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Sensitivity and Specificity of macroscopic criteria for diagnosing *Hematodinium* sp. infections in snow crabs

Sensibilité et spécificité des critères macroscopiques pour le diagnostic des infections à *Hematodinium* chez le crabe des neiges

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ABSTRACT

The sensitivity of a diagnostic test is the probability the test will give a positive result given that the animal has the disease. The specificity is the probability of a negative result given the animal is not diseased. We estimated the sensitivity and specificity of macroscopic examination of snow crabs for detecting infection with *Hematodinium* sp. by comparing the visual diagnosis with results from microscopic examination of hemolymph smears. Estimated sensitivity was 53% and estimated specificity was 100%. This indicates that a positive result from macroscopic examination is definitive, given that the observer is well trained, but that macroscopic examination will fail to detect many infections. Because the incidence (i.e., occurrence of new) of *Hematodinium* infections may follow a seasonal trend, and newly infected animals may not exhibit symptoms of the disease, these estimates pertain to the Fall, the time of year at which the observations were made. Macroscopic observations made a few months earlier in the year may well miss more infections due to a higher proportion of infected animals being asymptomatic. Lastly, we note an alarming increase in prevalence of BCD in snow crabs from Conception Bay, Newfoundland. The prevalence was highest in female crabs; a finding that may indicate increasing costs to the snow crab fishery.

RÉSUMÉ

La sensibilité d'un test diagnostique est la probabilité d'obtenir un résultat positif pour un animal atteint de la maladie, tandis que la spécificité désigne la probabilité d'obtenir un résultat négatif lorsque l'animal n'a pas la maladie. Nous avons estimé la sensibilité et la spécificité de la détection de l'infection à *Hematodinium* par examen macroscopique du crabe des neiges en comparant cette méthode avec l'examen microscopique de frottis d'hémolymphe. La sensibilité a été estimée à 53 %, et la spécificité, à 100 %, indiquant qu'un résultat positif pour un examen macroscopique effectué par un technicien bien formé est concluant, mais que de nombreux cas d'infection ne seront pas détectés par un tel examen. Comme l'incidence (c.-à-d., le nombre de nouveaux cas) d'infections à *Hematodinium* pourrait varier selon les saisons et que les animaux nouvellement infectés ne présentent pas nécessairement de symptômes, ces estimations ne sont valables que pour l'automne, saison à laquelle les observations ont été faites. Il est fort possible qu'un plus grand nombre de cas d'infection ne soient pas détectés par des observations macroscopiques faites quelques mois plus tôt dans l'année, en raison d'une proportion plus élevée d'animaux infectés asymptomatiques. Finalement, nous relevons une hausse alarmante de la prévalence de la maladie du crabe amer chez le crabe des neiges de la baie Conception (Terre-Neuve). La prévalence était plus élevée chez les femelles, ce qui pourrait accroître les coûts pour les pêcheurs du crabe des neiges.

INTRODUCTION

The snow crab (*Chionoecetes opilio*) is currently the most commercially important species harvested in Newfoundland and Labrador. Landings for this fishery peaked in 1999 at 69,000 mt with a value in excess of $300,000,000 (Can.). Since the collapse of the groundfish fishery in 1992, snow crab has been the mainstay of the Province's fishing economy. In 1990, the presence of bitter crab disease (BCD) was first observed in snow crabs off Newfoundland. It has since been reported in increasing numbers of commercial catches off Newfoundland (Taylor and Khan 1995). The causative agent of BCD, *Hematodinium* sp., is a parasitic dinoflagellate that lives in the hemolymph, or blood, of infected crabs (Meyers et al., 1987). The parasite has previously damaged snow and Tanner crab fisheries in southeast Alaska (Meyers et al., 1987; 1990; 1996); hence, there is some cause for concern.

Epizootics of *Hematodinium* sp. have impacted several crustacean fisheries. The American blue crab (*Callinectes sapidus*) fishery has suffered significant annual mortalities in high salinity waters (Messick 1994; Messick and Shields 2000). The Norway lobster (*Nephrops norvegicus*) fishery in Scotland has suffered estimated losses of £L2-4 million to a *Hematodinium*-like parasite (Field et al.,1992; Field and Appleton, 1995). The French velvet crab (*Necora puber*) fishery suffered a catastrophic decline due *to H. perezi* (Wilhelm and Boulo, 1988; Wilhelm and Miahle, 1996), and the edible crab (*Cancer pagurus*) fishery was affected by the same or a similar agent (Latrouite et al., 1988). At present, it is not clear if the same species of *Hematodinium* occurs in both Alaska and Newfoundland, but given the effects of *Hematodinium* spp. on other fisheries, we suspect that the dinoflagellate represents a significant threat to the region's snow crab industry.

Preliminary data indicate that since 1990, the prevalence of *Hematodinium* in snow crabs off Newfoundland appears to have increased. In 1997, we initiated a project to (1) develop a sampling design and sampling protocol to estimate the prevalence of the disease, (2) obtain the first quantitative estimates of prevalence in a pilot study in Conception Bay, (3) compare the performance of two methods used in detecting *Hematodinium* infections (macroscopic visual examination versus microscopic evaluation of hemolymph smears), and (4) examine relationships between certain host factors and prevalence of infection. In this paper we report only on the relative performance of macroscopic and microscopic criteria for determining infection with *Hematodinium* sp.

MATERIALS AND METHODS

Sampling design and sampling protocol

The prevalence survey took place in Conception Bay, Newfoundland, during the annual stock assessment survey from 28 September to 9 October, 1998. The Bay was stratified into two depth strata (stratum 1, > 183 m depth, 81 nautical square miles; stratum 2, 91 – 183 m, 72 square miles). However, because of logistical problems, only two locations from stratum 2 were sampled for snow crabs. Consequently, although the sampling design presented is applicable to multi-stratum surveys, we examine the data as if it were from stratum 1. Based on past survey results, stratum 1 comprises the bulk of the snow crab population in the Bay (Miller and O'Keefe 1981). Stations were chosen randomly, and crabs were sampled at each station with either a trawl or a fleet of traps or both. A lined No. 36 shrimp trawl with a "tickler" chain and SCANMAR sensors was dragged across the bottom for 10 minutes at a speed of 2.5 knots. Trapping was conducted using fleets of eight standard Japanese conical traps, five of which had 13.1 cm mesh and three of which had 3.1 cm mesh. Each trap was baited with 3 kg of previously frozen northern shortfin squid (*Illex illecebrosus*) and Atlantic mackerel (*Scomber scombrus*). The standard soak time for traps was 24 hours but because of weather conditions in the autumn actual soak times varied from 16 to 139 hours.

Most of the snow crabs captured were examined but, because of logistical constraints associated with the handling of thousands of animals, some crabs were only counted. When traps were retrieved, the procedure was to place the contents of each trap into a separate basket. When a basket was filled, the crabs within were examined. The remaining crabs from the trap were sexed, counted and discarded. Using this procedure, over 80 % of the captured crabs were examined. For the trawl survey, the catch was rinsed with sea water, snow crabs were sorted randomly into baskets, and all non-target species were discarded. Baskets containing snow crabs were randomly selected and processed as for traps. Snow crabs in baskets that were not selected were counted by sex. A subsample of hemolymph was taken from every eighth crab processed in the trawl survey, except for certain days in which no hemolymph sampling was done. No bias arises from not sampling on some days because each day's station locations were selected randomly and the decision to samplw was based on the availability of a student rather than on the day's location. A previous pilot study indicated that one in eight crabs could be subsampled by one person without slowing the standard survey sampling during trawl operations. In addition, a hemolymph sample or a smear was obtained from every crab that showed macroscopic signs of infection on those days when the disease survey was in effect. No hemolymph samples were taken from crabs in the trap survey.

Sex, carapace width, shell condition and macroscopic signs of bitter crab disease were noted for all of the examined crabs. Shell conditions were determined using criteria described by Taylor *et al.* (1989). The primary

macroscopic sign of infection with *Hematodinium* is a distinct color change to the carapace that gives the crabs a cooked appearance. Infected crabs also have an opaque ventrum, listless or lethargic behavior, and milky, discolored hemolymph (Taylor and Khan, 1995).

Laboratory procedures

Hemolymph samples were drawn with a 22 ga. needle on a 3-ml tuberculin syringe from the arthrodial membrane at the juncture of the basis and the ischium of the cheliped or a walking leg. Hemolymph samples were handled in one of three ways. (1) Hemolymph was placed on an acid-cleaned, poly-l-lysine-coated microslide, fixed immediately in Bouin's fixative, and transported to the laboratory for further processing. (2) Small crabs were bled by detaching the dactyl from a walking leg, squeezing out a drop of hemolymph onto a slide, and drying the thick smear for later processing. (3) Due to the difficulty of handling fixatives in Coplin jars at sea, we also used a cold fixation method with good results. Briefly, a uniquely coded 3-ml syringe with 23 ga. needle was preloaded with 1 ml ice-cold, 10% formalin in filtered seawater. Crab hemolymph was drawn into a syringe at a proportion of approximately 1:2 to 1:5 hemolymph to fixative. The fixed hemolymph was gently shaken, stored on ice for transportation, and refrigerated until processed. In the laboratory, aliquots of fixed hemolymph were placed on poly-l-lysine coated slides, allowed to set for 45 s, fixed in Bouins solution for 24 hr, and transferred to 70% ethanol for holding. All of the smears were hydrated, stained with Jenner-Giemsa for 10 - 20 min (Presnell and Scheibmann, 1997), dehydrated through an acetone series, cleared in a xylene series, and mounted in cytoseal.

Stained smears were examined with a light microscope at 400x for 5 min each in random order and with hidden identification numbers to prevent bias. Smears were classified as "infected" if at least one clearly identifiable cell of *Hematodinium* sp. of any stage was found. Identification was based on cell size, presence of condensed chromatin in the nucleus (a feature of the dinoflagellates), and the typical "notched" appearance of the chromosomes.

Comparison of diagnostic procedures

Although hemolymph smears do not provide a perfectly reliable test because recently infected animals may have too few parasite cells to be detected, we treat the smears as a gold standard to which macroscopic observations can be compared. The sensitivity and specificity of the macroscopic test can be estimated in two ways.

The systematic sample of every eighth crab was examined by both diagnostic methods and the results can be summarized in a cross-classified 2x2 table (Table 1). From this, the sensitivity and specificity of the macroscopic approach can be estimated as

$$\textit{sensitivity} = \frac{\textit{number infected according to both methods}}{\textit{number infected according to smear}} \tag{1}$$

and

$$\textit{specificity} = \frac{\textit{number not infected according to both methods}}{\textit{number not infected according to smear}}. \tag{2}$$

Additional information was available about the sensitivity and specificity of the macroscopic approach because every crab designated positive for the disease according to macroscopic criteria was further tested by examination of hemolymph. We incorporated this additional information by viewing the macroscopic examination of all crabs as providing a (post-)stratification of the observations into two categories ("macroscopically positive" and "macroscopically negative"). Each of these two categories was then subsampled with hemolymph slides prepared for about half of the "macroscopically positive" crabs and for one eighth of the "macroscopically negative" crabs.

Accordingly, let Q_o be the number of animals visually examined that are macroscopically negative and Q_1 be the number macroscopically positive. Let q_o and q_1 be the number of animals drawn from the Q_o and Q_1 animals, respectively, that are examined microscopically. The results of the microscopic examinations are summarized as counts, R_{ij}, for i and j taking values 0 and 1, with i denoting the result of the microscopic test, j denoting the result of the macroscopic test, 0 denoting uninfected, and 1 denoting infected. Thus, for example, R_{o1} denotes the number of animals classified as uninfected by the microscopic method and infected by the macroscopic method.

The sensitivity and specificity are then estimated by

$$\textit{sensitivity} = \frac{(r_{11}/q_1)Q_1}{(r_{11}/q_1)Q_1 + (r_{10}/q_0)Q_0} \tag{3}$$

$$\textit{specificity} = \frac{(r_{00}/q_0)Q_0}{(r_{00}/q_0)Q_0 + (r_{01}/q_1)Q_1}. \tag{4}$$

These equations make use of the relationship (Bayes Rule)

$$\Pr(J = j \mid I = i) = \frac{\Pr(I = i \mid J = j)\Pr(J = j)}{\sum_{h=0}^{1} \Pr(I = i \mid J = h)\Pr(J = h)}.$$

where *I* and *J* are random variables representing the outcome of the microscopic and macroscopic tests, respectively, when the tests are applied to a randomly selected individual. In the above equation, the numerator represents the probability of the joint outcome of the two tests (joint probability = conditional probability of microscopic result given the macroscopic result x probability of getting the macroscopic result). The denominator represents the marginal probability of getting outcome *i* with the microscopic test.

RESULTS

The systematic sample of every eighth crab in the trawl survey resulted in a sample of 355 crabs (Table 1). Nine of these crabs showed macroscopic signs of the disease while 346 did not, giving a prevalence of 2.53%. Hemolymph smears were examined from all nine macroscopically positive and 333 of the macroscopically negative crabs. (Samples from 13 macroscopically negative animals were lost.) All of the 9 apparently infected animals according to the macroscopic criteria tested positive for the presence of *Hematodinium* when hemolymph smears were examined. Of the 333 apparently uninfected animals by macroscopic criteria that were examined microscopically, 7 (2.10%) contained *Hematodinium* cells in the smears. Because 13 slides from the apparently uninfected category were lost, it was not valid to apply the simple formulae for sensitivity and specificity (equations (1) and (2)). However, application of equations (3) and (4) gave the following estimates:

Sensitivity = 0.527

Specificity = 1.000

Overall, 10,165 crabs were examined macroscopically: 4,707 crabs in the trawl survey and 5,458 crabs in the trap survey. Of these, 108 crabs in the trawl and 31 crabs in the trap surveys were macroscopically positive for infection for an apparent prevalence (according to macroscopic criteria) of 1.36%. However, the apparent, or macroscopic, prevalence of infection was 0.57% in the trap survey (Table 2) and 2.29% in the trawl survey (Table 3), indicating significant differences between survey methodologies in predicting prevalence of macroscopic infections (Chi-square = 56.838, df = 1, $P<0.001$).

In the trawl survey, the prevalence of cryptic infections, or macroscopically negative but microscopically positive infections, was 7 out of 333 crabs or 2.10%

(Table 1). We adjusted the prevalence based on the macroscopic and microscopic data for false negatives and positives to give the following: macroscopically positive (108) x specificity (1.00) + macroscopically negative (4599) x macroscopically negative but microscopically positive infections (7) / macroscopically negative examined microscopically (333) = 204.7 / 4707 = 4.35%. This value corresponds closely with the intuitive estimate of 4.39% (2.29% macroscopically positive + 2.10% cryptic infections).

Host sex was a distinct factor in infections of *Hematodinium* sp. In the trawl survey, male snow crabs had a signficantly lower prevalence than females (Chi-square = 56.19, df = 1, $P<0.001$, Table 3). Whether this was a result of fishing on male crabs, a result of predilection of the parasite, or an increased vulnerability of for female and juvenile crabs was not determined.

DISCUSSION

In Conception Bay, Newfoundland, the prevalence of *Hematodinium* sp. infections has increased markedly over time. In the 1992-1993 fishery assessment, the prevalence of BCD in trapped male crabs was 0.037% (n = 12,012) with a single sample of 135 crabs having a relatively high prevalence of 3.7% (Taylor and Khan 1995). In the present study, the 1998 fishery assessment, the prevalence of overt, macroscopically diagnosed BCD in trapped male crabs was 0.55% versus 1.63% in the trawl survey (n = 5,439 and 4,044, respectivly) with an additional 2.10% prevalence in crabs with asymptomatic infections. These prevalences are noticeably higher than that previously reported and indicate a disturbing trend in the prevalence of BCD in the region's commercially important snow crab industry.

The trap and trawl data also indicate a possible bias in estimating the prevalence of disease. Similar biases were noted for velvet crabs, *Necora puber*, infected with *Hematodinium* sp. off of Brittany, France. Trawl samples had significantly higher prevalences than pot (trap) samples primarily due to non-selective catching of healthy and listless, diseased crabs in trawls (Wilhelm and Miahle 1996). In the present study, the trawl samples had higher prevalences because the trawl had a lower minimum size of retention than the traps, and sublegal males and females are not removed by the fishery. There is therefore no removal of diseased animals from the female population other than increased mortality to disease.

Hematodinium infections in snow crabs are most reliably diagnosed from microscopic examination of hemolymph or other tissues. Comparison of diagnostic procedures based on macroscopic criteria and microscopic examination of hemolymph smears reveal that the macroscopic procedure had high specificity (estimate = 1.0). That is, macroscopic signs of infection are specific to the disease. On the other hand, the sensitivity of the macroscopic procedure was around 0.53, suggesting that many infected animals could be missed if the macroscopic

procedure alone were used. The relatively low sensitivity estimate highlights two important phenomena. (1) Prevalence based solely on macroscopic diagnosis is underestimated by approximately 50%; a finding that has significant ramifications for estimating the impact of the disease on the fishery. (2) Crabs exhibiting macroscopic signs of infection are in advanced stages of the disease; detection and diagnosis of the parasite and study of the disease processes must focus on the asymptomatic or "cryptic" infections that are reliably diagnosed only through microscopic examination.

The duration of infection and the ability to detect microscopic infections (detectability) has been examined in snow and blue crabs experimentally infected with *Hematodinium* spp. (Meyers et al. 1987, Shields and Squyars 2000). Infections were detectable in the hemolymph of experimentally infected snow crabs after 55, 69, and 97 days post inoculation (Meyers et al. 1987), but sample sizes were too low for statistical representation. In the temperate-water blue crab, infections were detectable in 30 to 35% of experimentally infected hosts after 14 to 18 days, reaching 80 to 85% after 26 to 32 days, and 100% after 35 days (Shields and Squyars 2000). Histological examinations of the heart indicated 80 to 100% detection after 3 to 5 days post inoculation. The time-to-development of the pink carapace, i.e., a massive end-stage infection, has not been reported in snow crabs, and discoloration of the carapace rarely occurs in infections in the blue crab. Thus, while we do not directly report on the ability to detect microscopic infections from infection challenges, our data indicate that microscopic infections are at least as common as those observed macroscopically, and may fall along the time scale of 50 to 100 days post initial infection, one possibility indicated by Meyers et al. (1987).

We speculate that macroscopic signs of BCD become more pronounced as epizootics progress. In snow crabs from Newfoundland, anecdotal observations based on macroscopically-diagnosed BCD infections indicate that BCD appears to be more prevalent at the end of the commercial fishing season (October) than at the beginning of the season (May-June) (Taylor unpubl. data). However, because the macroscopic method is not very sensitive, it may be that there are just as many animals infected in the spring as in the autumn but the infections may not be detectable by macroscopic methods. By contrast, in the Tanner crab fishery of southeastern Alaska, the prevalence and intensity of BCD infections increased through spring into late summer, then declined through autumn falling to zero in late winter (Eaton et al., 1991; Love et al. 1993). This implies that prevalence estimates made from macroscopic examinations at different times of the year are not comparable and meaningful comparisons should be based on standardized surveys. Therefore, estimates should be based on unbiased collection methods that include females and pre-recruits, and seasonal samples that include periods with peak prevalences.

Infections with *Hematodinium* spp. in other species of crabs follow cycles that may be associated with host molting (Eaton et al. 1991) or maturation (Messick 1994; Messick and Shields 2000). Summer peaks in *Hematodinium* sp.

infections occur in Tanner crabs just after molting (Eaton et al. 1991). Summer peaks in *Hematodinium* sp. infections in blue crabs occur well after the main molting period. The disease is more common in juvenile than adult blue crabs and may be related to frequency of molting in juveniles (Messick 1994; Messick and Shields 2000). Thus, surveys for BCD infections should collect data on host factors such as size, maturity, molt stage, and physical factors such as depth and temperature.

We conclude that macroscopic diagnosis is only useful to indicate the relative prevalence of crabs in end stages of the disease. Visual, macroscopic identification is highly repeatable if the observations are made by trained observers; however, prevalence will likely be underreported. A more complete assessment of prevalence must rely on microscopic examination of hemolymph or histological preparations. Microscopic assessment is critical to understanding seasonal factors or host-parasite relationships that can influence the mortality of the host as we do not know the duration or length of progression of the disease to the terminal stage.

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Table 1. Summary of prevalence data from the systematic survey of the trawl data for sensitivity and specificity.

Source	**Microscopic-ally positive**	**Microscopic-ally negative**	**Total**	**Microscopic-ally positive (%)**
Macroscopically positive	9	0	9	100.000
Macroscopically negative	7	326	333	2.102
Total	**16**	**326**	**355***	**4.507**

* 13 slides were inadvertently lost from the analysis by one of the junior authors.

Table 2. Nominal macroscopic prevalence of *Hematodinium* sp. in snow crabs by station for 19 fleets of 8 traps fished during the 1998 Conception Bay assessment cruise. Variance estimated for a proportion estimated from cluster sampling as in Dawe et al. (1993).

Station number	Depth (m)	Number infected	Total number	Prevalence (%)
1	196	2	352*	0.568
2	209	2	342	0.585
3	190	0	392*	0.000
4	222	1	392	0.255
5	228	4	356	1.124
6	262	2	291	0.687
7	272	3	359	0.836
8	175	0	246*	0.000
9	226	2	298	0.671
10	214	1	199	0.503
11	277	1	266	0.376
12	258	7	268	2.612
13	267	0	193*	0.000
14	257	2	166	1.205
15	263	0	339	0.000
16	257	0	336	0.000
17	223	0	260	0.000
18	246	3	199*	1.508
19	244	1	204*	0.490
Total		**31**	**5458**	**0.57**
Variance				**0.206**

*Includes in descending order 1, 10, 1, 1, 1 and 5 female crabs. One infected female crab is included in Station 18.

Table 3. Nominal macroscopic prevalence of *Hematodinium* sp. in snow crabs by station for 24 trawl hauls during the 1998 Conception Bay assessment cruise. N_P is the number parasitized, N_T is the total number sampled per station. Variance estimated for a proportion estimated from cluster sampling as in Dawe et al. (1993).

Station number	Depth (m)	Males N_P/N_T	Prevalence (%)	Females N_P/N_T	Prevalence (%)
1	201	0/266	0.000	3/23	13.043
2	229	4/452	0.885	0/16	0.000
3	224	0/60	0.000	0/1	0.000
4	245	2/325	0.615	1/62	1.613
5	226	1/229	0.437	0/7	0.000
6	260	4/195	2.051	3/41	7.317
7	270	2/346	0.578	1/1	100.000
8	152	1/84	1.190	2/16	12.500
9	224	2/203	0.985	2/27	7.407
10	224	2/161	1.242	0/4	0.000
11	282	3/113	2.655	1/3	33.333
12	231	0/131	0.000	0/12	0.000
13	279	5/171	2.924	2/21	9.524
20	91	0/160	0.000	0/89	0.000
21	243	1/206	0.485	0/6	0.000
22	250	11/228	4.825	9/95	9.474
23	235	0/30	0.000	0/1	0.000
24	252	1/54	1.852	2/14	14.286
25	247	7/135	5.185	3/58	5.172
26	235	3/95	3.158	4/47	8.511
27	248	6/154	3.896	2/33	6.061
28	213	5/90	5.556	2/40	5.000
29	204	6/199	3.015	5/46	10.870
30	206	0/67	0.000	0/0	0.000
Total		**66/4044**	**1.632**	**42/663**	**6.335**
Variance			**1.152**		**2.294**