Application of Hemolymph Smear Histology Diagnosis to Detect Disseminated Neoplasia in Basket Cockles (Clinocardium nuttallii)

Authors: Vandepas, Lauren E., Crim, Ryan N., Gilbertson, Emily, Yonemitsu, Marisa A., Unsell, Elizabeth, et al.

Source: Journal of Shellfish Research, 43(2) : 239-244

Published By: National Shellfisheries Association

URL: https://doi.org/10.2983/035.043.0211

Journal of Shellfish Research, Vol. 43, No. 2, 239-244, 2024.

APPLICATIO OF HEMOLYMPH SMEAR HISTOLOGY DIAGNOSIS TO DETECT DISSEMINATED NEOPLASIA I BASKET COCKLES *(CLINOCARDIUM NUTTALL//)*

LAURENE. VANDEPAS,'h RYA N. CRIM,3 EMILY GILBERTSON,2 MARISA A. YONEMITSU,[4,5] ELIZABETH UNSELL,6 MICHAEL J. METZGER,4 ADAM LACY-HULBERT[2] AND FREDERICK W. GOETZ[1]

[1]Northwest Fisheries Science Center, National Oceanographic and Atmospheric Administration, Montlake Boulevard E, Seattle, WA 98112; [2]Benaroya Research Institute at Virginia Maso1z, 9th Ave1zue, Seattle, WA 98101; [3]Puget Sound Restoratio1zFu1zd, Day Road W, Bainbridge Island, WA 981JO; [4]Pacific Northwest Research Institute, Broadway, Seattle, WA 98122; [5]Molecular and Cellular Biology Program, University of Washington, NE Campus Parkway, Seattle, WA 98195; [6]Fisheries Departme1zt, Suquamish Tribe, Suquamish, WA 98392

ABSTRACT Disseminated neoplasia (DN)isa form of cancerin bivalve molluscsthatcan be transmissible between individuals and in some cases across species. Neoplastic cells are highly proliferative, and infection is usually lethal. Commercially valuable bivalve species (mussels, cockles, softshell clams, and oysters) are affected by outbreaks of DN, making disease diagnosis and mitigation an important issue in ecological restoration efforts and aquaculture. Basket cockles *(C/inocardium nuttalli1)* are native to the North American Pacific coast from California to Alaska. Recent concern from some Coast Salish Tribes regarding an observed long-term decline in basket cockle populations in Puget Sound, WA has increased interest in monitoring efforts and subsequent collection for aquarium-reared broodstock. Disseminated neoplasia was detected in Puget Sound basket cockle populations, delaying aquaculture efforts so that potential broodstock could be assessed for the presence of DN. This study details a minimally invasive, inexpensive, nonlethal method for high-throughput screening for DN in adult basket cockles. The hemolymph smear screening method to diagnose DN in *C nuttallii* can be applied at field sites at low financial cost. Results of the hemolymph smear technique were validated against whole tissue histology, the standard method for DN diagnosis. Due to the similar cellular morphologies of DN in different bivalve species, it is proposed that hemolymph histology can likely be applied for diagnosing DN in other bivalves.

KEY WORDS: disseminated neoplasia, *Clinocardium nuttallii,* disease screening, tissue histology, basket cockle

INTRODUCTION

Diverse bivalve species worldwide are susceptible to disseminated neoplasia{DN), a disease in which cancerous cells proliferate inthe hemolymph- the blood-like fluid in molluscs containing a mixture of nutrients, respiratory gases, and circulating cells - of the infected individual and eventually disperse through unknown mechanisms intosolid tissuesin advanced stagesof disease (Cooper et al. 1982b, Barber 2004, Alderman et al. 2017). Recently, DN in multiple bivalve taxa is transmissible between individuals or, in somecases, between species(Metzger et al. 2015, 2016, Murchison 2016, Yonemitsu etal.2019). In cases where **DN** has been determined to be a transmissible cancer, the lineages of contagious cancerous cells usually arise independently in each species (reviewed in Metzger et al. 2016). Disseminated neoplasia has been reported in oysters, mussels *(Mytilus trossulus),* softshell clams *(Mya arenaria),* common cockles *(Cerastoderma echlle),* and golden carpet shell clams *(Polititapes aureus)* (Fanley 1969, Frierman & Andrews 1976, reviewed in Metzger et al. 2016). Disseminated neoplasia infection is generally lethal (Elston et al. 1988, Barber 1990, Dairain et al. 2020), though recovery in some infected individuals has been reported (Cooper et al. 1982a).

Cytological diagnostic characteristics of neoplastic cells include largecell size, high nucleus-to-cytoplasm ratio, a prominent nucleus or several nuclei, rounded shape, and nonadherent behavior, in contrast to highly adherent hemocytes (Cooper et al. 1982a, Elston et al. 1992, Barber 2004, Carballal et al. 2013, Alderman et al. 2017, Dairain et al. 2020). Several methods are commonly applied to diagnose DN in bivalves. Fluorescence-activated flow cytometry is an established diagnostic technique to detect low levels of **DN** (<12% of total cells in hemolymph), as DN cells are typically polyploid, which can be detected with DNA-staining dyes (da Silva et al. 2005, Smolarz et al. 2005, Vassilenko & Baldwin 2014). Histological analyses of paraffin-embedded tissue sections from dissected animals or cytospin-generated hemolymph preparations can reliably detect neoplastic cells in advanced stages of disease (Carballal et al. 2013, 2015, Dairain et al. 2020, Garcia-Souto et al. 2022). Because neoplastic cells are often observed in the hemolymph except in late-stage disease when other tissues are infiltrated by neoplastic cells, tissue sections can be of limited use as an early diagnostic tool in some systems (Barber 2004).

Wide-scale mortality events due to DN outbreaks have been reported in mussels *(Mytilus* sp.) in the United States (Bower 1989, Barber 2004, Galimany & Sunila 2008), softshell clams on the Atlantic coast of North Anlerica (Brown et al. 1978, Farley etal. 1991, Barber 2012, Muttrayetal. 2012), Baltictellin {Dairain et al. 2020), and the common cockle in Europe (Le Grand et al. 2010). In the Pacific Northwest of the United States, a variety of bivalve species, some commercially important, are known to be susceptible to neoplasia (House & Elston 2006). The pathogenicity and prevalence of DN and its potential ecological impacts on these bivalve populations are not well understood.

Basket cockles are a traditional food staple for Coast Salish tribal communities (Apeti et al. 2013) and are also popular

*Correspondingauthor. E-mail: lvandepas@benaroyaresearch.org
DOI: I0.2983/035.043.021I

among recreational harvesters. There is growing interest in basket cockle aquaculture and potential population enhancement among some Coast Salish tribes and the commercial shellfish industry (Dunham et al. 2013a, 2013b). Recent routine health screening of basket cockles by local governmental agencies in Puget Sound, WA (USA) identified ON in wild populations (R. Elston, WA Department of Fish and Wildlife, personal communication), complicating the viability of aquaculture projects. To test large numbers of individual adult basket cockles for use as broodstock, a quick, minimally invasive, and inexpensive screening method for ON was required.

This study details a hemolymph smear method for rapid live-screening of basket cockles for ON using small volumes of hemolymph and inexpensive, widely accessible cell staining materials. Whereas the application of stained hemolymph as a diagnostic tool for the presence of ON has been well established in the softshell clam *Mya arenaria* (Cooper et al. 1982a, Farley et al. 1986) and the mussel *Mytilus* (Elston et al. 1988), hemolymph smears have not been previously described as a technique for the diagnosis of ON in cockles. The cockle hemolymph smear method presented here iscompared with standard ON diagnostic techniquesthat uselethal tissuesampling and histological sectioning; demonstrating that H&E staining of hemolymph smears can be used to reliably identify ON in basket cockles.

MATERIALS AND METHODS

A11imal Col/ectio11

Adult *Clinocardium nuttallii* (Fig. IA) were collected from beaches in Puget Sound, WA in 2019: Semiahmoo (location code: SM) in mid-December and Agate Pass (location code: Y) in late March 2019. Previous routine disease assessments on cockles from this region showed a prevalence of neoplasia at roughly 10% (R. Elston, WA Department of Fish and Wildlife, personal communication). All cockle samples used in this study were collected in coordination with Suquamish Tribe shellfish biologists. Following collection, cockles were transported in coolers and processed for disease screening immediately upon arrival at the National Oceanographic and Atmospheric Administration's Manchester, WA station.

No11/etl,aJ Hemo(vmpl, Collectio11 fi·om Clinocardium nuttallii

To prevent stress-induced spawning or injury, a small volume of hemolymph was extracted from the adductor muscle using a 1-mL syringe and 26-gauge I-inch (2.54 cm) needle (Fig. IB, C). This is done by gently handling an individual that had been resting undisturbed to minimize the risk that the valves are tightly shut, and inserting the needle whereas the valves are in a semi-open (relaxed) state. A total of 300 µL of hemolymph wascollected from each cockle.

Sample Preparation for Histology

Hemolymph Smear

To ensure hemocytes adhere to glass slides during staining, the collected hemolymph was applied to poly-L-lysine slides (Electron Microscopy Sciences, Hatfield, PA) and allowed to rest for 10 min at room temperature on a flatsurface (Appendix, Fig. **AJA). A** "blood smear" was performed by gently removing excess hemolymph using a coverslip (Appendix, Fig. **AIB,** C). The slide was allowed to air dry at room temperature on a flat surface. The adhered hemocytes were then fixed by submerging slides in 100% methanol for I5 min. The slides were removed from the methanol and air-dried at room temperature. The slides were then in a covered container to prevent dust debris from attaching to the slides. Methanol-fixed hemolymph slides can then be stored at room temperature indefinitely or used immediately for histology. Representative images were taken on a Leica DMR upright microscope equipped with an Insight 4-megapixel color CCD camera (Diagnostic Instruments)

Hemolymph Smear Hematoxylin and Eosin Staining

Note: AJl H&E reagents acquired from Leica

1. Hydrate gradually in distilled water (70% EtOH 50% EtOH 25% EtOH) so fixed cells are not osmotically shocked and damaged.
2. Apply adequate Hematoxylin, Mayer's (Lillie's Modification) to completely cover smears on slides and incubate for 5 min.
3. Rinse the slide in two changes of distilled water to remove excess stain.
4. Apply adequate Bluing Reagent to completely cover the sample and incubate for 10--15 sec.
5. Rinse slides in two changes of distilled water.
6. Dip slides in 95%-100% alcohol and blot excess ethanol off the edges of slides.
7. Apply adequate Eosin Y Solution (Modified AJcoholic) to completely cover the sample and incubate for 2-3 min.
8. Rinse the slide in 95% alcohol for I min.

Figure 1. Demonstration of hemolymph draws in from *Cli11ocai·di1111111ttallii.* (A) Live basket cockle clam with siphons extended. (B) Schematic representation of needle (red) position for drawing out hemolymph from the adductor muscle. (C) Demonstration of minimally invasive hemolymph drawn from a lh'e cockle. White arrowhead denotes point of needle insertion.

9. Rinse the slide in 100% alcohol for I min. Repeat twice more.
10. Dehydrate slides in three changes of ethanol (95% 95% 100%).
11. Clear slides in three washes of Hemo-De (d-Limonene) Xylenes substitute (Electron Microscopy Services) for one minute per wash.
12. Mount in synthetic resin, such as Permount (Fisher Scientific).

Fixation, Embedding, and Sectioning of Whole Tissues

1. Excise a thin section (approximately 2 mm) of tissue from the gonad, stomach, foot, and gill using a scalpel.
2. Place in a tissue cassette, filling no more than l/2-2/3 of the cassette volume.
3. Transfer tissue cassettes to Davidson's Solution {For 1 L: 111 mL acetic acid, 320 **mL** 99% + ethanol, 222 mL 10% phosphate-buffered formaldehyde solution, 367 mL de-ionized water) for 24--48 h. Note: The volume of fixative should be l0X the volume of tissue.
4. Remove tissue cassettes from the fixative and transfer them to 70% ethanol. Cassettes can be stored at room temperature indefinitely.

RESULTS

*ldemificatio11of DN i11*Clinocardium nuttallii *Using Hemolympl, A11a(vsis aud Histological Methods*

Following the detection of ON in basket cockle populations from Puget Sound, WA, during a routine environmental monitoring survey, a minimally-invasive hemolymph smear protocol was developed to examine hemolymph for the presence of cells with neoplastic morphology which did not require lethal tissue sampling (Fig. I). Phase-contrast light microscopy was first used to observe live hemocytes extracted from cockles that were collected from Agate Pass in Puget Sound. Nonneoplastic cells in the hemolymph {hemocytes) exhibit diverse cell morphologies, with most cells being adherent and displaying pseudopodia, as has been observed in other bivalves {Fig. 2A; Cooper et al. 1982b, Carella et al. 2017). In contrast, live neoplastic cells do not readily adhere to tissue culture dishes and are rounded in shape, with an abnormally high density of cells in the hemolymph during advanced disease states compared with nonneoplastic individuals (Fig. 2B; Cooper et al. 1982b).

Hemolymph was sampled from 29 individual basket cockles collected from Semiahmoo, WA, and examined using the hemolymph smear method and H&E staining. High-resolution images of hemolymph smears of all sample replicates may be found in this image repository: https://zenodo.org/records/11153963. Disseminated neoplasia was not observed in 27 individuals. Hemolymph drawn from nonneoplastic cockles showed normal hemocytes displaying an expected array of cell morphologies and sizes (Fig. 2C; de la Ballina et al. 2022). Two samples were flagged as containing neoplastic cells following imaging of the H&E-stained hemolymph smear: specimens SM-06 and SM-26. In contrast to nonneoplastic individuals, the hemolymph of a cockle with advanced neoplasia contained cells of uniform size and round shape with enlarged nuclei, the typical morphology of neoplastic cells in other bivalve species (Fig. 2D; Barber 2004). Given the readily identifiable differences between neoplastic cells and normal hemocytes using a combination of

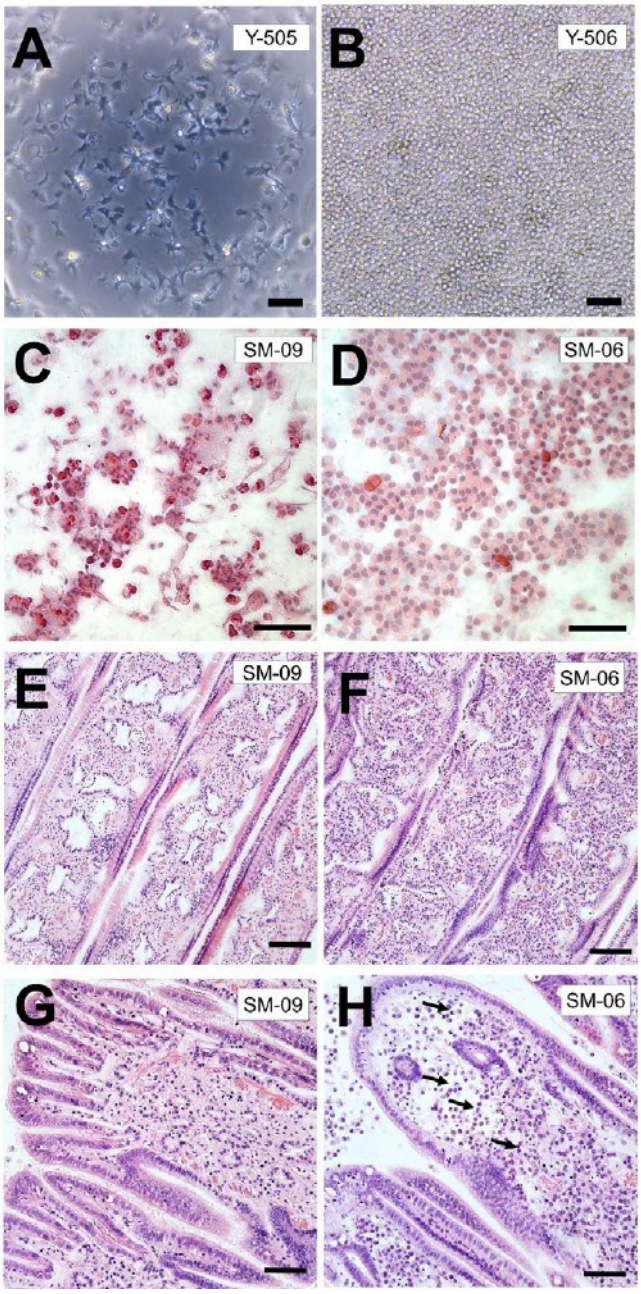

Figure 2. Comparison of live unstained, and H&E-stained hemolymph-smears and tissue sections from nonneoplastic and neoplastic exemplar cockles. (A) Live normal hemocytes in a cockle sampled from Agate Pass WA (sample Y-505). (B) A mixture of lh·e normal (normeoplastic) hemocytes and neoplastic cells in a cockle infected with ON sampled from Agate Pass WA (sample Y-506). (C) H&E stained hemoq1es from a normeoplastic cockle collected from Semiahmoo WA (sample SM-09). (D) Cells stained by H&E displaying the distinctive rounded morphology and enlarged nuclei of neoplastic cells in a cockle collected from Semiahmoo WA (sample SM-06). (E and F) Hematoxylin and eosin histology of *Oit1ocardi11111 1111ttalliig*ill tissuesections fromanin1alscollected fromSemiahmoo WA.(E) onneoplastic gill tissue displaying normal tissue structure. (F) GiJJlamella of a cockle showing disorganized gross tissue structure and broad infiltration of neoplastic cells. (G) High magnification of healthy gill tissue. (H) High magnification of gill lamella from a cockle ,,ith advanced D . Arrows point to neoplastic cells. (A-D) Scale bar = 50 11111. (E and G) Scale bar = 200 **µm.** (F and **H)** Scale bar= **100** µm. Full-size images of slide smear and tissue sections of all samples used in this sh1dy may be found in this image repository: https://zenodo.org/records/11153963.

nonlethal hemolymph extraction, slide smear, and H&E staining protocols, the authors suggest that the hemolymph smear method described here is adequate for the detection of ON in *Clinocardium nuttallii.*

Validatio11of Hemolympl, Smeal" Protocol by Direct Compariso11 with Tissue Sectio11Histology to Detect DN i11 Basket Cockles

To establish that the detection of ON using hemolymph smear analysis was comparable in accuracy to tissue section histology, whole tissues were sampled from the same 29 cockles previously used to assess hemolymph via slide smear. The whole tissues (comprising gill, gut, gonad, and foot) werefixed, embedded in paraffin, and sectioned usingestablished histological techniques (Carballal et al. 2013, Carella et al. 2017). Sections were then stained with H&E, imaged, and examined for the presence of neoplastic cells within the tissues (Fig. 2E-H; Supplemental Dataset 1; https://zenodo.org/records/11153963).

Representative images from stained gill tissue sections from a nonneoplastic cockle (SM-09; Fig. 2C) show normal tissue morphology and cell density with no visible neoplastic cells, congruent with its paired hemolymph smear (Fig. 2E, G; compare with Fig. 2C). Conversely, tissue sections taken from a cockle with neoplasia (SM-06) show gill tissue with abnormally high cell density and visible round cells with large nuclei (Fig. 2F, H). Neoplastic cells from this individual cockle were also detected by hemolymph smear (Fig. 2D).

Histological analysis of tissue sections thus confirmed the presence of neoplastic cells in the gill tissue in the same individual cockles for which neoplastic cells had been detected by hemolymph smear (samples SM-06 and SM-26). Further, no evidence of neoplasia was detected in the other 27 cockles that had been categorized as nonneoplastic using the hemolymph smear. High-resolution images of **H&E** stained gill tissue sections from all sample replicates used in this study may be found in the image repository (Supplemental Dataset 1).

DISCUSSION

Disseminated neoplasia can be diagnosed using tissue histology, hemocytology, or fluorescence-activated flowcytometry (Reno et al. 1994, Carballal et al. 2013, Alderman et al. 2017). Few research efforts have focused on large-scale nonlethal screening methods to survey wild populations. Rapid, inexpensive techniques for the diagnosis of ON in bivalves could be applied in disease ecology, fisheries management, population restoration efforts, and aquaculture.

Although considered common throughout their coastal Northeast Pacific Ocean range, *Clinocardium nuttallii* is reported to have a patchy distribution that may vary seasonally, complicating population monitoring through established intertidal bivalve surveys used by Washington tribal and state fisheries. Available data suggest that C *nuttallii* population dynamics are highly stochastic with temporally and spatially asynchronous population "booms" and "busts" (Barber et al. 2019); however, the factors regulating these population dynamics remain largely unknown.

The presence of **ON** in Puget Sound *Clinocardium nuttal/ii* populations compelled the development of rapid, live-sampling diagnostic methods to determine whether the disease was present in individual cockles intended for use as brood stock. Tissue histology is considered a standard approach for diagnosing **ON** in bivalves; it was therefore critical that the accuracy of **ON** detection via the live-collected hemolymph smear method was compared with classic tissue section histology (Barber 2004, Carballal et al. 2013, Carella et al. 2017). Basket cockles collected from Semiahmoo, **WA** were screened *(n* = 29) and ON was identified in two animals using the hemolymph smear method (6.7% of samples). Methanol-fixed hemolymph smears show striking differences in cellular morphology between nonneoplastic and neoplastic individuals in both isolated hemocytes and within infected whole tissues (Fig. 2; Supplemental Dataset!).

Examination of embedded and sectioned whole tissues compared with hemolymph smears from the same individuals shows that the two techniques yield identical diagnostic results within the samples (Supplemental Dataset I). The authors posit that the minimally invasive hemolymph sampling and slide smear methods used in this study can be applied to detect ON in cockle clams, equivalent to classical whole-tissue sampling and histology methods. Further, based on prior ON imaging efforts in diverse bivalve species, it is possible to use other commonly available DNA stains and counterstains (e.g., Giemsa, Feulgen stain) to hemolymph smears for the assessment of hemocyte morphology and detection of neoplastic cells (Cooper et al. 1982a, Farley et al. 1986, Elston et al. 1988, De Vera et al. 2005).

Hemolymph smears are advantageous for several reasons including cost, speed, and requiring relatively few technical investments; this method does not require access to specialized flow cytometry equipment or fluorescent dyes, significantly reducing costs associated with disease diagnosis. In addition, hemolymph smears are relatively simple to execute, and neoplastic cells can be discerned via visual diagnosis with any compound light microscope. Lastly, this protocol does not require lethal sampling, allowing for repeated sampling of the same cockle for research or diagnosis of individuals destined for use as broodstock or in captive display. This combination of sample collection and diagnostic tools has the potential to be generally applied to other taxa allowing for rapid, minimally invasive, and inexpensive diagnosis of ON in bivalves.

ACKNOWLEDGMENTS

The authors are grateful to Ralph Elston for technical support; Paul Hershberger and USGS Disease Ecology Laboratory, Marrowstone, WA; Suquamish Tribe Fisheries Department; Washington Department of Fish and Wildlife. The authors are also grateful to Pamela Johnson and the Benaroya Research Institute's Histology Corefacilityfor technical support.This work was supported by BIA Tribal Resiliency Grant #Al9AP00215, National Academy of Sciences-National Research Council postdoctoral fellowship to L. E. V., and NSF EEID Grant 2208081 to M. J. M. Hatchery work was funded by the Suquamish Tribe.

CONFLICT OF CNTERFBT

The authors have no conflicts of interest to declare.

DATA AVATLABfLITY STATEMENT

All data referenced in this study is available in the figures herein and in this imaging repository: https://zenodo.org/records/I1153963.

LITERATURE CITED

Alderman, D. **J., M.** Green, G. Balouet, T. Renault & S. Ford. 2017. Disseminated neoplasms in bivalves. ICES Identification Leaflets for Diseases and Parasites of Fish and Shellfish. No. 67, 6 pp.

Apeti, D. A., S. I. Hartwell, **M.** Myers, J. Hetrick & J. Davenport. 2013. Assessment of contaminant body burdens and histopathology of fish and shellfish species frequently used for subsistence food by Alaskan Native communities. North Pacific Research Board Final Report, 63 pp.

Barber, B. J. I990. Seasonal prevalence and intensity and disease progression of neoplasia in soft shell clams, *Mya arenaria,* from the Shrewsbury River, New Jersey. *Pathol. Mar. Sci.* 377-386.

Barber, B. J. 2004. Neoplastic diseases of commercially important marine bivalves. *Aqua/. Living Resour.* 17:449--466.

Barber, B. J. 2012. Seasonal prevalence and intensity and disease progression of neoplasia in soft shell clams, *Mya arenaria,* from the Shrewsbury River, New Jersey. In: Perkins, F.0. & T. C. Cheng, editors. Pathology in marine science. San Diego, CA: Academic Press, Inc. pp. 377-386.

Barber, **J.** S., C. P. Ruff, J. T. McArdle, L. L. Hunter, C. A. Speck,D. W. Rogers & C. **M.** Greiner. 2019. Intertidal clams exhibit population synchrony across spatial and temporal scales. *Limnol. Oceanogr.* 64:S284--S300.

Bower, S. **M.** 1989. The summer mortality syndrome and haemocytic neoplasia in blue mussels (Mytilus edulis) from British Columbia. *Can. Tech Rep. Fish Aquat. Sci.* 1703:65.

Brown, R. S., R. E. Wolke, S. B.Saila &C. W. Brown. 1978. Prevalence of neoplasia in JO New England populations of the soft-shell clam *(Mya arenaria). Ann. N Y. Acad. Sci* 298:522-534.

Carballal, M. J., B. J. Barber, D. Iglesias & A. Villalba. 2015. Neoplastic diseases of marine bivalves. *J. Invertebr. Pathol.* 131:83-106.

Carballal, M. J., **D.** Iglesias, S. Diaz & A. Villalba. 2013. Disseminated neoplasia in clams *Venerupis aurea* from Galicia **(NW** Spain): histopathology, ultrastructureand ploidy of the neoplastic cells, and comparison of diagnostic procedures. *J. Invertebr. Pathol* 112:16--19.

Carella, F., G. De Vico & G. Landini. 2017. Nuclear morphometry and ploidy of normal and neoplastic haemocytes in mussels. *PLoS* Onel2:e0173219.

Cooper, **K. R., R.** S. Brown & **P. W.** Chang. 1982a. Accuracy of blood cytological screening techniques for the diagnosis of a possible hematopoietic neoplasm in the bivalve mollusc, *Mya arenaria. J. Invertebr. Pathol.* 39:281-289.

Cooper, **K. R., R. S.** Brown & **P. W.** Chang. 1982b. The course and mortality of a hematopoietic neoplasm in the soft-shell clam, *Mya arenaria. J. Invertebr. Pathol.* 39:149-157.

da Silva, **P. M.,** P. Soudant, M. J. Carballal, C. Lambert & A. Villalba. 2005. Flow cytometric **DNA** content analysis of neoplastic cells in haemolymph of the cockle *Cerastoderma edule. Dis. Aquat. Organ.* 67:133-139.

Dairain, A., **M. Y.** Engelsma, **J.** Drent, **R.** Dekker & D. W. Thieltges. 2020. **High** prevalences of disseminated neoplasia in the Baltic tellin *Limecola balthica* in the Wadden Sea. *Dis. Aquat. Organ.* 138:89-96.

De la Ballina, N. R., F. Maresca, A. Cao & A. Villalba. 2022. Bivalve haemocyte subpopulations: a review. *Front. Immunol.* 13:826255.

De Vera, A. Z., S. E. McGladdery, A. A. Herrera, M. F. Stephenson, M. Maillet & D. Bourque. 2005. Occurrence of hemic neoplasia in slipper oyster, *Crassostrea iredalei* (Faustino, 1928), in Dagupan City, Philippines. Disease in Asian Aquaculture V. 321-325.

Dunham, A., H. Gurney-Smith, N.Plamondon,S.Yuan&C. M. Pearce. 2013a. Aquaculture potential of the basket cockle *(Clinocardium nuttalli1).* Part I: effects of stocking density on first year grow-out performance in intertidal and off-bottom suspended culture. *Aquac. Res.* 44:1236--1253.

Dunham, **A., H.** Gurney-Smith, N. Plamondon, S.Yuan&C. **M.**Pearce. 2013b. Aquaculture potential of the basket cockle *(Clinocardium nuttalli1).* Part 2: effects of stocking density and depth on second year grow-out performance of three size cohorts in intertidal and off-bottom suspended culture. *Aquac. Res.* 44:1277-1299.

Elston, **R.** A., M. L. Kent & A. S. Drum. 1988. Progression, lethality and remission of hemic neoplasia in the bay mussel *Mytilus edulis. Dis. Aquat. Org.* 4:135---142.

Elston, **R. A., J.** D. Moore & **K.** Brooks. 1992. Disseminated neoplasia of bivalve mollusks. *Rev. Aquat. Sci.* 6:405---466.

Fanley, C. A. 1969. Probable neoplastic disease of the hematopoietic system in oysters, *Crassostrea virginica* and *Crassostrea gigas. Natl Cancer Inst. Monogr.* 31:541-555.

Farley,C. A., S. V Otto&C. L. Reinisch. 1986. New occurrence of epizootic sarcoma in Chesapeake Bay soft shell clams, *Mya arenaria. Fish Bull.* 84:851--857.

Farley, C. A., D. L. Plutschak & R. F. Scott. 1991. Epizootiology and distribution of transmissible sarcoma in Maryland softshell clams, *Mya arenaria,* 1984-1988. *Environ. Health Perspect.* 90:35--41.

Frierman, E. **M.** & J. **D.** Andrews. 1976. Occurrence of hematopoietic neoplasms in Virginia oysters (*Crassostrea virginica). J. Natl Cancer Inst.* 56:319-324.

Galimany, E. & I. Sunila. 2008. Several cases of disseminated neoplasia in mussels *Mytilus edulis* (L.) in Western Long Island Sound. *J. Shellfish Res.* 27:1201-1207.

Garcia-Souto, D., **A. L.** Bruzos, S. Diaz, S. Rocha, A. Pequeiio-Valtierra, C. F. Roman-Lewis, J. Alonso, R. Rodriguez, D. Costas, J. Rodriguez-Castro& A. Villanueva. 2022. Mitochondrial genome sequencing of marine leukaemias reveals cancer contagion between clam species in the Seas of Southern Europe. *Elife* 11:e66946.

House, M. & R. A. Elston. 2006. Disseminated neoplasia of bivalve molluscs. American Fisheries Society Diagnostic Procedures for Finfish and Shellfish Pathogens 5.2.11.

Le Grand, F., E. Kraffe, X. de Montaudouin, A. Villalba, Y. Marty & P. Soudant. 2010. Prevalence, intensity, and aneuploidy patterns of disseminated neoplasia in cockles *(Cerastoderma edule)* from Arcachon Bay: seasonal variation and position in sediment. *J. Invertebr. Pathol.* 104:110--118.

Metzger, **M.** J., C. Reinisch, J. Sherry & S. P. Goff. 2015. Horizontal transmission of clonal cancer cells causes leukemia in soft-shell clams. *Cell* 161:255---263.

Metzger, **M.** J., A. Villalba, **M.** J. Carballal, D. Iglesias, J. Sherry, C. Reinisch, A. F. Muttray, S. A. Baldwin & S. P. Goff. 2016. Widespread transmission of independent cancer lineages within multiple bivalve species. *Nature* 534:705-709.

Murchison, E. P. 2016. Transmissible tumours under the sea. *Nature* 534:628-629.

Muttray, A., C. Reinisch, **J.** Miller, W Ernst, P. Gillis, **M.** Losier & J.Sherry. 2012. Haemocytic leukemia in Prince Edward Island (PEI) soft shell clam *(Mya arenaria):* spatial distribution in agriculturally impacted estuaries. Sci. Total Environ. 424:130--142.

Reno, P. **W., M.** House & A. Illingworth. 1994. Flow cytometric and chromosome analysis of softshell clams, *Mya arenaria,* with disseminated neoplasia. *J. Invertebr. Pathol.* 64:163-172.

Smolarz, K., T. Renault, P. Soletchnik & **M.** Wolowicz. 2005. Neoplasia detection in *Macoma balthica* from the Gulf of Gdansk: comparison of flow cytometry, histology and chromosome analysis. *Dis. Aquat. Organ.* 65:187-195.

Vassilenko, E. & S. A. Baldwin. 2014. Using flow cytometry to detect haemicneoplasiain mussels(*Mytilustrossulus)* fromthePacificCoast of Southern British Columbia, Canada. *J. Invertebr. Pathol* 117: 68-72.

Yonemitsu, **M. A., R. M.** Giersch, **M.** Polo-Prieto, **M.** Hammel, A. Simon, F. Cremonte, F. T. Aviles, N. Merino-Veliz, E. A. Burioli, A. F. Muttray & J. Sherry. 2019. A single clonal lineage of transmissible cancer identified in two marine mussel species in South America and Europe. *Elife* 8:e47788.

APPENDIX

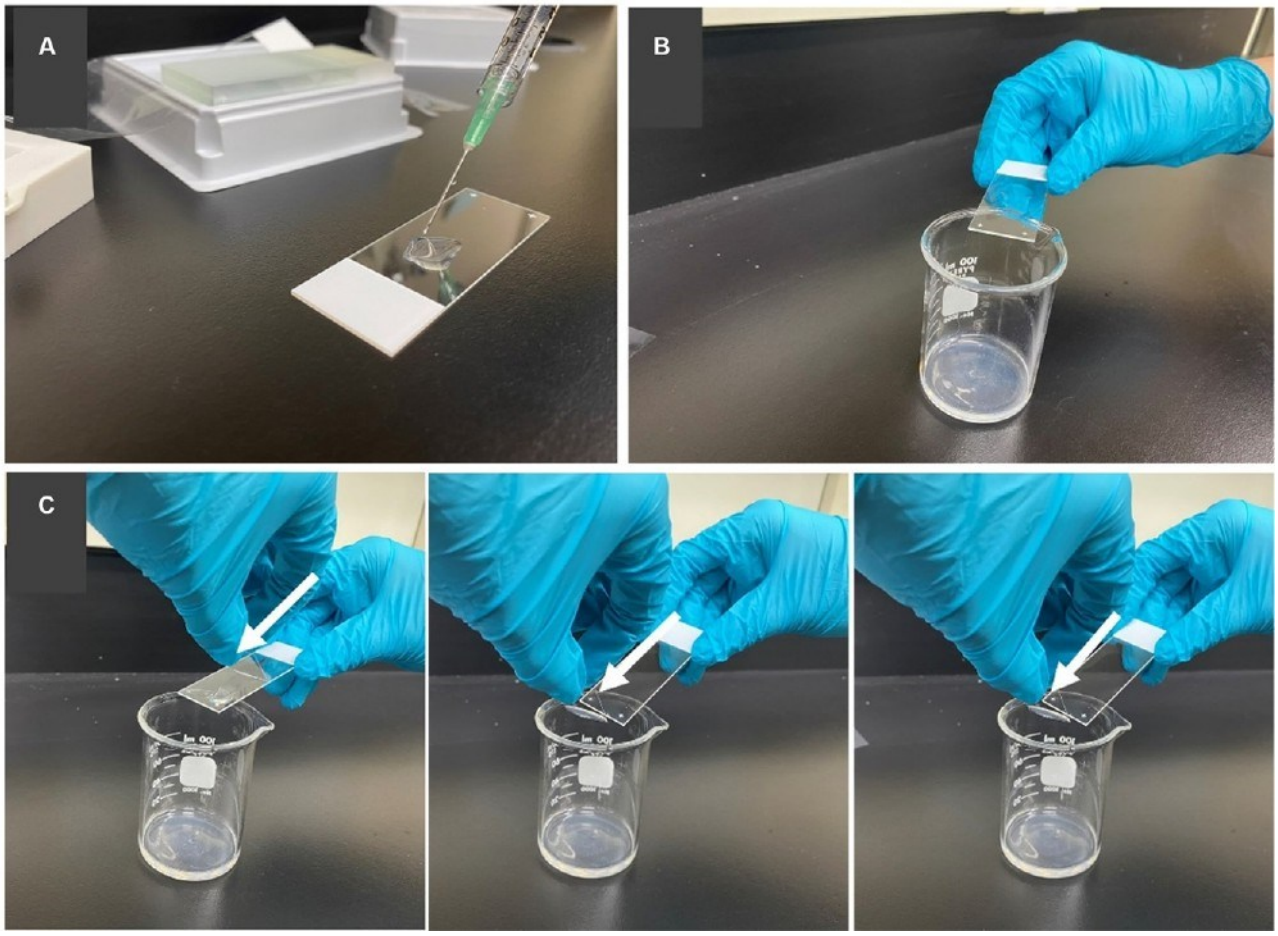

Figure Al. Demonstration of a hemolymph smear. (A) Approximately JOOµL of hemolymph are applied to a poly-L-lysine-treated slide and allowed to rest for 10 min on a flat surface. (B) Position the slide O\·er a waste container to collect excess hemolymph. (C) Use a square cO\·erslip to remove excess hemolymph by gently moving the co,·erslip across the slide surface from one end to the other taking care not to make direct contact with the slide. Arrow shows direction of movement of coverslip.