

1 CHAPTER 3.2.1.

2
3 **SSO DISEASE**
4 *(Haplosporidium costale)*
5

6 **GENERAL INFORMATION**

7 *SSO disease is caused by the protistan Haplosporidium costale (= Minchinia costalis) (13) of the*
8 *phylum Haplosporidia. Haplosporidium costale, commonly known as SSO (seaside organism), infects*
9 *the oyster Crassostrea virginica (3).*

10 *The geographical distribution of H. costale is in high salinity bays (>25 part per thousand) along the east*
11 *coast North America from Virginia, USA to Nova Scotia, Canada (2, 7). Significant oyster mortalities*
12 *attributable to SSO disease have historically been restricted to coastal Virginia, Maryland and Delaware*
13 *(2, 3), but significant mortality attributable to SSO recently occurred in Massachusetts.*

14 *The plasmodial stage of H. costale occurs intercellularly in connective tissue of the digestive gland, mantle*
15 *and gonad (3, 11); spores occur throughout connective tissue in the same organs (2, 3, 10).*
16 *Haplosporidium costale has a limited seasonality. Oysters become infected in May and June at the time*
17 *when spores are released from previously infected moribund oysters (3, 6). Infections remain subclinical*
18 *through the autumn and winter, then plasmodia develop the following March/April, followed by*
19 *synchronous sporulation and oyster mortalities in May/June (2, 3, 6). This seasonal epizootiology may be*
20 *more complicated than historically noted; H. costale sporulation recently has been detected, and verified*
21 *using molecular tools, in oysters collected in October (11, 12).*

22 *The distribution of H. costale and H. nelsoni (the causative agent of MSX disease) overlaps in high*
23 *salinity areas from Virginia, USA to Nova Scotia, Canada, and co-infections with both pathogens have*
24 *been reported (5, 6, 11). The pathogens can be readily differentiated during sporulation. Haplosporidium*
25 *costale sporulates throughout the connective tissue of most organs, whereas H. nelsoni sporulates only in*
26 *the epithelium of the digestive tubules (1, 3, 5). The plasmodial stages of these two pathogens, however,*
27 *cannot be reliably differentiated based on morphology (5), and definitive diagnosis in the absence of spores*
28 *requires molecular tools (11, 12).*

29 *Sporulation disrupts connective tissue and the high prevalence of spores in dead oysters suggests that*
30 *sporulation results in the death of the oyster. Sporulation is believed to be the end result of all H. costale*
31 *infections (1) and thus infections are invariably fatal (7).*

32 *It has not been possible to transmit H. costale experimentally in the laboratory. No life cycle has been*
33 *elucidated for any member of the phylum Haplosporidia, but an intermediate host is suspected (7).*

34 *For diagnosis, the recommended guidelines for sampling are those stated in Chapter I.2. of this Manual.*

35 **EXAMINATION PROCEDURES**

36 **1. SCREENING METHODS**

37 **1.1. Histological examination**

38 General histological procedures are detailed in Chapter I.2. of this *Manual*. A transverse section is
39 cut through the visceral mass and placed in a fixative such as Davidson's or Carson's fluid (the
40 latter enables the samples to be re-used for electron microscopy, if necessary). The ratio must be
41 no more than one volume of tissue to ten volumes of fixative. The sections are subsequently
42 treated by conventional histological procedures (4). Haplosporidia are revealed by many
43 nonspecific stains, such as haematoxylin & eosin (H&E).

44 Multinucleate plasmodia of *Haplosporidium costale* (usually 5–8 µm in diameter) occur throughout
45 the connective tissue. Plasmodia are only easily detectable between March and June. Synchronous
46 sporulation of *H. costale* can be observed throughout the connective tissue of the digestive gland,
47 mantle and gonads; sporulation does not occur in the epithelia of the digestive tubules, as seen
48 with *H. nelsoni* spores (1, 10). Spores (3 × 5 µm in size) are commonly found in gaping, moribund,
49 oysters. The parasite cannot readily be detected between July and March (1, 3); however, *H. costale*
50 infections, co-occurring with *H. nelsoni*, recently have been reported in October (11, 12).
51 Plasmodia of *H. costale* cannot reliably be distinguished from those of *H. nelsoni* on the basis of
52 morphology.

53 Infection intensity has been rated (4) as follows: localised (LO) = any infection where plasmodia
54 are localised in a small area of one tissue type; rare (R) = systemic infections with less than ten
55 plasmodia in the entire section; light (L) = systemic infections with less than two plasmodia per
56 field at ×400 magnification, but more than ten plasmodia in the entire section; moderate (M) =
57 systemic infections with 2–5 plasmodia per field at ×400 magnification; heavy (H) = more than
58 five plasmodia per field at ×400 magnification; sporulation (S) = any infection where spores are
59 present.

60 2. PRESUMPTIVE DIAGNOSTIC METHODS

61 2.1. Histology

62 When spores are present, *H. costale* can be presumptively diagnosed in the oyster *Crassostrea*
63 *virginica* if the spores are the correct size and occur throughout the connective tissue. For detailed
64 histological procedures see Section 1.1. of this Chapter and Chapter I.2. of this *Manual*.

65 2.2. Polymerase chain reaction of DNA from oyster tissue

66 A positive polymerase chain reaction (PCR) amplification is only a presumptive diagnosis because
67 it detects DNA and not necessarily a viable pathogen. Other techniques, preferably *in-situ*
68 hybridisation, must be used to visualise the pathogen.

69 Two sets of PCR primers have been developed for *H. costale* detection: SSO1358F (5'-TAC-TGC-
70 TAG-CGC-TTG-TTC-GCA-AGA-T-3') and SSO1507R (5'-TCG-GGT-CGG-CCC-GCT-GAC-
71 TGG-GT-3') (8) and SSO-A (5'-CAC-GAC-TTT-GGC-AGT-TAG-TTT-TG-3') and SSO-B (5'-
72 CGA-ACA-AGC-GCT-AGC-AGT-ACA-T-3') (11). Both of these primer pairs target the small
73 subunit ribosomal DNA and have been shown to be sensitive and specific for this pathogen (8,
74 11). A multiplex PCR (9) for *H. costale* and *H. nelsoni* (MSX) has been developed using the former
75 SSO primers (8), but it has not been validated. PCR reaction mixtures contain reaction buffer (10
76 mM Tris, pH 8.3; 50 mM KCl; 1.5 mM MgCl₂; 10 µg/ml gelatin), 400 µg/ml bovine serum
77 albumin, 25 pmoles each of SSO1358F and SSO1507R or SSO-A and SSO-B, 200 µM each of
78 dATP, dCTP, dGTP, dTTP, 0.6 units *AmpliTaq* DNA polymerase (Applied Biosystems), and
79 template DNA, in a total volume of 25 µl. The reaction mixtures are cycled in a thermal cycler.
80 The programme for the GeneAmp PCR System 9600 thermal cycler (Applied Biosystems) using
81 SSO-A and SSO-B is: initial denaturation at 94°C for 4 minutes, 35 cycles of 94°C for 30 seconds,
82 59°C for 30 seconds, and 72°C for 1.5 minutes, and a final extension at 72°C for 5 minutes. The
83 cycle programme is identical using SSO1358F and SSO1507R except that the annealing
84 temperature is 55°C. An aliquot (10% of reaction volume) of each PCR reaction is checked by
85 agarose gel electrophoresis and ethidium bromide staining for the 150 base pairs (bp)

86 amplification product of SSO1358F and SSO1507R or the 557 bp amplification product of SSO-
87 A and SSO-B.
88

88 **3. CONFIRMATORY IDENTIFICATION OF THE PATHOGEN**89 **3.1. *In-situ* hybridisation examination of *Haplosporidium costale***

90 *In-situ* hybridisation is the method of choice for confirming identification because it allows
91 visualisation of a specific probe hybridised to the target organism. DNA probes must be
92 thoroughly tested for specificity and validated in comparative studies before they can be used for
93 confirmatory identification.

94 *In-situ* hybridisation has recently been developed to differentiate plasmodia of *H. costale* from
95 those of *H. nelsoni* in tissue sections (11). Species-specific labelled oligonucleotide probes
96 hybridise with the small subunit ribosomal RNA of the parasites. This hybridisation is detected by
97 an antibody conjugate that recognises the labelled probes. Substrate for the antibody conjugate is
98 added, causing a colorimetric reaction that enables visualisation of probe–parasite RNA
99 hybridisations.

100 The procedure for the *in-situ* hybridisation is conducted as follows. Positive and negative controls
101 must be included in the procedure.

- 102 i) Cut a transverse section through the visceral mass that includes mantle, gill and digestive
103 gland and place it in Davidson's AFA fixative (glycerin [10%], formalin [20%], 95% ethanol
104 [30%], dH₂O [30%], glacial acetic acid [10%]) for approximately 24 hours, then transfer to
105 70% ethanol until processed by histological procedures (step ii). The ratio must be no more
106 than one volume of tissue to ten volumes of fixative.
- 107 ii) The samples are subsequently embedded in paraffin by conventional histological procedures.
108 Sections are cut at 5–6 µm and placed on positively-charged slides or 3-aminopropyl-
109 triethoxylane-coated slides. Histological sections are then dried overnight in an oven at 40°C.
- 110 iii) The sections are deparaffinised by immersing them in xylene or another less toxic clearing
111 agent for 10 minutes. The solvent is eliminated by immersion in two successive absolute
112 ethanol baths for 10 minutes each and rehydrated by immersion in an ethanol series. The
113 sections are then washed twice for 5 minutes in phosphate buffered saline (PBS).
- 114 iv) The sections are treated with proteinase K, 50 µg/ml in PBS, at 37°C for 15 minutes. The
115 reaction is then stopped by washing the sections in PBS with 0.2% glycine for 5 minutes.
116 The sections are then placed in 2' SSC (standard saline citrate) for 10 minutes.
- 117 v) The sections are prehybridised for 1 hour at 42°C in prehybridisation solution (4' SSC, 50%
118 formamide, 5' Denhardt's solution, 0.5 mg/ml yeast tRNA, and 0.5 mg/ml heat-
119 denaturated herring sperm DNA).
- 120 vi) The prehybridisation solution is then replaced with prehybridisation buffer containing
121 5 ng/µl of the digoxigenin-labelled oligonucleotide probe. The sequence of the probe
122 designated SSO1318 (11) is 5'-CGA-ACA-AGC-GCT-AGC-AGT-ACA-T-3'. The sections
123 are covered with *in-situ* hybridisation plastic cover-slips and placed on a heating block at
124 90°C for 12 minutes. The slides are then cooled on ice for 1 minute before hybridisation
125 overnight at 42°C in a humid chamber.
- 126 vii) The sections are washed twice for 5 minutes each in 2' SSC at room temperature, twice for
127 5 minutes each in 1' SSC at room temperature, and twice for 10 minutes each in 0.5' SSC at
128 42°C. The sections are then placed in Buffer 1 (100 mM Tris, pH 7.5, 150 mM NaCl) for 1–
129 2 minutes.
- 130 viii) The sections are placed in Buffer 1 (see step vii) supplemented with 0.3% Triton X-100 and
131 2% sheep serum for 30 minutes. Anti-digoxigenin alkaline phosphatase antibody conjugate is
132 diluted 1/500 (or according to the manufacturer's recommendations) in Buffer 1
133 supplemented with 0.3% Triton X-100 and 1% sheep serum and applied to the tissue

- 134 sections. The sections are covered with *in-situ* hybridisation cover-slips and incubated for
135 3 hours at room temperature in the humid chamber.
- 136 ix) The slides are washed twice in Buffer 1 for 5 minutes each (see step vii) and twice in Buffer 2
137 (100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 5 minutes each. The slides are then
138 placed in colour development solution (337.5 µg/ml nitroblue tetrazolium, 175 µg/ml 5-
139 bromo-4-chloro-3-indolylphosphate p-toluidine salt, 240 µg/ml levamisole in Buffer 2) for 2
140 hours in the dark. The colour reaction is stopped by washing in TE buffer (10 mM Tris, pH
141 8.0, 1 mM EDTA [ethylene diamine tetra-acetic acid]).
- 142 x) The slides are then rinsed in dH₂O. The sections are counterstained with Bismarck Brown Y,
143 rinsed in dH₂O, and cover-slips are applied using an aqueous mounting medium. The
144 presence of *H. costale* is demonstrated by the purple-black labelling of the parasitic cells.

145 REFERENCES

- 146 1. ANDREWS J.D. (1982). Epizootiology of late summer and fall infections of oysters by *Haplosporidium*
147 *nelsoni*, and comparison to annual life cycle of *Haplosporidium costalis*, a typical haplosporidan. *J.*
148 *Shellfish Res.*, **2**, 15–23.
- 149 2. ANDREWS J.D. (1988). *Haplosporidium costale* disease of oysters. *In: Disease Diagnosis and Control in*
150 *North American Marine Aquaculture*, Sindermann C.J. & Lightner D.V., eds. Elsevier, Amsterdam,
151 The Netherlands, 296–299.
- 152 3. ANDREWS J.D. & CASTAGNA M. (1978). Epizootiology of *Minchinia costalis* in susceptible oysters in
153 seaside bays of Virginia's eastern shore, 1959–1976. *J. Invert. Pathol.*, **32**, 124–138.
- 154 4. BURRESON E.M., ROBINSON M.E. & VILLALBA A. (1988). A comparison of paraffin histology and
155 hemolymph analysis for the diagnosis of *Haplosporidium nelsoni* (MSX) in *Crassostrea virginica*
156 (Gmelin). *J. Shellfish Res.*, **7**, 19–23.
- 157 5. COUCH J.A. (1967). Concurrent haplosporidian infections of the oyster, *Crassostrea virginica* (Gmelin).
158 *J. Parasitol.*, **53**, 248–253.
- 159 6. COUCH J.A. & ROSENFELD A. (1968). Epizootiology of *Minchinia costalis* and *Minchinia nelsoni* in
160 oysters introduced into Chincoteague Bay, Virginia. *Proc. Natl. Shellfish. Assoc.*, **58**, 51–59.
- 161 7. FORD S.E. & TRIPP M.R. (1996). Diseases and defense mechanisms. *In: The Eastern Oyster*
162 *Crassostrea virginica*, Kennedy V.S., Newell R.I.E. & Eble A.F., eds. Maryland Sea Grant College,
163 College Park, Maryland, USA, 581–660.
- 164 8. KO Y.-T., FORD S.E. & FONG D. (1995). Characterization of the small subunit ribosomal RNA gene
165 of the oyster parasite *Haplosporidium costale*. *Molec. Marine Biol. Biotechnol.*, **4**, 236–240.
- 166 9. PENNA M.-S., KHAN M. & FRENCH R.A. (2001). Development of a multiplex PCR for the detection
167 of *Haplosporidium nelsoni*, *Haplosporidium costale* and *Perkinsus marinus* in the eastern oyster (*Crassostrea*
168 *virginica*, Gmelin, 1791). *Molec. Cell. Probes*, **15**, 385–390.
- 169 10. PERKINS F.O. (1969). Electron microscope studies of sporulation in the oyster pathogen *Minchinia*
170 *costalis* (Sporozoa: Haplosporida). *J. Parasitol.*, **55**, 897–920.
- 171 11. STOKES N.A. & BURRESON E.M. (2001). Differential diagnosis of mixed *Haplosporidium costale* and
172 *Haplosporidium nelsoni* infections in the eastern oyster, *Crassostrea virginica*, using DNA probes. *J.*
173 *Shellfish Res.*, **20**, 207–213.

- 174 12. SUNILA I., STOKES N.A., SMOLOWITZ R., KARNEY R. C., & BURRESON E.M. (2002). *Haplosporidium*
175 *costale* (Seaside Organism), a parasite of the eastern oyster, is present in Long Island Sound. *J. Shellf.*
176 *Res.*, **21**, 113–118.
- 177 13. WOOD J.L. & ANDREWS J.D. (1962). *Haplosporidium costale* (Sporozoa) associated with a disease of
178 Virginia oysters. *Science*, **136**, 710–711.

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