EVOSTC Annual Project Report

Project Number: **12120111-J**
Project Title: **PWS Herring Program - What is the age at first spawning for female herring in PWS?**
PI Names: Ron Heintz, JJ Vollenweider
Time Period Covered: February 1, 2012 - February 15, 2013
Date of Report: February 15, 2013
Report Prepared By: R. Heintz, J. Vollenweider
Project Website: N/A

Work Performed:
Work is on schedule as planned.

Histological examination of lab gonad samples were completed by Dr. Gary Marty in July (See Appendix for report). Among the 177 gonads collected from post-spawned fish in the laboratory, 113 were ovaries that had features consistent with previous mature egg development, three were testis that had features consistent with previous sperm development, and one was an ovary that had follicle development (i.e., it probably would have spawned next year) but no good evidence of previous mature egg development (this was diagnosed as an immature female; alternatively, it might be a female that spawned but no longer has microscopic evidence of having spawned).

Ovarian microscopic features most useful for assessing whether the fish had spawned include postovulatory follicles and postovulatory eggs. Other findings that might be useful include (*i*) pigmented macrophage aggregates and (*ii*) nonspecific inflammation. In my experience, ovaries that have not spawned usually do not have either pigmented macrophage aggregates or nonspecific inflammation. In contrast, ovaries that have spawned might have nonspecific inflammation longer than obvious postovulatory follicles. The sensitivity and specificity of "nonspecific inflammation" as a biomarker of previous spawning needs to be validated under controlled conditions.

Scales and gonads were collected from ~200 herring in PWS in July 2012. The gonad samples have been sent to Dr. Gary Marty for histological analysis. Scale analysis will commence in early October. Scale aging and growth increment measurements from laboratory and field collections will be compared to histological results to determine if scales can be used to detect age-at first spawn and skip spawning in Pacific herring.

Future Work:
We are currently in the process of histological analysis & scale examination of the herring samples caught in July 2012 in Prince William Sound. Upon completion, we will relate histological results to scale growth increments. The final sample collection will be made from herring spawning aggregations in PWS this spring, and a scale analysis contract will be put in place for their examination to occur, including aging and measurement of growth increments.

Coordination/Collaboration:
Summer 2012 field collections were made with the collaboration of many, including 1) Rob Campbell and the PWSSC for vessel support, 2) Evelyn Brown for locating herring schools during aerial surveys (we also ground-truthed several fish schools for the aerial surveys), 3) Yumi Arimatsu (USGS) for sharing herring school locations and catching opportunistic herring samples while conducting LTM acoustic-trawl surveys, 4) Rich Brenner and Steve Moffit (ADF&G) for sharing whale and herring school locations, and 5) Dave Janka (M/V Aukelet) for sharing whale and herring school locations.

Additionally, while catching herring samples, we collected photo-ID's of humpback whales for the LTM humpback study, which constitutes one of only a few summer observations across PWS.

Community Involvement/TEK & Resource Management Applications:
The results from this study have significant implications for resource management. The goal of this study is to determine the age when herring first spawn in PWS. The predictive capabilities of the ASA population model for herring in PWS may be improved by validating the assumption that herring first spawn at age three. Knowing the proportion of fish in each age class that spawn and the proportion of primiparous individuals in each age class will provide a means for adjusting estimates of the total post-spawning biomass in the ASA by estimating the proportion of each age class that was not on the spawning grounds in the previous year. Data regarding the proportions of spawners by age class would improve the accuracy of model estimates of spawning stock biomass.

Information Transfer:
Preliminary results will be presented at the AK Marine Science Symposium in January 2013. Final results will be published in a journal.

Budget:
Budget expenditures are proceeding as per projections.

***EXXON VALDEZ* OIL SPILL TRUSTEE COUNCIL**
ANNUAL PROGRAM REPORT YEAR 1

Budget Category:	Proposed Year 1	Actual Year 1	TOTAL Difference
Personnel			$0
Travel	$4,000	$4,741	($741)
Contractual	$38,000	$39,790	($1,790)
Commodities	$3,500		$3,500
Equipment			$0
Indirect Costs (*will vary by proposer*)			$0
SUBTOTAL	$45,500	$44,531	$969
General Administration (9% of subtotal)	$4,095	$4,008	$87
PROJECT TOTAL	$49,595	$48,539	$1,056
Other Resources (Cost Share Funds)	$0	$0	$0

COMMENTS:

FY12	**Program Title: HRM Age at First Spawn** **Team Leader: Heintz**	**SUMMARY**

APPENDIX 1.

Interim Histopathology Report

2011 Pacific Herring Ovarian Development Study - NOAA Auke Bay Laboratory

Pathologist: Gary D. Marty, DVM, Ph.D., Diplomate, A.C.V.P.

Date: July 12, 2012

Diagnoses: For details of the diagnoses, see the spreadsheet included with the report <2011ABL_Herring_histopath-INTERIM.xlsx >.

Interim Comment: Among the 177 gonads received, 113 are ovaries that have features consistent with previous mature egg development, three are testis that have features consistent with previous sperm

development, and one is an ovary that has follicle development (i.e., it probably would have spawned next year) but no good evidence of previous mature egg development (this was diagnosed as an immature female; alternatively, it might be a female that spawned but no longer has microscopic evidence of having spawned).

Ovarian microscopic features most useful for assessing whether the fish had spawned include postovulatory follicles and postovulatory eggs. Other findings that might be useful include (*i*) pigmented macrophage aggregates and (*ii*) nonspecific inflammation. In my experience, ovaries that have not spawned usually do not have either pigmented macrophage aggregates or nonspecific inflammation. In contrast, ovaries that have spawned might have nonspecific inflammation longer than obvious postovulatory follicles. The sensitivity and specificity of "nonspecific inflammation" as a biomarker of previous spawning needs to be validated under controlled conditions.

This report has been written without knowledge about the actual exposure history of the fish. This report will be revised after the history of each fish is revealed.

Histopathology: Cassetted gonads from 117 Pacific herring (*Clupea pallasii*) fixed in formalin were received on 5 May 2012 by the British Columbia Animal Health Centre for routine processing to H&E slides. The 117 cassettes have labels progressing discontinuously from 311001 through 314267 (see spreadsheet <2011ABL_Herring_histopath-INTERIM.xlsx> for details). After the gonads were processed routinely into paraffin, each was transected once near the cut edge. The transected piece was embedded transversely to the original piece, yielding one sagittal and one transverse section through each gonad. Slide labels are the same as the original cassette labels. The paraffin blocks from which these tissues were sectioned are retained by the BC Animal Health Centre and filed under the Animal Health Centre's case number 2012-1956; therefore, any requests for recuts need to include the Animal Health Centre's case number. After the analysis contract was awarded on 5 July 2012, the slides were transferred to me (Dr. Gary Marty, Fish Pathology Services).

I examined each slide in ascending numerical order by slide number (= original Auke Bay number) without knowledge of the history of the fish (i.e., blinded study). All gonads were completely scanned using a 4× objective lens, with scans at higher magnification as needed. Each gonad was assessed for maturity and sex, quality control variables, and microscopic findings. Variables were either assigned a category (male vs. female, immature vs. mature, absent "0" vs. present "1"), a number (e.g., # of postovulatory eggs in the section), or a semiquantitative score selected from none (0), mild/small numbers (1), moderate (2), or severe/abundant (3). Good examples or "Type Specimens" for each variable are summarized (Table 1). Abbreviation and case definitions for each are described in Appendix 1, and the results are in spreadsheet <2011ABL_Herring_histopath-INTERIM.xlsx>.

The scientific literature contains different terms used for stages of ovarian development in herring. I use the following, in order of developmental stage (Bucholtz et al. 2008):

Oogonia – the smallest and least developed of the oocytes, they range from 30 - 100 µm in diameter, with a central nucleus (pale, eosinophilic, oval, and slightly foamy) that is surrounded by deeply basophilic cytoplasm. The diameter of the nucleus is about half of the diameter of the oocyte.

Primary oocyte – morphologic features are similar to oogonia except that they are larger, up to 160 µm in diameter. Primary oocytes are the earliest stage of ovarian maturation, and they do not occur in juvenile fish.

Secondary oocyte, cortical alveoli stage (see slide 314262) – With continued growth of the oocyte (200 - 300 µm diameter), the cytoplasm develops peripheral vacuoles that are up to 30 µm in diameter. The margin of the nucleus is more angular and the nucleoplasm is more homogenous and dull-eosinophilic. Nuclear diameter is slightly larger than in oogonia, but is comprises only 25 - 33% of total oocyte diameter.

Secondary oocyte, yolk granule stage (no examples in these samples) – With continued growth of the oocyte (>300 µm in diameter), the oocyte cytoplasm develops brightly eosinophilic yolk granules between the nucleus and the cortical alveoli.

Other terms – structures scored for each ovary include postovulatory follicles, postovulatory eggs, and oocyte/follicular atresia; they are described in Appendix 1.

Quality Control: Tissue autolysis (a measure of decomposition after the fish dies) is "none" for all 117 gonads. Preservation and staining quality are excellent for two groups of gonads (311001 - 311092, 42 fish; and, 314238 - 314267, 30 fish) but of marginal quality for another group of gonads (311093 - 314237, 45 fish). Among the marginally preserved gonads, the deeply basophilic contents of immature (nonyolked) follicles are displaced by large oval to bilobed vacuoles. The vacuoles are most pronounced at the periphery of the ovaries (i.e., central regions of large ovaries have minimal artifact). No other cell types are vacuolated. In affected slides, erythrocyte cytoplasm stains poorly, and sometimes the nuclei of the immature eggs stains pale basophilic instead of deeply basophilic (e.g., slide 314216). This is probably a specific type of artifact that might be a result of exposure to an incorrect chemical during fixation. For example, transfer of tissues from formalin to alcohol and then back to formalin often decolorizes erythrocyte cytoplasm; however, I am not aware that this transfer causes vacuoles in immature ovarian follicles. If the tissues were air lifted before they were preserved, the artifacts might be air vacuoles resulting from decreased pressure during transfer; however, the lack of vacuoles within vessels makes this possibility unlikely. This problem can probably be avoided by ensuring the tissues are immersed 24 hours in only 10% neutral buffered formalin for fixation. After tissues are preserved; they can be safely transferred to tap water for transport to the histology laboratory.

Most slides have mild artifact (tissue folds and splits), as expected with paraffin sections. Tissues have no acid hematin deposits and no postfixation dehydration.

Literature Cited:

Bucholtz, R.H., Tomkiewicz, J. & Dalskov, J. (2008) Manual to determine gonadal maturity of herring (*Clupea harengus* L.). DTU Aqua-report 197-08, Charlottenlund: National Institute of Aquatic Resources. 45 p.

Table 1. Summary of type specimens for variables in Pacific herring gonads.

Abbreviation[1]	None
	Score = 0
		Mild/small #s
		Score = 1
			Moderate
			Score = 2
				Severe/abundant
				Score = 3

Atly	311001	none	none	none
ART	311001, 314262	311004	none	none
VIF	311001	none	none	311093
PFD	311001	none	none	none
AHT	311001	none	none	none
POF	314238	311033, 314246	not applicable	
MPE	311001		311067	
ICH	311001	311062	314244	314257
ANI	311001	311011	none	none
GGR	311001	311004	311087	none
EGC	310079	311064	311047	311001
NSI	none	314262	311029, 311078	311080, 311091
GMA	311001	311047	none	none
HVW	311001	311004	none	none
OAI	311001	311065	314241	314258
OAM	311001	311069	none of quality	none
RCF	311001	none	none	none
TIP	none	311002	none	none

[1]For explanation of abbreviations, see case definitions in Appendix 1.

Appendix 1. Case definitions for each variable related to Pacific herring gonad histopathology.

Maturity:

Juvenile - juvenile ovaries have no oocytes developed beyond oogonia, and they have no evidence that oocytes had ever developed beyond oogonia.

Developing - developing ovaries have developing oocytes but no evidence that follicles ever developed mature eggs. Ovaries in this category have no postovulatory follicles (POF), no postovulatory eggs in the section (POE), and no oocyte atresia of mature follicles (OAM).

Postspawning - postspawning ovaries have evidence that follicles have developed mature eggs, and postspawning testis have evidence that seminiferous tubules have developed mature sperm. Ovaries in this category have at least one score greater than zero for postovulatory follicles (POF), postovulatory eggs in the section (POE), or oocyte atresia of mature follicles (OAM).

Quality control:

1) Atly = Autolysis. Changes in membrane integrity begin immediately after death.

Score = 0; no membrane changes, erythrocytes stain intensely.

Score = 1; loss of membrane integrity; erythrocytes are pale.

Score = 2; none are moderate.

Score = 3; none are severe.

2) ART = Artifact. Tissue changes that are not inherent in the tissue sampled. Sources of artifact included handling at necropsy, processing, sectioning, and staining. Artifact is scored on the basis that it impeded interpretation of tissue morphology. Examples of artifact include splits, bubbles, folds, or knife marks. During spawning, eggs are nearly free within the ovary; therefore, representative cross sections are nearly impossible to obtain and artifact is often severe.

Score = 0; sections have no tissue alterations that would impede analysis or photography of any part of the sections.

Score = 1; tissue alterations are present, but most areas could still be photographed without artifact, and analysis for lesions is unaffected.

Score = 2; tissue alteration prevented analysis for lesions in some areas and photography would be unacceptable anywhere.

Score = 3; tissue alterations are too extensive for histopathologic analysis.

3) VOC = vacuolation of oocyte cytoplasm. In affected oocytes, the deeply basophilic cytoplasm is displaced by a single vacuole that is oval to bilobed and usually larger than the nucleus. These vacuoles are different from the multiple, small vacuoles that occur normally at the periphery of the

cytoplasm in secondary oocytes, cortical alveoli stage. No other cell types are vacuolated. Another change in affected slides, erythrocyte cytoplasm stains poorly, and sometimes the cytoplasm of primary oocytes stains pale basophilic instead of deeply basophilic (e.g., slide 314216). This is a specific type of artifact. I do not know the cause, but it might be a result of exposure to an incorrect chemical during fixation. For example, transfer of tissues from formalin to alcohol and then back to formalin often decolorizes erythrocyte cytoplasm; however, I am not aware that this transfer causes vacuoles in oocyte cytoplasm.

Score = 0; the section has no vacuoles in oocyte cytoplasm.

Score = 1; the section has vacuoles, but ≤10% of the oocytes are affected.

Score = 2; 10% - 33% of the oocytes are vacuolated.

Score = 3; >33% of oocytes are vacuolated.

4) PFD = postfixation dehydration. This is a specific type of artifact. The margins of affected organs have evidence of dehydration after fixation (e.g., nuclei stain dull blue; erythrocyte cytoplasm stains yellow instead of red; cytoplasm of other cells stains pale blue or not at all). The most common cause is removal of tissues from liquid for more than a few minutes (e.g., during shipment or trimming). Other possible causes include fixation in formalin that is too concentrated (e.g., 100% formalin instead of 10% formalin), or transfer to ethanol that is too concentrated (e.g., >70% ethanol) before processing to paraffin, or immersion in hypertonic saltwater formalin.

Score = 0; no postfixation dehydration.

Score = 1; postfixation dehydration limited to total sectional area less than 500 µm in diameter.

Score = 2; total sectional area of postfixation dehydration >500 µm, but no 10× objective-lens field is completely affected by PFD.

Score = 3; total sectional area of postfixation dehydration fills at least one 10× objective-lens field.

5) AHT = Acid hematin. This is a specific type of artifact. Acid hematin is a granular brown pigment that accumulates when tissues are not fixed in neutral buffered fixative and when tissues become acidic during fixation. Acid hematin is birefringent under polarized light; the primary differential, melanin granules, are not birefringent under polarized light. Acid hematin granules are most common where erythrocytes accumulate (e.g., anywhere in the spleen, and around congested blood vessels in the liver). In cases of decreased blood flow, acid hematin can be a "useful artifact" (i.e., consistent with lactic acid as a result of decreased perfusion).

a) Score = 0; no acid hematin deposits.

b) Score = 1; acid hematin deposits are limited to total sectional area <500 µm in diameter.

c) Score = 2; total sectional area of acid hematin deposits >500 µm, but deposits do not prevent analysis of tissues for lesions.

d) Score = 3; extensive acid hematin deposits impair analysis of tissues for lesions.

Microscopic findings:

I. POF = postovulatory follicle. Postovulatory follicles are what remain of the follicle after the egg is released. Recent ovulation is sometimes associated with vascular congestion. After the egg is released from the follicle, the follicular epithelium contracts and the lumen eventually disappears. Follicular cells tend to have foamy cytoplasm and postovulatory follicles are surrounded by an irregular thin band of collagen. Granulomatous inflammation commonly occurs with postovulatory follicles; the inflammation can be sterile, and it is scored separately in the GGR category.
   A. Score = 0; no postovulatory follicles.
   B. Score = 1; postovulatory follicles are present.
   C. Score = 2; not applicable.
   D. Score = 3; not applicable.

II. POE = # of postovulatory eggs in the section. This score is used for eggs that have been released from their follicle but not spawned. The chorion might be present, but many these eggs have rupture and are being cleaned up by inflammatory cells.
   A. Score = 0; no postovulatory eggs in the section.
   B. Score = ≥1; the number of postovulatory eggs in the section (any sections with more than 10 eggs are designated as ">10").

III. ICH = gonadal *Ichthyophonus hoferi*. *Ichthyophonus hoferi* is a primitive parasite associated with chronic systemic disease and mortality in Pacific herring (Marty et al. 2010). The gonad is the least common organ to be infected (Marty et al. 1998); therefore, any occurrence in the gonad is evidence of much greater prevalence in the population.
   A. Score = 0; sections have no *Ichthyophonus hoferi*.
   B. Score = 1; *Ichthyophonus* present, but <1 per 10× objective-lens field and minimal inflammation.
   C. Score = 2; ≥1 and <3 *Ichthyophonus hoferi* per 10× objective-lens field, but minimal inflammatory reaction, or <1 per 10× objective-lens field with moderate to abundant inflammation.
   D. Score = 3; ≥1 *Ichthyophonus hoferi* per 10× objective-lens field, with prominent granulomatous inflammation, or ≥3 *Ichthyophonus hoferi* foci per 10× objective-lens field, regardless of amount of inflammation.

IV. ANI = *Anisakis*. A nematode parasite common in the visceral cavity of Pacific herring.
   A. Score = 0; no *Anisakis* in the slide.
   B. Score = 1; the slide contains 1-8 sections of *Anisakis*.
   C. Score = 2; the slide contains 9-15 sections of *Anisakis*.
   D. Score = 3; the slide contains >15 sections of *Anisakis*.

V. GGR = gonadal granulomas (or focal granulomatous inflammation). Foci of granulomatous

inflammation are distributed throughout the interstitium. GGRs occasionally contain eosinophilic granular cells (EGCs). This lesion does NOT include inflammation scored as part of the *Ichthyophonus* score.

A. Score = 0; no granulomatous inflammation in sections examined.
B. Score = 1; sections have <1 focus of granulomatous inflammation per 10× objective-lens field.
C. Score = 2; sections have ≥1 but <3 foci of granulomatous inflammation per 10× objective-lens field.
D. Score = 3; sections have ≥3 foci of granulomatous inflammation per 10× objective-lens field.

VI. EGC = eosinophilic granular cells. EGCs are a type of inflammatory cell that are often scattered throughout the ovary in low numbers (i.e., a score of 1 might be normal), but sometimes they occur in higher numbers. With a similar appearance, follicular cells with eosinophilic granules are considered normal and are NOT scored as EGCs. EGCs associated with ICH or GGR are incorporated into the ICH or GGR scores and are NOT included in the EGC score. EGCs are not directly associated with any foreign material/body, although that might be responding to something outside of the plane of section examined.

A. Score = 0; ≤2 (and usually zero) EGCs in every 40× objective-lens field.
B. Score = 1; >2 EGCs in at least one 40× objective-lens field scored, but ≤25 in all 40× objective-lens fields.
C. Score = 2; 15 - 25 EGCs in several 40× objective-lens fields, with an occasional f40× objective-lens with >25 EGCs.
D. Score = 3; >25 EGCs in several 40× objective-lens fields.

VII. NSI = nonspecific inflammation. Postspawning ovaries commonly have a range of inflammatory changes that can include proteinaceous edema, lymphocytes, and histiocytes. A few cases have focal inflammation that forms a lattice-like structure that is about the size of a mature egg (e.g., slides 311061 and 314250); these structures have no remnants of yolk, so they might not be of egg origin. Most of this inflammation is probably related to previous spawning. Along with pigmented macrophage aggregates, this inflammation is probably the most persistent evidence of previous spawning (i.e., it remains longer that postovulatory follicles or postovulatory eggs). This lesion does NOT include inflammation scored as part of ICH, ANI, GGR, or EGC.

A. Score = 0; no nonspecific inflammation.
B. Score = 1; nonspecific inflammation comprises <5% of the volume of the section.
C. Score = 2; nonspecific inflammation comprises 5 – 20% of the volume of the section.
D. Score = 3; nonspecific inflammation comprises >20% of the volume of the section.

VIII. GMA = gonadal pigmented macrophage aggregates. Macrophage aggregates are pigmented yellow-brown to green-brown, and occasionally contain lymphocytes and EGCs. They are probably a good marker of previous follicle maturation and spawning. Fish that have not matured eggs are less likely to have gonadal macrophage aggregates, but massive atresia of immature follicles might lead to accumulation of pigmented macrophage aggregates.

A. Score = 0; no pigmented macrophage aggregates.
B. Score = 1; sections have ≤2 GMAs > 60 µm in diameter per 10× objective-lens field.
C. Score = 2; sections have >2 but ≤5 GMAs > 60 µm in diameter per 10× objective-lens field.
D. Score = 3; sections have >5 GMAs > 60 µm in diameter per 10× objective-lens field.

IX. HVW = hyalinized vessel walls. Vessel walls are thickened with dull, amorphous, eosinophilic

material. Hyalinization is usually limited to ovaries immediately postspawning.

A. Score = 0; no hyalinization of vessel walls.
B. Score = 1; at least one vessel wall is hyalinized and the wall is ≤40 µm thick.
C. Score = 2; at least one vessel wall is hyalinized and the wall is >40 µm but <80 µm thick.
D. Score = 3; none are severe.

X. OAI = atresia of unyolked (immature) follicles; a change/lesion in ovaries based on the estimated average number of atretic follicles per 10× objective-lens field. Initial stages of "immature" atretic follicles have irregularities and breaks in the outer margin of the oocyte. Advanced stages have complete fragmentation of the oocyte cytoplasm. Final stages of atresia have marked shrinkage, complete fragmentation of nucleus, and variable infiltration with macrophages.

A. Score = 0; no atretic unyolked follicles in the section.
B. Score = 1; <1 atretic unyolked follicle per 10× objective-lens field.
C. Score = 2; 1-3 atretic unyolked follicles per 10× objective-lens field.
D. Score = 3; >3 atretic unyolked follicles per 10× objective-lens field.

XI. OAM = atresia of yolked (maturing or mature) follicles; a change/lesion in ovaries based on the estimated average number of mature atretic follicles per 4× objective-lens field. Initial stages of "mature" atretic follicles have irregularities and breaks in the chorion and disruption of the cytoplasmic membrane. Advanced stages have complete fragmentation of the chorion, degeneration of lipid vacuoles and yolk protein droplets, increased cytoplasmic eosinophilia, and complete dissolution of the nucleus. Final stages of atresia have marked shrinkage, complete fragmentation of both chorion and nucleus, replacement of the cytoplasm by granular, eosinophilic necrotic debris, and variable infiltration with macrophages.

A. Score = 0; no atretic follicles in the section.
B. Score = 1; <1 atretic follicle per 4× objective-lens field.
C. Score = 2; 1-3 atretic follicles per 4× objective-lens field.
D. Score = 3; >3 atretic follicles per 4× objective-lens field.

XII. RCF = rupture/collapse of yolked follicles; a change/lesion in ovaries based on the estimated average number of ruptured follicles per 4× objective-lens field. Unlike atretic follicles, where individual fragments are usually <10 µm in diameter, the zona radiata of ruptured follicles is broken in only a few spots. The fragments, often >100 µm long, are folded and surrounded by variables numbers of macrophages and EGCs.

A. Score = 0; no ruptured follicles in the section.
B. Score = 1; <1 ruptured follicle per 4× objective-lens field.
C. Score = 2; 1-3 ruptured follicles per 4× objective-lens × field.
D. Score = 3; >3 ruptured follicles per 4× objective-lens field.

XIII. TIP = testicular intratubular protozoan. Protozoa are usually first identified as a 60-µm-diameter clear space within seminiferous tubules. The clear space sometimes contains sporulated on unsporulated oocysts of *Eimeria sardinae*. Females = NA.

A. Score = 0; no coccidians within seminiferous tubules.
B. Score = 1; <5 coccidia per 10× objective-lens field.
C. Score = 2; ≥5 but <15 coccidia per 10× objective-lens field.
D. Score = 3; ≥15 coccidia per 10× objective-lens field.

Literature Cited:

Marty, G.D., E.F. Freiberg, T.R. Meyers, J. Wilcock, T.B. Farver, and D.E. Hinton. 1998. Viral hemorrhagic septicemia virus, *Ichthyophonus hoferi*, and other causes of morbidity in Pacific herring *Clupea pallasi* spawning in Prince William Sound, Alaska, USA. Dis. Aquat. Org. 32:15-40.

Marty, G.D., P.-J.F. Hulson, S.E. Miller, T.J. Quinn II, S.D. Moffitt, and R.A. Merizon. 2010. Failure of population recovery in relation to disease in Pacific herring. Dis. Aquat. Org. 90:1-14. doi:10.3354/dao02210