Vol 7 Issue 3 2019

HATCHERYFEED

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ADVANCES IN MICROBIAL MANAGEMENT

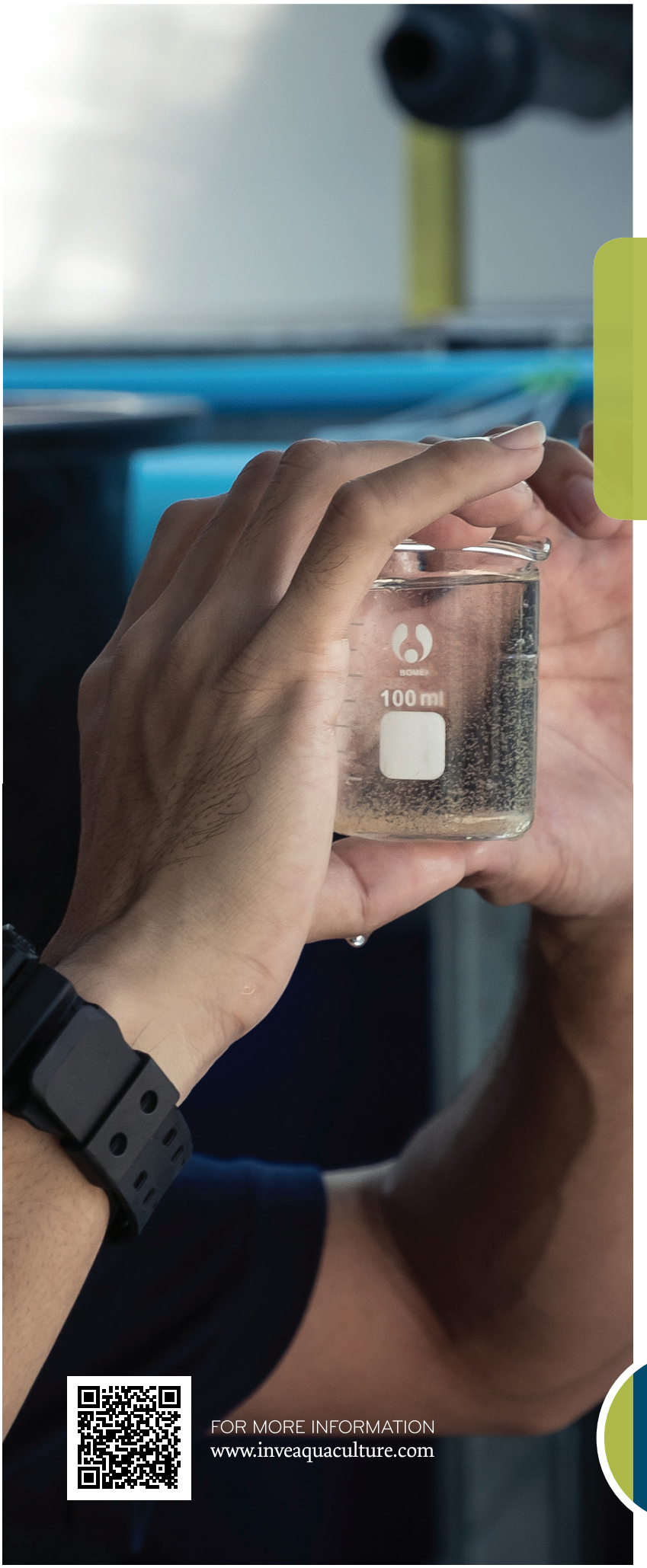
Probiotic Use in Hatcheries

New Line of Live Feed Disinfectants

Advances in Shrimp Microdiets

Genetic Editing and Management

Published by: Aquafeed.com LLC. Kailua, Hawaii 96734, USA www.aquafeed.com info@aquafeed.com



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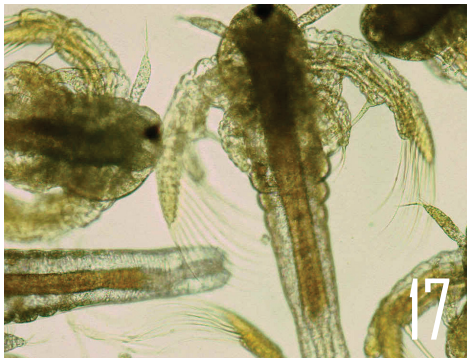
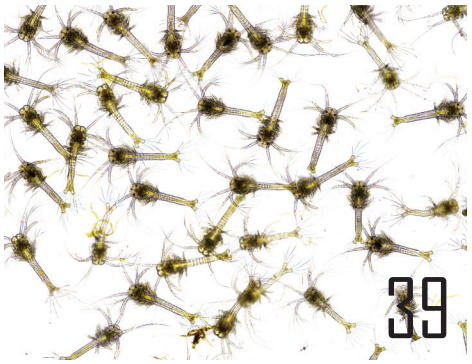
Contents





HATCHERYFEED VOL 7 ISSUE 2 2019

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Hatcheryfeed magazine is published by Hatcheryfeed, a division of Aquafeed.com LLC.

Aquafeed.com, LLC.,
Kailua, Hawaii 96734, USA.

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INVE AQUACULTURE develops new technological innovations for *Artemia*

INVE Aquaculture has developed two new additions to its set of Artemia technologies, as a culmination of long-standing efforts in revolutionizing the sourcing, characterization, enrichment and ease-of-use of *Artemia* cysts.

The first innovation is SMArt (Sensitivity Modified *Artemia*), a new technology that allows cysts to hatch in the dark. One of the main concerns in achieving a consistent *Artemia* output is the cysts' sensitivity to external factors such as light, temperature and storage. SMArt is a new technology that allows the cysts to hatch in the dark, avoiding decreased hatching performance due to lower light exposure.

The second addition is D-FENSE, a new built-in protection for the best hatchery biosecurity. It is an additional process to the *Artemia* cysts, forming a specific coating that suppresses the growth of bacteria such as Vibrio sp. during the hatching. This important bacterial reduction results in better larval survival and reduced contamination risks.

These new innovations will be added to the SEP-Art concept, a technology for easy separation, making no less than three state-of-the art enhancement technologies that will be available for INVE Aquaculture's prime *Artemia* brands.

By simplifying the process of harvesting high quality *Artemia* nauplii, all of these innovations again represent major milestones in improving hatchery performance, rationalizing manpower and resources and increasing the sustainability and environmental impact of the industry, the company stated.

USA partnership to boost domestic farm-raised fish

Scientists from Florida Atlantic University's Harbor Branch Oceanographic Institute partnered with U.S. Department of Agriculture (USDA) Agricultural Research Service (ARS) to boost the U.S. aquaculture industry in a $2.4 million project.

FAU's Harbor Branch faculty is skilled in warm water finfish research and development, and has led the way in advancing aquaculture in Florida and globally for more than 40 years. The USDA-ARS' National Program for Aquaculture mission is to conduct research and deliver technologies that improve domestic aquaculture production efficiency and product quality while minimizing impacts on natural resources. FAU's Harbor Branch will host three new USDA scientists on their campus who have expertise in genetics, fish nutrition and health to develop novel technologies that will ensure a steady supply of warm water marine fish seedstocks optimized for commercial production.

Focused on improving market competitiveness for domestic aquaculture producers, the project will initially focus on Florida pompano (*Trachinotus carolinus*) and red drum (*Sciaenops ocellatus*) as model species for improvement. The key objective of the project is to develop year-round spawning strategies for captive broodstock and larviculture methods for seed production of marine finfish. In addition, the scientists will develop methods for genetic improvement of warm water marine finfish for optimum production efficiency. This approach will help to increase profitability and ensure environmental sustainability by producing less waste, posing less risk of disease, and using feed more efficiently.

Benchmark's *Streptococcus*-resistant tilapia fry available soon

Benchmark discovered a significant quantitative trait locus ("QTL") for *Streptococcus iniae* resistance in tilapia. *Streptococcus* infections are among the most critical disease challenges in tilapia production and this technological breakthrough presents a significant opportunity for the industry to reduce this infection and the use of antibiotic treatments.

Benchmark's genomic analysis from controlled disease resistance trials has shown that a significant proportion of the genetic variation for resistance is caused by a small region of DNA – a Quantitative Trait Locus or QTL. Benchmark has made a patent application in relation to its discovery.

The QTL identified will be used to select broodstock with high levels of *Streptococcus iniae* resistance for the production of commercial fry. Currently, Benchmark selects broodstock for improved resistance to *Streptococcus agalactiae* using genomic selection, and for resistance to *Streptococcus iniae* by Marker-Assisted Selection using the S. iniae QTL. Benchmark's commercial Spring Tilapia fry will be available to producers during early 2020.

Benchmark state-of-the-art broodstock and ova facility

The world's most advanced land-based facility for broodstock and ova production for Atlantic salmon, SalmoBreed Salten, was opened over three full days at the end of May this year. Benchmark opened the doors to more than 250 guests from all over the world, including customers, investors, politicians and the local community.

A long way from start to goal

The opening marked the end of a relatively long planning and construction timeline. The project, already launched back in 2012 was initiated by today's 25% shareholder in SalmoBreed Salten AS, Salten Stamfisk AS. Preparations of the land was done, but when the time was up to decide on construction planning, the salmon prices had dropped below production cost putting severe constraints on the financing of the broodstock plant. It took seven more years and a new owner of SalmoBreed to get the plans realized as Benchmark took over the company in 2014 and understood the potential of the operation. The NOK 400 million investment (USD 45 M), the single most significant investment in Benchmark's history, puts the company at the forefront of the development of Atlantic salmon genetics.

Best in the world

So what makes Benchmark describe the facility as the world's most advanced plant for broodstock and ova production? First of all, the location has access to great water sources, both seawater from the Sørfjord taken from 15 m and 70 m depths and freshwater from Sørfjordvatnet next to the national park. Secondly, the facility runs on three different systems, flow-through, reuse and recirculation depending on which water temperature is needed and to secure the best water quality at all times. By adjusting light and temperature, SalmoBreed Salten can manipulate seasons and thereby make the broodfish spawn regardless of the actual season outside the buildings. Last, but not least, the facility is designed to hold broodstock on land the entire life cycle, giving the highest levels of biosecurity offered in the industry and a production scheme that will define the standard for the future of ova production.

Jan-Emil Johannessen, Head of Benchmark Genetics said that "the opening of our new facility in Salten is a significant milestone for Benchmark which will allow us to capitalize on our leading market position in salmon genetics and the favorable long-term market trends in the industry. Producing on land means that we are in complete control of the spawning season and thereby able to supply our customers with high-quality salmon eggs every week of the year."

Localization as a success factor

The location of the facility is in proximity to the airport in Bodø, the main roads crossing Norway and the North University. As the industry growth is mainly expected to come from the Northern regions of Norway in the coming years, the location in Sørfold is also of commercial importance to Benchmark.

World-class biosecurity

The plant, divided into four independent hygienic zones, holds the most stringent procedures for biosecure production. Meaning that if one zone is affected by a disease, the other zones can continue to operate regardless of the problem zone.

Stig Joar Krogli, General Manager of SalmoBreed Salten AS, said that "although cost-driven for the

project, we believe that we have chosen the best possible solution. Our customers must be confident that the eggs they get from us are the best in class concerning biosecurity."

Three days of celebration

The opening ceremony lasted three full days. The first day, designated to key stakeholders, international guests from Asia and the Americas, politicians including the Norwegian Minister of Petroleum and Energy and investors, included a seminar focusing on how breeding and genetics can contribute to the sustainable growth of the aquaculture. Among the list of guest speakers were Erland Bullvåg, Dean of Nord University; Kristian Eikre, Investment Director at Ferd; Klemet Steen, Chief Advisor Smolt at Lerøy, Knut Rønningen, Senior Advisor at the Food Safety Authority and Morten Rye, Benchmark's genetics expert.

The facility was officially opened the following day on May 22 by Kjell-Børge Freiberg, Norway's Minister of Petroleum and Energy. In his speech, he pointed out the importance of establishing high-tech facilities and jobs in the Northern parts of Norway. As former mayor in the community of Hadsel, he still holds a great concern about the region and the opportunities for salmon farming as a significant industry for the future. Salutes concluded his speech from an old cannon conducted by costumed soldiers from the local fort, Skandsen.

Stig Joar Krogli said that "our facility consists of 10,000 m^2 building mass and a water capacity of 8,000 m^3. The capacity when in full operation is over 150 million ova per year and the first batches of eggs have already been delivered to customers last November, exactly according to the initial plans".

The event concluded on May 23 in Kjerringøy, north of Bodø, with a full-day seminar with customers focusing on genetics, production and product innovation.

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Artemia solutions for fish and shrimp farms

Luk Van Nieuwenhove, I&V BIO

Many aquaculture species still rely on live food as the main nutritional source during the early stages. Farmers need to secure enough *Artemia* cyst stocks which means cash drain in many cases. There are more than a hundred different *Artemia* brands available in the market which make the choice difficult for farmers. The hatching percentage as guaranteed on the packaging is seldom achieved and hatching conditions such as light, temperature and aeration are difficult to control. These conditions can result in daily oversupply or shortage of *Artemia* nauplii.

Separation of swimming nauplii from the non-hatched cysts and empty shells is a major challenge for every hatchery managers. Bacteria blooms fast during hatching and are difficult to control, often resulting in heavily contaminated *Artemia* which negatively affects the health of the shrimp and quality of the tank water.

Artemia solutions

I&V-BIO developed a patented separation technology which combined with specific hatching and quality protocols ensure the production of *Artemia* nauplii on an industrial scale. Separation technology, disinfection and packaging are equally important to produce consistent quantity and quality. Customized programs in combination with trained staff guarantee on-time deliveries 365 days per year.

The company provides INSTART 1, *Artemia* nauplii with no impurities, no damaged animals, no *Vibrio* and are offered in a consistent live-paste (800g per tray) setting a new standard in the shrimp and fish industry.

These Nauplii enable the hatcheries to follow strict biosecurity protocols relieving them from the burden of hatching *Artemia* cysts in often sub-optimal conditions.

To further strengthen the health of shrimp PL, the company also offers INSTART E, (enriched *Artemia* nauplii) to hatcheries, nurseries and grow-out. Instart E is produced in a 3-step enrichment process: enrichment with high quality DHA emulsion; enrichment with algal extracts high in amino acids and carotenoids; and enrichment with ELVAN, a blend of herb extracts, proven for its powerful anti-*Vibrio* effect and its prebiotic properties.

Decapsulated cysts (M-BRYO) are also available in a fresh ready-to-use paste and a range of high quality dry diets are also available.

I&V-BIO Nauplii Centers are GMP certified. The facilities all have their own water treatment systems to exclude EHP spore contamination. Daily *Vibrio* analysis are performed as well and RT-PCR analysis for EHP and EMS.

Artemia Nauplii Center

The first pilot *Artemia* Nauplii Center was established in Thailand in 2013 by Frank Indigne and Luk Van Nieuwenhove. Afterwards I&V-BIO opened a brand new, state of the art facility in Kakinada, India in 2018 followed by a smaller one in Lampung, Indonesia. Construction in Vietnam and Ecuador is finished and both Nauplii Centers will be operational in October 2019. Bangladesh will be operational beginning 2020. Most of these facilities will have a production capacity of 700-800 trays of 800 g leaked Instar1 *Artemia*. Each tray contains about 60 million Nauplii which is the equivalent of one can of 70% hatching.

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Faster growth, healthier fish, targets met

Andrew Lawrence, Ewos Canada Ltd.

EWOS Prebiosal® is a multi-component prebiotic supplement used to stimulate and optimize intestinal microflora in a wide variety of species. EWOS Prebiosal® has no medicinal ingredients or live bacteria and will not interfere with existing certifications like BAP, OSC, ASC or Whole Foods. Over years of commercial, field and laboratory studies at Cargill Innovation Centers in Norway and Chile, EWOS Prebiosal® has become our most researched and documented dietary support product. The many benefits of using EWOS Prebiosal® include:

Optimized gut health

The digestive tract of all fish species harbor complex microbial communities, collectively referred to as gut microbiota. The gut microbiota has a tremendous impact on feed digestion, the development of intestinal epithelium, immunity, and disease. Furthermore, studies have revealed that the gut microbiota of fish is far more variable than previously realized and is heavily influenced by diet, starvation, pathogen, system type, geographical location, water temperature, seawater transfer and antibiotics. EWOS Prebiosal® works by encouraging the colonization of commensal, or interdependent, bacteria on the intestinal microvilli which stimulates the regeneration of intestinal cells and the fish's mucosal system. The rapid regeneration of gut epithelial cells provides a greater surface area for nutrient absorption, which stimulates the fish's natural immune system and reduces the possibility of colonization of pathogenic bacteria.

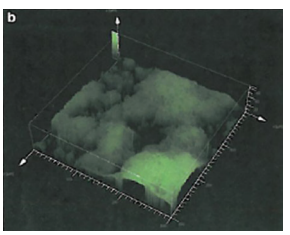

Figure 1. The commensal bacteria of the gut constitute a biofilm or society, i.e. an organ in itself acting as a multicellular entity. Disorganization of the gut microbiota means a loss of synergistic partners and disturbance of the biofilm collective.

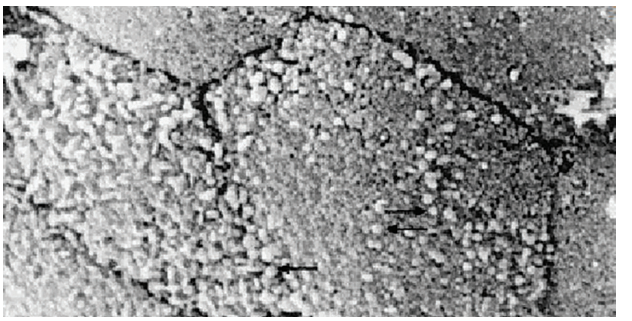

Figure 2. Electron micrograph of bacteria colonizing enterocyte microvilli in the lower intestine of salmon, magnified 7500x. (Photo: E. Ringø).

Gaps within the biofilm provide an entry point for opportunistic bacteria but with EWOS Prebiosal® an improved mucosal barrier is formed which helps to prevent this.

Improved performance

The gut microbiota of fish is an essential element in the digestive system and consequently has a profound effect on fish performance. Bacteria in the gut breaks down and metabolizes nutrients, thereby stimulating pancreatic secretions which contain enzymes vital to digesting proteins, carbohydrates, and fats. Gut bacteria cause fermentation of dietary fibers in the fish's colon, which produces short-chain fatty acids that are easily absorbed by the fish and reduce the pH in critical areas of the gut. Through this process, EWOS Prebiosal® increases digestion efficiency and nutrient uptake, which results in improved fish growth and performance in the range of 9 to 14 percent.

Disease resistance

The effect of dietary components on the gut microbiota is also an important consideration as the gastrointestinal tract has been suggested as one of the major routes of infection in fish. EWOS Prebiosal® works to reduce the possibility of infection by

pathogenic bacteria in two ways: i) by reducing pH in critical areas of the gut, colonization by pathogenic bacteria that are not well adapted to acidic environments is prevented, and ii) increased mucosal production reduces the risk of settlement of non-commensal and pathogenic bacteria. If populations of these bacteria are able to settle and develop, toxic compounds that damage the gastrointestinal tract can be produced and this can render the fish more susceptible to pathogenic bacterial infections.

Improved physiology

The greatest potential benefit of EWOS Prebiosal® for today's fish culturist is the opportunity to maintain optimal gut microflora. The maintenance of optimal gut microflora and preventing colonization of non-commensal species has positive downstream effects on the gastrointestinal tract. As discussed previously, things like diet, starvation, pathogen, different lifestyles, geographical location, water temperature, seawater transfer and antibiotics all have profound effects on the gut microbiota. Sudden changes in levels and species of bacteria can have negative effects and EWOS Prebiosal® has been shown to maintain the species balance (abundance and variety) in the gut, which contributes to improved intestinal physiology, particularly in the distal region, with reduced risk of enteritis. Furthermore, liver physiology is improved in fish fed EWOS Prebiosal® and this is positively correlated to more effective utilization of lipids.

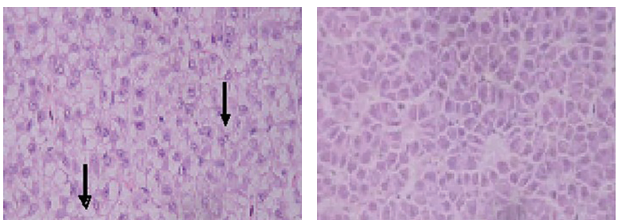

Figure 3. Large vacuoles displayed with a diet containing EWOS Prebiosal® (left). Small vacuoles displayed in a control diet (right).

EWOS Prebiosal® improves growth and feed assimilation through increased uptake of nutrients in the intestine, the results of which may be seen in the liver. Liver cells in fish fed EWOS Prebiosal® supplemented feed display large vacuoles indicating higher levels of nutrient uptake and metabolism compared to fish fed the same feed without EWOS Prebiosal®.

Improved flesh quality

Atlantic salmon (*Salmo salar*) fed EWOS Prebiosal® are shown to have a healthier liver physiology, with reduced atrophy and improved vacuolation. Improved liver physiology, particularly where bile production is concerned, correlates to more efficient lipid utilization in salmon, meaning that lipids are more effectively used as an energy source. More effective nutrient and lipid utilization correlates to significantly improved growth as well as final harvest quality.

Improved pigmentation uptake

In a study conducted by CIC, fillets from Atlantic salmon fed EWOS Prebiosal® were compared with fillets from fish fed a control diet. These samples were chemically analyzed for fat and astaxanthin levels. Across all samples, astaxanthin levels in the fish fed EWOS Prebiosal® compared to those fed the control were found to have over 10 percent more astaxanthin deposited in the musculature.

Gareth Butterfield, Sales Director for EWOS Canada Ltd. stated that "Prebiosal has been a long-standing product for EWOS and a great support tool with various applications for our customers. I stand by Prebiosal as a product that can help grow fish faster and more efficiently with peripheral benefits to further support production success. The microbial community in the stomach of fish greatly influences its physiology, its ability to fight disease, how food is digested and absorbed, and can even influence final product quality through improved flesh characteristics. As a non-medicinal product, we recommend feeding EWOS Prebiosal® throughout the life cycle in order to benefit from the improved growth performance and feed conversion and as part of an integrated program to prevent disease and promote wellbeing. It also carries the benefit that it can be added to any of our product ranges and sizes."

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Probiotic use in hatchery production systems to increase PL stress resistance

Richard Carpenter, BiOWiSH Technologies

The shrimp industry depends on hatchery production of post larvae (PL). For farmers to achieve successful production, they need access to quality PL that are stress-resistant and disease-free. PL quality is so critical, the FAO (Food and Agriculture Organization of the United Nations) has designated access to healthy, disease-free PL as one of the top issues facing the industry today.

Production cycles can be ruined by unpredictable stressors such as sudden environmental changes in temperature, salinity and pH. These environmental stressors coupled with abundant disease outbreaks are leading the industry to look for novel strategies to combat these issues. Production managers are increasing the use of post hatchery-controlled nurseries to reduce grow out time and increase stocking densities, while also recognizing the need for added biosecurity. Hatcheries are continuing to provide disease-free assurances while implementing new strategies such as developing alternative strains of PL that are faster growing and less susceptible to stress.

Probiotics in hatchery production

Probiotic technology is an increasingly important tool in hatchery production with the potential to address many of these issues. In the context of shrimp production, the WHO (World Health Organization) defines probiotics as live microorganisms which, when administered in adequate amounts, confer a health benefit on the PL. Probiotics may be administered through feed, either in-feed or top coated onto feed, to provide significant benefits, for example, to gut health. They may also be delivered into the water column to help maintain water quality.

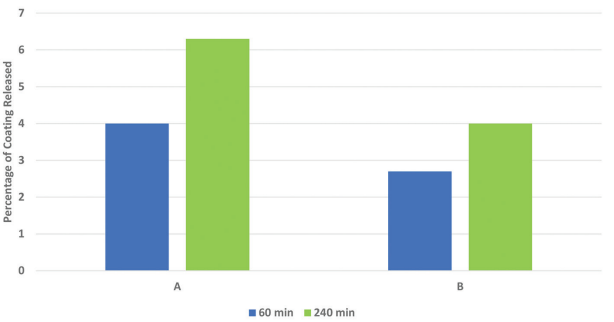


Figure 1. When coated onto 1 mm feed, BiOWiSH® MultiBio 3PS provides a stable, coated product with high retention, greater than 94-97% retention in water (Treatment A: ambient temperature (21˚C) feed coated with BiOWiSH® MultiBio 3PS; Treatment B: 40˚C feed coated with BiOWiSH® MultiBio 3PS).

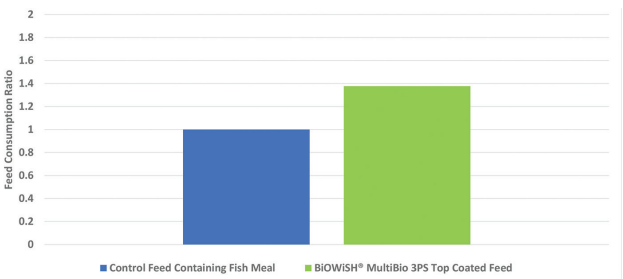


Figure 2. Shrimp feed attractability testing shows BiOWiSH® MultiBio 3PS increases rate of feed consumption by 38% when top-coated onto commercial feed (34% protein/21% fish meal).

In order to provide sustained economic value to the industry, however, probiotics must overcome historical issues such as inadequate stability, variable performance, and poor production quality. To address these issues, different microbial species have been introduced into probiotics products. In addition, the probiotics industry is developing more rigorous standards for optimizing species and strain selection, product formulation levels, and dosages for different

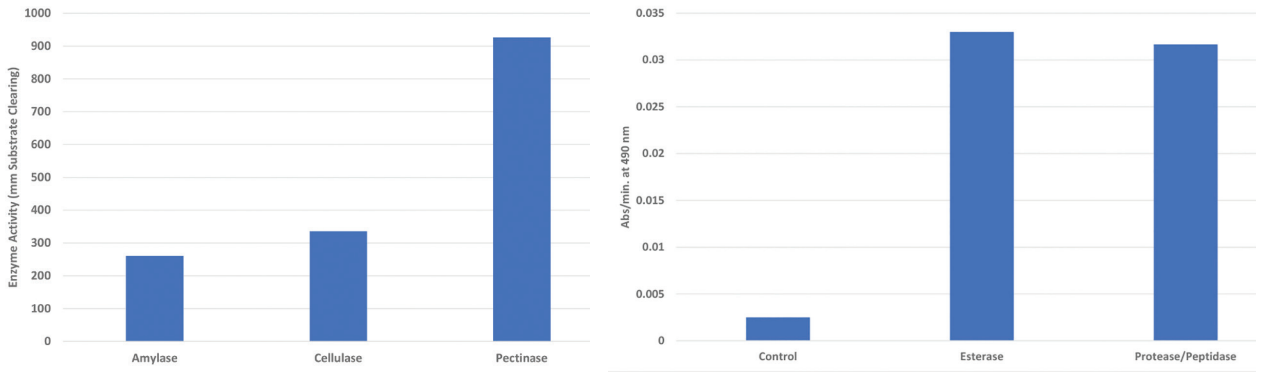


Figure 3. Relative activities determined versus controls on dyed starch, cellulose, and pectin substrates via plate clearing zone assays (left). Protease and esterase activities determined via diacetyl fluorescein hydrolysis assay (right).

use conditions. Building a technical foundation for the use of probiotics in hatchery production is critical if the industry is to overcome previous challenges and begin to adopt probiotics as a sustainable aspect of hatchery management.

Bacillus probiotics

In regard to composition, probiotic products today may include a single species or a blend of microbial species with varying levels of activity. Typically, *Bacillus* probiotics are delivered in an inactive spore form, which is more stable. To be effective, these products require application conditions that can induce microbial germination. Germination can occur either in the water column or host animal. Probiotic microbial species that are not *Bacillus* typically exist as vegetative organisms. These may be susceptible to damage from heat exposure or other storage conditions. The latter is particularly important for how probiotics are applied in feed. Depending on feed process conditions and temperatures, probiotic products are more often top-coated onto processed feed.

BiOWiSH Technologies has identified several species of naturally occurring lactic acid bacteria and *Bacillus* microorganisms that are very beneficial in hatchery production. The company utilizes a standardized blend of these microorganisms to deliver probiotics for use in both water quality maintenance and feed enhancement.

BiOWiSH® MultiBio 3PS is a proprietary feed probiotic that can be blended into hatchery feed, or top-coated onto feed, depending on the feed type and stage of hatchery production. When applied to feed, it is stable and adheres well (Fig. 1). When coated onto feed, this

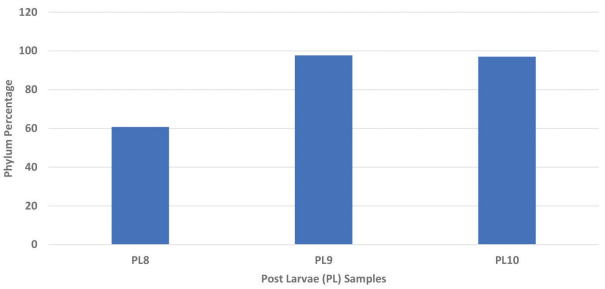
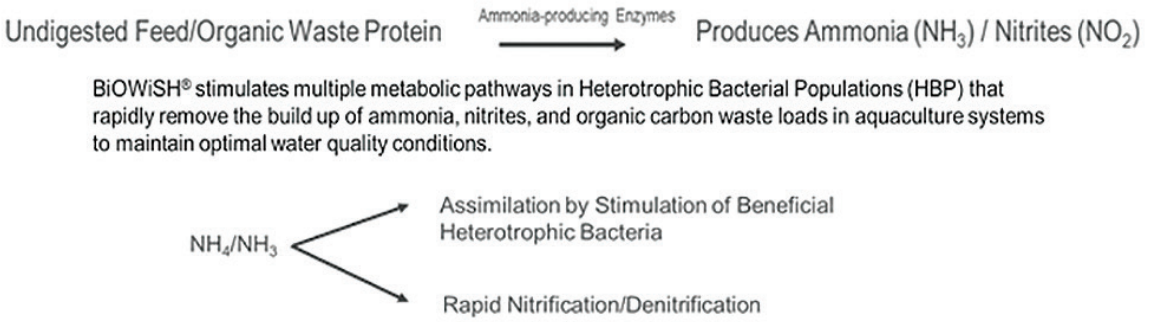


Figure 4. 16s Ribosomal RNA microbiome phyla analysis. As post-larvae develop, their microbiome changes/shifts. With the BiOWiSH® two-step approach, these changes include an increase in beneficial heterotrophs such as Firmicutes phyla containing *Lactobacillus* and *Bacillus* with no detectable *Vibrio*.

probiotic assemblage delivers three major performance benefits in hatchery production.

First, it provides an additional element of feed attractability, which increases feed consumption rates (Fig. 2). If feed sits in tanks unconsumed, vital nutrients can be leached out, reducing the nutritional value of the feed. Undigested feed can also contribute to an increasing organic waste and ammonia load during production, diminishing water quality and causing additional stress on PL. Increasing feed consumption rates helps ensure that the feed being consumed is higher in nutritional value to PL. Higher feed consumption rates help reduce the amount of undigested feed in the water column.

Second, when activated it produces a broad range of hydrolytic enzymes (Fig. 3) that can breakdown complex carbohydrates and protein present in feed. This helps improve digestibility and optimize nutrient availability, which is important to PL that have a short feed retention time. Lastly, a transcriptome analysis of PL during growth from PL8 – PL10 (Fig. 4) shows that this feed probiotic



helps ensure development of a healthy microbiome in PL during hatchery production. This benefit has been confirmed further in the shrimp gut when used in the later stages of nursery and grow-out production.

To help reduce chemical stress and maintain optimum water quality, BiOWiSH® AquaFarm added routinely to the tank water column reduces ammonia and nitrite buildup (Fig. 5). This proprietary formula induces a healthy beneficial heterotrophic bacteria population in the water column. These microorganisms maintain tank water quality through efficient nitrification of ammonia and a rapid, novel form of aerobic denitrification that reduces the levels of these harmful materials even as feed rates increase as PL grow.

The combination of these two products helps ensure successful production with increased PL size and uniformity. It also produces PL that are more resistant to unexpected environmental stressors during later stages of the production cycle. When applied the two product approach also helps reduce dependency on chemical additives which are becoming increasingly problematic for the industry.

Summary

Probiotics and other microbial technologies are demonstrating viable and beneficial strategies to overcome several challenges the hatchery industry faces. Building a stronger technical foundation for their application and mode of action, while ensuring consistent performance and product quality, is critical to their continued use in hatcheries and overall shrimp production. As with all new technologies, the outlook for their continued sustainability in hatchery management and value in the market will depend on their ability to deliver quality PL and successful production with a viable economic value proposition.

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Effectiveness of a new line of products to disinfect live feed and larval culture

Mònica Rius, CENAVISA and **Santiago Cabaleiro**, **Mª Belén Fandiño**, CETGA

Rotifer and *Artemia* are essential for success in the intensive production of fish aquaculture. However, the use of live feed greatly contributes to high larval mortality due to the opportunistic pathogenic bacteria that proliferate in these cultures. Specifically, *Vibrio alginolyticus* was identified as one of the most important pathogens. Although in the past, this bacterial load was reduced by using antibiotics, these treatments are not efficient enough, nor appropriate nowadays.

Material and methods

CENAVISA and CETGA (Technological Center of the Aquaculture Cluster) have been collaborating to develop and test a wide number of products to improve the microbiological quality of live food. After preliminary trials on toxicity and efficacy against *Vibrio alginolyticus*, BIO-ROTICEN (BR) and the combination of BIO-ROTICEN with AQUACEN O2 POLVO (AQ.P) were identified as the most promising candidates for the treatment of rotifer and for Artemia and larvae, respectively, so, further *in vivo* and *in vitro* studies were performed.

Microbiological analysis was carried out from samples of rotifer and *Artemia* cultures, and water from larval cultures before and after exposure to the candidate products, in TSA and TCBS agar plates and incubated at 25 °C for 24 hours. The viability of the pathogens contained in the samples before and after exposure was analyzed.

Rotifer trials

Several tanks containing 500 rotifer/mL were treated with 2ml/L of BR for 60 minutes, on aeration. Before adding it to the larval cultures, rotifer was rinsed for five minutes with seawater to eliminate any trace of the product. Bacterial load in treated and control tanks were compared.

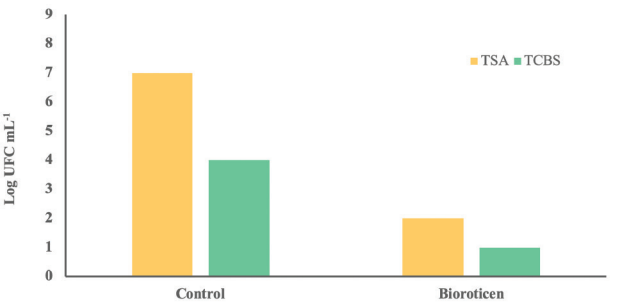

Figure 1. Efficacy of BR applied during rotifer enrichment. Bacterial count in TSA and TCBS (500 rotifer/mL). No treatment for Control. Test group: BIO-ROTICEN (2mL/L) for 60 minutes.

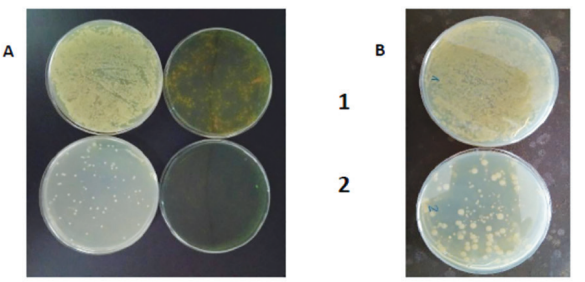

Figure 2. Effect of BR treatment during rotifer enrichment. A. Rotifer enriched during 4 hours (500 rot/mL). TSA (right) and TCBS (left) plates. A1, no treatment; A2, BR (2mL/L) for 45 minutes. B. Comparison between antibiotic and BR treatment. Rotifer enriched for 4 hours (400 rot/mL). TSA plates. B1, treatment with oxytetracycline (100 ppm) for 4 hours; B2: BIO-ROTICEN (1.5 mL/L) for 45 minutes.

A 5-log reduction (from 10^7 to 10^2 CFU/mL) in TSA was observed, as well as 2-log reduction in TCBS (Fig. 1, 2A).

BR has higher efficacy than antibiotic treatment in reducing the bacterial load of the enriched rotifer

(Fig. 2B). Rotifer density and motility was not affected by the disinfection process with BR. Moreover, enrichment of the rotifer was not affected by the treatment either, as it is shown in Figure 3, where the stomach of the rotifer remains full of feed. Therefore, it can be concluded that the treatment with BIO-ROTICEN does not negatively affect the condition or the enrichment of the rotifer. Furthermore, this reduction of the bacterial load allows higher survival rate and larval growth.

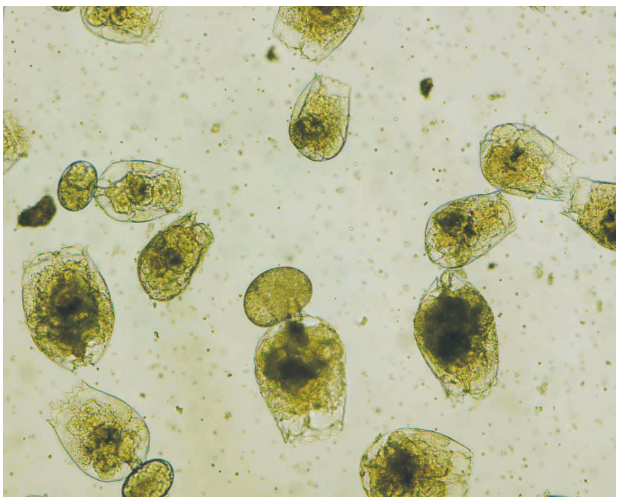

Figure 3. Rotifer (*Brachionus plicatilis*) enriched after 45 min with BR.

Metanauplii trials

Several tanks containing 200 enriched Metanauplii/mL were treated with different combinations of BR (1.2, 2.4 and 3.6 mL/L) and AQ.P (15, 30 and 45 mL/L of a dilution of 20 g/L). After a 45 minutes incubation, Metanauplii was rinsed for 10 minutes with seawater and five minutes with freshwater to eliminate any trace of the products. Bacterial load in treated and control tanks were compared.

Figure 4 shows the effectiveness of all disinfectant treatments in comparison with the control group. All of the combinations of BR and AQ.P (A, B and C) produce a reduction of the total bacterial count, the A combination being the one with the higher effectiveness with less dose. Moreover, A and B combinations got a 2-log reduction in TCBS. Treatment does not affect the motility of *Artemia*, which is even more active after the treatment with AQ.P. No mortality was observed in the treated Artemia as it was in control tanks. As in the case of the rotifer, there is no effect in the enrichment of the *Artemia* (Fig. 5).

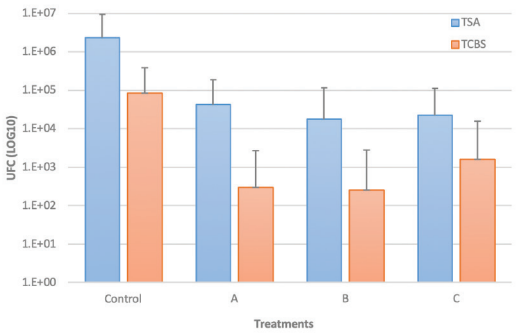

Figure 4. Efficacy of BN and AQ.P after the metanauplii enrichment. Bacterial count in TSA and TCBS (200 *Artemia*/mL). No treatment for control group. A: BIO-ROTICEN (1,200 ppm) and AQ.P (300 ppm); B: BR (2,400 ppm) and AQ.P (600 ppm); C: BR (3,600 ppm) + AQ.P (900 ppm). All treatments were applied for 45 minutes before harvesting the enriched *Artemia*.

Figure 5. Enriched *Artemia* after 45 minutes treated with BR and AQ.P.

Nauplii trials

Although the bacterial load of nauplii is lower due to the absence of enrichment, it is also necessary to reduce it before adding them as live feed to the larvae. Taking into account the results obtained in the metanauplii tests, the combination of 1.2 mL/L of BIO-ROTICEN + 15 mL/L of a dilution of 20g/L of AQUACEN O2 POLVO for 45 minutes was used for nauplii, following the same methodology already described for metanauplii. After the treatment, a 2-log reduction (from 10^5 to 10^3 CFU/mL) in TCBS was observed, without negative effects on the nauplii viability (Fig. 6).

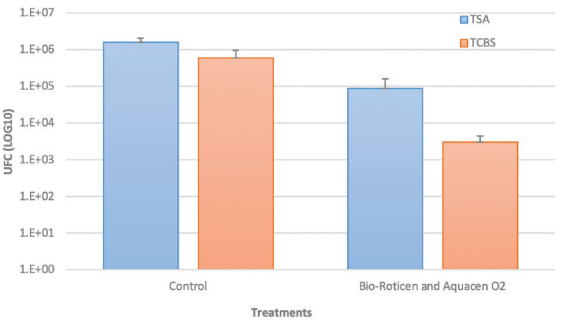

Figure 6. Efficacy of BR and AQ.P in Nauplii. Bacterial count in TSA and TCBS (200 artemia/mL). No treatment for control group. Test group: 1.2 mL/L BR + 15 ml/L of a dilution of 20 g/l of AQ.P for 45 minutes before harvesting.

Turbot larvae (29 days) after treatment with BR and AQ.P.

Larval trials

Trials were also performed in turbot larval culture. Two treatments were applied: A, 0.1 mL/L of BR; B, 0.2 mL/L. Both treatments also included 2.5 mL/L of an AQ.P dilution (20 g/L). These disinfectants were added to larvae culture tanks on day 6 and day 8, with a daily water removal of 50% and 75% of, respectively.

A reduction of the bacterial load was observed in the treated tanks in comparison with the control, in both TSA and TCBS (Fig. 7). This reduction implies a higher survival rate and larval growth.

Conclusions

Disinfection of rotifer with 2 mL/L of BIO-ROTICEN for 60 minutes was shown to be very effective in reducing the bacterial load without any effect on the viability of the enriched rotifer as well as 1.2 mL/L of BIO-ROTICEN + 15 ml/L of a dilution of AQUACEN O2 POLVO for 45 minutes in *Artemia*. In larvae culture tanks, 0.1-0.2 mL/L of BIO-ROTICEN and 2,5 ml/L of a dilution of AQUACEN O2 POLVO at days 6 and 8 with water removal, reduces the bacterial load in the larvae tanks.

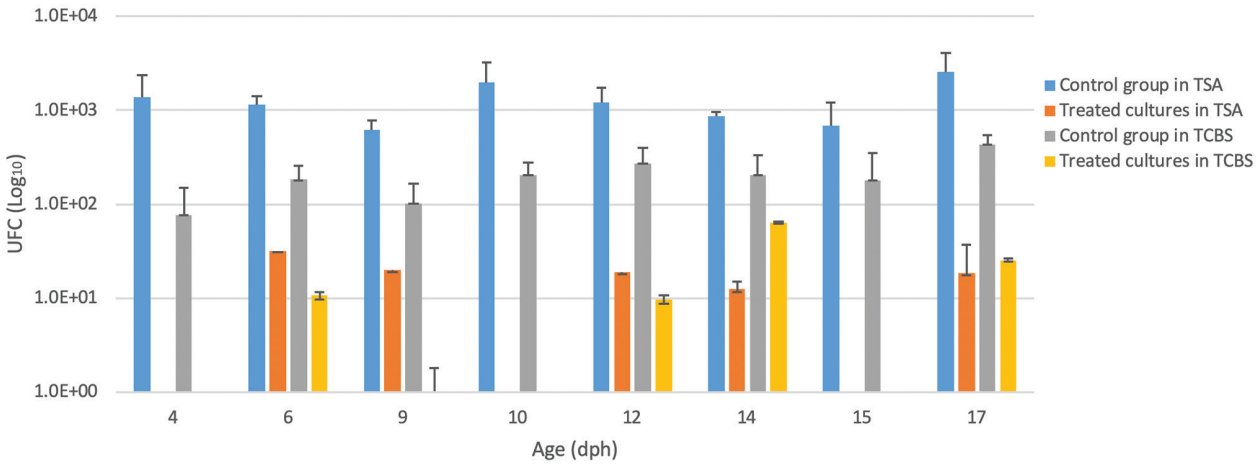

Figure 7. Effect on larvae cultures. Bacterial count on TSA and TCBS.

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Manipulation of the microbiota in a shrimp hatchery

Stephen G. Newman, Aquaintech

The terms microbiome and microbiota are frequently confused with each other. The microbiota is a subset of the microbiome. It refers to the microorganisms that are present in a given environment (1). The microbiome refers to the total genomes and gene products of all of the bacteria, bacteriophage, fungi, protozoa and viruses that are present in a specific environment (1). This can be in and on an animal or a plant or it can refer to any element of the environment including objects. As applied to aquaculture most often it is used in reference to what is present internally as well as the external surfaces of a given aquatic animal. It can also refer to the specific composition of individual elements of a production environment, such as the sediments, the water column, etc. A given microbiome is really just a subset of a much larger ecosystem, just as the microbiota are components or elements of the microbiome.

Not all that long ago much of the microbiome was hidden from science. It was thought that we could grow most of the components on artificial media and many workers reported broad changes on the microbiome based on observations on how specific experimental protocols altered what could be cultured. Using much more sophisticated tools we can now characterize all of the elements of a given microbiome, the vast majority of which, we now know, cannot be cultured. This, for the first time, has allowed us to see how complex the microbiota/microbiome is and how it can be affected by the use of many different materials in the feed and in the environment. We have a much clearer, albeit evolving, idea of what microbiota are elements of the microbiome (2).

Shrimp hatchery production

The exact tonnage of farmed shrimp being produced currently is not known. Production is somewhere between 3.8 and 5 million MTs depending on the source (3, 4). Using the higher figure, to produce the 5 million MTs of shrimp that were farmed globally in 2018, an estimated 500 billion post larvae (PLs) would have had to be produced (assumes 20 g average weight at harvest, 50 percent survival in the hatchery). This production is spread across thousands of hatcheries in dozens of countries. The state of the art for the production of these post larval shrimp remains for the most part relatively primitive. Even though larger operations may produce a billion or more PLs a month there are many smaller hatcheries. Biosecurity is weak in general regardless of the size of the facility and those who operate them all too often neglect the tools of science (Newman personal observations).

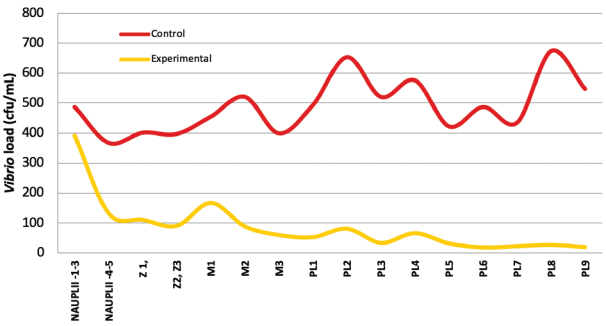

Figure 1. Impact of *Bacillus* (PRO4000X) on (yellow on TCBS) *Vibrio* loads in hatchery tanks.

Vibriosis in shrimp culture

One of the most common problems in hatcheries is vibriosis due to infections with *Vibrio* bacteria. Vibrios are ubiquitous in marine and, to a lesser extent, freshwater environments (5). They serve an important function in the recycling of chitin, the structural component of crustacean exoskeletons, among other things. Some are human pathogens (*Vibrio parahaemolyticus, Vibrio cholerae*) although

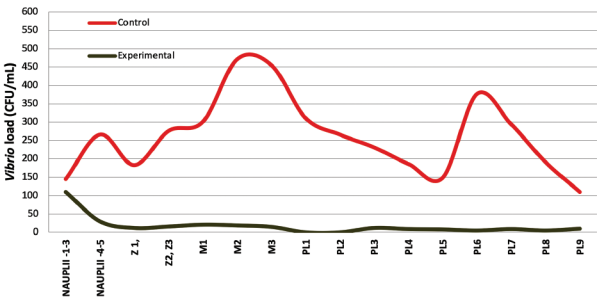

Figure 2. Impact of *Bacillus* (PRO4000X) on (green on TCBS) *Vibrio* loads in hatchery tanks.

many more are aquatic animal pathogens (*Vibrio parahaemolyticus, Vibrio anguillarum*, etc.) (6). As of this writing some 147 distinct species have been identified with many hundreds of variant strains (7).

One of the common problems caused by Vibrios in shrimp hatcheries is known as the zooea syndrome. Zooea are the first feeding stage of larval shrimp and large mortalities can occur at this highly sensitive stage if *Vibrio* loads are not adequately controlled (8). The first effort to control the impact of Vibrios in shrimp hatcheries via the use of microbiota manipulation was developed by Giovanni Chasin and published by Garriques in Ecuador in the early 1990s (8). They reported that *Vibrio* strains from the wild that were able to degrade sucrose on the widely used selective media for Vibrios, thiosulfate-citrate-bile salts-sucrose agar (TCBS), i.e. they formed yellow colonies, when added in large numbers to hatchery tanks were able to dramatically reduce the severity of the disease. Unfortunately, the assumption was that these Vibrios, typically *Vibrio alginolyticus*, were not virulent. Animals were routinely tested for susceptibility to verify this. However, failure to understand how diverse this taxon is and how readily they exchange genetic material has resulted in hatcheries losing entire production runs.

Microbiota manipulation

This practice continues even though it can have a serious downside. The use of a bacterial species that are much less likely to cause acute problems as a result of the exchange of genetic material with virulent strains makes more sense. With this in mind, the ability of several spore farming gram positive bacteria were tested for their ability to impact *Vibrio* loads in a shrimp hatchery in India. A tableted product (PRO4000X) containing equal proportions of specifically selected proprietary strains of *Bacillus subtilis* and *B. licheniformis* was added to hatchery tanks and the impacts on total *Vibrio* loads determined (Fig. 1, 2).

These tests demonstrated that these proprietary Bacillus strains when added daily to shrimp hatchery tanks were able to dramatically lower the total Vibrio loads and keep them low. This was an example of microbiota manipulation in a production environment that had a dramatic impact on the presence of presumptive pathogens that routinely negatively impact shrimp hatchery production. The hatchery personnel noted that the tanks were much cleaner, that they were able to stop exchanging water and that the animals in the experimental tanks were cleaner, stronger, grew better and were free from vibriosis problems.

This example of microbiota manipulation shows a clear cut, although not permanent impact. The *Bacillus* must be added daily to ensure that levels do not decline to the point where they no longer result in the desired outcome, i.e. reduction of *Vibrio* loads (Habeeb Rahaman-personal communication). When the *Bacillus* spores are no longer being added the impact will disappear. It is not likely that the *Bacillus* have become a stable component of the microbiota. One would expect that daily addition would not be needed if the added *Bacillus* strains were able to become stable elements of the microbiota/microbiome.

These tests demonstrate that it is possible in shrimp hatchery tanks to alter the microbiota in a manner that has a beneficial impact on the overall production, significantly altering the composition of the bacteria normally present. While the exact mechanisms remain to be elucidated, the evidence suggests that we are looking at a simple case where the added Bacillus spores upon germination compete against resident species for nutrients. This effect has also been noted in shrimp ponds as well (Habeeb Rahaman personal communication).

References available on request

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Moving beyond standards: how improving early life feeding strategies can boost finfish aquaculture

Alessandro Moretti, INVE Aquaculture

Seabass and seabream farming are the cornerstone of Mediterranean aquaculture. Fry production of these two species has been steadily increasing over the past few decades and currently represents more than 90 percent of all fry produced in the Mediterranean. While this has been driven by well-established larviculture and weaning protocols, there is still room for improvement. Developing more efficient protocols that optimize the use of live food creates a window of opportunity that can enhance the quantity and quality of the produced fry while simultaneously reducing operational costs. Moreover, hatchery protocols can also be manipulated to positively modulate the robustness and growth response of future fish. As reported in the following sections, even the smallest breakthroughs in fish larviculture and nursery rearing protocols can excel performance at the farm gate, which is the ultimate goal of fry production.

Larval rearing protocols

The use of specific products to balance the nutritional and microbiological profile of live feeds is paramount to safeguard a premium larval performance and maximize stress resistance. A scientific trial was performed with seabream larvae cultured using an optimized rearing protocol with newly developed enrichment for rotifers and Artemia, live feed conditioners, probiotics, and optimized dry feed protocol, and compared with conspecific larvae raised with standard commercial products. While similar survival was recorded at the end of weaning, the biomass achieved using the optimized rearing protocol was significantly higher (Fig. 1).

Survival after salinity stress tests was also notably higher when larvae were raised using the optimized rearing protocol (Fig. 1). It is also noteworthy that deformity levels were notably reduced in larvae reading using the optimized protocol: 2.3 percent, 1.0 percent, and 4.0 percent average operculum, tail, and severe deformities, respectively, were recorded in the optimized rearing protocol, which contrasts with the 9.3 percent, 6.5 percent, and 15.8 percent average deformities, respectively, observed in larvae reared in the control experimental treatment.

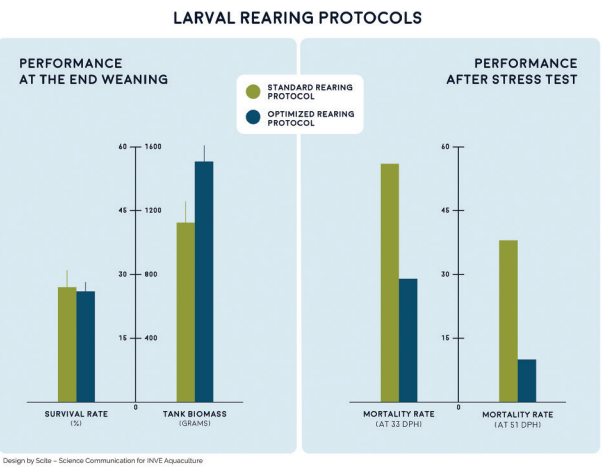

Figure 1. Survival (%) and biomass (g) at the end of weaning (left) and survival performance after the stress test (right) in seabream larvae raised using FRY 2.0 protocols and ingredients versus conspecifics raised with standard commercial products. The stress test was performed at 59 ppt for 33-day post-hatching (dph) larvae and at 69 ppt for 51-dph larvae.

Promoting long-lasting effects during the early life can positively affect the physiology and immunology of subsequent life stages, thereby resulting in a superior performance beyond the hatchery gate.

These findings clearly emphasize that one must look beyond traditional end-points, such as survival at weaning, to truly understand the impact of improved larviculture protocols. Overall, by optimizing larval rearing protocols one can significantly improve growth, stress resistance, and fry quality at the farm gate.

Boosting the immune system through live feeds

For decades, enrichment products were only perceived as a tool to correct nutritional deficiencies of the most commonly used live feeds in marine fish larviculture, such as rotifers and Artemia. As detailed in the previous section, modern hatcheries no longer solely focus on the use of premium enrichment products to fine-tune the nutritional profile of their live feeds. Hatcheries also rely on state-of-the-art microbial management protocols that boost the quality and performance of their larvae to unprecedented levels.

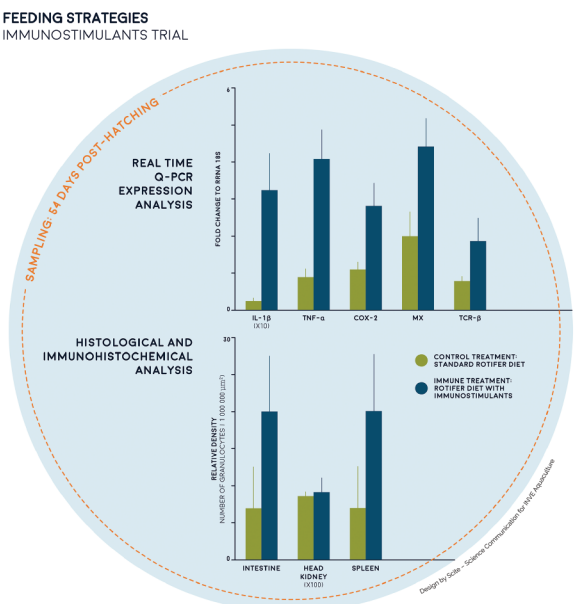

Figure 2. Gene expression, histological, and immunohistochemical analysis of seabream larvae fed a standard rotifer diet or a diet with rotifers supplemented with immunostimulants.

Early-life specific ingredients are essential to safeguard fry quality. Promoting long-lasting effects during the early life can positively affect the physiology and immunology of subsequent life stages, thereby resulting in a superior performance beyond the hatchery gate. This was validated in a scientific trial assessing the effect of manipulating rotifers as a pathway to boost the immune system of fry. Similar survival and growth were observed for seabream larvae fed a standard rotifer diet or a diet with rotifers supplemented with specific ingredients. However, larvae fed the supplemented rotifers displayed a higher expression level of several immune modulating genes at the end of weaning, with no gut tissue lesions or signs of local inflammation (Fig. 2).

These features reveal that specimens provided with this optimized diet are better prepared to respond against stressors, pathogens and diseases. This was confirmed in a salinity stress test at 33 days post-hatching (dph) where seabream supplied with an optimized rotifer diet displayed a notable reduction in the Cumulative Stress Index (CSI) compared to those fed a standard rotifer diet, from 124 to 19 CSI. Overall, results showed that the potential to enhance the immune response of seabream larvae through fine-tuning rearing protocols and nutritional strategies is only starting to be untapped.

The importance of careful nursery feeds

There is increasing awareness that early-life fish rearing protocols and feed quality can affect performance at later stages. State-of-the-art larval rearing must not only be combined with superior genetic background of fish, but close attention must also be given to the nursery phase. In fact, seabream feeding conditions experienced at nursery are decisive for fish performance during cage grow-out. Healthy larvae with premium quality can be easily destroyed if the wrong nursery formulation is used. Feed formulation is paramount to safeguard that larvae can grow in a harmonious, healthy, and uniform way, rather than growing fast. This may only be achieved if optimal feed formulations are employed for each production step, from hatching to post-weaning, nursery, and grow-out.

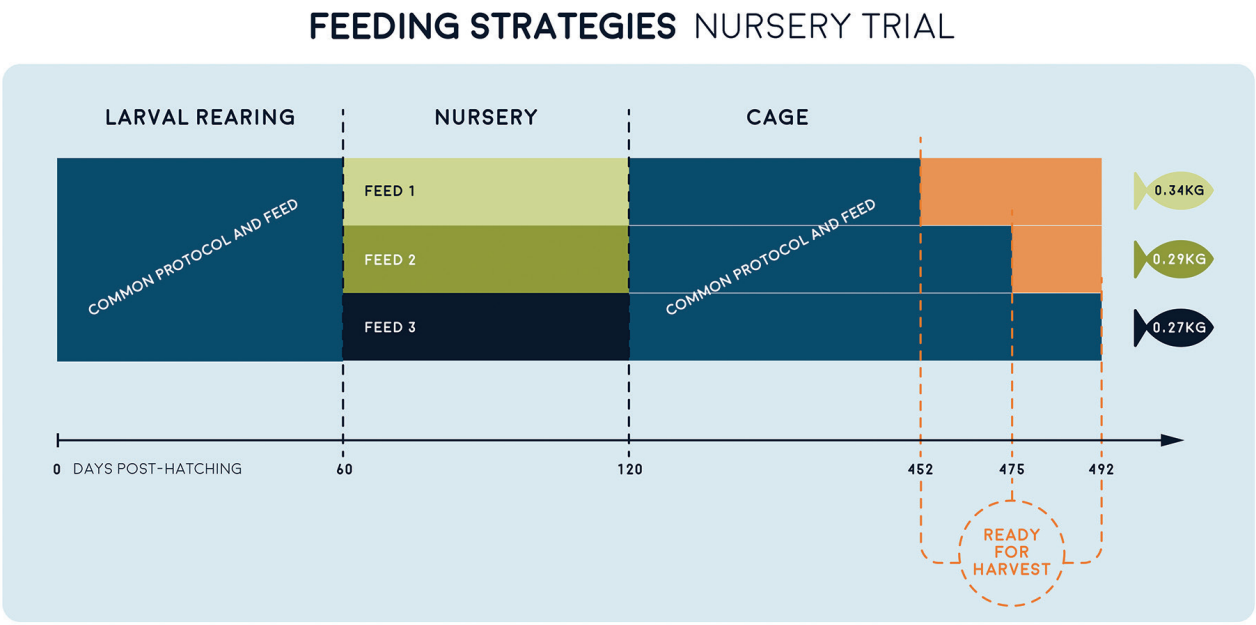

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Figure 3. Effect of different nursery feeds on seabream grow-out performance and time to reach commercial size.

However, feed formulation at the post-weaning and nursery stages often erroneously considers a nutritional demand of fish similar to that during grow-out while nursery stages represent an important step in between larval and grow-out stages. This misinterpretation still endures in marine fish hatcheries and is often the cause of poor performances. Indeed, one must acknowledge that enduring a 5-day delay at the nursery stage is a good trade-off if later on one can secure a 50-day gain at grow-out. This was validated in a scientific trial where seabream that underwent identical larviculture procedures until 60 dph displayed contrasting grow-out performances in cages if distinct nursery feeds were used (Fig. 3).

Nursery feeds supplied during a few weeks (from 60 to 120 dph) dramatically affect the time that fish take to reach commercial size. Indeed, if a premium nursery feed is used, seabream can be ready for harvest up to 40 days before conspecifics fed on standard grow-out feeds that force fry to grow without a continuous support of the strategy followed during larval rearing. By safeguarding the supply of an optimal diet at the nursery stage, fish farmers may choose to either harvest earlier or harvest at the standard time-point but with a 30 percent weight gain. This weight gain represents a notable reduction in production costs of about 20 percent, which results in a net cost savings of up to 1000€ per metric ton of fish produced. It is thus crucial to support a good management during pre-ongrowing to guarantee that the strategy used during larval rearing is continuously supported and addresses the varying fish demands throughout their life cycle.

Concluding remarks

To foster the farming of seabass and seabream, and thus leverage Mediterranean and EU aquaculture production towards the goals set for 2030, it is paramount that the industry moves beyond the standardized production protocols currently in use. The partial or even full replacement of live food, the modulation of the immune response, and the promotion of positive carry-over effects from one production stage to the next will play a key role to increase the productivity and profitability of seabass and seabream aquaculture.

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Prospects for the use of inert microdiets in shrimp hatcheries and nurseries

André Barreto, Wilson Pinto, Renata Serradeiro and **Luis Conceição**

The Pacific white shrimp (*Penaeus vannamei* Boone, 1931) has become one of the primary species produced in aquaculture. Although traditionally farmed in Asian and South American countries, production is expanding to European territory with a revamped approach, where quality is prioritized over quantity. The provision of high-quality adults relies on optimized husbandry practices during the early life-stages of shrimp, which still require to be enhanced. Shrimp hatchery production still depends on the costly live-prey production chain, generally based on *Artemia*, as the carnivorous stage mysis in traditional protocols requires a constant supply of the latter. The benefits of using live-prey are overshadowed by sub-optimal nutritional values and the potential to act as vectors for pathogenic agents, affecting the predictability of larval production. Therefore, the design of a high-quality inert microdiet that can completely, or partially in co-feeding regimes, replace live feeds, as well as improve PL nutritional status, would represent significant economic and ecological benefits for shrimp aquaculture. Although a great deal of work is still required, a similar path has been paved for some relevant cultured fish species with great success, which can inspire the shrimp industry. A better understanding of the nutritional requirements and digestive capacities at different larval stages will enable to progressively enhance inert diet formulations to better suit the needs of a developing shrimp organism. Nutritional studies on the first developmental stages of shrimp found in the literature are promising but scarce, with some reports of partial and/or total successful replacements of live feeds with inert diets.

Figure 1. One of Riasearch Lda RAS systems where shrimp larval trials are performed (left) and Pacific white shrimp post-larvae feeding on inert microdiets in one of the trial tanks (right).

Most microdiet formulation tests have been done on post-larvae and early juvenile shrimp and a wide range of ingredients has been contemplated. The rapid expansion of shrimp farming has generated increases in the demand for commercial feeds for the on-growing phase of production, that represents the bulk of

Shrimp's high tolerance to the inclusion of different ingredients in formulated diets opens prospects for an introduction of inert diets at earlier developmental stages and high live-prey replacement and the production of more eco-friendly diets for the post-larval/juvenile stage.

inert diet consumption. These microdiets commonly include fish and squid meals as the primary protein sources due to their balanced composition in essential amino acids, fatty acids, vitamins and minerals, but also due to their high palatability, which increases ingestion at early developmental stages. However, fishmeal supply has become limited and costly due to environmental concerns. Accordingly, the partial or total replacement of fish meal in aquaculture diets for more accessible and economical high-quality ingredients has been a prioritized field of study. Nonetheless, use of alternative ingredients at early life stages of shrimp should be regarded with caution. Soybean meal is one of the most widely available plant protein sources used in alternative aquafeed formulations, with demonstrated capacity to replace fishmeal in high proportions in practical juvenile shrimp diets.

In comparison with fishmeal, marine macro and microalgae have also been shown to produce similar or higher shrimp growth performances when incorporated as whole or broken biomasses in low dietary inclusion levels. Bioflocs, a by-product of biofloc technology systems, have also been successfully converted to meal form and used as an alternative protein source for *P. vannamei* diets. These consist of heterotrophic bacteria, phytoplankton, organic matter and grazers of the bacteria. Although research efforts have been focused on testing the effectiveness of different protein sources, lipids and carbohydrates also represent the important parcels of shrimp diets indicating that even more changes to formulations are possible. These can minimize the protein requirements with no adverse effects if properly chosen and enable the reduction of production costs as they are the least expensive form of dietary energy.

Optimizing microdiets for shrimp post-larvae/juveniles

Recently, two experimental trials were performed in Portugal by SPAROS Lda - RIASEARCH Lda aiming at evaluating growth and survival of *P. vannamei* post-larvae/juveniles fed microdiets formulated with diferent ingredients. In the first trial, three different experimental premium microdiets were tested: 1) mix of plant-based and marine proteins; 2) marine and vegetable protein mix rich in plant-based phospholipids; and 3) macroalgae-rich and formulated to have high water-stability and high performance (Table 1).

TRIAL 1	Marine + Plant proteins	Marine + Plant proteins and Plant Phospholipids	Macroalgae
Main Ingredients	Krill meal, squid meal, fish protein hydrolysate, wheat gluten, pea protein	Krill meal, squid meal, fish protein hydrolysate, wheat gluten, pea protein, soy lecithin	Krill meal, squid meal, fish protein hydrolysate, wheat gluten, pea protein, macroalgae mix
Crude protein (g/100 g)	63	63	62
Total lipid (g/100 g)	17	17	16

Table 1. Main ingredients and crude protein and total lipid levels of the shrimp experimental microdiets tested on the first trial.

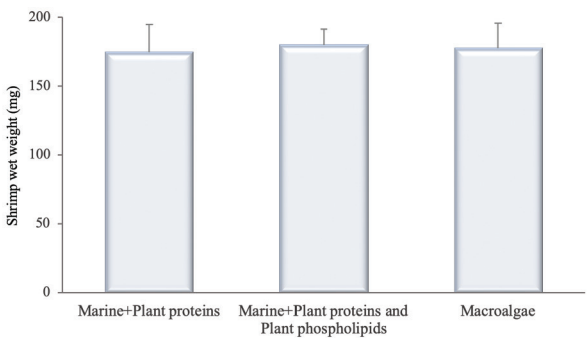

Figure 2. Final body wet weight of Pacific white shrimp reared for 21 days under three dietary treatments. 1) experimental diet rich in marine and plant-based proteins; 2) experimental diet rich in marine and plant-based proteins and plant-based phospholipids; 3) experimental diet rich in macroalgae and formulated to have high water-stability and high performance (n=3 experimental units).

TRIAL 2	Squid meal + Krill meal	Krill meal	Fish meal
Main Ingredients	Squid meal, krill meal, fish protein hydrolysate, wheat gluten, pea protein	Krill meal, squid meal, fish meal, fish protein hydrolysate, wheat gluten	Fish meal, squid meal, fish protein hydrolysate, wheat gluten
Crude protein (g/100 g)	62	66	66
Total lipid (g/100 g)	17	13	13

Table 2. Main ingredients and crude protein and total lipid levels of the microdiets tested in the second trial.

Pacific white shrimp post-larvae (2 mg wet weight) were reared for 21 days in 40L tanks part of a RAS system (Fig. 1) at a density of 5000 shrimp/m^3. Shrimp were co-fed with *Artemia* nauplii and microdiets the first three days, with shrimp promptly exhibiting inert diet acceptance (Fig. 1).

The different experimental microdiets had very good performances, with no significant effects on growth and survival between them. At the end of the trial, shrimp from all treatments achieved final wet weight values around 180 mg (Fig. 2), feed conversion ratios (FCR) values around 0.67, relative growth rates (RGR) around 23 % day^{-1} and survival around 97 %.

On a second trial, a premium commercial weaning microdiet designed for flatfish, rich in squid and krill meals (SPAROS, Portugal) was tested together with two experimental microdiets: 1) one rich in krill meal, and another with a marine protein mix rich in fishmeal followed by the microdiet rich in krill meal in the second half of the trial (Table 2).

Pacific white shrimp juveniles (0,328 g wet weight) were reared for 29 days in 40L tanks part of a RAS system at a density of 2500 shrimp/m^3. It was possible to observe a diet impact on shrimp weight (Fig. 3). Shrimp fed the microdiet rich in krill meal had a higher weight than those from the remaining treatments.

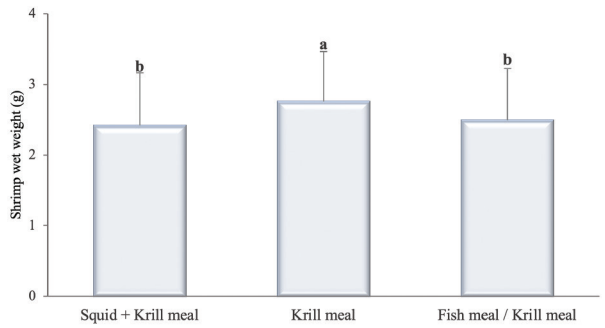

Figure 3. Final body wet weight achieved by Pacific white shrimp reared for 29 days under three dietary treatments. 1) premium commercial weaning microdiet diet for flatfish, rich in squid and krill meals; 2) experimental microdiet rich in krill meal; 3) experimental microdiet rich in fishmeal and experimental microdiet rich in krill meal the second half of the trial (n=4 experimental units). Different subscript letters indicate significant statistical differences between treatments ($p>0.05$).

However, no significant differences were observed on the remaining growth parameters analysed, with all treatments achieving FCR values around 1.12 and RGR around 7.3 % day^{-1}. As for survival, shrimp fed both experimental microdiets had a significantly lower survival than those fed the commercial flatfish diet rich in squid and krill meals (94 ± 2 % and 97 ± 0.8 %, respectively).

The inclusion of krill meal as the main protein source instead of fishmeal marginally enhanced shrimp growth. Nevertheless, results from both trials suggest that the *P. vannamei* has a high tolerance to changes in diet formulations and good growth can be achieved utilizing a wide range of ingredients in the microdiets. Additionally, results suggest the importance of designing species-specific microdiets, as utilizing a diet for flatfish did not produce identical growth results as a diet designed for *P. vannamei*.

Future prospects

Awareness has increased to the importance of early nutrition as a critical component for larval production, as sub-optimal nutrition during early developmental stages has long lasting effects on quality criteria. Although further research needs to be conducted to better understand the nutritional requirements of shrimp during the early developmental stages, its high tolerance to the inclusion of different ingredients in formulated diets opens prospects for an introduction of inert diets at earlier developmental stages and high live-prey replacement and the production of more eco-friendly diets for the post-larval/juvenile stage, contributing to tackle the ecological challenges faced by shrimp farming.

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Insights on hatchery feed for *Penaeus monodon* postlarvae: custard diets supplemented with microbial biomass Novacq™ improve survival and enhance growth of larger animals

Artur N. Rombenso, Ha Truong, Cedric Simon, CSIRO

The use of microbial biomass as a complimentary ingredient or feed additive has been highlighted as a promising strategy to promote growth and health of Penaeid prawns (*Litopenaeus vannamei* and *Penaeus monodon*) and sustainability of aquafeeds (Burford et al., 2004; Kuhn et al., 2008, 2009; Glencross et al., 2013, 2014, 2015; Arnold *et al*., 2016; Qiu and Davis, 2018). Currently, there is a variety of microbial origin products including pro- and pre-biotics, immune-stimulants, and proteins, among others. Their distinct microbial composition and manufacturing procedures generally result in different nutritional characteristics used in aquafeeds and aquaculture systems.

Novacq™, a microbial biomass-based dry ingredient, has been successfully applied in juvenile (3-8g) *P. monodon* diets at inclusion rates ranging from 5-10 percent supporting better growth (up to 50 percent increase), increasing feed intake, and improving performance on suboptimal diets (lower protein content or with no marine products like fishmeal) (Glencross *et al*., 2013, 2014, 2015; Arnold *et al*., 2016). However, there is no report on the usefulness of microbial biomass in *P. monodon* postlarvae.

Egg custard diets have been investigated in the freshwater prawn *Macrobrachium rosenbergii* larval rearing and postlarvae due to their excellent nutritional profile and high digestibility (Alam *et al*., 1995, Nair et al., 2007; Shailender et al., 2012; Sin and Shapawi 2017). However, there is a lack of nutritional studies evaluating the suitability of formulated custard diets in *P. monodon* postlarvae. Accordingly, we assessed the suitability of custard diets (experiment 1) and the effects of Novacq™ supplementation in custard diets (experiment 2) on *P. monodon* postlarvae survival and growth performance.

Material and methods

Experiment 1

Two dietary treatments were tested, a control custard and a commercial diet (Frippak, INVE). For the control custard diet, all ingredients (Table 1) were mixed using a food processor and cooked in the microwave for approximately 15 minutes until a spongy texture was achieved. The dough was then spread evenly onto a tray and oven dried for 12 hr at 60°C. Once cooled, the diet was ground and sieved to three crumble size-ranges (125-250 µm, 250-350 µm, 350-500 µm).

Approximately 3,840 postlarvae (PL3) were individually counted and randomly allocated to sixteen 6-L tanks at a stocking density of 40 PL/L (240PL/tank) at Bribie Island Research Centre (BIRC, Queensland, Australia). The experimental system was set up with a flow-through system with continuous aeration and

These findings highlight the importance of using highly digestible ingredients and raise questions about the most appropriate dietary levels of protein and lipid, and protein to energy ratio for P. monodon PLs.

water temperature between 29-30 ºC. Photoperiod was set at 12L:12D with 30 percent light intensity. Water quality parameters including temperature = 29.3 ± 0.0 °C and D.O. = 6.64 ± 0.0 mg/L were assessed daily, whereas salinity = 32.1 ± 0.0 ppt was measured every two days. Postlarvae were fed seven times daily with auto feeders (18:00, 21:00, 24:00, 03:00, 06:00) and hand-fed (09:00 and 15:00) for 21 days. Feeding ratio

	Experiment 1	Experiment 2	
Ingredients (grams as is)	**Control**	**Control**	**Novacq™**
Whole eggs	490	490	490
Fish oil	53	50	50
Baker Yeast	48	48	48
Lecithin	6	6	6
Frozen polychaetes	95	95	95
Vitamin mix	6	6	6
Whole FD squid	0	13	13
Casein	38	38	38
Novacq™	0	0	127
Fishmeal	135	127	127
Wheat flour	53	51	0
Diatomaceous earth	76	76	0
Total	1000	1000	1000

Table 1. Dietary formulations.

was based on the stocking density and it was slightly overestimated to ensure feed availability for all PLs. Feed was delivered on top of the air stones to facilitate dispersion throughout the tank. Artemia was also provided at 09:00 and 15:00 during the first three days of the experiment based on CSIRO in-house protocol to ensure weaning onto formulated diets. From trial-day 1 to 7 crumble size was between 125-250 µm, day 8-14 crumble size 250-350 µm, and day 15-21 crumble size 350-500 µm. Tanks were daily siphoned to remove the uneaten feed and feces.

	Experiment 1		Experiment 2	
	Control	Commercial	Control	Novacq™
Dry matter	97	94	83	79
Protein	40	50	45	45
Lipid	24	12	22	24
Ash	24	12	19	21
Carbohydrate	9	20	14	10
Gross energy (MJ/Kg)	21	21	22	22

Table 2. Proximate composition (percent) of experimental diets.

Experiment 2

Two treatments were tested, the control and Novacq™ (microbial biomass) custard diets. The control treatment was a custard-based formulation, which served as a basal formulation for the second treatment in which Novacq™ was supplemented at 12.7 percent (Table 1). Custard diets were manufactured as mentioned in experiment 1. However, in this trial diets were sieved into two crumble sizes <500 µm and between 500-750 µm. Images of the commercial diet from experiment 1 and the control and Novacq™ custard diets from experiment 2 taken from a real-view perspective and under a light microscope are shown in Figures 1(a-f).

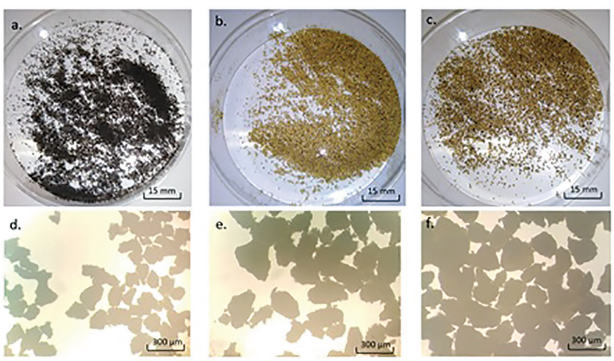

Figure 1. a, b, c depict images taken from a real-view perspective and images d, e, f depict images taken under a light microscope of the dietary treatments including the commercial diet from experiment 1, control custard diet from experiment 2 and Novacq™ custard diet from experiment 2, respectively.

A total of 2,880 postlarvae (PL15) were randomly allocated to twelve 80-L tanks at a stocking density of 3 PL/L (240 PL/tank) at BIRC. The experimental tanks were set up with a flow-through system with continuous aerated and water temperature between 29-30 ºC. Photoperiod was set at 12L:12D. Water quality was maintained within the suitable for this species with an average water temperature of 29.3 ± 0.1 °C and dissolved oxygen of 6.31 ± 0.01 mg/L.

Postlarvae were fed seven times daily with auto feeders (18:00, 21:00, 24:00, 03:00, 06:00) and hand-fed (11:00 and 15:00) for two weeks. Feeding rates started

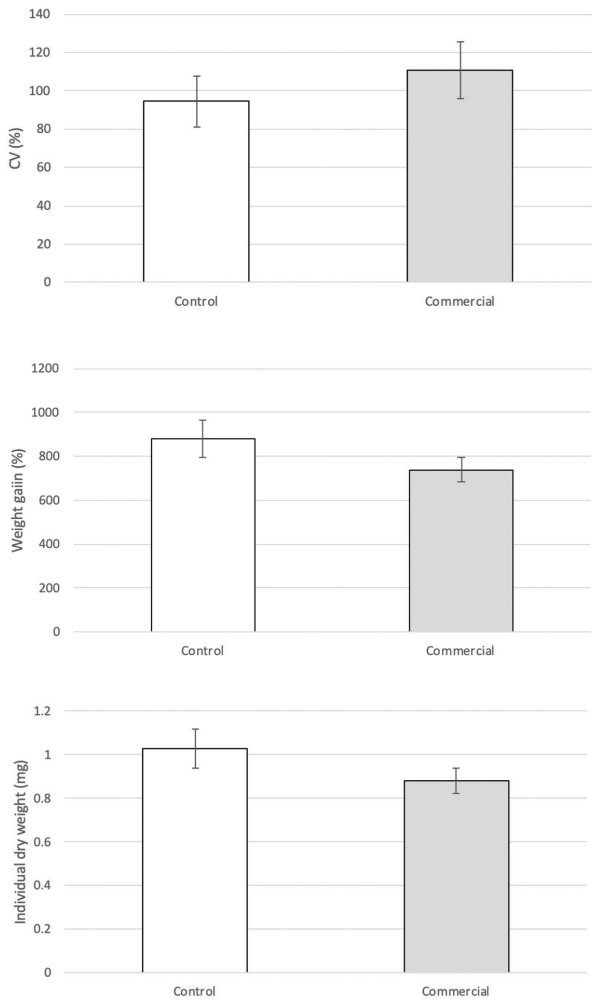

Figure 2. Individual dry weight (mg), weight gain (percent) and coefficient of variation (CV; percent) of *P. monodon postlarvae* (3-24) fed the control custard and commercial diets (experiment 1). No statistical difference was observed by t-test ($p > 0.05$).

at 100 percent of biomass during the first week, and 75 percent during the second week. From trial-day 1 to 7 crumble size was between <500 µm, and day 8-14 crumble size 500-750 µm. Tanks were siphoned every other day to remove the uneaten feed and feces. No other feeds nor live feeds were used.

Data from experiments 1 (n=8) and 2 (n=6) were analyzed by t-test using NCSS 12.

Results and discussion

Experiment 1

Postlarvae readily accepted the control custard diet, which performed equivalent to the commercial diet with no differences in survival and growth performance (individual dry weight, weight gain, and coefficient of variation; Fig. 2-4). This was achieved despite the differences in dietary proximate composition, i.e. the commercial diet displayed higher protein levels (50 percent vs. 40 percent protein) and reduced lipid

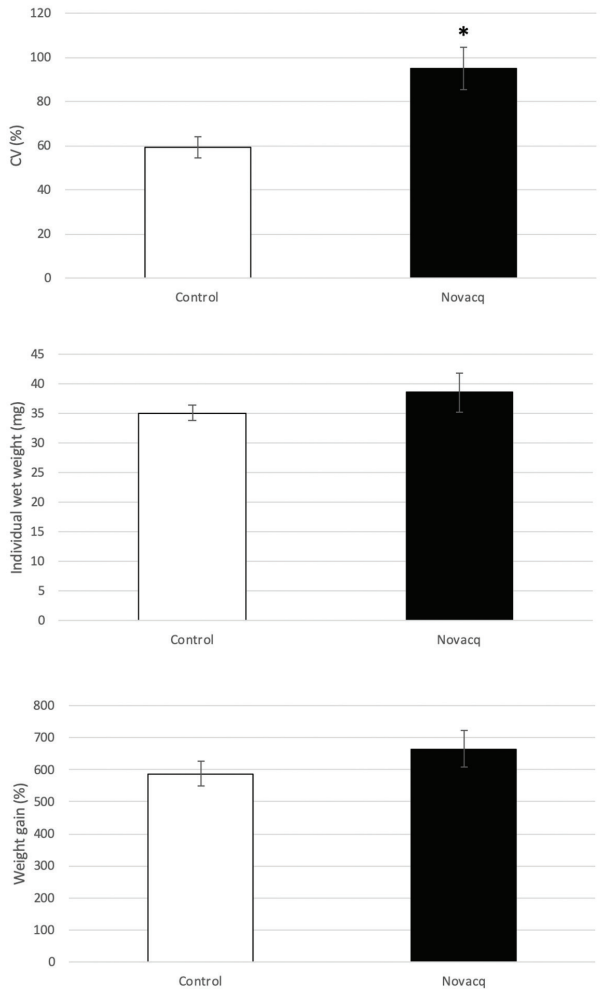

Figure 3. Individual dry weight (mg), weight gain (percent) and coefficient of variation (CV; percent) of *P. monodon postlarvae* (3-24) fed the control and Novacq™ custard diets (experiment 2). No statistical difference was observed by t-test ($p > 0.05$).

content (12 percent vs. 24 percent lipid) compared to the control custard diet (Table 2). Postlarvae were able to maintain suitable growth with 20 percent less protein in the custard diets, suggesting the increased availability of better-quality protein which improved digestibility and utilization of nutrients by PLs. These findings highlight the importance of using highly digestible ingredients and raise questions about the most appropriate dietary levels of protein and lipid, and protein to energy ratio for *P. monodon* PLs.

Experiment 2

Custard diets (control and Novacq™) were accepted and utilized by PLs with no differences in mean individual wet weight and weight gain (Fig. 3). Interestingly, Novacq™ custard diet presented higher survival (7 percent increase) and CV (95 percent vs. 59 percent; Figure 3; $p<0.05$) compared to the control custard diet and enhanced the growth of the bigger PLs (Fig. 4). Indeed, the average of the biggest 15, 10 and 5 PLs fed Novacq™ custard diets were superior to those fed the control custard diet ($p<0.05$; Fig. 5). As a diverse source of marine microbes, Novacq™ could closely mimic the food sources of prawns in nature, thus supplying an unknown nutrient deficiency or growth factor which enhances the robustness of PLs in hatchery systems. Considering these PLs were produced from non-selected broodstock, large variation in size and the presence of runts with limited scope for growth was expected. It appeared for animals that were predisposed to grow fast, Novacq™ provided a significant boost in their performance. It was not possible to accurately measure feed intake due to the very small animal and feed biomass, but higher intake, especially in the better performing PLs, is a possible explanation for these findings.

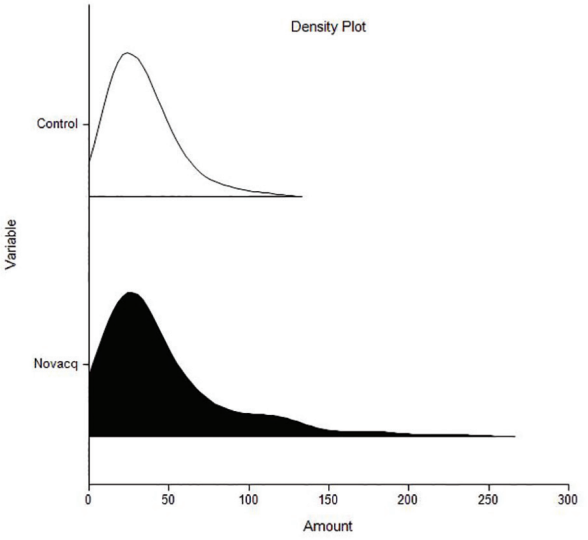

Figure 4. Density plot displaying individual weight distribution of *P. monodon postlarvae* (3-24) fed the control and Novacq™ custard diets (experiment 2).

Summary

Our findings demonstrate the suitability of custard diets in *P. monodon* postlarvae (PL3 to PL24) and that Novacq™ supplementation (12.7 percent) in custard diets is beneficial in PL nutrition, improving survival and enhancing the growth of better performing PLs.

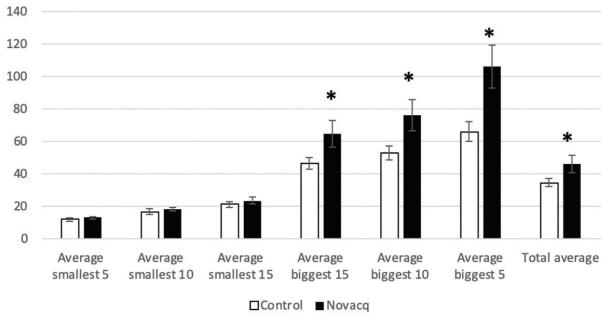

Figure 5. Average individual weight (mg) per weight class of 150 randomly selected *P. monodon postlarvae* (3-24) fed the control and Novacq™ custard diets (experiment 2). Statistical differences were observed by t-test ($p < 0.05$).

Acknowledgments

We would like to thank Dean Musson, Brian Murphy, Isaak Kadel, Laura Markham, Simon Irvin, and David Blyth for experimental and animal maintenance as well as Nicholas Bourne and Sue Cheers for composition analyses.

References available on request

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Recent results from research in microdiet performance

Bernd Ueberschär & **Ingmar Høgøy,** Molofeed

Molofeed, a Norwegian start-up which is dedicated to developing microdiets that aim to substitute live feed for fish and shrimp larvae in early stages, was involved in the process of improving inert diets and has developed a new generation of microencapsulated feed.

In marine larviculture, microparticulate diets have begun to rule the early larval stage hatcheries and the complete replacement of live feed in marine larval rearing is probably one of the most desired improvements in marine larval fish production. Part of the desire for a full replacement of Artemia nauplii in larval nutrition is the increasing shortage in good quality Artemia cysts coupled with an ever-increasing price and the "natural" deficiencies in common live feed (i.e. rotifers and Artemia nauplii), which need to be boosted with several enrichments to achieve acceptable results. But this approach has its limitations and apparently, technically well-designed microdiets can be used as a "transporter" for any kind of macro-, micronutrients and more.

In this article, Molofeed presents some of the recent results from their research activities in this area.

Methods

Microdiets will become widely accepted if the same performance as in the common live feed protocols are achieved. The major parameters which are considered in hatcheries are the growth rate, occurrence of malformations and survival rates throughout the larval stages. In the process of developing microdiets, specifically these three issues were thoroughly considered and tested in a number of research trials. The results were compared to the performance of larval groups fed with the standard live feed protocol (*Artemia* nauplii). In these trials, seabass larvae were used as the "model species" throughout the research and developmental process of the various recipes. A typical feeding schedule of these trials is depicted (or shown) in Figure 1. Four different feeding regimes were applied with *Artemia* feeding throughout the entire trial period (standard live feeding regime) and compared with an optimized recipe, R 1, amalgamating all knowledge gathered in the developmental process of microdiets.

Another recipe, R 2, designed with higher caloric content in order to support the requirements of larvae specifically in the early ontogenetic stage was used to compare the effects of an early and late weaning schedule.

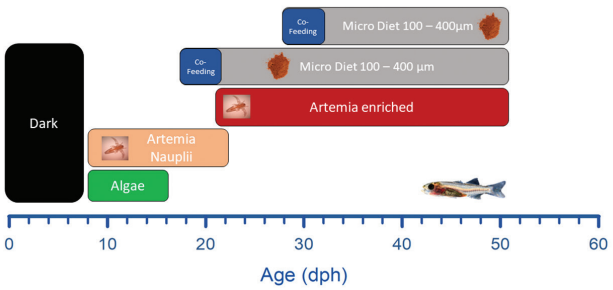

Figure 1. Typical feeding regime in research trials with sea bass larvae. First feeding was 7 days after hatching. First feed was newly hatched *Artemia nauplii* (AF 430, INVE), followed by 48h-old enriched Artemia nauplii (from 21 dph). Complete weaning to microdiets was conducted at 21 dph (early weaning, R 2) and 32 dph respectively (late weaning, R 1 & R 2). A co-feeding period of 3 days was applied in each microdiet group. The rearing trial was finished at 50 dph.

Survival

The survival rate beyond metamorphosis is one of the important indicators of the quality of the rearing conditions and a suitable feeding regime. The survival rate of the late weaning groups fed with Molofeed microdiets (R 1 & R 2) is comparable (Fig. 2) with the standard live feeding protocol (*Artemia* nauplii). The group with early weaning (R 2) shows, however,

a significantly lower survival rate, which is still a typical effect of early weaning. Part of the reasons for significantly lower survival may be attributed to the weak acceptance of microdiets in early larval stages. At this age, there are a significant number of larvae which are not able to switch quickly from live feed to microparticulate feed which resulted in a mortality peak in this treatment shortly after switching to microdiets. Another reason could be the limited digestion capacity in this age which prevents these larvae to digest microdiets as efficiently as in later stages and may contribute to the elimination of those larvae which are "naturally" less viable and disappearing at a later stage anyway. There is certainly the need for further elaboration on how to optimize microdiets for a more successful early weaning.

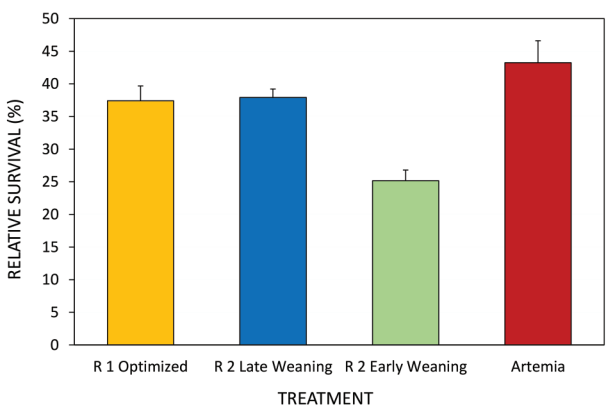

Figure 2. Comparison of the mean mortality for each treatment at the end of the trial (50 dph). The mean values were calculated from 3 replicates for each recipe (all surviving larvae counted at the end of the trial).

Growth rate

The growth rate or weight of the larvae at the end of an experiment is an overall-indicator of the rearing settings. It integrates the entire rearing conditions, such as biotic and abiotic regimes (i.e. water parameter, feeding regime, feed quality and quantity). The results from the experiments clearly depict that the optimized microdiets (R 1) in the late weaning group achieved a higher weight compared to the live feed group at 50 dph (Fig. 3). The groups with early and late weaning fed with the microdiet recipe R 2 achieved the same weight at the end of the trial, demonstrating, that early weaning is not necessarily a disadvantage with properly designed microparticulate feed, considering the growth rate of the larvae.

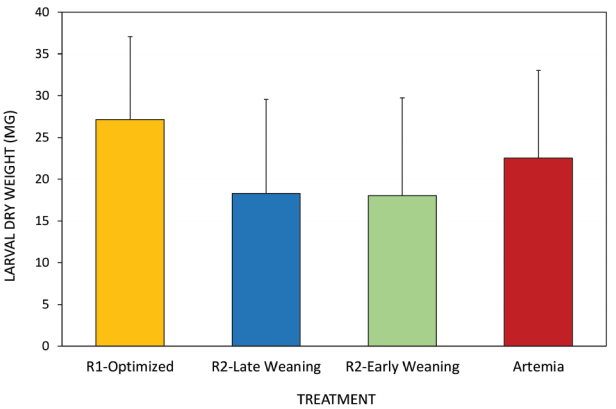

Figure 3. Mean dry weight per larva from the final sampling at 50 dph. Larvae were individually freeze-dried and weight measured with a microbalance. Mean values with standard deviation of 45 individually measured larvae per treatment from 3 replicates (corresponds to 15 individual larvae from each tank).

Skeletal anomalies

Malformations are among the most difficult issues to tackle when feeding marine fish larvae with microdiets. This used to be a major problem, specifically if early weaning (i.e. < 20 dph) was applied. Malformations are an important issue in the rearing of marine fish larvae since the fingerlings which show obvious deviations from the usual morphology are normally not accepted from on-growing farms and hatchery operators put a lot of attention on the occurrence of malformations. The following results provide evidence on the occurrence of malformations in the groups fed with microdiets (optimized recipe R 1, and R 2 for early and late weaning, compared to *Artemia*). Only kyphosis and lordosis were considered.

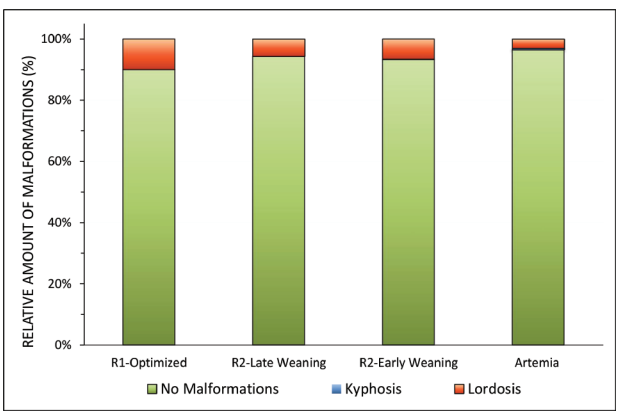

Figure 4. Relative amount of malformations for all treatments. Mean value of 3 replicates for each treatment. About 100 larvae per replicate were examined for malformations (about 300 per treatment). Lordosis is the most prominent malformation for all treatments. Kyphosis was mainly visible in the live feed group, but in a small amount.

The results demonstrated, that the rate of malformed larvae in the microdiet groups is close to the live feed group and even the early weaning did not result in a higher rate of malformations at the end of the trial. It is obvious that lordosis was the most prominent deformation, kyphosis was not an issue, with the exception of a small segment in the *Artemia* group. A small amount of malformations is usually inevitable under aquaculture conditions and can be considered as "white noise".

In order to demonstrate, that the shape and size of the larvae fed with R 1 are similar to the Artemia group examples of photos from the final evaluation of the larval size and occurrence of malformations are presented (Fig. 5 a,b). There is almost no difference in the morphology, size and shape of the larvae.

Figure 5 a-b. Arrangement of larvae at the end of a rearing trial for the measurement of individual length and rating of malformations. The example shows part of the about 100 larvae arranged per tank, in this case R 1 (left) compared with the live feed group (right, *Artemia*).

Technical quality of microdiets

The technical quality has a significant impact on the overall performance of microdiets and is often an underestimated issue. The Molofeed products use a specific and patented microencapsulation technology which reduces leaching and results in an excellent technical quality of the product. The technical properties of Molofeed microdiets help to minimize the deterioration of the water quality due to remaining food particles in the rearing tanks. The Molofeed microdiets do not disintegrate in the water and excessive feed can easily be removed from the tank bottom. The uniformity of the particle size and shape and the surface structure are quality indicators and can facilitate acceptance and feeding success (Fig. 6a).

In addition, the behavior of microdiets under humid air conditions, such as is common in tropical areas is an important feature when considering the use of microdiets under these conditions. The behavior of the Molofeed products was tested in a tilapia hatchery in Malawi (Fig. 6b). It was demonstrated that even under high air humidity, the microdiets maintain their powder-like structures and stay pourable.

Molofeed achievements

In summary, the results which were presented here demonstrate that replacement of live feed with Molofeed microdiets from 28 dph yields reasonable results which are comparable with the performance of the live feed group. Even early weaning has yielded in this trial comparable good results, although the trade-off is apparently still a higher mortality rate but the weight was the same in both treatments for R 2. Further research and improvements in the Molofeed microdiet recipes will show, if the deficiencies for early weaning can be diminished. It would be a great advantage for hatchery operators, if early weaning (e.g. around day 18 in seabass) is a reasonable option in the future.

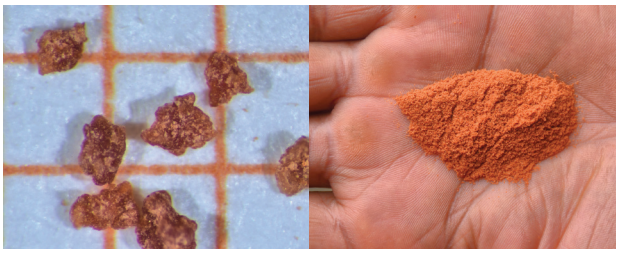

Fig. 6a (left) and Fig 6b (right). Visual inspection of a microdiet batch(left). In this picture, the size class 200 - 400µm was monitored. Scale size is 0.5mm. The special feature of Molofeed microdiets is the capsule-like character. There are some variabilities in the surface of the capsules, which disappear in the re-hydration process. Under high air humidity, the Molofeed products maintain their pourable character, thus making it suitable also for feeding larvae under tropical conditions.

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The unrivaled benefits of fresh natural high-density live algae in aquaculture hatcheries for larval production

Tara Wyman & **Philippe Bois,** Algafeed

Live microalgae benefits in larval productions are second to none. Many attempts have been made to substitute it with dry feed, algal paste and various substitutes but nothing to date can replace natural live microalgae in larval diet without compromising animal health and survival. Shellfish exclusively consume microalgae all their lives. The first five to six days of a shrimp larvae from Zoea to Mysis is exclusively relying on nutritious microalgae and no substitute has yet been identified. Finally, fish larvae greatly benefit from having live algae earlier on from efficient green water that allows to keep oxygen level high and provide the little food they need as well as enriched zooplankton feed (rotifer, copepod, Artemia) in order to attain healthy early growth.

Algafeed photobioreactor

Algafeed has developed a modular patented photo-bioreactor system that allows for the semi-continuous growth (10 to 20 percent of each tank algal biomass is harvested once daily) of highly concentrated strains typically 10 to 50 times denser that is generally observed in traditional hatcheries. This helps maintain the algal biomass in an exponential growth state virtually indefinitely as well as maintaining it at high density. Typical daily stable densities achieved are shown in Table 1 for five common strains used by the aquaculture industry. High algal density comes with addi-tional benefits since minimal volumes are required to reach the targeted cell density. This greatly dilutes nutrient (nitrate, phosphate) to virtually insignificant levels allowing to keep high water quality at all time.

Rotifer trials

Availability of a robust source of live microalgae combined with an adequate feeding regimen can significantly improve aquaculture practices. As a first example, rotifer cultivation was improved using a mix of 75 percent /25 percent *Nannochloropsis*/T-*Isochrysis lutea* microalgae when compared to algal paste using the same cell numbers. As shown in Figure 1, nearly 2.5x more rotifer densities increased were observed between live algae and algal paste. Not only quantitative improvement was observed but also qualitatively rotifer populations showed significant increase in gravidity by 2-fold with many rotifers having 2, 3, or 4 eggs in the live algae populations when only rotifers with one eggs were seen in the algal paste tanks. It is suspected that the live algae is readily available for rotifer consumption as it remains easily in the water column, in particular these two small algae strains. In comparison, most of the algal paste ended at the bottom of the rotifer growth tanks, making it unavailable for consumption. The quality of the resulting rotifer biomass fed with live microalgae also increased by 30 percent the fish larvae survival from 50 percent to 80 percent demonstrating the benefits associated with using live algal biomass.

Table 1

	T-Isochrysis lutea	*Chaetoceros muelleri*	*Thalassiossira weissflogii*	*Tetraselmis sp.*	*Nannochloropsis gaditana*
Density range (million cell/mL)	25-35	30-40	5-10	8-10	100-300
Size range (micron)	5-10	5-20	15-30	10-20	3-5

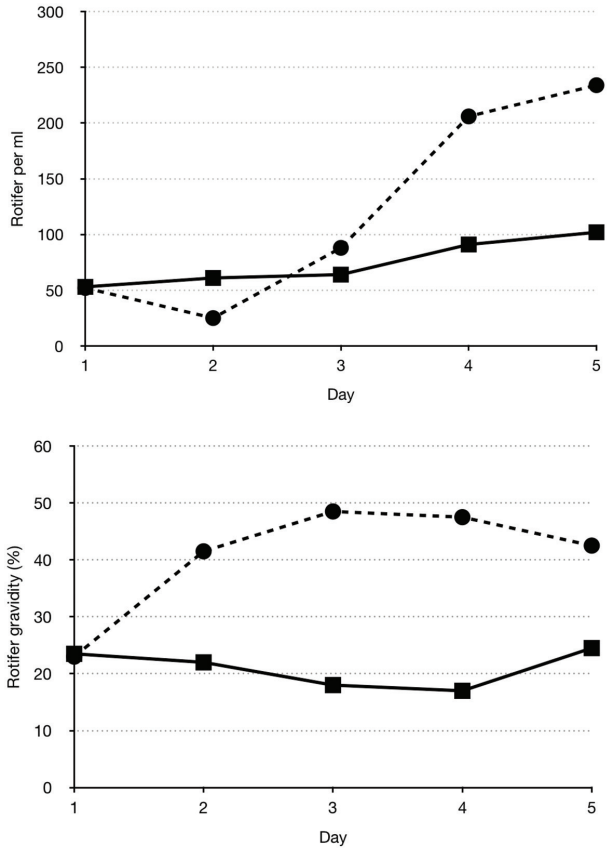

Figure 1. Comparative growth of rotifer is shown. Rotifer density per mL (left) and gravidity (rotifer seen with eggs) (right) are graphed. The average of multiple experiments is shown. Live algae are shown with a dash line and rounds, algal paste is displayed with solid line and squares.

Shellfish trials

Significant improvements were also observed in shellfish hatcheries. Using a feeding table as a guideline (Table 2), significant survival was achieved as well as extremely mobile and healthy animal using hard clams. Once a baseline microalgae density between 25,000 to 75,000 cells/mL was achieved, the density was maintained by adding the necessary algae required as per the consumption rate indicated in Table 2. Two types of mixes were used. A pre-set mix of 60 percent T-*Isochrysis lutea* and 40 percent *Chaetoceros muelleri* per volume with average densities ranging from 25 to 30 million cells per mL and a post-set mix of 60 percent T-*Isochrysis lutea*, 20 percent *Chaetoceros muelleri*, 10 percent *Tetraselmis sp*. and 10 percent *Thalassiosira weissflogii* per volume with average densities ranging from 20 to 25 million cells per mL. Again, the live algal biomass remained in suspension in the water column, allowing the hard clam larvae to thrive. Due to sufficient algal density and the quality of the semi-continuously grown algae, clam larvae were ex-tremely active showing homogeneous size and digestive track food content (Fig.2).

Life stage	Age/size	Average consumption rate (cell/clam/day)
Veliger	1-2 days	3,000
	3-5 days	7,500
	5-8 days	12,500
Pediveliger (pre-set)	8-14 days	20,000
Early post-set	300-400 microns	35,000
Mid post-set	400-600 microns	60,000
Late post-set	600-1200 microns	100,000

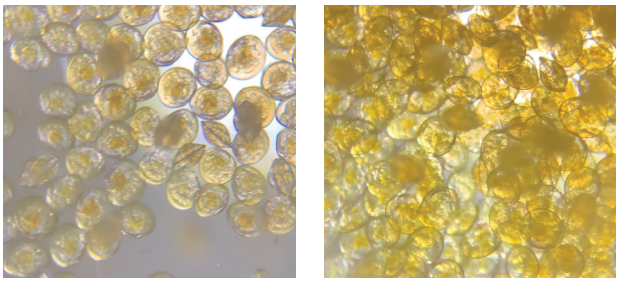

Figure 2. Two different densities of hard clam 7 days old larvae are shown. Note the homo-geneous size and digestive track coloring indicative of suffcient food availability. Also, no empty shells are readily visible.

Shrimp trials

Finally, following a similar strategy, a live microalgae mix consisting of T-*Isochrysis lutea* and diatoms (*Chaetoceros muelleri* & *Thalassiosira weissflogii*) was used for the first four to six days of shrimp larvae from Zoea 1 to Mysis 1. The high algal density allowed for very little volume being added therefore reducing addition of excessive nutrients in the larvae water. Algae was added 12 hours prior to the shrimp Nauplii in the larval tanks in order to pre-condition the water. Microalgal biomass was readily visible in the larval gut (Fig. 3) as well as significant amount of fecal matter. Shrimp larvae were very active as well as a very robust growth. The ability from shrimp hatchery farmers to easily and reliably grow the necessary algal biomass required for their crop is paramount to their success. Current traditional methods are unreliable and a paradigm shift in algal cultivation is required in order to address the growing demand and growth of the shrimp industry.

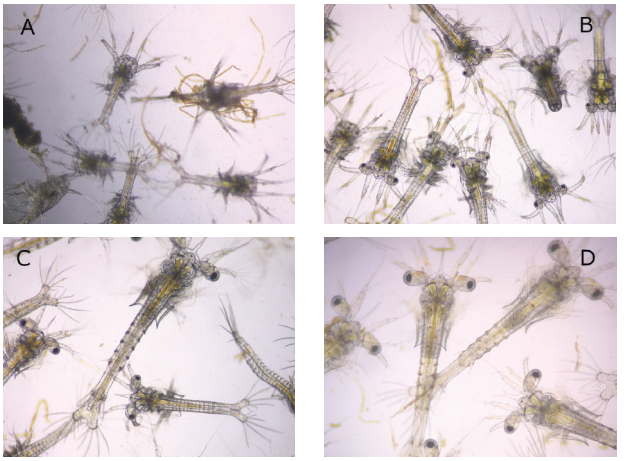

Figure 3. Representative pictogram of live microalgae fed shrimp larvae at day 1 (A), day 2 (B), day 3 (C), and day 4 (D). Note the straight larval gut full of golden algae as well as the fecal matter.

A robust and reliable source of microalgae is the foundation of any success in modern aquaculture hatchery. Large hatcheries will always require on-site production of high quality microalgae tailored to their needs. Algafeed technology allows hatchery farmers to thrive by limiting inefficiency, reducing excessive larval mortality, gaining reliability and ultimately growing their business.

Acknowledgements

This study was entirely financed by Algafeed. We are most grateful to Nicole Kirchhoff (Live Advantage Bait), Lee Bryan (Bryan Clams), and Carlo Palmese Bio Larva Laboratory) for their support with our full scale commercial testing and validation.

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Developments in aquaculture

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Aquaculture Ghent University

Improving broodstock and larval robustness: nature and nurture

Like in any other animal production sector, aquaculture has been profiting greatly from genetic selection programs which have been aiming especially at higher growth rate, disease resistance and adaptation of species to an aquaculture environment. The central concept behind any breeding program is identical. At the start, available genetic diversity is identified and captured as much as possible in a founding population. The genetic diversity lies at the basis of phenotypic diversity. Yet phenotypic diversity can also be the result of environmental influences. Provided phenotypic variance, as influenced by the environment, is minimal and kept under control, selection of favorable phenotypes will result in the retention of favorable alleles and/or genotypes which can be passed on to the next generation resulting in a broodstock population with an adapted genetic make-up (nature).

Darwinism is the basis of this approach, namely that selection of the most adapted individuals to a certain environment (here aquaculture) and/or selection of the most favorable genotypes is possible. Yet, there is more. As already mentioned, phenotypes are influenced by environmental conditions. It is now becoming apparent (but already suggested a long time ago by Lamarck) that acquired phenotypes can, to a certain degree, be passed on to the next generation. Or in other words, acquired phenotypes can be lasting and persisting in the next generation, independently from genetic variation (by nurture). Because of the fragmented scientific information on the mechanisms involved, a lot of different definitions and terminologies have been coined. Often the phenomenon is called 'transgenerational inheritance of phenotypes by epigenetic mechanisms'. Epigenetic modifications include DNA methylation, histone modification and non-coding RNA production. All these mechanisms modulate gene expression in an organism resulting in, for instance, cell differentiation or organismal adaptation to environmental circumstances. Some of these modifications, as instigated by environmental cues, are passed on to the next generation. This discovery adds another layer of complexity to genetic selection, yet from a practical point of view it opens up opportunities. It suggests that it should be possible to expose broodstock to certain environmental conditions, resulting in an acquired phenotype with the potential of the phenotype to be passed on (at least partly) to the next generation. However, the process is poorly understood at the moment and it is not clear which phenotypes can be passed on, or, for instance, which time window of opportunity during development should be targeted. It is also not clear if the phenomenon is equally maintained across species. With respect to robustness, it is becoming clear that prior exposure to an immune challenge in invertebrates can increase the response later in life (this is called trained immunity), but that the enhanced response can also be transmitted to the next generation, increasing, for instance, larval robustness. Using *Artemia* as a model organism, we could demonstrate that exposing animals during their development to a non-lethal heat shock twice a day, increases their tolerance to a lethal heat shock and to *Vibrio* challenge, and this phenotype is passed on to the following three generations although the effect is fading away with generations.

It is likely that in the near future epigenetic markers will become established with a clear link to certain phenotypes. This will generate the possibility to use genetic (nature) and epigenetic (nurture) markers in conjunction to characterize broodstock populations. Epigenetic markers could become especially useful in steering broodstock husbandry practices. It might help, for instance, to establish the type of stress a broodstock should be exposed to, to become more robust and to pass on this phenotype to the next generation producing, for instance, more robust larvae.

Precision shrimp genetics for local success

Robbert Blonk, Hendrix Genetics

In our previous article in Hatcheryfeed Magazine, May 2019, we showed that precision farming needs precision feeding and precision breeding. We explained how different environments and feeds require different breeding strategies to optimize performance. This is caused by what breeders call “Genotype by Environment” (GxE) or “Genotype by Diet” (GxD) interactions. We gave several examples based on our own experience and literature. For example, trials with the Hendrix Genetics’ Kona Bay soy tolerant strain of whiteleg shrimp showed 24 percent faster growth on soy diets compared to regular shrimp diets based on fishmeal. Conversely, shrimp selected for growth on regular diets do not thrive as well on diets with a high level of soy. Based on this research, we concluded that specific diets need a specific shrimp or vice versa.

In this article, we will take this concept further to a global level. We will show there is a significant value of breeding for the entire shrimp value chain. We will show the impact of interactions for this value. In addition, we will show this justifies investment in central, expert organized breeding programs with local breeds and feeds.

Global shrimp, local solutions

Global shrimp production is dominated by two species, namely white leg shrimp and black tiger shrimp. However, the environments where these animals are cultured are diverse. Although the common denominator is the equatorial band as main production area, that’s really where the similarities end. Shrimp are produced in Europe, Middle East, Africa, India, Bangladesh, South East Asia, China, Latin America, Central America; even in RAS systems which can be located virtually everywhere. Other factors as high or low-density growth conditions, different feed quality and types, and pressure from different diseases add to the mix that justifies precision breeding.

The benefits of using the right strain

For a breeding company, it is tempting to breed one solution - one strain - to supply the industry, as obviously this is cheaper with investment and R&D costs spread out over a large volume. However, if one considers the value of a precision breeding program for the shrimp value chain, different conclusions should be drawn. For example, let’s have a look at the typical output of a breeding program, called the genetic response. The genetic response is the improvement of a population compared to the previous generation due to genetics. An achievable value for long term genetic response for growth in a typical well-organized

multi-trait breeding program is 10 percent or higher with each generation. This means that compared to an arbitrary base population, new generations are improving with 10 percent each generation. In the first new generation, growth of shrimp will increase with a factor 1.10. In the second generation, growth improves again with 1.10. Thus, compared to the base population, the second generation will be 21 percent (1.10^2) faster in growth. For survival traits, we generally see genetic improvements of up to approximately 5 percent per generation. Cumulative gains made in aquaculture in long term breeding programs show more than doubling growth rates (Janssen *et al*., 2017). With everything else being equal, it is a permanent improvement in the performance of the population.

When animals are not cultured in the environment where they were bred for, i.e. when GxE or GxD interactions play a role, one can easily lose 50 percent of the expected gain (in some cases even as far as resulting in negative trends). In that case, one would end up with 5 percent additional growth instead of 10 percent. We challenge the readers to calculate the impact of this 5 percent missed growth improvement per generation in their business. Multiplied with the total production volume, it is easy to see we are talking big numbers here. We conclude that tailored strains from precision breeding programs have a major impact on economic performance of production.

Breeding for impact - scale makes the difference

Well-organized breeding organizations do everything to get the most out of their animals, respecting long term breeding goals, animal welfare, and keeping a balance between traits of interest and levels of inbreeding. This requires specialist know-how, significant investment in facilities, people, IT, and new technologies such as genomic selection, data recording equipment on animals, and reproduction technology.
Still, breeding is a fixed cost business, especially in the case of shrimp where very few broodstock animals are needed to enable tons of production volume. The production of improved PL's or broodstock has more or less fixed-requirements for facilities and infrastructure. A critical mass of human capital is needed to get the job done but it does not really matter how large the output of the program is beyond the break-even point. Therefore, centralized large-scale breeding programs are likely to be the most successful as they can invest most in R&D at the lowest unit cost.

Global local shrimp breeding and feeding in Ecuador

Clearly, both precision breeding for tailored strains and centralized large-scale breeding have a major impact on production performance. This calls for breeding programs to be developed under local conditions, production types, and feeds, but at large scale and with input of global genetics expertise. An excellent incarnation of this idea is the recently launched Ecuadorian joint venture between global multi-species breeding company, Hendrix Genetics; global feed producer Skretting; and local shrimp producer, Ecuacultivos. The newly established company is aimed at production of genetically improved whiteleg shrimp PL's for the local market, building on R&D capacity and expertise of both multinationals, and local know how.

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Genetic engineering in aquaculture

John Buchanan, Center of Aquaculture Technologies

The first significant reports on the application of genetic engineering in animals appeared in the 1980s. The description of techniques for the creation of transgenic mice by microinjection of DNA launched the field of genetic engineering by providing a practical, if labor intensive, method for delivery of specific, exogenous DNA into the genome (Palmiter *et al,* 1982). Work in earnest in other species followed soon thereafter. The first reports of transgenic fish appeared in 1984 and 1985, using microinjection of exogenous DNA into the cytoplasm of fertilized eggs (Maclean and Talwar, 1984; Zhu *et al,* 1985). Fish and shellfish make appealing targets for genetic manipulation as they produce large numbers of eggs that are typically easily manipulated, and fertilization can typically be accomplished externally. Accordingly, successful creation of transgenic lines has been reported for over 40 species of finfish and shellfish.

Figure 1. Transgenic and non-transgenic Atlantic salmon at same age. Picture courtesy of AquaBounty Technologies.

The AquAdvantage salmon

AquaBounty Technologies was one of the first companies to attempt to commercially deploy a transgenic food animal, the AquAdvantage Salmon. The salmon line was created as part of experiments in 1989 to add an additional transgenic growth hormone gene into the salmon genome. A salmon with an incredibly rapid growth phenotype was identified as part of those experiments (Fletcher *et al,* 1992). This salmon line descends from that original founder animal, and the company moved forward through the regulatory review process with the aim of bringing the fish to commercial aquaculture.

After decades of regulatory review from the US Food and Drug Administration, in 2015 the salmon received regulatory approval as safe to eat as any other food with no difference from conventionally grown salmon. This genetically engineered salmon is required to be grown in secure, land-based facilities reviewed by US FDA, and as a sterile and all-female population for containment purposes. This line has a demonstrated accelerated growth rate (Fig. 1) compared to non-transgenic siblings, reaching market size 10 to 12 months faster, and also has an estimated 20% better feed efficiency. With the salmon finally approved, AquaBounty has added a focus on farming and grow-out to the company, leveraging their experience in operation of recirculating aquaculture systems (RAS) to take advantage of the growth rate and feed conversion benefits in the salmon. The first cohort of genetically engineered salmon are currently in grow-out in the United States and are expected to enter into commerce in 2020.

Genome editing

With this commercialization of the first genetically engineered food animal, the aquaculture industry has entered a new era in genetic engineering. In the ensuing decades since this salmon line was founded in 1989, advances in methods for DNA delivery, control of transgene expression, and precise engineering of the genome have been substantial. These technologies are currently being used to address pressing problems in aquaculture and to improve productivity.

Foremost among these new technologies is use of the CRISPR/Cas9 system (and related technologies) to

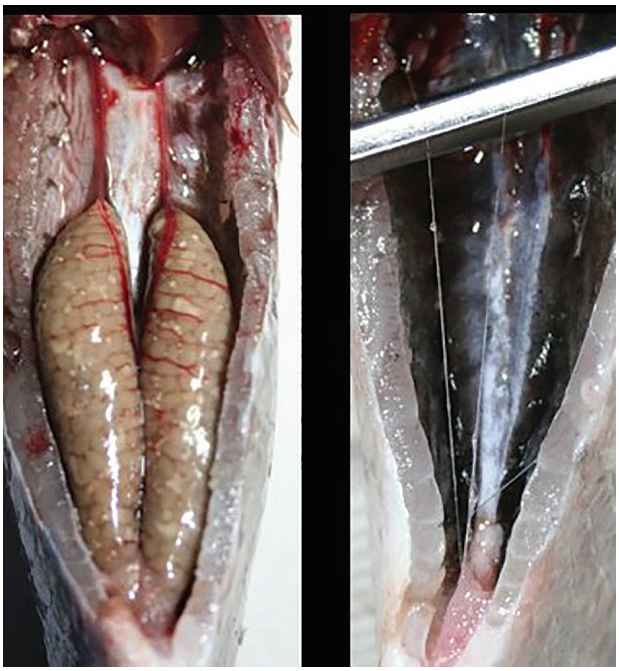

Figure 2. Dissected gonads of sibling Nile tilapia without (fertile) and with (sterile) genome edited changes in a gene involved in egg development.

create "genome editing" (Jinek *et al*, 2012). Genome editing works by creating a targeted and predictable modification to the DNA of an organism. Specific enzymes are used to induce these changes, and if these changes are made to the genome of a newly fertilized embryo, then the changes are heritable. This technique can be used to create precise changes in DNA that could have occurred naturally. Breeding programs are designed to select for natural changes in DNA associated with improved performance and enrich them in a population. Genome editing allows for the beneficial specific changes to be directly created in a breeding population, saving significant time and money.

In contrast to transgenic approaches where exogenous DNA is added to the genome of an organism to beneficial effect, with some genome editing approaches no new DNA is added to the cell or taken up in the genome. Whether this new technology falls under regulations for transgenic organisms is an interesting question for regulatory bodies. This is an important point, as research on new technologies to improve food productivity is dramatically impacted by the regulatory palatability of such approaches. Governments around the world are currently assessing whether such genome edited products will be regulated under existing frameworks for products produced with transgenesis, through existing frameworks for conventional products, or with new regulations.

Breeding programs are designed to select for natural changes in DNA associated with improved performance and enrich them in a population. Genome editing allows for the beneficial specific changes to be directly created in a breeding population, saving significant time and money.

Producing sterile fish

At least partly due to these perceived regulatory advantages, application of genome editing in aquaculture in aquaculture has been the primary focus for research efforts in fish and shellfish genetic engineering in recent years. In 2018, a group at the Center for Aquaculture Technologies announced patent filings around genome editing approaches for producing sterile fish and shellfish for aquaculture grow-out. The goal of this approach is to allow biological containment and minimize environmental impact of escapee fish, while also improving productivity by preventing energy lost to sexual maturation during grow-out. Using genome editing to address this goal has proven successful (Fig. 2). The ability to culture sterile fish and shellfish with equivalent or better performance compared to fertile stocks solves many of the problems in the aquaculture industry. As no DNA was used in the genome editing process, the sterile lines contain no new or added DNA.

Progress towards commercialization

As another example, Intrexon Corporation and AquaBounty Technologies successfully collaborated on the creation of a line of tilapia using genome editing. Changes were created in a gene controlling mussel growth. The resulting line of tilapia, designated FLT 01,

is reported to boast an improved filet yield of 70%, and improved growth and FCR of greater than 10%. The FLT 01 line was developed using genome editing with no foreign DNA or new combinations of genetic material. Importantly, in December 2018, the regulatory agencies in Argentina ruled that the genome editing changes made in the tilapia genome to produce the FLT 01 line of tilapia would not be regulated as a genetically modified food product. In September 2019, Brazil similarly ruled that the FLT 01 line of tilapia would not be regulated under their genetically modified food regulations. The regulators found that the regulations did not apply as the fish contained no exogenous DNA, exogenous DNA was not used to engineer the fish, and the changes engineered could have occurred naturally. Both of these decisions provide significant optimism that regulatory pathways to applying this technology to aquaculture applications are reasonable.

On a final note, selective breeding, broadly defined as the selection of broodstock for improvement in traits across time and generations, is sometimes thought of as alternative approach to genetic engineering. Genetic engineering provides the potential for rapid and abrupt improvement in a trait. However, selective breeding is an important tool in combination with genetic engineering, with potential to both improve the overall background of the genetically engineered organism and also to select for an improved, consistent response to the engineered genetic change.

With the advances in genome editing in recent years, exciting new possibilities in both genetic engineering and the regulatory process are upon us. Genome editing is increasingly considered as "precision breeding" and as part of the toolbox for genetic improvement in aquatic species.

References available on request

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