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Collection techniques for the analyses of pathogens in crustaceans

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ABSTRACT

Outbreaks of diseases have been reported from a number of ecologically or commercially important crustaceans in tropical, temperate, and boreal waters. The etiology of a disease is often unknown prior to these outbreaks and the effect of the pathogen on the host population is poorly understood. Various techniques can be used to collect, identify, and monitor host populations for pathogens. These include classical methods, such as visual or histological assessment, to more refined techniques, such as simple and quantitative polymerase chain reaction assays. The strengths and weaknesses of the different methods are presented as well as some general guidelines for managing data associated with disease surveys in conjunction with field collections.

Key words: crabs, diagnostics, diseases, electron microscopy, field collections, histopathology, lobsters, pathogens, shrimps

INTRODUCTION

Most of our knowledge of pathogens and diseases in crustaceans has come from the accidental discovery of discolored or misshapen hosts in museum collections, cryptic infections from unrelated studies, or from natural outbreaks that have killed commercially important hosts. Identification of the pathogens can be difficult because of poor fixation and improper handling of appropriate host specimens collected for other reasons. In the case of museum collections, the parasitic or pathogenic agents are often poorly fixed for preservation because they were not identified as such until the hosts have been examined in the laboratory. In addition, the methods used to preserve museum specimens or to prepare hosts for other studies are not always appropriate for proper fixation and handling of their pathogens. In the case of fisheries collections, the etiological agents are typically unknown prior to an outbreak, and that can lead to poor preservation and identification of the proper agent (Shields, 2012). During outbreaks in fisheries, managers typically collect dead hosts, which are very difficult to necropsy properly. Live diseased or moribund hosts are typically culled at sea, leaving the pathologist with few specimens for diagnostic studies. Moreover, the pathogens of crustaceans represent a diverse biota, and disparate pathogens require different methods for proper assessment and identification. Although some of the pathogens of crustaceans have counterparts in well-studied vertebrates, such as Platyhelminthes, most are specialists on Crustacea,

and thus may be more difficult for the non-specialist to identify, such as parasitic dinoflagellates and rhizocephalans.

Given the myriad ways in which crustaceans are used and studied, one can imagine that there are several methods for analyzing their tissues for pathogens and associated pathologies. The simplest method for diagnosis is to use macroscopic, or visual, assessment for disease, but this relies on the pathogen having specific, or pathognomonic, signs of gross infection. Macroscopic assessment can work quite well for some infectious disease agents, especially when combined with more refined diagnostic techniques to confirm the findings. Additional methods include cytological, histological, and molecular techniques; and these typically require development, testing, and comparison with visual assessments. Here I briefly cover methods used in collecting and diagnosing pathogens from crustaceans with an emphasis on field surveys and proper handling for further processing and diagnosis in the laboratory setting.

VISUAL ASSESSMENT METHODS

Macroscopic diagnostics can be as simple as reporting the number of discolored hosts in a sample (e.g., Pestal *et al.*, 2003), or counting hosts with obviously misshapen features, such as bopyrid isopods that inflate the gill chambers of their hosts (e.g., Chaplin-Ebanks & Curran, 2007), or rhizocephalan barnacles that protrude from

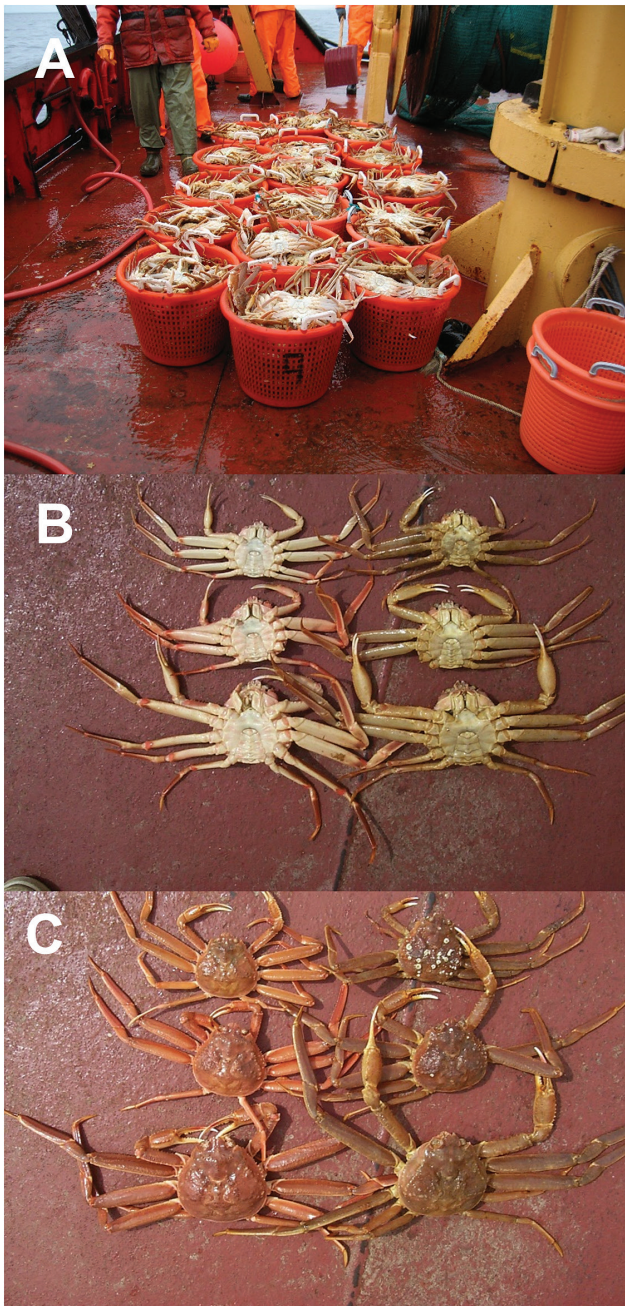


Figure 1. **A**, Snow crabs, *Chionoecetes opilio*, captured by crab pots and awaiting post-capture processing by personnel from the Department of Fisheries and Oceans, Canada. Crabs were caught in Conception Bay, Newfoundland. In cases where too many crabs were caught, five baskets would be randomly selected and all of the crabs therein would be processed (Pestal *et al.*, 2003). Photo by D. Taylor. **B**, Dorsal view of crabs with bitter crab disease (left) caused by infection with *Hematodinium* sp. Crabs with heavy infections can be visually diagnosed by their cooked appearance. **C**, Ventral view of the same crabs for comparative purposes. Photo P. O'Keefe from Pestal *et al.* (2003).

the marsupium (Figs. 1, 2) (e.g., Sloan *et al.*, 1984; Hawkes *et al.*, 1985). Field-based surveys and a few long-standing monitoring programs that implemented visual assessments have provided significant insights into the biology of several pathogens in biomass surveys from trap or trawl hauls (Meyers *et al.*, 1987; Field *et al.*; 1992; Castro & Angell, 2000; Stentiford *et al.*, 2001). With the exception of epizootic shell disease, these studies have relied

on previous identification of the agents. In most cases, the agents must have pathognomonic signs of infection, which means that the macroscopic sign of infection is specific to the pathogen causing the disease. It is imperative that the epidemiological sensitivity and specificity be determined for the diagnostic method, particularly when using macroscopic or visual assessment, as this will have bearing on the estimation of prevalence in the host population (Pestal *et al.*, 2003; Shields *et al.*, 2015a). Visual assessment is particularly effective when combined with other methods to verify the specificity of the condition as well as to account for the prevalence of subpatent infections. By way of example, *Hematodinium*-like infections discolor the carapace of the snow crab, *Chionoecetes opilio* (O. Fabricius, 1788), but the discoloration occurs only in hosts with advanced infections (Fig. 1). Using just the discolored carapace for assessment under-reports the actual prevalence by as much as 50%, but the discoloration is highly specific to the pathogen in this system making it useful for diagnostic purposes (Pestal *et al.*, 2003).

A significant advantage to macroscopic, or visual, diagnosis is that the method is not lethal and prevalence data can be readily incorporated into shipboard or field sampling protocols. Where possible, macroscopic signs of disease should be incorporated into field surveys, particularly those involving commercial fisheries. One example will demonstrate the powerful insights that can be gained by incorporating disease data into field surveys. Epizootic shell disease (ESD) emerged in lobsters, *Homarus americanus* H. Milne Edwards, 1837, from Long Island Sound in the late 1990s (Fig. 2F) (Castro & Angell, 2000). The etiology of the disease and its effect on the host population were unknown at that time (see Shields, 2013 for review). It was originally thought that the disease would have minor effects on the lobster population because the affected animals could molt out of it; however, over time the landings in Long Island Sound declined precipitously and in negative correlation with increasing prevalence of ESD (Wahle *et al.*, 2009). Dominion Resource Services, Inc., undertook biweekly mark-recapture studies of lobsters starting in 1982 as part of their mandated environmental monitoring services. They incorporated the presence of ESD into their routine data collection for lobster surveys in the late 1990s (Landers, 2005). Their dataset now encompasses over 35 years of mark-recapture data and allows for enhanced statistical analyses to estimate relative survival of lobsters with ESD in relation to healthy lobsters. The analyses show that ESD imposes a significant increase in mortality rates on affected lobsters and likely resulted in the precipitous decline in landings in Long Island Sound (Hoenig *et al.*, 2017). Improved estimates of natural mortality were possible because of the field data on ESD. The natural resource agencies of states bordering Long Island Sound also incorporated data on ESD into their surveys, which helped to establish important environmental relationships with ESD (Howell *et al.*, 2005; Glenn & Pugh, 2006).

Microscopic assessment of pleopods, or pleopodal staging, is another non-lethal technique used to evaluate disease in crustaceans. For this technique a lightly sclerotized, translucent pleopod is removed from the host and examined immediately with a compound microscope. This method can be used to detect systemic protozoal infections before they cause obvious discoloration to the carapace of the host (Field & Appleton, 1995). *Hematodinium*-like infections in the Norway lobster *Nephrops norvegicus* (Linnaeus, 1758) have been assessed by pleopodal staging with a stereo or compound microscope (Field *et al.*, 1992, 1998; Stentiford *et al.*, 2001). Infections were even categorized by their relative intensity in the host. The swimming leg, or fifth pereopod, of the blue crab, *Callinectes sapidus* Rathbun, 1896 has been evaluated for detecting *Hematodinium* infections in live juvenile crabs (Messick, 1994), but the method is not routinely used for this species and still requires a microscope for evaluation. It is easier and more reliable in practical terms to bleed the animal and evaluate the hemolymph directly, either shipboard or in the laboratory (pers. obs.).

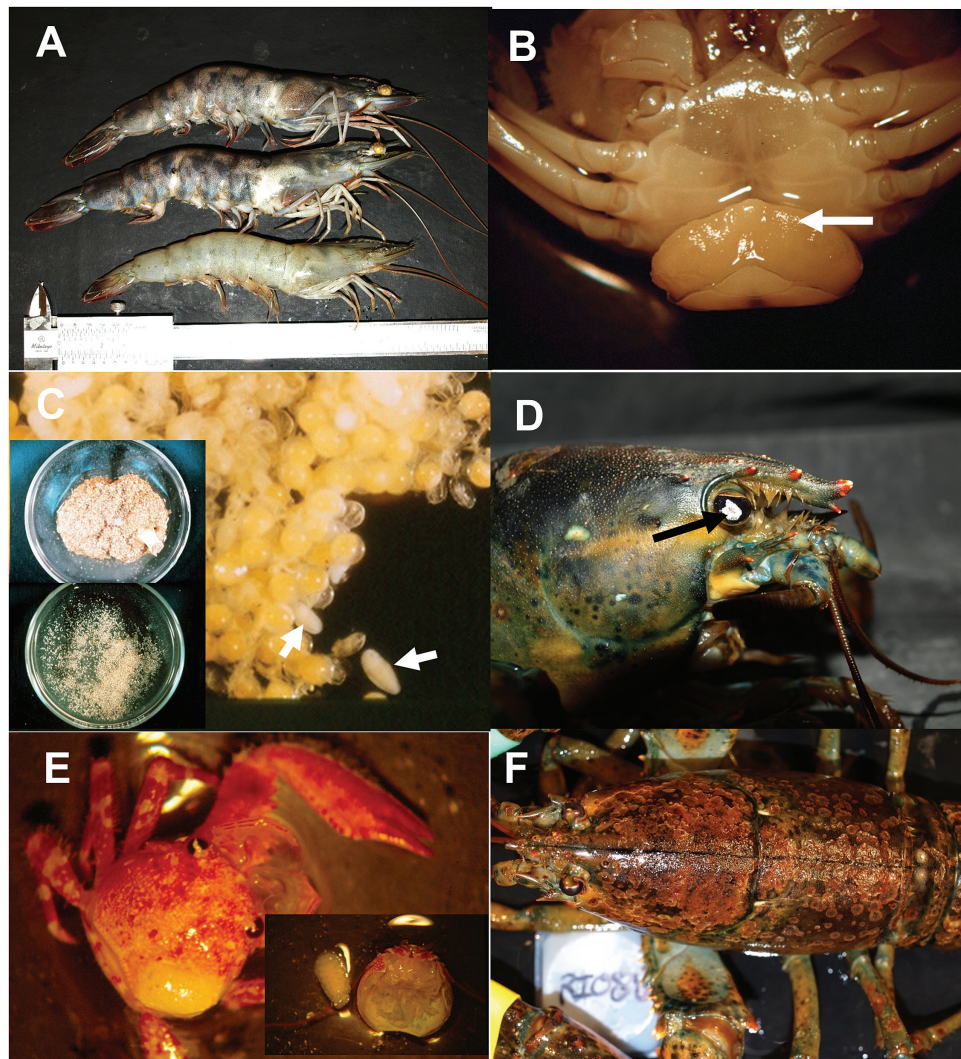


Figure 2. Examples of external signs of infection in shrimps, crabs, and lobsters. **A**, *Penaeus aztecus* Ives, 1891 from Wachapreague Creek, VA. The shrimp on the bottom has a white body indicative of a microsporidian infection (image brightness increased slightly to enhance effect). See also Fig. 3A. Photo: H.J. Small. **B**, *Thalamita* sp. from off Lizard Island, Great Barrier Reef, Australia, with a patent externa of a rhizocephalan barnacle (arrow). **C**, Embryos in the clutch of *Paralithodes camtschatica* (Tilesius, 1815) showing empty eggs eaten by *Carcinonemertes regicides* Shields, Wickham, & Kuris, 1989 (arrows). Insets show the fixed pleopod (top) and the plethora of worms shaken free from a formalin-fixed pleopod (bottom). As many as 600,000 worms have been estimated from one crab clutch (Kuris *et al.* 1991). **D**, *Homarus americanus* from Narragansett Bay, RI, showing a white lesion in the eye indicative of blindness (arrow). Such obvious external signs of this disease are rare. **E**, *Petrolisthes lanarki* (Leach, 1820) from Heron Island, Great Barrier Reef, Australia, with a deformed carapace due to infection by the bopyrid isopod *Aporobopyrina* sp. Inset shows the isopod dissected away from the carapace. **F**, *Homarus americanus* from Narragansett Bay, RI, exhibiting a heavy level of epizootic shell disease. This individual was part of the “100 Lobsters” project (Shields *et al.*, 2012b). This figure is available in color at *Journal of Crustacean Biology* online.

Visual and microscopic assessment can be used to find egg predators such as nemertean worms or nicotoid copepods that live on the eggs of crabs and lobsters (Wickham & Kuris, 1988; Kuris *et al.*, 1991). Embryos on setae or whole pleopods from affected clutches can be removed with scissors and fixed in 10% neutral buffered formalin for further microscopic evaluation (Fig. 2C). Formalin is the fixative of choice for embryos because the pigments within the developing larvae are retained, whereas they are washed out with ethanol preservation. Some specimens should, however, be fixed and preserved in 95% ethanol for later molecular identification and diagnostics. Depending on the study objectives, whole pleopods can be removed and examined microscopically as the distribution of predators within individual pleopods can give clues to the life history of the parasites and other symbionts (Shields *et al.*, 1990a, b) or provide information regarding the host-symbiont association (Kuris *et al.*, 1991).

HEMOLYMPH COLLECTION AND SMEARS

There are several methods for collecting hemolymph from crustaceans, and these can be adapted easily for the study of different pathogens. Hemolymph samples from larger crustaceans (> 15 mm carapace width or carapace length) can be obtained with a 27 ga syringe from the arthrodistal membranes in the leg joints or from between the juncture of the carapace and the abdomen. The sample area should be swabbed with 70–95% ethanol if possible to avoid potential contaminants such as ciliates or diatoms that are often found on the external surfaces. Probably the simplest method to analyze hemolymph is to prepare wet smears and view them directly for altered cells or pathogens (Fig. 3A, B). One can alternately use a vital stain such as 0.3% neutral red or Janus green B in buffer or in invertebrate saline to help differentiate

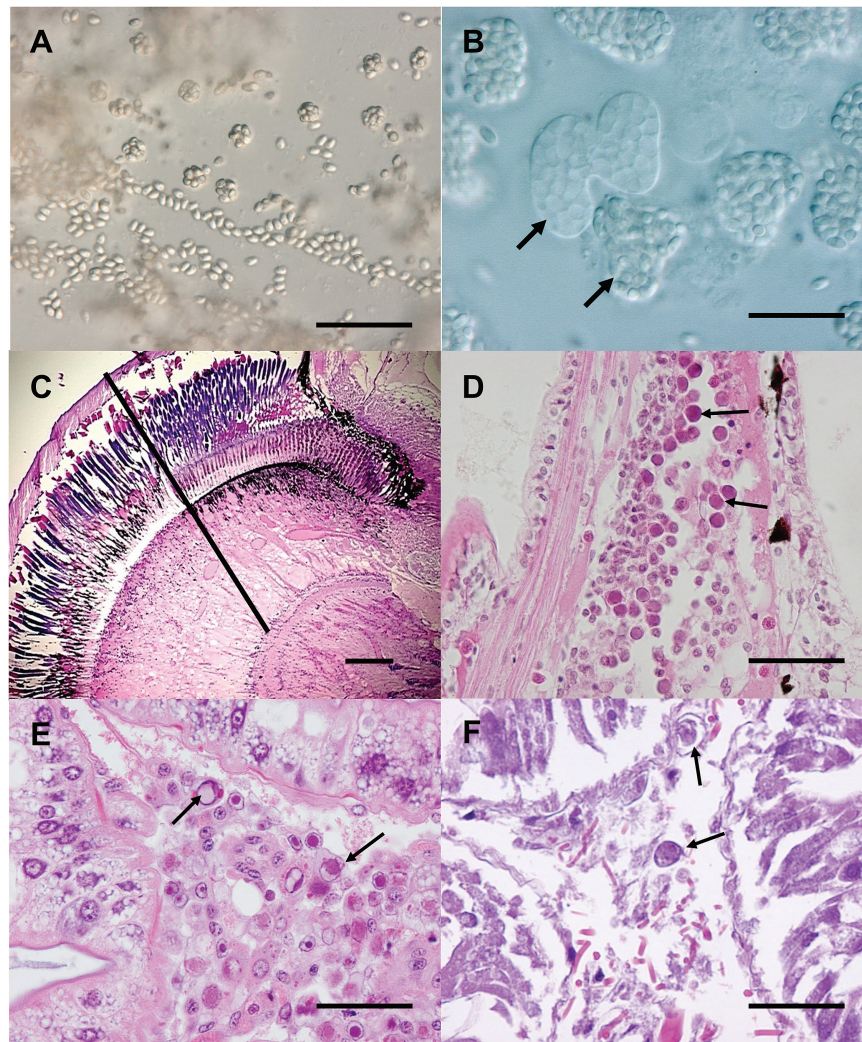


Figure 3. **A**, Wet smear of muscle tissue from a brown shrimp, *Penaes aztecus*, infected with the microsporidian *Perezia nelsoni* (Sprague, 1950) (see Fig. 1A). The pansporoblast is a packet of eight spores. Nomarski optics. Scale = 20 μ m. **B**, Wet smear of *Pleistophora* sp. in the muscle of an infected blue crab. The pansporoblast surrounds the developing spores within the sporont (arrow). Nomarski optics. Scale = 20 μ m. **C**, A histological section through the eye of *Homarus americanus* exhibiting blindness (left of line) and normal ommatidia (right of line). Hematoxylin and eosin stain. Scale = 250 μ m. **D**, A histological section from near the hindgut of an early benthic juvenile *Callinectes sapidus*. The connective cells are exhibiting classical signs of occlusion bodies (arrows) indicative of a viral infection. Hematoxylin and eosin stain. Scale = 100 μ m. This is an example of an opportunistic finding as crabs were being studied for another reason. **E**, A well fixed and preserved sample of hepatopancreas from a spiny lobster, *Panulirus argus* (Latreille, 1804), with a moderate infection of PaV1. The “rosette” of fixed phagocytes around the arteriole is well preserved, showing a few infected cells (arrows). The basement membrane of the hepatopancreas is tightly adherent to the tubule cells. All of the host nuclei and viral nuclear inclusions are well defined. Hematoxylin and eosin stain. Scale = 50 μ m. **F**, A poorly fixed and preserved sample of hepatopancreas from a spiny lobster with PaV1 showing an arteriole with fixed phagocytes as in **E**. The tissue features are badly preserved, making it difficult to interpret for the presence and intensity of infection. Presumptive virally-infected cells cannot be diagnosed properly (arrows). Hematoxylin and eosin stain. Scale = 50 μ m. This figure is available in color at *Journal of Crustacean Biology* online.

between hemocytes and parasites (Fig. 4) (e.g., Chatton & Poisson, 1931; Stentiford & Shields, 2005). Neutral red is taken up differentially by phagosomes within cells, and many protozoan parasites take up the dye, whereas host hemocytes (mainly granulocytes) show only a little uptake, and stray connective tissue cells have modest uptake. These dye preparations tend to form small crystals in buffer preparations and may need coarse filtration to remove them. Direct observation of wet smears is one of the easiest methods for observing pathogens in the hemolymph, but it does require advanced training to diagnose some agents, particularly parasitic dinoflagellates, amoebae, and microsporidians. Epifluorescence microscopy can be used to observe bacteria in moderate infections and special fluorochrome dyes can be used to enhance diagnoses of other pathogens as well (see below). Direct observation requires good photographic documentation at different magnifications for

later reference; and this can be difficult aboard a ship or in rustic field conditions.

If fresh smears are unsuitable, prepared slides are relatively easy to make and provide a permanent record. An easy but less preferred method is to make air-dried hemolymph smears (Fig. 4A). Thin smears of hemolymph are placed on ethanol-cleaned, poly-L-lysine or gelatin-subbed slides, air dried, fixed in 100% methanol, then stained with Giemsa stain or other Wright-stain derivatives (e.g., Humason 1979). Air-dried smears of crustacean hemolymph should, however, be avoided if possible, because unlike mammalian blood smears, dried hemolymph introduces too many artifacts that can make interpretation difficult, particularly when looking for cytoplasmic inclusions or nuclear alterations. Wet-fixed smears give much better results (Fig. 4B). For these, thin smears are made on poly-L-lysine-coated slides. The smears are placed horizontally

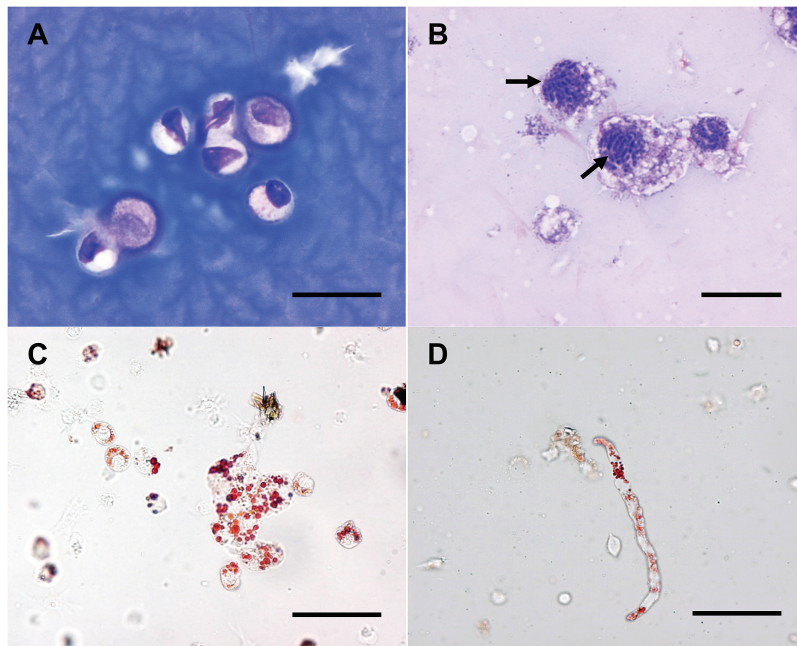


Figure 4. Different types of staining procedures for hemolymph preparations. **A**, Air-dried, methanol-fixed hemolymph smear from an uninfected snow crab, *Chionoecetes opilio* (O. Fabricius, 1788), showing cracks in the ground substance and artifacts from the drying process. The nuclei of the host cells are discernible but have few features. Giemsa stain. Scale = 20 μm . **B**, Wet-fixed hemolymph smear of a *Hematodinium*-like parasite from the snow crab processed through a Giemsa procedure modified for use with methanol dehydration (Pestal *et al.*, 2003). In this stage of the infection the nuclei (arrows) of the parasite possesses condensed chromatin with the appearance of metaphase making it relatively easy to diagnose it. Host hemocytes rarely undergo mitosis in the hemolymph. Scale = 20 μm . **C**, Live clump colony of *Hematodinium perezii* Chatton & Poisson, 1930 in the hemolymph of an infected blue crab (*Callinectes sapidus*) showing neutral red uptake. With few exceptions, host hemocytes and tissues show only weak uptake of neutral red. Scale = 50 μm . **D**, A large filamentous trophont of *H. perezii* in the hemolymph of an infected blue crab showing neutral red uptake. This stage is weakly motile and diagnostic for this pathogen. Scale = 50 μm . This figure is available in color at *Journal of Crustacean Biology* online.

in a humid chamber for 2–3 minutes to allow adherence of the cells, and then the preparation is fixed in Bouin's solution or 10% neutral buffered formalin in a Coplin jar. The slides are then returned to the laboratory and processed through a routine hematoxylin and eosin or other staining procedures (e.g., Messick & Shields, 2000; Pestal *et al.*, 2003). Many staining procedures can be adapted from standard histology texts (e.g., Luna 1968; Humason 1979) depending on specific pathogens and their staining attributes. These methods can be used in the field and aboard ships, and the only significant problem is taking suitable safety precautions when transporting fixatives or using them in confined, poorly ventilated spaces.

Quantitative assessments of pathogens can be made from hemolymph samples preserved with a fixative. Using a 27 ga syringe, 100 μl aliquots of hemolymph can be fixed in a 1:10 ratio with ice-cold 10% neutral-buffered formalin (900 μl) or other fixatives (e.g., 2% glutaraldehyde in 0.2M sodium cacodylate buffer). Quantitative cell counts of hemocytes or pathogens can then be made using a hemacytometer or flow cytometer. Total hemocytes counts are relatively easy to quantify with a hemacytometer, but differential cell counts require training. For pathogens that can be differentiated from host hemocytes (e.g., take up vital stains), cell counts using a hemacytometer can provide key data on intensity of infections, the presence of different life history stages and the relative abundance of pathogens in relation to host cells (e.g., Shields & Squyars, 2000).

The collection of preserved hemolymph samples can be adapted to field collections; however, there are some significant pitfalls to consider. Cells can adhere to the container used for fixation, they can clump due to handling, or the hemolymph constituents can form clots or precipitates with the fixatives, thus skewing the results or making the preparations difficult to assess. Hemolymph with high protein or lipid content, such as that from a female host

undergoing oogenesis, can form flocculants in fixatives making cell densities difficult to estimate in fixed samples (JDS, unpubl. data). In practical experience, polypropylene containers reduce adherence of cells and higher ratios of fixative can help reduce clotting, but the type of plastic container and the type of fixative should be tested prior to use (JDS, unpubl. data). Storage periods should also be considered as some fixatives require refrigeration or additional post processing and cells can degrade or clump if held too long (i.e., longer than 1–2 weeks).

Epifluorescence microscopy may also be useful for rapid diagnosis of pathogens. Hemolymph samples can be fixed directly in cold 10% neutral-buffered formalin or 5% paraformaldehyde. The cells are stained in the laboratory with various fluorescent stains such as fluorescein isothiocyanate (FITC) or 4',6-diamidino-2-phenylindole (DAPI), gently centrifuged, washed in buffer, and viewed as wet smears with epifluorescence microscopy. DAPI works particularly well for examining nuclear details for *Hematodinium* infections, because the nucleus of the parasite is often in a metaphase-like state, and the dye shows this character quite well. Fluorochrome dyes also work well for ciliate infections, because their macro- and micronuclei can be visualized as well as dye uptake within the basal bodies of the kineties. Janus green and fast green may provide some contrast to the nuclei of formalin-fixed cells with suitable results for some protozoans (JDS, pers. obs.).

Agar isolation

Bacterial infections are ubiquitous in crustaceans. In fact, many decapods do not have sterile hemolymph (i.e., Shields *et al.*, 2015b for Brachyura). Bacterial infections can become pathogenic when the host is stressed by handling or environmental stressors, and infected hosts can die quickly to bacterial infections. The bacterial

flora also overgrows the hemolymph of dead crustaceans very quickly rendering it difficult to determine the underlying cause of death. The classical method for examining bacterial infections is to streak sterily-collected hemolymph onto agar plates or broth. Some species such as *Aerococcus viridans* Williams, Hirsch & Cowan, 1953, specifically *A. viridans* var. *homari*, the causative agent of gaffkemia in lobsters, and *Vibrio* spp. are isolated and grown on specific agars (Lavallée *et al.*, 2001; Shields *et al.*, 2012a) that are commercially available. Others are isolated on a general medium such as marine agar (Difco® 2216, Thermo Fisher, Waltham, MA, USA) and then purified or assessed on other media. For larger crabs and lobsters, hemolymph is taken from the juncture of the basis and ischium of the fifth pereopod with a 27 ga needle on a 1 ml tuberculin syringe. The sample site is swabbed with 95% ethanol prior to the hemolymph draw and a few drops of hemolymph are expressed directly on agar plates and incubated at room temperature. Plates are assessed for colony growth after 48–72 hrs. For smaller crustaceans, glass pipettes or capillary tubes can be drawn out into very small syringe-like needles over an open flame and then used to obtain microliter quantities of hemolymph from an arthroal membrane or heart puncture. These mini-syringes require some skill to make and the procedure is best done in the laboratory setting following good laboratory practices and safety protocols. Bacterial isolates can be stored frozen in sterile 10% glycerol for later reconstitution and identification.

HISTOLOGICAL METHODS

Histology remains a standard assessment tool for disease diagnosis in histopathology and disease assessment. It provides information on the state of the host tissues, the etiology of disease, the level of infection, and pathological alterations of affected tissues. Histological identification and assessment of diseases requires proper fixation and handling of the tissues of interest. There are several concerns for tissues collected from field samples. Appropriate dissection and handling of the tissues must be conducted to ensure proper penetration of fixative into the tissues of interest and several tissues or organs have specific requirements with respect to fixation and embedding. For example, an important feature of nearly all crustaceans is that their cuticle provides an impervious barrier to fixation; hence dissection is usually required to provide good penetration of the fixatives into the tissues of interest. Cuticle, foregut, or gill preparations may also require a short decalcification period for proper sectioning, whereas gonads often require different fixatives as well as longer embedding times in paraffin. This necessitates the use of multiple cassettes, specific cataloging systems for identification numbers and specimen tracking, assorted fixatives for different tissues, storage containers, and management of hazardous wastes when collecting specimens for analysis. There are excellent texts on histological techniques (e.g., Luna, 1968; Humason, 1979) and ideally more than one should be used for reference.

Fixatives and fixation

The correct fixation of the tissues is one of the most important steps for proper diagnosis, because poor fixation can render tissues useless for histological assessment. This often happens in field situations when there is an incomplete understanding of the role of proper fixation, or when resources are limited. Three issues are absolutely critical to proper fixation: appropriate dissection, correct choice of fixative, and a suitable fixation method that includes proper ratios of tissue to fixative. Appropriate dissection means that specimens are dissected and processed such that the fixatives penetrate the tissues of interest. The fixative must penetrate rapidly into the tissues

to produce good results. For small crustaceans such as copepods or small amphipods, no dissection may be necessary or small pin pricks may provide suitable entry points for fixation. For larger specimens, a break in the cuticle is required for the proper penetration of the fixatives; even larger specimens, such as market-sized crabs or lobsters, must be dissected and their tissues placed directly into fixative. Sometimes field sampling requires fixing whole specimens. In such cases, large amphipods, small crabs (< 30 mm carapace width) and juvenile lobsters (< 30 mm carapace length) should be cut in half prior to fixation. For larger specimens, placing tissue samples directly into histology cassettes will save time and effort as they can be processed through from fixation to paraffin embedding with little additional handling. Many cassettes are designed to fit directly into the chuck on the microtome, further reducing handling time.

The choice of fixative is important because tissues have different requirements for appropriate fixation and preservation. Bouin's solution and Davidson's fixative are good general fixatives because they have good penetration into the tissues, prepare the tissue for histological stains, and give superior staining results with hematoxylin and eosin stains and other staining techniques. Bouin's solution, however, has two significant limitations: picric acid can be difficult to work with in certain situations, because when it dries out it can form unstable crystals that are unstable and potentially explosive, and it hydrolyzes DNA more rapidly than other fixatives, making it less desirable for molecular-based *in situ* hybridization techniques (e.g., visualization of a pathogenic virus). The picric acid in Bouin's solution nonetheless serves as a counter stain and provides outstanding color to tissues prepared for hematoxylin and eosin stains. Tissues should be fixed in a few different fixatives, such as Bouin's solution, 10% neutral buffered formalin, Z-fix® (Anatech, Battle Creek, MI, USA) or SafeFix® II (Thermo Fisher, Waltham, MA, USA) to provide good assessment of the fixation protocol. SafeFix® II is easier to employ in field settings because it has less volatility. Neutral-buffered formalin, Z-fix®, and SafeFix® II are fixatives of choice for molecular applications, such as *in situ* hybridizations (e.g., Bruce *et al.*, 1993; Carnegie *et al.*, 2003; Li *et al.*, 2006; Small *et al.*, 2007); however, commercial fixatives can be significantly more expensive than 10% neutral-buffered formalin. Davidson's fixative can be modified with seawater to provide a buffered fixative. It is an excellent fixative for histology as well as for whole mounts of helminthes.

Proper fixation is the most important step in histological assessment (Fig. 3C–F). The ratio of tissue to fixative is critical, and it should never be less than 1:10. The size of the tissue sample and the container used in fixation are also important because the fixative has to penetrate throughout the tissue for proper fixation. For paraffin histology, pieces of tissue no larger than 10–20 mm in size are ideal, but larger sizes can be processed depending on the nature of the tissue. If possible, the tissues should be moved out of the fixative into a preservative (70% ethanol) no later than 48–72 hours after fixation, but fixatives such as 10% neutral buffered formalin can be used to hold specimens for longer periods (weeks) if necessary.

Some tissues require special care and handling for good histological assessment. For example, the hepatopancreas degrades very rapidly and is highly sensitive to weak fixation, poor buffering, and poor penetration of fixatives (Fig. 3F). The hepatopancreas of crustaceans that have been dead for longer than 10–15 minutes rapidly degrade due to autolysis, so post-mortem changes can render this organ difficult to assess even after short periods. Muscle and spongy connective tissues, however, can often provide useful information in post-mortem situations. Lipid-rich tissues such as ovaries may require fixatives with good penetration, such as alcohol-formalin-acetic acid (AFA), longer paraffin infiltration times, or longer times in vacuum-assisted paraffin infiltration.

Steedman's ester wax method is a useful alternative embedding technique for tissues rich in lipids (Humason, 1979).

Cuticular structures and decalcification

The crustacean cuticle presents a significant barrier to the histologist. For lightly sclerotized cuticles, such as gills and pleopods, short decalcification periods (3–6 hours) in decalcification fluid (e.g., the formic acid - sodium citrate method of Luna, 1968) may be all that is required. For heavily sclerotized pieces, such as eyestalks (Fig. 3C), sections of carapace, claw, and whole bodies, longer decalcification is necessary (overnight or > 12 h). After decalcification, specimens may require additional trimming or processing. For example, after decalcification, lobster eyes can be safely halved with a single-edged razor blade (not a scalpel) with much less damage to the delicate support network holding the ommatidia in place (Maniscalco & Shields, 2007; Magel *et al.*, 2009; Shields *et al.*, 2012a). Bisecting the eye without decalcification ruins these delicate structures. In another example, an ongoing epidemic of shell disease in the American lobster (see Castro & Somers, 2012; Shields, 2013) required examining the cuticle of affected animals in some detail. Using bone shears or heavy scissors, cuticle samples were taken from the dorsal and ventral surfaces and other locations from symptomatic and asymptomatic hosts. Cuticle pieces included a portion of the underlying epidermis, which revealed clues to the presence of a pseudomembrane that partially defined the disease syndrome (Smolowitz *et al.*, 2005a, b; Shields *et al.*, 2012a). One problem with decalcification is that the reagents, particularly formic acid, can damage the integrity of nucleic acids, and thus interfere with *in situ* hybridization techniques, so decalcification times should be reduced if the technique is to be used. Some histological procedures cause tissues such as the cuticle to become brittle, but limiting preservation time and reducing dehydration times may resolve this development.

ELECTRON MICROSCOPY

For certain diseases, transmission electron microscopy (TEM) is a critical diagnostic tool in support of field collections (e.g., Stentiford & Feist, 2005); and seminal papers on viral etiologies in crustaceans include TEM as a primary tool for identification. Although the method has lost its prominence in many diagnostic laboratories, it still offers a powerful means of identifying microbial pathogens such as viral and rickettsial agents. Many of the methods used in TEM can be difficult to employ, and tissues require tedious embedding in plastics as well as significant technical training in ultramicrotomy. Some methods, however, can be quite simple, such as in the negative staining of hemolymph samples for spiroplasms in *Eriocheir sinensis* H. Milne Edwards, 1853 as in Wang & Gu (2002), or negative staining of viral particles from some types of viral purifications as in Adams & Bonami (1991).

Histological assessment, with additional sampling for TEM, should be included in field studies or field-based collections where known or suspect viral pathogens are present. There are significant constraints to using TEM for processing large numbers of samples. The fixatives employed in TEM are hazardous, particularly glutaraldehyde and osmium tetra-oxide. A single good fixative for both paraffin histology and TEM is problematic, but 1G:4F (1 part glutaraldehyde to 4 parts formalin; Luna, 1968) has been used with mixed results in surveys of oyster diseases (e.g., Carver *et al.*, 2010). Tissues often can be left in the primary fixative in the refrigerator for short periods, up to a few weeks, so it is possible to use glutaraldehyde in short-term field situations, but it can be difficult to transport as it presents a significant chemical hazard. It is best to ship whole live animals showing signs of infection to the diagnostic laboratory where tissues can be processed for TEM as well as other assessments. It may not be possible to

ship infected hosts across international boundaries, however, even if the specimens have been biologically fixed. One solution is to contact a research hospital in the area of collection because they may offer services for TEM fixation and plastic embedding (e.g., Xu *et al.*, 2007). Once embedded in paraffin or plastic, specimens no longer represent a chemical hazard and can be shipped safely and easily.

MOLECULAR METHODS

Molecular methods are now routine for disease diagnosis. Molecular assays, such as the polymerase chain reaction (PCR), *in situ* hybridization (ISH) and quantitative PCR, are extremely valuable diagnostic tools. These methods give information on a specific pathogen but provide little to no information on other organisms that may be present in a sample. It is therefore beneficial to include other methods, such as direct observation and histology, in the repertoire of routine diagnostic techniques for assessment of pathogens. High-throughput sequencing has been used in some situations to help identify a pathogen but it is not practical for use in most field or fishery applications (Hewson *et al.*, 2014).

As with histological fixation, nucleic acid analysis requires correct preservation of tissues and pathogens. There are two standard methods for preserving tissues for later molecular analysis: freezing specimens at -20°C or lower or preserving samples in 95% ethanol. For work with microbial pathogens, hemolymph or other tissue samples can be adequately preserved by these methods, and the preservation can last many years. For most DNA-based diagnostics, hemolymph samples can be stored frozen in anticoagulants such as citrate-EDTA buffers (see Söderhäll & Smith, 1983; Durliat & Vranckx, 1989), or stored neat in frozen 1 ml aliquots, or by preserving 1:10 in 95% ethanol and storing at room temperature. A minimum ratio of 1 part tissue to 10 parts preservative is best for proper preservation. Other fixatives, such as rum or isopropyl alcohol, may work in short-term or emergency situations. For example, we have used rum to successfully preserve samples as a short-term alternative to avoid purchasing and shipping reagent-grade ethanol to and from overseas locations (Moss *et al.*, 2013). Note that rum or 70% ethanol is not an adequate long-term preservative for DNA diagnostics (Dean *et al.*, 2001; Vink *et al.*, 2005), and lysis buffers used in many DNA extraction kits are not preservatives but rather buffers used in extraction protocols. Samples to be preserved or archived must be stored either in the freezer or in 95% ethanol with an eye to long-term curation. For expression or transcriptomic work, tissues should be stored routinely in fixatives such as RNAlater® (Thermo Fisher, Waltham, MA, USA) that allow gene expression studies (e.g., Hasson *et al.*, 1997). Freshly collected tissue samples in RNAlater® can be stored for several months at -80°C for later gene expression studies (Beale *et al.*, 2008).

Validation of molecular tools

Molecular tools are valuable for establishing the presence of a pathogen, but they do require interpretation. Pathogens can for instance be present within or on a prospective host organism, but they may not necessarily be infecting that organism; hence, it is critical to confirm that the pathogen is indeed within the tissues of the host (Burreson, 2008). This is crucial because many laboratories rely heavily on polymerase chain reaction (PCR) methods, which simply give presence or absence of a pathogen, and sometimes the primers are not designed or tested adequately to diagnose a pathogen correctly (Claydon *et al.*, 2004). Moreover, PCR-based assays often have a very high specificity to strains of an organism from a specific region. If an assay is to be used on samples from outside the parameters used for its development, then samples and parameters should be re-validated to avoid the

possibility of false negatives (Carnegie *et al.*, 2016). Validation includes sequencing samples to prove that the DNA from the pathogen of interest is being amplified appropriately (Claydon *et al.*, 2004; Burreson, 2008).

Where possible, one should evaluate hosts using a variety of techniques, including *in situ* hybridization (ISH) methods to confirm infections (e.g., Carnegie *et al.*, 2003; Li *et al.*, 2006; Small *et al.*, 2007; Burreson, 2008). ISH can be used to validate the presence of a pathogen in the tissues or whether it is adhering to the carapace of exposed animals or is passing through the digestive tract without actually infecting host tissues. This is important for microbial agents that may be present in the water column, perhaps adhering to potential hosts but not infecting them. ISH is also useful for attempting to piece together complex life cycles, tissue affinities, or to find rare life history stages that may use several host species (e.g., Audemard *et al.* 2002).

Molecular and immunological methods allow for the rapid screening of large numbers of samples. It can nevertheless be costly to perform PCR assays on hundreds of individual samples, particularly if prevalence of a pathogen is very low. With the caveat that one must define presence *versus* infection as indicated above, pooling samples is an excellent way to conserve resources as well as screen large numbers of samples. Several pooling algorithms have been developed for estimating disease prevalence in host populations (Worlund & Taylor, 1983; Litvak *et al.*, 1994; Hedt & Pagano, 2008). Simple pooling is efficient when prevalence is low (< 5%), and the lowest confidence intervals can be obtained from equal pool sizes (Litvak *et al.*, 1994; Williams & Moffitt, 2001). There can be issues, however, with sample size, quantity of host *versus* pathogen DNA, and equal *versus* unequal sample sizes in pooled samples (Worlund & Taylor, 1983; Thorburn, 1996; Williams & Moffitt, 2001). Unequal sample sizes can be used to improve the estimates of confidence intervals when prevalence approaches 10% or more (Williams & Moffitt, 2001). The size of the sample pool and the efficiency (sensitivity) of the PCR method as well as the quantity of host *versus* pathogen DNA has to be optimized for the method to work well.

Even with the advent of molecular diagnostics, suitably preserved reference specimens may still be difficult to obtain. Such was the case during the outbreak of *Neoparamoeba pemaquidensis* (Page, 1980) in the American lobster in 1999, when there was a lack of properly preserved samples of the closely related *Paramoeba pernicioso* Sprague, Beckett and Sawyer, 1969 for much needed comparisons (Mullen *et al.*, 2004). With the emergence of new pathogens, retrospective studies of properly preserved specimens of related species can provide important insights into diagnostics. *Paramoeba pernicioso* is a rare parasite of blue crabs, and it is difficult to diagnose using cytological methods (Messick, 2002). Samples of *P. pernicioso* were initially not available in suitable fixatives for molecular comparisons. My existing samples of *P. pernicioso* were unfortunately lost when an ultracold freezer malfunctioned; and this, further demonstrates that collections used in field samples must be properly preserved, stored, and curated to retain their value (e.g., Shields *et al.*, 2012b).

FIELD COLLECTIONS

Populations of many commercially important crustaceans are monitored through intensive pre- or post-harvest biomass surveys. Nonetheless, when disease outbreaks arise in commercially exploited crustaceans, it can be difficult to obtain well-fixed and preserved tissues that are suitable for diagnosis of the pathogens involved. Further issues often arise in terms of sample size, observer bias, and the management of data, including important metadata, or the resulting data stream can overwhelm existing storage resources. Some thought must therefore be given to host and sample collection, the number of samples to be collected and

processed, and data management and collective sharing of the information.

For estimates of prevalence, it is important to have reasonable sample sizes and to minimize sample or collection biases (e.g., Gregory & Blackburn, 1991, Jovani & Tella, 2006, Shields *et al.* 2017). Prevalence can be biased by low sample sizes; hence, requisite sample sizes must be considered before beginning any field assessments of disease. It also can be difficult to process large sample sizes of hosts in a timely manner, both in the field and in the laboratory. Pilot studies or trial runs can help determine bottlenecks in processing or provide insights into methods to avoid. One relatively straightforward means to sample large field collections is through randomization of host individuals. Randomization can be as simple as sampling every other host or every x^{th} host in a trap or trawl, with the caveat that the first host in the sample stream must be randomly selected. Trap or trawl hauls also can be similarly randomized for sampling and all individuals in the selected trap or trawl then sampled. More focused or biased collection of diseased animals is also important as it provides material for identification and documentation of the pathogen(s) and allow for the optimization of additional diagnostics (e.g., Pestal *et al.*, 2003; Shields *et al.*, 2005, 2007).

Managing the data stream

Data management can be one of the more difficult aspects of coordinating large field-based programs. A collection of large numbers of animals from disparate workers can rapidly generate an overwhelming amount of data. I prefer to use a project abbreviation with consecutive accession numbers for specimens, much like that used in museum collections. Other methods also work well. For example, one large field-based study on PaV1 in the Caribbean spiny lobster used the initials of the boat captains followed by consecutive numbers for each host animal that was sampled (JDS, unpubl. data). The boat crews were recorded separately by date making it relatively easy to cross reference and access the data.

For large studies, it is often necessary to assign several identification numbers to samples because of the different data generated by collaborators. That is, field and host data may reside in one data set whereas histological or PCR data may reside in another, and the identification numbers are cross-referenced for ease in reference and quality assurance. Although this may seem trivial, it can be difficult to implement and track data sets through time, particularly after the completion of the data collection component of a study. A good identification system is thus required. Although computer databases can be specifically designed for such tracking, their implementation requires additional labor, training and documentation.

Field collections often have a veritable “boat load” of environmental or collection data that adds to the complexity and wealth of information needed to assess the ecology of the hosts and their diseases. The associated metadata must be curated properly so it is not lost or poorly managed between collaborators. Data sets and databases must be designed to incorporate and archive metadata collected for field samples (e.g., environmental conditions, station data) as well as for that generated in the laboratory (e.g., different methods for diagnosis, different observers). Such databases require significant design and layout to incorporate the varieties of data to be stored and analyzed (e.g. Fig. 5). We routinely scan all data sheets and retain them in a “cloud”-based storage service with photographic documentation of histological samples and field specimens. Processed data files are stored similarly when they are completed, thus maintaining a coherent storage area for all of the data.

By way of example, in response to the severe decline of the American lobster, *H. americanus*, in the waters of Long Island Sound, NY, USA, concomitant with increased prevalence of epizootic shell disease, a \$2.3 million research initiative was

LOBSTER SHELL DISEASE PROJECT

ID# _____ Date of collection _____
 Collection location _____ Collector _____
 CL _____ Shell Disease _____
 Injury _____ Disease Intensity _____
 Sex (M, F) _____ Molt stage (Pr, I, Ps) _____
 Date of Necropsy _____ TCBS positive or negative _____
 Photograph of carapace _____

| Sample ID | Sample ID | Weight | Storage | Shell pieces | Sample ID | Storage |
|---------------------|------------|--------|------------------------|--|------------|----------------------|
| Hemolymph | 4ml | | Acetonitrile | Carapace – disease | 2 samples | 95% EtOH |
| Hemolymph | 1ml | | RNA later | Carapace – healthy | 2 samples | 95% EtOH |
| plasma | 1ml | | Spun/frozen | Carapace – disease | 2 samples | Frozen |
| cells | | | Spun/frozen | Carapace – healthy | 2 samples | Frozen |
| Hemolymph | 1ml x 2 | | Frozen (bank) | | | |
| Hepatopancreas (HP) | 2-4g | | Acetonitrile | Claw | 2 samples | Frozen |
| Gill | 2-4g | | Acetonitrile | Dorsal abdomen (D. abd.) | 2 samples | Frozen |
| Muscle | 2-4g | | Acetonitrile | Telson/Uropod (T/U) | 2 samples | Frozen |
| Gonad | 2-4g | | Acetonitrile | Ventral abdomen (V. abd.) | 2 samples | Frozen |
| Epidermis | 1-2g | | Acetonitrile | Ventral sternum (V. st.) | 2 samples | Frozen |
| Epidermis | X2 | | Frozen | Leg piece (leg) | 2 samples | Frozen |
| Hepatopancreas | Cassette 1 | | Bouin's | Soft Tissues for bank (epi., HP, gonad, heart, gill) | 2 samples | Frozen |
| Heart | Cassette 1 | | Bouin's | Carapace – disease | | 10%NBF |
| Gill | Cassette 2 | | Bouin's | Carapace – disease | | Acetonitrile |
| Eyestalks | Cassette 3 | | 1 in Bouin's, 1 frozen | Carapace – disease | Cassette 5 | Bouin's |
| Epidermis | Cassette 1 | | Bouin's | Carapace – healthy | Cassette 4 | Bouin's |
| Gonad | Cassette 1 | | Bouin's | | | |
| Muscle | | | RNA later | Pleopod | | 95% EtOH |
| Gonad | | | RNA later | Tail or claws and gills | ~20g | Whirlpack/frozen -20 |
| Hepatopancreas | | | RNA later | Hepatopancreas | ~20g | Amber jar/frozen -20 |

Weigh samples to go into acetonitrile. For plasma and cell separations: Spin hemolymph, low speed, 4C, and separate. Use anticoagulant if required.

Figure 5. Sample data sheet from the “100 Lobsters” project used by laboratory personnel to facilitate tissue sampling, storage, shipment and receipt of samples (Shields *et al.*, 2012b). The data sheet was vetted among research groups to include tissues of interest to all collaborators.

funded to monitor and study lobster health. Part of the initiative included the “100 Lobsters” project in which one laboratory served as a central point for dissecting, distributing, and archiving tissues as well as for data storage (Shields *et al.*, 2012a, b). Project goals were to sample 100 lobsters for joint analyses among several participating laboratories. Carapace, hemolymph, and various tissues were dissected, preserved accordingly and sent to several

collaborators for further assessment (histology, gene expression, metal contamination, contaminants exposure). A data sheet was developed prior to the work that served as a checklist to ensure that tissues and data were obtained for each animal entering the study (Fig. 5). It also facilitated the later distribution of tissue samples. A component of the project involved coordinating data access for the 100 lobsters to researchers, fisheries managers,

fishermen, and laypeople in the form of a website (www.uglylobster.org). The project is a work in progress but it serves as a useful tool for understanding the complexity of this disease phenomenon in Long Island Sound.

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