Chapter 4

Amyloodinium ocellatum

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1. Background

Amyloodinium ocellatum (Brown, 1931) is an ectoparasitic dinoflagellate (Dinophyceae, Peridiniales, Thoracosphaeraceae) that is endemic in temperate and tropical areas worldwide. *A. ocellatum* is capable of successfully adapting to a variety of different aquatic environments, making its environmental control difficult. Although, in general, its optimal temperature range is between 23 and 27°C and its optimal salinity range is 30–35‰, this dinoflagellate is also virulent in extreme conditions. In fact, lethal outbreaks have been registered at high temperatures (>35°C) in both full seawater (46‰) (Kuperman and Matey, 1999) and brackish environments (7‰) (Beraldo et al., 2017). Furthermore, because this dinoflagellate is characterized by low host-species specificity, it can potentially infect several aquatic organisms living within its ecological range. Amyloodiniosis is a major threat for semi-intensive aquaculture (valliculture, or inland brackish farming) in southern Europe and in different aquaculture facilities worldwide, especially in the warmest months, causing serious morbidity and mortality. If the concentration of dinospores is very high, death of the host can occur in <12 h (Lawler, 1980).

In general, the life cycle can be completed in 5 to 7 days, depending on salinity and temperature that strongly modulate its duration. The life cycle of *A. ocellatum* is direct and divided into three phases:

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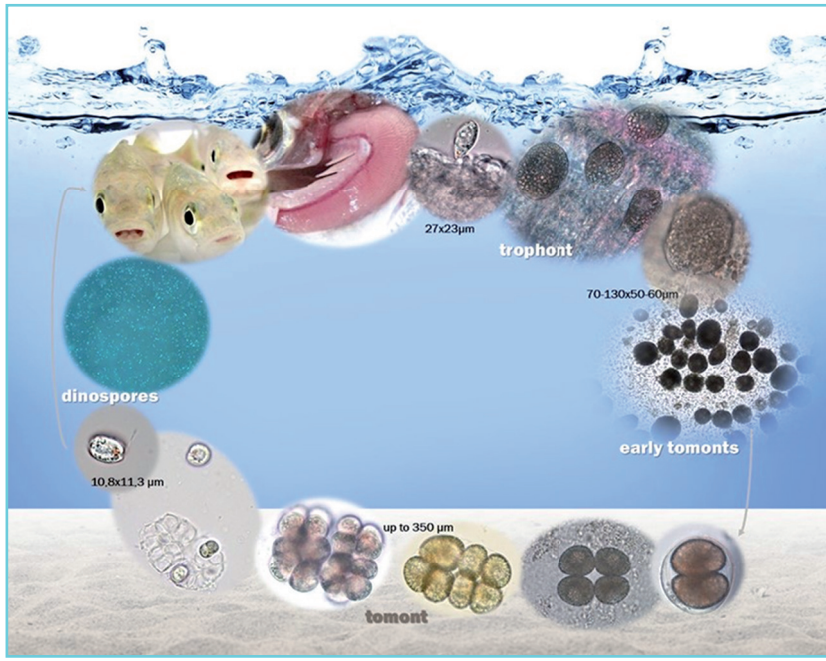

Fig. 4.1 Diagrammatic representation of the life cycle of *Amyloodinium ocellatum*. Credit: Paola Beraldo.

(1) the trophont (parasitic) stage that feeds directly on the host and is predominantly present in the gills and skin epithelia (adhesion sites); in the European sea bass (ESB, *Dicentrarchus labrax*), trophonts have also been found attached to the epithelium of the oro-pharyngeal cavity (Beraldo et al., 2017); (2) the tomont (free-living cystic reproductive stage) that can asexually produce up to 256 new specimens (dinospores); and (3) the dinospore (free-swimming infective stage) that hatches from the tomont. *A. ocellatum* transmission is direct from fish to fish by dinospores, which are free floating and look for new hosts to infect. The infective stage is easily spread in the water and on rearing equipment (fishing nets, filtration material of recirculation systems, parameter probes, etc.). Moreover, it has been experimentally confirmed that dinospores can also be transmitted in aerosol droplets (Roberts-Thomson et al., 2006), thus explaining how, following windy atmospheric events (e.g. storms or typhoons), some fish farms with no previous reports

of amyloodiniosis can be affected by *A. ocellatum* (Beraldo et al., 2017; Dequito et al., 2015). Moreover, it is noteworthy to mention that just a few dinospores in a water droplet are sufficient to start an infection.

2. General facilities, fish and *Amyloodinium ocellatum*

For both *in vitro* and *in vivo* infection trials, stage 1 *A. ocellatum* trophonts, collected during spontaneous outbreaks on fish farms or from experimentally infected fish, are necessary. Parasite collection is performed as described in section 4. Stage 2 tomonts are then incubated at 16 ± 0.5°C, in a solution composed of sterile seawater (35‰) and a mixture of different antibiotics (detailed in section 3).

For *in vitro* studies, the G1B gill cell line (ATCC® CRL-2536TM) can be used to maintain the *A. ocellatum* life cycle. All procedures must be performed under sterile conditions beneath a biological hood. Cells are incubated at 25 ± 0.2°C and regularly supplied with fresh L-15 (Leibovitz) complete medium. After reaching confluence status, the cells are then progressively adapted to the same medium with increasing doses of IO2/HBSS medium (Noga, 1989).

For *in vivo* infection, healthy ESB juveniles should be obtained from a hatchery with no previous record of *A. ocellatum* infection. ESB are acclimatized for two weeks prior to infection in a recirculating system. Water physico-chemical parameters must be measured daily during acclimatization (temperature 22.5 ± 2°C, salinity 35 ± 2‰, pH 8.0, NH4-N 0.02–0.03 mg/l, NO2-N <0.015 mg/l, natural photoperiod). The fish are then transferred to aquaria (300 l, 31‰ salinity) equipped with aerators, water pumps and a temperature control system (22–25°C). All experimental procedures must be conducted in compliance with the Guideline of the European Union Council (Directive 2010/63/EU) and individual national laws governing the use of laboratory animals.

3. Equipment and reagents

- Anaesthetic MS-222 (E10521, Sigma Aldrich – Merck)
- Antibiotics: amoxicillin (0.5 µg/ml; CT0061B, OXOID, Thermo Scientific), flumequine (0.6 µg/ml; CT0666B, OXOID, Thermo Scientific), penicillin/streptomycin (2%; P4333, Sigma Aldrich – Merck), sulphamethoxazole/trimethoprim (0.5 µg/ml; CT0052B, OXOID, Thermo Scientific), tetracycline (0.2 µg/ml; CT0053B, OXOID, Thermo Scientific)
- Automatic pipettes and (sterile/non-sterile) pipette tips
- Biological hood
- Centrifuge
- Centrifuge sterile tubes (15 and 50 ml)
- Cell-counting chamber (S50 Sedgewick–Rafter Cell, Pyser – SGI)
- Dissection tools
- Funnel (siliconized) and Nitex mesh (100 µm)
- G1B gill cell line (ATCC® CRL-2536TM)
- Glass jar or beaker (siliconized)
- Glass slides and coverslips
- Heaters
- Incubator set at the desired temperature
- IO2/HBSS medium (Noga, 1989)
- Leibovitz (L5520, Sigma Aldrich – Merck) complete medium [L-15 with the addition of 10% heat-inactivated fetal calf serum (F4135, Sigma Aldrich – Merck), 1% penicillin/streptomycin solution (P4333, Sigma Aldrich – Merck) and 1% L-glutamine (59202C, Sigma Aldrich – Merck)]
- Light, inverted and stereo microscopes
- Lugol's iodine solution
- Parafilm®M (HD234526B, Heathrow Scientific)
- Percoll® (P1644, Sigma Aldrich – Merck)
- Plastic non-sterile and sterile Pasteur pipettes
- Plastic non-sterile and sterile Petri dishes
- Sigmacote® (SL2, Sigma Aldrich – Merck)
- Sterile salt water (35‰)
- Sterile serological pipettes (1, 5 and 10 ml) and hand pipettor
- Sterile tissue culture flasks (25 and 75 cm^2).

4. *Amyloodinium ocellatum* collection

1. Collect trophonts from ESB gills during natural outbreaks, or from experimental infection, by placing the fish in a jar half-filled with freshwater to induce trophont detachment from the host epithelia. After 1 min, add an equivalent volume of 40‰ seawater to reach 20‰ salinity; fish are maintained in this condition for 5–6 min at 20–24°C.
2. Remove the animals from the jar (transfer them to rearing tanks), then pour the contents into a siliconized glass jar through a siliconized glass funnel lined with two layers of 100-µm nylon filter mesh, to remove large debris. Let the parasites settle in the siliconized glass jar and transform into early tomonts (20–30 min).
3. Aspirate the seawater overlay, then collect and transfer tomonts to centrifuge tubes (15 or 50 ml).
4. Wash tomonts by centrifugation for 10 min at 150 ×*g*, remove the supernatant and re-suspend the tomont pellets in sterile saltwater (20‰); follow with a second wash under the same parameters.
5. Gently layer 2 ml of the tomont suspension (in the sterile saltwater) onto 2 ml of Percoll® in 15-ml centrifuge tubes (Fig. 4.2). Centrifuge for 10 min at 180 ×*g* to separate tomonts from the debris.
6. Remove the supernatant and wash the tomont pellets three times by centrifugation for 5 min at 800 ×*g*, with 15 ml of sterile saltwater plus antibiotics (at the concentrations detailed in section 3); use tomonts immediately or preserve them for about 6 months (see section 5.2) in accordance with the objective of the research activity.
7. For hatching dinospores, tomonts are incubated at 24 ± 0.5°C in plastic Petri dishes in a solution composed of sterile saltwater and a mixture of the different antibiotics detailed in section 3. It is better to seal the Petri dish lid with Parafilm in order to avoid evaporation: drying out is lethal to *A. ocellatum*.

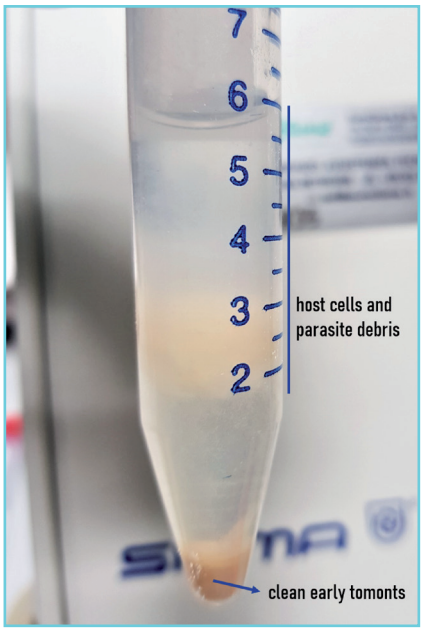

Fig 4.2 Purification of early tomonts in Percoll® gradient. Credit: Paola Beraldo.

NOTE 1. Freshwater determines detachment of trophonts from adhesion sites by osmotic shock. However, it is better not to prolong the treatment beyond 1–2 min since the absence of salt in the water is lethal for *A. ocellatum*.

NOTE 2. During the 'tomontization' process, tomonts cover themselves with a layer of sticky mucus. For this reason, in order to avoid their attachment to glassware surfaces it is recommended to siliconize all glass equipment before use.

NOTE 3. It is recommended to add antibiotics to the saltwater medium to avoid bacterial, fungal and/or protozoal contamination, since some microorganisms can inhibit the development of *A. ocellatum*.

NOTE 4. At 24°C, completion of the tomont reproductive cycle requires approximatively 2–4 days.

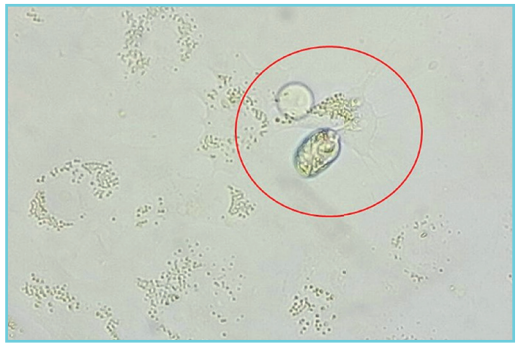

Fig. 4.3 Dinospore attached to the G1B cell monolayer. Credit: Paola Beraldo.

5. Standard operating procedure for *Amyloodinium ocellatum in vitro* maintenance

5.1. Maintenance with G1B cell culture

1. Propagate walking catfish gill (G1B) cells in sterile tissue culture flasks (in accordance with ATCC protocol) in L-15 complete medium. At confluence status, replace the L-15 complete medium with a mixture of L-15 complete medium and IO2/HBSS (conditioning medium), which has to be added initially in the proportion of 25% IO2/HBSS and 75% L-15 complete medium, then in the proportion 1:1 of the two media.
2. Aspirate a few *A. ocellatum* dinospores hatched from the collected tomonts with a sterile Pasteur pipette and transfer to the G1B cell monolayer (under sterile conditions).
3. Monitor the sterile tissue culture flask under the inverted microscope (Fig. 4.3).

5.2. Hibernation of tomonts

1. Collect and purify tomonts as detailed in section 4.
2. Then, maintain tomonts in an incubator (16 ± 0.5°C) in plastic Petri dishes in a solution composed of sterile saltwater and a mixture of the different antibiotics detailed in section 3. Just to make sure, seal the Petri dish lid with Parafilm in order to avoid evaporation; renew the medium monthly.

NOTE 1. G1B cells need approximatively 7–10 days to adapt to the conditioning medium. Therefore, it takes two weeks to reach the appropriate salinity to perform the *in vitro* infection.

NOTE 2. Although *A. ocellatum in vitro* infection and propagation is feasible, this system does not always allow for appropriate parasite numbers to be obtained. Besides, it is very laborious and relatively expensive. To solve this problem, we recommend preserving the parasite at the tomont stage (at 16 ± 0.5°C in an incubator). This provides a continuous supply of dinospores. In fact, at this temperature, hibernated tomonts remain viable for over 6 months and their infectivity is maintained when brought back to 20–25°C, as confirmed by the resumption of the reproductive process and dinospore hatching. Every 6 months it is best to renew the parasite stock, by obtaining new tomonts from an experimental infection.

6. Standard operating procedure for *Amyloodinium ocellatum in vivo* infection

1. Acclimatize healthy ESB juveniles, obtained from a parasite-free hatchery, to the experimental facilities in tanks with independent recirculation systems for 2 weeks. Divide fish over the tanks.
2. After acclimatization, transfer fish to aquaria (300 l) filled with saltwater (e.g. n = 10–15 fish/aquarium according to the size). Use one or more aquaria for the experimentally infected groups, and retain some aquaria for the control group (unexposed fish) if so required.
3. Increase water temperature to 24 ± 1°C during the last days of acclimatization. It is better to cover aquaria with plastic sheets to avoid dinospore contamination.
4. Count the number of dinospores hatching from hibernated/fresh tomonts with a cell-counting chamber and adjust them to a final concentration of approximately 10 dinospores/ml. This will be the inoculum.
5. Pour the inoculum into each individual infection aquarium and keep them there for 2 h.

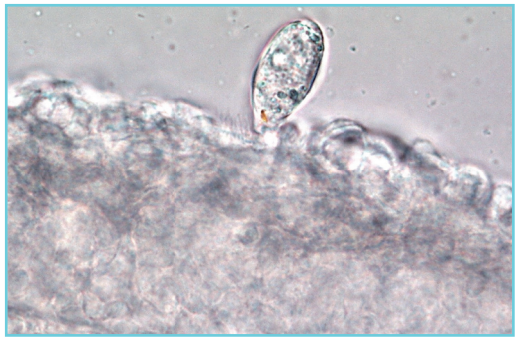

Fig. 4.4a An early trophont attached to ESB gill epithelium after 2-h infection. Credit: Paola Beraldo.

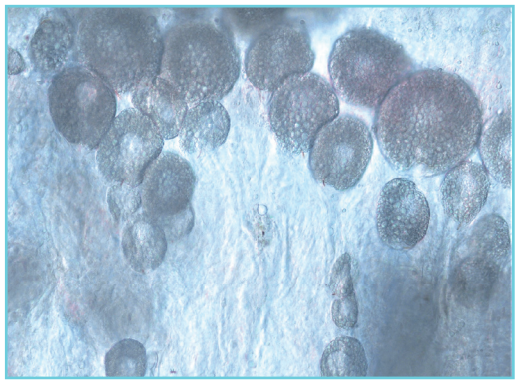

Fig. 4.4b Trophonts attached to ESB gill 48 h post-infection. Credit: Paola Beraldo.

6. Immediately after the 2-h infection, take one fish out of each aquarium (*intra vitam* gill biopsy) and check whether trophonts have adhered (Fig. 4.4a). Subsequently, monitor ESB in order to detect the appearance of clinical signs of amyloodiniosis.
7. Eventually, 48 h after infection, sacrifice some fish using a lethal dose of anaesthetic and examine gill samples with a light microscope to determine the parasite burden (Fig. 4.4b). Gills from control fish have to be examined to check that no contamination has occurred.
8. Monitor the evolution of the infection (Fig. 4.5).

NOTE 1. The dinospore concentration per ml was derived from the following formula: (number of dinospores in 50 grids × 1000/12.5) × dilution factor, as reported in Dehority (2003). To facilitate counting, we recommend adding a few drops of Lugol's iodine to the dilution solution to stain the dinospores.

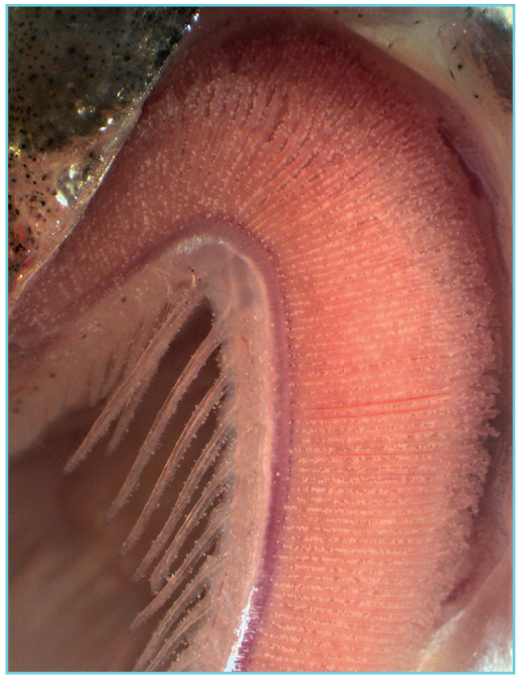

Fig. 4.5 ESB gills with a severe infection. Credit: Paola Beraldo.

NOTE 2. Amyloodiniosis is characterized by clinical signs including apathy, dyspnoea, increased respiratory rate with laboured breathing and gathering at the water surface. Jerky movements and pruritus have also been documented as typical symptoms of the infection. Although scattered trophonts on the skin could confer a dusty appearance (hence the name marine velvet disease), this is not a common finding in some fish species such as ESB (in massive infections, ESB die without obvious gross skin alterations).

7. Summary templates

***Amyloodinium ocellatum* Brown, 1931**	
Parasite isolation	
Fish or intermediate host species required for parasite collection	All brackish and marine fish can potentially be infected by *A. ocellatum*. The protozoan has no intermediate hosts

Susceptible host characteristics	Naïve fish and juveniles are the most susceptible. No sex differentiation
Environment of infected hosts	Salinity: *A. ocellatum* tolerates a wide range of salinities; however, the optimum is 30–35‰ Temperature: influences *A. ocellatum* tolerance to salinity. From 18°C and above the parasite can successfully complete its biological cycle. Avoid temperatures ≤14°C that negatively affect its viability Oxygen: low oxygen levels can indirectly reinforce the negative impact of *A. ocellatum* Atmospheric events: typhoons and storms can promote the spread of dinospores in aerosol droplets
Identification of infected individuals	Observation of clinical symptoms
Parasite stage(s) collected	Trophonts from the target sites
Size (µm) of parasite stages	Early trophont 27 × 23 µm, grown trophont 70–130 × 50–60 µm; tomont up to 350 µm; dinospores 9–10 × 11 µm
Site of infection, including target tissues if relevant	Dependent on the parasite burden. In general. gills are the primary site of infection followed by skin, fins and eyes. In ESB the oral cavity is also a confirmed site of infection
Isolation technique(s)	Freshwater bath. For fish species that cannot tolerate this procedure it is possible to collect trophonts by gill scrapings
Post-processing	Several washes with sterile saltwater plus antibiotics. The use of antibiotics will reduce the bacterial load and contamination by other microorganisms, which can interfere with *A. ocellatum* development
Collection media	Saltwater; alternatively it is possible to use IO2/HBSS medium
Transport/maintenance conditions	Maintain tomonts in saltwater + antibiotic medium in plastic Petri dishes in an incubator at 16 ± 0.5° or 22–25°C, according to the research purposes. Seal the Petri dish lid with Parafilm
Monitoring requirements	Check tomont development periodically with an inverted microscope
Optimal fixative(s) if using	Paraformaldehyde

***Amyloodinium ocellatum in vitro* infection**	
Axenic	Yes, on G1B monolayer
Source of the inoculum	Dinospores
Quantification of infective stages	In this case it is not fundamental to count the dinospores because a few specimens are sufficient
Dose of inoculum	A few dinospores (5–10 dinoflagellates)
Culture conditions	L-15 medium + IO2/HBSS at 25°C in an incubator
Monitoring of the culture	Daily
Parasite stages cultured	Trophonts/tomonts
Storage	Hibernation of the tomont stage at 16 ± 0.5°C in an incubator
Attenuation of virulence	Not feasible
Other relevant information	Although *in vitro* infection of *A. ocellatum* is possible, it is not always sufficient to acquiring the appropriate quantity of parasites. Therefore, for a continuous supply of dinospores, we recommend preserving the parasite at the tomont stage at 16°C in an incubator; at this temperature, tomonts remain viable and maintain their infectivity when transferred to 20–25°C

***Amyloodinium ocellatum* tomont *in vitro* culture (hibernation)**	
Axenic	Even if tomonts are purified as detailed in section 4, it is virtually impossible to avoid bacterial, protozoal and/or fungal contamination. However, by adding antibiotics to the medium it is possible to control the contaminant load
Source of the inoculum	Early tomonts from the gills
Quantification of infective stages	If necessary by cell-counting chamber (S50 Sedgewick–Rafter Cell)
Dose of inoculum	The number of tomonts can be determined by using a counting chamber, but it is not fundamental to count them. However, it is recommended to inoculate tomonts at lower concentrations, because higher concentrations could interfere with preservation and indirectly with development
Culture conditions	Saltwater added plus antibiotics
Monitoring of the culture	Every 2 weeks
Renewal of culture medium, timing and harvesting	It would be better to change the medium once per month

Parasite stages cultured	Tomonts
Storage	In plastic Petri dishes, in an incubator at 16 ± 0.5°C; seal the Petri dish lid with Parafilm
Attenuation of virulence	None
Other relevant information	This *in vitro* maintenance method does not affect the viability of tomonts or dinospores: the reproductive process is simply slowed down. Furthermore, this protocol is easier to apply and allows for greater numbers of dinospores to be obtained, compared with propagation in the G1B cell line

***Amyloodinium ocellatum in vivo* infection challenge**	
Host characteristics	ESB juveniles
Water conditions	Salinity 20–31‰, temperature 20–27°C, oxygen 7–8 mg/l
Challenge dose	5–10 dinospores/ml
Parasite stage	Dinospore
Challenge method	Bath, pouring a defined concentration of dinospores directly into the aquarium
Intermediates/vectors involved	None
Timing of infection	Dinospores attach to the fish immediately after being added; subsequently it is possible to check for the presence of trophonts in the gills, as well as any symptoms
Disinfection procedures	Freshwater that is lethal for the protozoan and fallowing of rearing facilities by drying
Type of facilities required	Aquarium and tanks
Diagnosis of infection	Trophont detection by fresh gill smears
Other relevant information	It is recommended to cover the aquaria with plastic sheets to avoid undesired contamination of the uninfected group (if set up) by dinospores. Moreover, it is essential to pay the utmost attention when manipulating fish of the two different groups, because a small number of dinospores is sufficient to trigger the infection process

8. References

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