezclas de microalgas vivas (Holt, 2003). En el caso específico de *Acartia tonsa*, su producción masiva mediante huevos subitáneos almacenados en frío ha ganado interés como alternativa prometedora en los criaderos de acuicultura (Drillet *et al*., 2006a, b, 2007, 2011; Ajiboye *et al*., 2011; Camus, 2012; Zeng *et al*., 2018). Sin embargo, estos embriones almacenados en frío pueden sufrir alteraciones bioquímicas, especialmente en su contenido de ácidos grasos esenciales como el EPA y el DHA, afectando negativamente la supervivencia y el desarrollo de las larvas de peces si no se enriquecen adecuadamente (Drillet *et al*., 2006b). A pesar de su importancia, existe información limitada sobre protocolos efectivos para el enriquecimiento nutricional de copépodos (Payne *et al*., 2001; Sun *et al*., 2013).

9.6 Larvicultura de ornamentales marinos

Un desafío persistente en la larvicultura de especies ornamentales marinas es la alimentación durante las etapas tempranas, especialmente en la transición de la alimentación endógena a la exógena (Holt, 2003; Olivotto *et al*., 2017; Callan *et al*., 2018; Hansen y Möller, 2021). Para lograr una cría exitosa, los sistemas de cultivo larval deben replicar de cerca el entorno oceánico abierto, donde el alimento es abundante y la calidad del agua es alta (Olivotto *et al*., 2017; Samat *et al*., 2020). La técnica conocida como "agua verde", que incorpora microalgas en el entorno de cría, ofrece varios beneficios: (i) mejora la calidad del agua, equilibrando el oxígeno disuelto y reduciendo compuestos nitrogenados y bacterias patógenas; (ii) mejora la capacidad de depredación de las larvas, al reducir la intensidad lumínica y estimular la actividad enzimática digestiva; (iii) aumenta la supervivencia de las presas vivas, incrementando su valor nutricional antes de ser consumidas por las larvas (Setu *et al*., 2010; Neori, 2011; Basford *et al*., 2021;

Eryalçin y Tınkır, 2024). El tamaño y la forma del tanque influyen significativamente en el éxito de la cría larval (Cañedo-Orihuela *et al*., 2023). Los microcosmos, tanques pequeños, permiten una alta concentración de larvas y presas, pero requieren una gestión cuidadosa de los parámetros del agua, especialmente de los compuestos nitrogenados como el amoníaco, que pueden aumentar rápidamente y causar altas tasas de mortalidad (Cañedo-Orihuela *et al*., 2023). En contraste, los mesocosmos, tanques más grandes, ofrecen mayor estabilidad en la calidad del agua debido a su mayor volumen, lo que facilita el control de variables ambientales (Shields, 2001). Aunque su producción de biomasa por unidad de volumen es menor, los mesocosmos proporcionan un entorno más estable y realista.

El pez *Pseudochromis fridmani*, perteneciente a la familia Pseudochromidae, es una especie de gran valor comercial en el comercio ornamental marino (Wittenrich, 2007). Sin embargo, la gestión de reproductores y las primeras etapas de desarrollo representan desafíos críticos (Mies *et al*., 2014; Chen y Zeng, 2021a, b; Chen *et al*., 2023). El comportamiento agresivo intraespecífico dificulta la formación de parejas reproductoras y, durante el desove, el macho puede canibalizar los huevos antes de la eclosión (Wittenrich, 2007; Mies *et al*., 2014; Chen *et al*., 2023). A pesar de los avances en la larvicultura mediante el uso de copépodos calanoides y técnicas de "agua verde", las tasas de mortalidad siguen siendo altas debido a deficiencias nutricionales y cambios fisiológicos (Chen y Zeng, 2021a, b). Esta investigación aborda estos desafíos, contribuyendo al desarrollo de técnicas de producción sostenible para esta especie.

9.7 Objetivos de la tesis

La presente tesis doctoral tiene como objetivo fortalecer la industria de ornamentales marinos mediante mejoras en Acuarios públicos, desarrollando técnicas sostenibles de cultivo de especies marinas ornamentales, incluida la gestión de la recolección de huevos y el desarrollo de presas vivas de alta calidad para mejorar la calidad larval. Además, también busca optimizar la

producción de especies de peces en un acuario público, utilizando al pez ornamental marino *Pseudochromis fridmani* como caso de estudio industrial.

Para alcanzar el objetivo general, se abordaron los siguientes objetivos específicos:

- Desarrollar un sistema avanzado de recolección de huevos de peces que responda a la necesidad de aprovechar eficazmente el potencial de los tanques de gran volumen en acuarios públicos, los cuales albergan una amplia gama de especies que desovan regularmente, optimizando la gestión de la cantidad y diversidad de huevos disponibles para apoyar proyectos de conservación e investigación.
- Mejorar el manejo de reproductores del pez *Pseudochromis fridmani* (Klausewitz, 1968), a través del acondicionamiento de los peces, la formación de parejas y nuevos conocimientos sobre la biología reproductiva de la especie.
- Desarrollar un protocolo de enriquecimiento de presas vivas basado en microalgas, evaluando los efectos sobre la supervivencia y la composición bioquímica del copépodo calanoide *Acartia tonsa* (Dana, 1849) en comparación con el rotífero *Brachionus plicatilis* (Müller, 1786).
- Mejorar la producción larval del pez ornamental *Pseudochromis fridmani* mediante el uso de presas vivas de alta calidad.

9.8. Resumen de los capítulos

9.8.1 Capítulo 3: Desarrollo de un nuevo sistema de recolección de huevos de peces

Una de las principales limitaciones en el desarrollo de protocolos de acuicultura para especies marinas radica en la gestión de los reproductores y la recolección de embriones fertilizados (comúnmente conocidos como huevos) para su examen y posterior desarrollo. La recolección eficiente de huevos es crucial para el éxito de las operaciones acuícolas, especialmente en especies con estrategias reproductivas pelágicas, donde los huevos flotan y son transportados por las corrientes de agua. En un entorno de tanque, estos huevos deben ser retirados

rápidamente para evitar que ingresen en los sistemas de filtración, donde podrían dañarse. El dispositivo innovador, denominado "Recolector de Huevos Venturi", facilita la recolección eficiente de huevos con mínima supervisión por parte de un solo técnico. Este sistema puede adaptarse en tamaño y forma según las necesidades específicas, como las dimensiones del tanque, el número y tamaño de los reproductores y el diámetro de los huevos. Además, puede funcionar mediante una bomba dedicada o conectarse directamente a la bomba principal del tanque. El desarrollo de esta patente surge de la necesidad de mejorar el sistema existente conocido como **"**Recolector Air-lift**"** (Patente US No. 2006/0130771 A1). Aunque funcional, este dispositivo presenta limitaciones, como un flujo de succión insuficiente en tanques de gran volumen y la turbulencia generada por las burbujas de aire, que puede dañar o atrapar los huevos recolectados. Para superar estas deficiencias, el nuevo modelo incorpora un tubo Venturi, impulsado por una bomba de agua. Este tubo presenta un estrechamiento significativo en su interior, aumentando la velocidad del flujo de agua y creando un vacío en una tubería lateral conectada al colector de succión. El Recolector Venturi mejora significativamente la tasa de succión, la abundancia y la viabilidad de los huevos recolectados, eliminando las burbujas de aire y el efecto de espuma característico del sistema anterior. Durante las pruebas comparativas, este dispositivo mostró una capacidad de succión cuatro veces mayor que el sistema air-lift, procesando 1,800 L/h frente a los 450 L/h del modelo anterior. Además, no se observaron huevos dañados ni atrapados en los bordes del recipiente de concentración. En términos operativos, el Recolector Venturi demostró ser más eficiente, fácil de usar y con menores requisitos de supervisión, permitiendo una recolección óptima con menor riesgo de daño embrionario. Estas mejoras representan un avance significativo para la industria acuícola y los acuarios públicos, ofreciendo un dispositivo adaptable con aplicaciones potenciales en programas de conservación e investigación de

especies marinas. Esta innovación puede documentarse en una nueva patente o modelo de utilidad, con un amplio rango de aplicaciones en la cría y reproducción de especies acuáticas.

9.8.2 Capítulo 4: Actividad de desove y canibalismo filial en reproductores de dottybacks orquídea *Pseudochromis fridmani (Klausewitz, 1968)* a diferentes temperaturas

El pez dottyback orquídea (*Pseudochromis fridmani*) es una de las especies más atractivas en el comercio ornamental marino debido a su color vibrante, resistencia y pequeño tamaño. Sin embargo, su cultivo aún enfrenta desafíos relacionados con el manejo de los reproductores y la actividad de desove, lo que limita su producción a gran escala. Este estudio aborda estas limitaciones, analizando la formación de parejas, los comportamientos reproductivos y la histología gonadal bajo condiciones controladas a diferentes temperaturas (25 °C y 28 °C).

Se observaron diferencias significativas en la actividad reproductiva y el canibalismo filial entre los grupos sometidos a diferentes temperaturas. A 28 °C, aunque la tasa de desove aumentó, también lo hizo el canibalismo filial, lo que resultó en una mayor pérdida de huevos. A pesar de esta diferencia, el valor total de desove disponible no varió significativamente entre las dos temperaturas. Por otro lado, se confirmó que la temperatura de 25 °C mejora la gestión de los reproductores, reduciendo la incidencia de canibalismo filial y optimizando la producción.

El análisis histológico de las gónadas demostró que la evaluación del largo total y la forma del cuerpo es una herramienta fiable para diferenciar visualmente el sexo de los individuos. Los ovarios presentaron estructuras lobulares con ovocitos en diferentes etapas de desarrollo, mientras que los testículos mostraron túbulos seminíferos activos con células espermáticas en diversas fases de espermatogénesis. No se observó evidencia de reversión sexual en los

especímenes analizados, aunque se sugiere que esta capacidad podría manifestarse bajo condiciones ambientales específicas.

El estudio del desarrollo embrionario a 25 °C reveló que los huevos de *P. fridmani* son esféricos, transparentes y están agrupados por filamentos adhesivos. El desarrollo embrionario siguió una secuencia predecible, con etapas bien definidas desde la segmentación inicial hasta la eclosión, mostrando una notable resiliencia a pequeñas variaciones de temperatura.

En conclusión, mantener la temperatura de cría a 25 °C resulta crucial para reducir el canibalismo filial y mejorar la eficiencia reproductiva en *P. fridmani*. Además, la observación detallada del comportamiento reproductivo, la formación de parejas y el análisis histológico proporcionaron información valiosa para optimizar los protocolos de cría de esta especie. Este estudio representa un avance significativo en el manejo de reproductores y contribuye al desarrollo sostenible de la acuicultura ornamental marina, reduciendo la presión sobre las poblaciones silvestres y fomentando prácticas más responsables en la industria acuícola.

9.8.3 Capítulo 5: Dietas a base de microalgas vivas como enriquecimiento para mejorar el perfil nutricional de los nauplios del copépodo calanoide *Acartia tonsa* (Dana, 1849).

Los copépodos, especialmente *Acartia tonsa*, han surgido como una alternativa prometedora debido a su perfil nutricional superior, rico en ácidos grasos poliinsaturados de cadena larga (LC-PUFAs), incluidos EPA y DHA.

El objetivo principal fue evaluar el impacto de tres dietas basadas en microalgas (*Rhodomonas salina*, *Isochrysis galbana* y *Tetraselmis suecica*), administradas como dietas monoalgales o binarias (I-R y T-R), sobre la composición bioquímica, supervivencia y perfil de ácidos grasos de *A. tonsa* en comparación con *B. plicatilis*. Los resultados mostraron que la dieta binaria I-R mejoró significativamente el contenido de proteínas y LC-PUFAs en ambas

presas, siendo *A. tonsa* siempre superior a *B. plicatilis* en términos de retención de EPA y DHA.

Se observó que las diferencias en la composición bioquímica entre *A. tonsa* y *B. plicatilis* son específicas de cada especie, con *A. tonsa* demostrando una mayor capacidad para retener n-3 LC-PUFAs, esenciales para el desarrollo larval de peces marinos. Además, el uso de dietas mixtas permitió optimizar el cultivo de copépodos, evitando deficiencias nutricionales comunes en dietas monoalgales.

El estudio también destacó la importancia de la calidad y cantidad del alimento, ya que influyen directamente en la supervivencia, crecimiento y reproducción de los copépodos. Se identificó que una dieta enriquecida con *I. galbana* y *R. salina* mejora significativamente el rendimiento nutricional de *A. tonsa*, ofreciendo un alimento vivo de alta calidad para las larvas de peces ornamentales.

En conclusión, este estudio proporciona evidencias sólidas sobre la eficacia de las dietas binarias basadas en microalgas para mejorar la composición bioquímica y la supervivencia de *A. tonsa*. Estos hallazgos contribuyen al desarrollo de técnicas avanzadas de enriquecimiento y cultivo de copépodos, promoviendo su uso como alimento vivo óptimo en la acuicultura marina ornamental y mejorando la supervivencia y el crecimiento de especies larvales sensibles.

9.8.4 Capítulo 6: Los copépodos calanoides enriquecidos *Acartia tonsa* (Dana, 1849) mejoran el crecimiento, la supervivencia, la composición bioquímica y el desarrollo morfológico durante la primera alimentación larval del pez dottyback orquídea *Pseudochromis fridmani* (Klausewitz, 1968)

El presente estudio aborda una de las principales limitaciones en la acuicultura de peces ornamentales marinos: la alimentación de las larvas durante sus primeras etapas de desarrollo.

Se evaluó el impacto de las dietas enriquecidas con microalgas en dos presas vivas, el copépodo *Acartia tonsa* y el rotífero *Brachionus plicatilis*, sobre las larvas del pez ornamental *Pseudochromis fridmani* desde el primer día post-eclosión (0 dph) hasta el día 15 (15 dph). Los resultados demostraron que el uso de *A. tonsa* enriquecido mejora significativamente el crecimiento, la supervivencia, la composición bioquímica y el desarrollo morfológico del hígado, intestino y sistema esquelético de las larvas, en comparación con las dietas basadas en rotíferos.

Las larvas alimentadas con copépodos mostraron mayores tasas de crecimiento y supervivencia (82% frente al 54% en el grupo alimentado con rotíferos), así como una menor incidencia de mortalidad temprana. Además, las larvas alimentadas con *A. tonsa* presentaron un perfil bioquímico superior, con mayores niveles de ácidos grasos poliinsaturados de cadena larga (LC-PUFAs), particularmente EPA y DHA, esenciales para el desarrollo larval.

El análisis histológico reveló que las larvas alimentadas con copépodos tenían un hígado mejor organizado, con menor incidencia de esteatosis hepática y picnosis nuclear. Asimismo, el desarrollo intestinal fue más avanzado, con un mayor número y tamaño de células caliciformes y una mayor longitud y anchura de las vellosidades intestinales.

En cuanto al desarrollo esquelético, las larvas alimentadas con copépodos mostraron una osificación temprana y más completa en comparación con aquellas alimentadas con rotíferos, especialmente en la región craneal, columna vertebral y la aleta caudal. Estas diferencias se atribuyen al perfil nutricional superior de *A. tonsa*, que parece satisfacer mejor las demandas específicas de las larvas durante sus etapas iniciales.

En conclusión, el uso de copépodos *Acartia tonsa* enriquecidos con microalgas representa una estrategia eficaz para optimizar la alimentación larval de *Pseudochromis fridmani*. Esta estrategia no solo mejora la supervivencia y el crecimiento larval, sino que también reduce los efectos negativos asociados con las dietas tradicionales basadas en rotíferos. Estos hallazgos

contribuyen significativamente al desarrollo de nuevos protocolos de acuicultura sostenible, ofreciendo una alternativa viable para la producción masiva de esta especie ornamental bajo condiciones controladas y disminuyendo la dependencia de individuos capturados en el medio silvestre.

9.9 Conclusiones generales

1. La implementación de sistemas avanzados para la recolección de huevos, como el colector Venturi, ha mejorado significativamente **la eficiencia en la recolección de huevos pelágicos** en tanques grandes al minimizar los daños mecánicos y garantizar una mayor viabilidad de los huevos en comparación con los sistemas tradicionales de elevación por aire. Este avance representa una mejora sustancial en la gestión de eventos de desove y en el aprovechamiento de recursos.

2. Mantener a los reproductores a una temperatura estable de 25°C ha demostrado de manera concluyente **reducir el canibalismo filial, mejorar la actividad de desove y aumentar la eficiencia reproductiva** general en *P. fridmani*. Esta temperatura constituye un factor crítico en la gestión de los reproductores.

3. **La longitud total y la forma corporal han demostrado ser un conjunto relativamente confiable y rápido de medidas para la identificación de sexo** en *P. fridmani*. Estos parámetros no invasivos ofrecen un método eficiente para la identificación de los reproductores, contribuyendo a una mejor formación de parejas y al éxito reproductivo.

4. El uso combinado de microalgas vivas, específicamente *I. galbana* y *R. salina* (I-R), ha demostrado una **efectividad excepcional como estrategia natural de enriquecimiento para**

presas vivas, mejorando significativamente su contenido de ácidos grasos poliinsaturados de ladena larga (LC-PUFAs), incluidos el ácido eicosapentaenoico (EPA) y el ácido docosahexaenoico (DHA).

5. El enriquecimiento de copépodos calanoides (*Acartia tonsa*) y rotíferos (*Brachionus plicatilis*) con microalgas (I-R) **mejoró las tasas de supervivencia y el valor nutricional** tanto de las presas vivas como de los copépodos enriquecidos.

6. Los copépodos enriquecidos **presentaron un mayor contenido de proteínas y lípidos, junto con una mejor retención de ácidos grasos esenciales** como el eicosapentaenoico (EPA) y el docosahexaenoico (DHA), los cuales son fundamentales para la salud y el desarrollo óptimos de las larvas.

7. La larvicultura de *P. fridmani* mostró **mejoras notables durante los primeros 15 días posteriores a la eclosión (dph) cuando se utilizaron copépodos enriquecidos** en lugar de presas vivas tradicionales. Esto resultó en un mejor rendimiento larvario, mayores tasas de crecimiento y una composición bioquímica más favorable.
8. El uso de copépodos enriquecidos en la larvicultura **promovió significativamente un adecuado desarrollo digestivo, hepático y esquelético** en las larvas de *P. fridmani*. Las larvas alimentadas con copépodos enriquecidos presentaron **estructuras hepáticas e intestinales bien organizadas, menor daño celular y una mejor salud fisiológica** en general, subrayando la importancia de una nutrición adecuada.

9. Esta investigación **proporciona una base sólida para los avances a largo plazo en la acuicultura de peces ornamentales, ofreciendo metodologías validadas para la gestión de**

reproductores, presas vivas y larvas, con aplicaciones industriales en conservación, investigación científica y cría comercial en un modo más sostenible.

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