Advances in spotted wolffish (*Anarhichas minor*) broodstock management

Research Council of Norway

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Broodstock – Management - Reproduction – Sperm - Spotted wolffish - Welfare

Progress in the Management of Spotted Wolffish (*Anarhichas minor*) Broodstock

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Progress in the Management of Spotted Wolffish (*Anarhichas minor*) Broodstock

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1.- Introduction

Fig. 1. The **spotted wolffish breeders:** a **female** (enlarged belly) with a yellow tag, and two **males** with green tags. And a **sampling team**. The synergies among these elements drives progress in understanding and refining the management strategies for this species within research and aquaculture contexts.

Key Reference

Le François NR. Et al. (2021) Spotted Wolffish Broodstock Management and Egg Production: Retrospective, Current Status, and Research Priorities. Animals 11(10):2849.

The **spotted wolffish (*Anarhichas minor*)**, a member of the family Anarhichadidae, inhabits marine environments, primarily within the North Atlantic Ocean, particularly the waters adjacent to Canada, Greenland, Iceland, Norway, and the northern territories of the United Kingdom. Owing to its sporadic distribution, it commonly appears incidentally in trawl and longline fisheries rather than being deliberately targeted. Nevertheless, endeavors directed at its aquacultural cultivation indicate the species' potential to emerge as a notable farmed candidate.

Distinguished by its distinctive morphology, the spotted wolffish boasts an elongated, cylindrical body accompanied by a sizeable, rounded cranium. It possesses a formidable oral apparatus replete with robust, pointed dentition aptly suited for the pulverization of mollusks and crustaceans. As implied by its name, the species is recognized by its intricate pigmentation pattern, marked by an arrangement of diverse shades of brown, black, and white, facilitating effective camouflage within its natural rocky habitat.

The spotted wolffish exhibits propitious attributes conducive to aquaculture research and development (**Fig. 1**). Nonetheless, **the incipient state of this species' cultivation** process remains evident. Its inherent traits render it an ideal contender for diversification within aquacultural enterprises. In accordance with this perspective, recent years have witnessed dedicated initiatives focused on **advancing the aquacultural potential of the spotted wolffish** in Norway. Notwithstanding, the expansion of this nascent industry necessitates a heightened understanding of diverse facets of broodstock management, encompassing gamete production and manipulation.

Recent times have witnessed intensive efforts aimed at refining ***in vitro* fertilization methodologies** specific to this distinctive species. Progress has been made in refining techniques for **sperm cryopreservation**, incorporating refrigeration and cryobanking procedures.

This compendium expounds upon the evolutions and innovations observed within **broodstock management**, encompassing the domains of **sperm collection**, **evaluation of sperm quality**, **storage**, and its subsequent **use to fertilize** eggs.

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2. Housing

Spotted wolffish broodstock are housed in onshore facilities and transferred to smaller tanks during the breeding season for better individual monitoring. The following guidelines are recommended:

Fig. 2. Tagged broodstock. These spotted wolffish, distinguished by the presence of fin clips, are maintained in densely populated conditions within tanks characterized by a substantial vertical water column.

- **Photoperiods**: Adjusting the amount of light the fish are exposed to can effectively influence their reproductive processes by manipulating their sex hormone levels. However, more research in this area is still necessary.
- **Feeding**: Spotted wolffish have a diet primarily composed of organisms residing on the ocean floor, such as crustaceans like crabs, lobsters, shrimp, and mollusks like clams and mussels, but also consume sea urchins with low lipids content. Therefore, the inclusion of lipids must be low. The development of suitable artificial feed for captive specimens is still ongoing.
- **Tagging.** It is common to use bachelor-button tags on or near the dorsal fin in large animals. Moreover, by taking advantage of their slow-moving pace, it is possible to determine the sex of individual fish in advance and tag them following a color code. This procedure will assist the farmer when performing breeding manipulations (**Fig. 2**).
- **Salinity** Currently, the spotted wolffish breeders are reared in a salinity range of 26–34 PSU with no detrimental effects reported.
- **Temperature**. In the spotted wolffish, the optimum temperature for growth is around 8–12°C. spotted wolffish breeders can be kept at a temperature of 9–10°C out of the spawning season but during final maturation (up to three months before spawning corresponding with the vitellogenesis), fish should be kept at temperatures below 6°C.
- **Stocking Density**: The ideal density for raising spotted wolffish varies depending on their size. Smaller fish are best reared at densities below 40 kg/m^2, while larger individuals thrive densities above 40 kg/m2. Adult fish are commonly maintained at densities exceeding 70 kg/m^2 (Tremblay Bourgeois et al., 2010 (**Fig. 3**).

Fig. 3. Broodstock confinement. A critical component of aquaculture breeding programs as it allows for the controlled propagation of desirable genetic traits and the production of healthy offspring.

2. Housing - Additional considerations

In addition to the provided information, it's worth noting the significance of maintaining precise environmental conditions and monitoring during the breeding and maturation phases of spotted wolffish in captivity.

Fig. 4. Environmental conditions are crucial for optimizing growth rates, enhancing reproductive success, and supports the overall health of the cultured spotted wolffish broodstock.

- **Environmental Conditions**: Aside from salinity and temperature, other factors such as water quality, pH levels, and oxygen saturation play pivotal roles in ensuring the health and successful reproduction of captive spotted wolffish. A comprehensive understanding of the species' ecological preferences allows for the recreation of a suitable habitat within aquaculture settings (**Fig. 4**).
- **Breeding Manipulations**: The utilization of color-coded tags to distinguish between genders facilitates more efficient breeding manipulations. This approach enables breeders to identify and select specific individuals for controlled mating, which can contribute to the propagation of desirable genetic traits and the optimization of breeding programs.
- **Behavioral Observations**: Given the species' slow-moving nature, careful behavioral observation can provide insights into their reproductive readiness and overall well-being. Changes in behavior, such as increased activity or courtship behaviors, can indicate that individuals are approaching their breeding season.
- **Health Monitoring**: Regular health assessments, including checks for diseases, parasites, and overall physical condition, are essential for maintaining the well-being of the broodstock. Early detection of health issues can prevent potential disruptions to breeding efforts (**Fig. 5**).
- **Research and Innovation**: Continued research into spotted wolffish aquaculture techniques is essential for refining current practices and developing new methodologies. Advancements in artificial feed formulation, breeding management, and reproductive physiology can lead to increased success rates and the sustainable growth of this species as a farmed candidate.

By incorporating these considerations into the captive management of spotted wolffish, farmers can further enhance the **success** of their breeding programs and contribute to the sustainable utilization of this unique marine species.

Fig. 5. An anesthetized male spotted wolffish is positioned within a temporary container. The purpose of anesthetizing the fish is to mitigate stress and streamline the process of health monitoring and sperm collection.

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2. Handling procedures

Consistent and comprehensive assessment of broodstock holds paramount importance in the oversight and curation of top-tier broodfish. Furthermore, an important aspect involves the **evaluation of the sperm production in males**. This evaluation coincides with the distension of the ventral portion of female fish resulting from the presence and maturation of eggs. Ultimately, this **synchronization** culminates in their artificial simultaneous release and fecundation.

Anaesthesia procedure

Before any fish handling animal should be deeply anesthetized as follows:

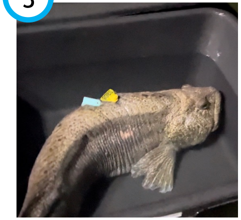
1

Arrange the sampling materials, prior to releasing the animal from the tank and initiating the anesthesia procedure.

2

Use anaesthesia for the sedation of wolffish. (14 mg/L of tricaine methanesulfonate, MS-222).

3



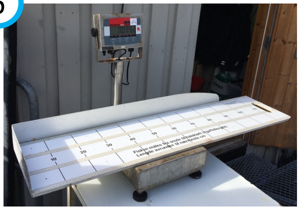
Carefully transfer one fish at a time into a small enclosure containing MS-222 (140 ppm) and allow it to lose consciousness.

4

Monitor anaesthesia deep. Optimal point is when animal stay bottom-up without agitation.

Screening procedure

5



Before handling the fish, ensure that the scale and measurement board are conveniently prepared.

6

Position the fish with its ventral side facing outwards, facilitating easy handling and visual inspection.

7

Annotate the bachelor-button tag number and diligently monitor each fish evaluated.

8

Record weight and length, monitor growth, and visually inspect the ventral side.

3. Male Reproductive System

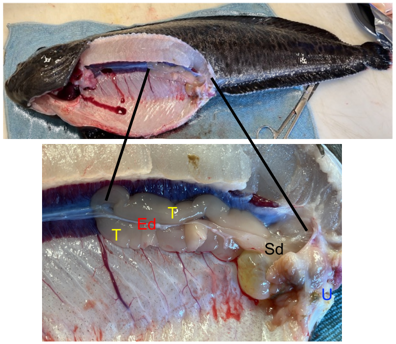


Fig. 6. Male reproductive system of a 3 kg juvenile spotted wolffish. Picture showcase key components including the testes (T), efferent ducts (Ed), spermatic ducts (Sd), and the location of urogenital pores.

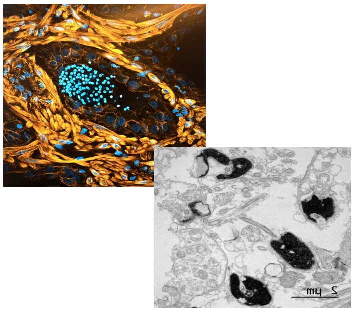


Fig. 7. Spotted wolffish sperm. Upper slide showing a fluorescent micrograph of the testes. Lower panel, sperm shown through transmission electron microscope.

The male reproductive tract in the spotted wolffish, like in many fish species, consists of various structures that play essential roles in the production, storage, and transfer of sperm (**Fig. 6**):

Testes: The testes are the primary organs responsible for producing sperm. In the spotted wolffish, there are typically two sets of testicular lobes: the ocular testicular lobe and the blind side testicular lobe. These lobes produce and store sperm cells.

Efferent Ducts: Sperm produced in the testes travel through efferent ducts, which are small tubes that connect the testes to the larger ducts of the reproductive system. These ducts facilitate the transport of sperm from the testes to other parts of the reproductive tract.

Spermatic Ducts: The spermatic ducts are larger tubes that receive sperm from the efferent ducts. They serve as conduits for transporting sperm towards the urogenital pore, which is the opening through which sperm and urine are expelled.

Urogenital Pore: The urogenital pore is the external opening through which sperm and urine are released from the body. It's located in the abdominal region and serves as the endpoint for the male reproductive tract.

In the case of the spotted wolffish, which exhibits unique reproductive characteristics, it's important to note that the sperm is already motile upon stripping from the fish, but it becomes inactive when exposed to seawater or high osmolality. Its motility is sustained within an osmotic range of 200 to 500 mOsm, indicating that it operates in an environment with similar values, likely the ovarian fluid. This suggests adaptations for internal fertilization, likely taking place within the female's reproductive tract, rather than external fertilization. Their testes are small, resulting in a low Gonadosomatic Index (GSI) that remains stable throughout the year and does not increase with age.

Overall, the male reproductive tract of the spotted wolffish is adapted to produce and transfer sperm (**Fig. 7**) effectively, enabling the species' reproductive success within its natural habitat.

4. Sperm striping

Sperm collection necessitates specialized training to: A) Identify testes on both ocular and blind sides, B) Empty the bladder of urine, and C) Gently extract sperm by applying slight pressure to testes and efferent ducts, all in a single step procedure.

1.- Identify mature broodstock, carefully inspect them using a mirror. Look for males with dilated urogenital openings.

2.- Immerse the fish in a water suspension containing 150 ppm of MS-222 into a small basin.

3.- The sedated fish is gently placed on a table with wet towels for the purpose of stripping.

4.- After releasing the metabolic waste, a Pasteur pipette is placed in the urogenital opening of the fish using a gentle suction.

5.- A lateral massage applying gentle but firm pressure is administered, moving from the dorsal to ventral direction beneath the testes. Ensure not stressing or harming the fish.

6.- The collected sperm in the pipettes is swiftly placed on ice to preserve its quality and prevent deterioration before analysis.

Photo credits: J. Galindo-Villegas & J. Superio.

4. Sperm evaluation

Computer-aided sperm analysis (CASA)

CASA is a sophisticated method used in reproductive biology and assisted reproductive technologies to **assess and quantify** various characteristics of spermatozoa. It involves using specialized software and digital imaging to analyze sperm movement and morphology in a standardized and automated manner, providing more accurate and consistent results compared to manual methods (**Fig. 8**).

In the case of the spotted wolffish, it's important to note that **sperm is naturally motile** and does not require any activator or suppressor of activity for quantification purposes.

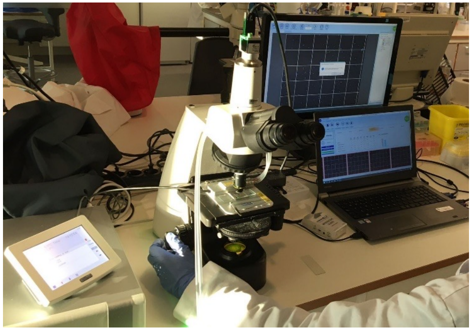


Fig. 8. On the left side, there is an optical microscope equipped with a cooling stage, along with the controls used for analyzing sperm quality. To the right, the CASA system.

Main quality parameters

Motility Parameters:

- **Progressive Motility**: Percentage of sperm with forward, purposeful movement.
- **Non-Progressive Motility**: Percentage of sperm with movement that isn't considered forward or purposeful.
- **Total Motility**: Percentage of sperm showing any type of movement, including both progressive and non-progressive motility.
- **Curvilinear Velocity (VCL)**: The average velocity along the actual sperm path.
- **Straight-line Velocity (VSL)**: The velocity of the sperm in a straight line.
- **Average Path Velocity (VAP)**: The average velocity along the smoothed sperm path. (See **Fig. 9** for graphical details).

Morphology Parameters:

- **Head Size and Shape**: Evaluates the size and shape of the sperm head, in addition to the length, width, and aspect ratio.
- **Midpiece Abnormalities**: Assesses abnormalities in the midsection of the sperm.
- **Tail Morphology**: Analyzes the shape and integrity of the sperm tail.

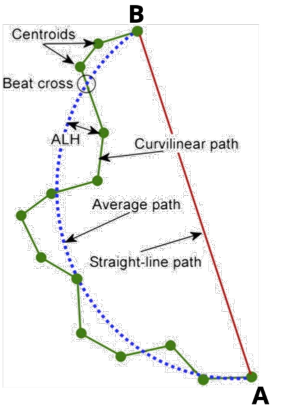


Fig. 9. Sperm motility pathways encompass a variety of trajectories. While A to B is the the closest path, few individuals adhere strictly to this path. Thus, variants exist.

5. Sperm storage - Refrigerated for short-term storage

Upon extraction, the sperm of spotted wolffish exhibits motility, featuring a unique attribute that currently prevents immobilization and reactivation. Hence, when considering refrigeration protocols for the sperm at temperatures of 4-8 °C, the primary focus should be on prolonging this process, extending it to the usual two-day period. This aligns with the observed maximum motility window reported so far.

Utilizing a plastic Pasteur pipette with a modified tip, sperm samples are acquired and connected to the urogenital opening through a suction technique outlined in the previous section. Only sperm samples exhibiting a motility level, as evaluated by CASA, exceeding 60%, are collected. These collected samples are then stored within the temperature range of 4-8 °C. Initially, the samples are positioned on a collection rack atop crushed ice. Subsequently, they are refrigerated for a period of up to 30 hours, employing a typical household refrigerator (**Fig. 10**).

If there are challenges in immobilizing and reactivating the sperm after extraction, an extender (a solution used to dilute cells outside the body) might play a role in preserving the sperm's motility and viability during refrigerated storage (**Fig. 11**).

Fig. 10. Sperm motility assessment and refrigeration are key aspects. Motility is systematically assessed using the CASA system. After stripping the fish, the sperm collected in Pasteur pipettes is stored at low temperature (4ºC) for up to 30 hours post-striping for egg fertilization.

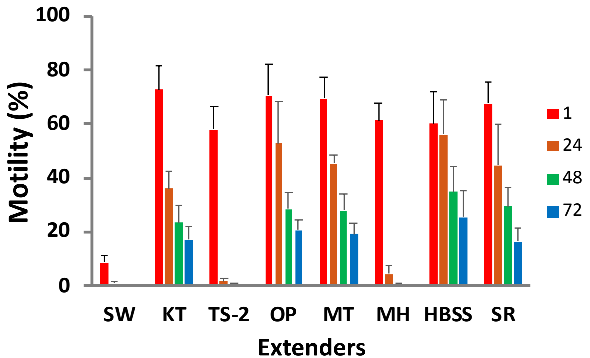


Fig. 11. The percentages of motility were monitored in testicular sperm suspensions diluted with different extender solutions and kept at 4ºC over a 72-hour duration. It's important to highlight the significant loss of viability observed in sperm motility starting from 24 hours after refrigeration. (Superio et al., 2023a).

5. Sperm storage - Markers & reference values

Assessing various parameters of sperm, such as volume, concentration, motility, and specific velocity components like VCL (curvilinear velocity), VSL (straight-line velocity), VAP (average path velocity), and live cell count, can provide comprehensive insights into the quality and functionality of sperm. The following basic parameters are commonly used as sperm markers in reproductive studies of species where few indicators exist (**Table 1**):

1.Volume and Concentration: The volume of ejaculate and the concentration of sperm within it are fundamental indicators of reproductive potential. They help in estimating the total number of sperm available for fertilization, which is crucial for understanding the fertility of a male individual.
2.Motility: Sperm motility refers to the ability of sperm to move, which is essential for reaching and fertilizing the egg. Assessing motility provides information about the overall health and functionality of sperm, as immotile or poorly motile sperm may have difficulty in successfully fertilizing an egg.
3.Velocity Components (VCL, VSL, VAP): These velocity components provide more detailed information about how sperm move. VCL is the actual path followed by the sperm, VSL is the distance between the start and end points, and VAP is the average velocity along the sperm's trajectory. These measures offer insights into the swimming patterns and potential efficiency of sperm movement.
4.Live Cell Count: Determining the proportion of live sperm cells in a sample is crucial since live cells have the potential to fertilize an egg. Live cell count indicates the viability of sperm, reflecting their ability to survive and function in the female reproductive tract.

By systematically testing different cryoprotectants and extender formulations, is possible to identify the most effective combination that optimally preserves spotted wolffish sperm during long-term storage. This process involves assessing various parameters, including post-thaw motility, viability, and fertilization potential. Successful cryopreservation methods assist in aquaculture breeding programs.

Table 1. Mean values of key markers observed in fresh spotted wolffish sperm with varying cryoprotectants and extenders.

			Cryoprotectant		Extenders			
	Average	**Fresh**	**DMSO** (10%)	**MeOH** (10%)	**KT**	**MT**	**HB**	**SW**
Parameters	Volume (mL)	0,5-10,0	na	na	na	na	na	na
	Sperm concentration (10^9 ml^{-1})	1,5 ± 0,2	0,6 ± 0,6	0.5 ± 0,8	0,5 ± 0,3	0,6 ± 0,1	0,4 ± 0,4	0,4 ± 0,6
	Total motility (%)	62,2 ± 1,8	40,6 ± 2,3	36,2 ± 1,9	23,2 ± 1,5	33,0 ± 0,6	16,6 ± 0,8	2,0 ± 0,1
	VCL (µm/s)	10,0 ± 0,8	4,1 ± 0,5	3,7 ± 0,4	4,0 ± 0,6	4,8 ± 0,6	4,0 ± 0,1	1,1 ± 0,4
	VSL (µm/s)	3,7 ± 0,7	1,6 ± 0,3	1,4 ± 0,6	1,8 ± 0,4	2,2 ± 0,4	1,7 ± 0,10	0,8 ± 0,2
	VAP (µm/s)	5,9 ± 0,5	2,2 ± 0,1	1,8 ± 0,3	2,4 ± 0,5	3,0 ± 0,5	2,4 ± 0,1	1,2 ± 0,5
	Live cells (%)	95,7 ± 0,8	nd	nd	91,3 ± 1,3	93,7 ± 0,7	87,5 ± 1,7	75,1 ± 1,6

DMSO – Dimethyl sulfoxide; **MeOH** – Methanol; **KT** – Kime & Tveiten; **MT** – Modified turbot; **HB** – Hank's balanced salt solution; **SW** – Sea water.

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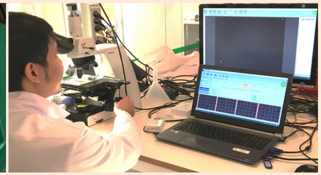
5. Sperm storage – Cryobanking long-term storage

Sperm cryopreservation is a technique used to preserve and store sperm cells at extremely low temperatures, typically in liquid nitrogen, to maintain their viability for an extended period. This process involves carefully preparing the sperm, adding cryoprotectants to safeguard against cell damage during freezing, and then gradually cooling the sperm to sub-zero temperatures.

Collect fish sperm and determine the quality for each sample using the CASA system





Incorporate the extender and cryoprotectant to each sperm sample, introduce the resulting solution into the selected container, and submerge it in liquid nitrogen for a duration of 30 seconds.

MIX

- Dimethyl sulfoxide (10%)
- Extender 1:3 (HBSS)*
- Sperm


Both cryogenic straws and cryotubes have the potential to be effective, but optimization is required!!

Regardless of whether the user opts for straws or vials as the chosen cryopreservation device, the samples are preserved in liquid nitrogen at extremely low temperatures until they are required for future use.



* Hank's balanced salt solution (HBSS)

6. Use of hormones

Hormones are commonly used in aquaculture to induce spawning in various fish species. In the case of the spotted wolffish, the use of hormones can help synchronize the reproductive process, making it more predictable and facilitating controlled breeding. One of the key hormones used for spawning induction in fish is Gonadotropin-Releasing Hormone analogs (GnRHa), which stimulates the release of gonadotropins that trigger maturation and spawning.

Key aspects to consider on the application of GnRHa for spawning induction in the spotted wolffish males (females eggs production is easily observed, and progression of maturation follows a timeline):

1) Hormone selection and administration, 2) Treatment regime, 4) Monitoring and timing, 5) Spawning, 6) Collection

It's worth noting that while hormone-induced spawning can be a valuable tool in aquaculture and research, it requires careful consideration of factors such as the species' natural reproductive behavior, individual responses to hormones, and ethical considerations. Proper research and application are essential to minimizing stress on the fish and ensuring the success of the breeding program (**Fig. 12**).

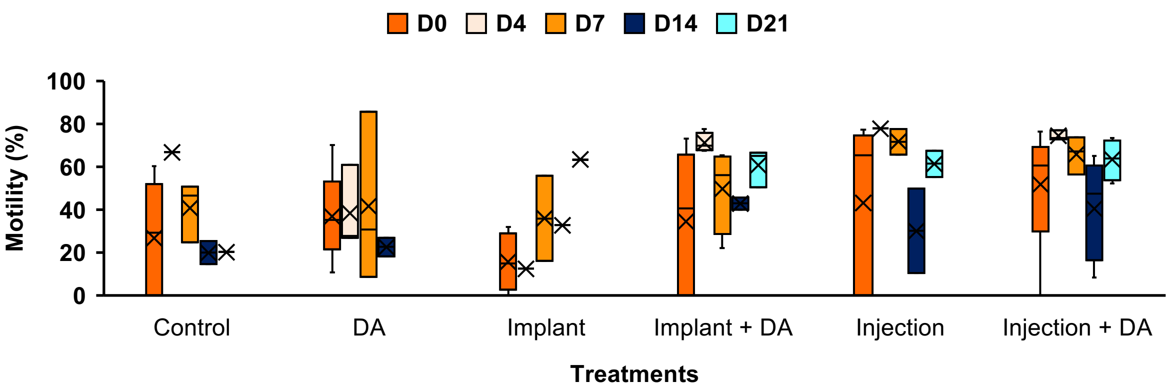


Fig. 12. Sperm Motility Percentage in Spotted Wolffish Broodstock after Treatment with Different GnRHa Preparations over a 21-Day Period through Muscular Implantation or Intramuscular Injection. The depicted data illustrates the Lower Quartile (Q1) and Upper Quartile (Q3), encompassing observations within the 9th to 21st percentile range. Please note that data outliers beyond this range (n=5) have been excluded from the plot. The influence of Dopamine Agonist (**DA**) is also considered. (Superio et al., 2023b)

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7. Reproductive cycle Under captivity

The **reproductive cycle** of the spotted wolffish under captivity involves distinct stages that correspond to their breeding behaviors and physiological changes. These stages can be summarized as follows (**Fig. 13**):

1. **Resting Phase**: This is a period of relative reproductive inactivity. During this phase, the fish may exhibit reduced feeding and minimal sexual activity.
2. **Maturation Phase**: As environmental conditions and cues become favorable, spotted wolffish begin to exhibit signs of maturation. Both males and females undergo physiological changes that prepare them for breeding. Males develop larger testes and produce mature sperm, while females' ovaries develop, and eggs mature.
3. **Courtship and Spawning Phase**: This is a critical phase where courtship behaviors and spawning occur. Males actively court females, displaying behaviors like nest-building, chasing, and fin displays. Once a receptive female is found, both male and female engage in spawning. The female releases eggs, and the male releases sperm, resulting in fertilization.

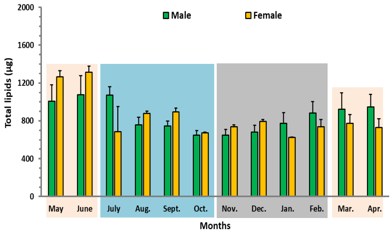

Fig. 13.- The reproductive cycle of the spotted wolffish. The three periods described in the text are presented in terms of total lipid content. Bars represent male and females. (Superio et al., 2023c).

It's important to note that the specific timing and duration of each phase can **vary** depending on factors such as environmental conditions, temperature, photoperiod, and the health of the fish. Monitoring and managing these factors in captivity are essential to successfully replicate and control the reproductive cycle of the spotted wolffish for breeding purposes.

Thereafter, once the fecundation is successful, the eggs takes between 5 to 7 months to hatch. After one year they become fingerlings (**Fig. 14**).

- **Egg Development Phase**: After the artificial fertilization, the eggs are typically laid in a breeding incubator, mimicking the nest. Oxygen saturation, continuous water flow, temperature, and darkness are all essential parameters required by the developing embryos. The eggs undergo embryonic development during this stage.
- **Hatching and Larval Phase**: The eggs hatch into larvae, which are initially dependent on yolk sac reserves for nourishment. As the larvae develop, they gradually transition to external feeding on small prey items
- **Post-Spawning Recovery**: After spawning, both males and females may experience some level of stress and physiological changes. They might require a period of rest and recovery before re-entering the reproductive cycle.

Fig. 14.- From egg to larval phase. The eggs are show in a typical incubator for this species. Then, the hatched larvae, and the early fingerlings are presented.

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